ECOLOGICAL-ECONOMIC BALANCE IN FINING ENVIRONMENTAL POLLUTION SUBJECTS BY A DYADIC 3-PERSON GAME MODEL

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(Received 6th Oct 2018; accepted 28th Nov 2018)

Abstract. A problem of rationalizing industrial wastewater treatment is considered. It issues from that industrial enterprises may violate conventions about water treatment, so then they are fined. Those fines are directed to control water pollution by measuring it and treating wastewater additionally, if necessary. Under threat of heavy fines, however, an enterprise may reduce or stop its manufacturing resulting in a budget cut for water resources conservation and recirculation. Therefore, fining for under-treating industrial wastewater is balanced using an environmental protection model in the form of a dyadic 3-person game. According to this game, in which three subjects of water pollution (industrial enterprises) are obliged to treat industrial wastewater, the ecologically healthy water balance of the reservoir does not worsen if just one subject does not apply the treatment. Such model implies that about one third of the polluted water recovers without treatment. If none of the subjects applies a water treatment system, they are fined (with a 3-fine for each). If only two subjects do not treat wastewater, all the three subjects are fined (with a 2-fine for each) due to impossibility of finding those two violators. Application of a water treatment system costs a conventional unit for each subject per a period of time (a day, a week, or a month). The game solution is searched on the regular finite lattice of situations, which is obtained by sampling the continuous set of those situations. Dealing with either non-symmetric or non-profitable situations, an approximate solution is found using concessions in the equilibrium. By this solution, the water treatment system is turned off for 3 periods of 10, and the 2-fine is optimally set at 0.34 units, whereas the 3-fine is set at 1.394 units. Under threat of the heavy 3-fine, the subjects will definitely come to a convention of scheduling the water treatment systems. Switching from "clean" to "polluting" manufacturing and backwards is to be controlled exactly once per those 10 periods. Eventually, the cost of 8.018 units for a subject is just the expected spending, whereas the least cost in 7 units is still possible under the corresponding water treatment schedule.
Keywords: *industrial wastewater, water treatment, fining, dyadic game, equilibrium situations*

Introduction

Water resources are intensively used in industries. For their conservation and recirculation, subjects of water pollution are obliged to treat industrial wastewater. The treatment should be executed as much as needed for maintaining the ecologically healthy water balance. A part of polluted water recovers without treatment (Beiras, 2018; Parimal, 2017).

Some industrial enterprises may violate conventions about water treatment. Then the government fines them for under-treating wastewater. Those fines are directed to control water pollution by measuring it and treating additionally, if necessary (Jiménez et al., 2018; Rey et al., 2018). In a certain sense, fining for under-treating is a way for strengthening the state treasury. However, an enterprise may reduce or stop its activity (manufacturing) under threat of heavy fines. The treasury subsequently will receive less. Then a budget for water resources conservation and recirculation will be cut. Therefore, fining for under-treating industrial wastewater should be balanced (Arguedas, 2013; Heyes, 2000; Regens et al., 1997).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14511474

Currently an imbalance in a water treatment convention is a common phenomenon. Primarily, this is about the absence of compelling factors which would force parties to comply with a convention. For instance, the Convention on the Protection and Use of Transboundary Watercourses and International Lakes, also known as the Water Convention, has a joint ad hoc expert group on water and industrial accidents (see [unece.org/env/water.html]). This group deals with the prevention of the accidental pollution of transboundary waters. There is a Protocol on civil liability for damage and compensation caused by transboundary effects of industrial accidents on transboundary waters. Its aim is to give individuals affected by the transboundary impact of industrial accidents on international watercourses a legal claim for adequate and prompt compensation. The financial limits of liability as well as the minimum amount of financial securities have been agreed by all the actors of the Protocol negotiation, including the insurance sector. Despite this, the Protocol has been ratified only by Hungary and it is not in force. The Protocol will enter into force once 16 states have ratified it. This is a partial but demonstrative evidence of the force lack in a convention. But it is obvious that such conventions can be reinforced by financial influence. Therefore, the role of budgeting to achieve a balance in a water treatment convention is principal (Heyes, 2000; James, 2017; Wolf, 2009).

An environmental protection model in the form of a dyadic 3-person game

A lot of economical factors influence how the budget for water resources conservation and recirculation is formed (Sgroi et al., 2018; Cui et al., 2018; Zikos and Hagedorn, 2017). So, no exact formulae exist for counting the fines. Only simple and rough models can be economically reliable and practically executable (Zomorodian et al., 2018; Regens et al., 1997; Arguedas, 2013). One of such models is a 3-person dyadic game firstly considered by Vorob'yov (1984). According to this game model, three enterprises manufacturing roughly the same production use similar water resources of a reservoir. The used water is polluted through the manufacturing process (e.g. it is shown by Skouteris et al., 2018; Hussain and Wahab, 2018; Mousavi et al., 2015). Sometimes an enterprise is called an environmental pollution subject. Application of a water treatment system costs a conventional unit for each subject per a period of time (a day, a week, or a month). If just a one subject does not apply the treatment, it does not worsen the ecologically healthy water balance of the reservoir owing to recovering. Such model implies that about one third of the polluted water recovers without treatment. If two or three subjects do not apply the treatment, then the polluted water cannot recover and all the three subjects are fined. Along with the fundamental research by Vorob'yov (1984), the fine set at 3 units was also researched by Romanuke (2009, 2010a) continued trying the equilibrium approach, which recently has been intensively developed for improving resource management policies (Xu et all, 2018; Guo, 2018; James, 2017). With fining the subjects in such a way, the corresponding dyadic game has four equilibrium situations in pure strategies and five equilibrium situations in mixed strategies (Romanuke, 2010a). However, no one of these situations is simultaneously symmetric and profitable. An equilibrium situation at which the subjects work without treating wastewater (a symmetric situation) is totally senseless, because the subsequent fines in 3 units are far worse than just applying three water treatment systems. Another symmetric equilibrium situation is in mixed strategies, at which a cost for every environmental pollution subject is equal to $2-\sqrt{3}/2$, i.e. this cost is about 13.4% higher than applying three water treatment

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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systems. At this situation, about 21.13% of the water-for-manufacturing is discharged back into the reservoir without treatment. A promising situation, though neither equilibrium nor very profitable, is a case when only 5.9% of wastewater is not treated whereas it costs 0.971 units for every subject. Despite these costs are very close to costs for applying the water treatment system, there are no any other symmetric situation at which the costs would be less.

A generalization of fining was researched by Romanuke (2010b). The article considered the fine greater than the unit. If the fine is less than 2 units then the corresponding dyadic game does not have equilibrium situations. For the fine equal exactly 2 units, there is a single symmetric equilibrium situation at which every environmental pollution subject treats just a half of the discharged wastewater. Obviously, this situation is unprofitable as the reservoir is polluted irreversibly whereas it costs 1.5 units for every subject. For fines greater than 2 units, the game has a single symmetric equilibrium situation at which the cost for a subject decreases down to 1 unit as the fine increases. Theoretically, threat of heavy fines may force the environmental pollution subjects to totally convert their manufacturing to treating wastewater as every subject will pay 1 unit anyway. Although such “clean” situation is not a Nash equilibrium, its equilibration is forced by the threat of heavy fines. On the other hand, those heavy fines threaten with that some subjects may reduce their manufacturing because of the cost in 1 unit may be initially unprofitable or economically irrational.

Goal of the article and tasks to be performed

Inasmuch as heavy fines may ruin an economical balance among those three environmental pollution subjects and government, subsequently leading to cutting a budget for water resources conservation and recirculation, economically more reasonable fines should be substantiated. Thus, fining for when all subjects do not apply treatment must be heavier than fining when only two subjects do simultaneously not treat wastewater. Along with that, no less than two thirds of the polluted water is to be treated. To find a solution to such a problem, there are six tasks to be performed:

1. To state costs in the game explicitly.
2. To substantiate a range of the fine for when only two subjects do simultaneously not treat wastewater (let it be called a 2-fine).
3. To substantiate a range of the fine for when no one treats wastewater (let it be called a 3-fine).
4. In order to find an applicable solution, to sample the continuous set of situations in mixed strategies.
5. To find symmetric equilibria (over the sampled set), at which the cost for every subject is less than 1 unit.
6. For the found equilibria, to select those fines by which no less than two thirds of the polluted water is treated costing the least.

Materials and methods

Costs of wastewater treatment in the dyadic 3-person game with generalized fines

Denote a pure strategy of the k-th subject by x_k, where $x_k \in \{0, 1\}$. Strategy $x_k = 0$ implies that the k-th subject totally applies its water treatment system, whereas $x_k = 1$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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implies that all wastewater is discharged without treatment. Henceforward, a mixed strategy of the k-th subject can be represented as a probability $d_k \in [0;1]$ of choosing pure strategy $x_k = 1$. This is why strategies $x_k = 1$ and $d_k = 1$ coincide (implying the same action or decision), and strategies $x_k = 0$ and $d_k = 0$ coincide also.

There are eight situations in pure strategies written as $\{x_1, x_2, x_3\}$ in the dyadic 3-person game. A cost for the k-th subject in situation $\{x_1, x_2, x_3\}$ is $c_k(x_1, x_2, x_3)$ or, shortly, $c_k^{\langle x_1 x_2 x_3 \rangle}$. If the 2-fine is a and the 3-fine is b, then (see the cube of situations and costs in *Fig. 1*):

$$\begin{gathered}
c_1^{\langle 000 \rangle} = c_2^{\langle 000 \rangle} = c_3^{\langle 000 \rangle} = 1, \\
c_1^{\langle 010 \rangle} = c_3^{\langle 010 \rangle} = 1,\ c_2^{\langle 010 \rangle} = 0, \\
c_1^{\langle 010 \rangle} = c_3^{\langle 010 \rangle} = 1,\ c_2^{\langle 010 \rangle} = 0, \\
c_2^{\langle 100 \rangle} = c_3^{\langle 100 \rangle} = 1,\ c_1^{\langle 100 \rangle} = 0, \\
c_1^{\langle 011 \rangle} = 1 + a,\ c_2^{\langle 011 \rangle} = c_3^{\langle 011 \rangle} = a, \\
c_2^{\langle 101 \rangle} = 1 + a,\ c_1^{\langle 101 \rangle} = c_3^{\langle 101 \rangle} = a, \\
c_3^{\langle 110 \rangle} = 1 + a,\ c_1^{\langle 110 \rangle} = c_2^{\langle 110 \rangle} = a, \\
c_1^{\langle 111 \rangle} = c_2^{\langle 111 \rangle} = c_3^{\langle 111 \rangle} = b.
\end{gathered} \quad \text{(Eq.1)}$$

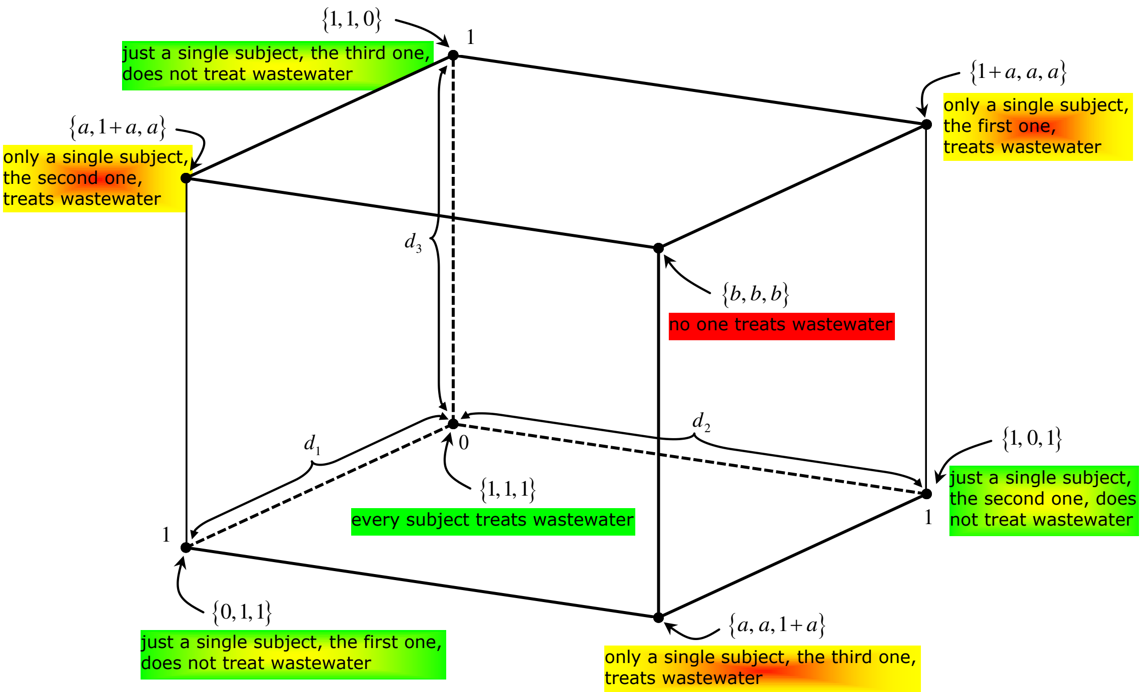

***Figure 1.** The cube of situations and costs in the dyadic 3-person game with generalized fines (see Romanuke, 2010b)*

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A cost for the k-th subject in mixed strategies situation $\{d_1, d_2, d_3\}$ is:

$$\begin{gathered} c_k(d_1, d_2, d_3) = \\ = (1-d_1)(1-d_2)(1-d_3)c_k^{\langle 000\rangle} + \\ +(1-d_1)(1-d_2)d_3 c_k^{\langle 001\rangle} + \\ +(1-d_1)d_2(1-d_3)c_k^{\langle 010\rangle} + \\ +(1-d_1)d_2 d_3 c_k^{\langle 011\rangle} + \\ +d_1(1-d_2)(1-d_3)c_k^{\langle 100\rangle} + \\ +d_1(1-d_2)d_3 c_k^{\langle 101\rangle} + \\ +d_1 d_2(1-d_3)c_k^{\langle 110\rangle} + \\ +d_1 d_2 d_3 c_k^{\langle 111\rangle},\ k = \overline{1, 3}. \end{gathered} \quad \text{(Eq.2)}$$

Plugging costs *Equation 1* into *Equation 2* gives:

$$c_1(d_1, d_2, d_3) = 1 + a(d_1 d_2 + d_2 d_3 + d_1 d_3) + (b - 3a) d_1 d_2 d_3 - d_1, \quad \text{(Eq.3)}$$

$$c_2(d_1, d_2, d_3) = 1 + a(d_1 d_2 + d_2 d_3 + d_1 d_3) + (b - 3a) d_1 d_2 d_3 - d_2, \quad \text{(Eq.4)}$$

$$c_3(d_1, d_2, d_3) = 1 + a(d_1 d_2 + d_2 d_3 + d_1 d_3) + (b - 3a) d_1 d_2 d_3 - d_3. \quad \text{(Eq.5)}$$

Every subject struggles to minimize its cost by adjusting the probability of discharging without treatment. If in situation $\{d_1^*, d_2^*, d_3^*\}$ costs (*Eqs. 3–5*) are such that

$$c_1(d_1^*, d_2, d_3) \leqslant c_1(d_1, d_2, d_3) \text{ by } d_k \in [0; 1] \ \forall k = \overline{1, 3}, \quad \text{(Eq.6)}$$

$$c_2(d_1, d_2^*, d_3) \leqslant c_2(d_1, d_2, d_3) \text{ by } d_k \in [0; 1] \ \forall k = \overline{1, 3}, \quad \text{(Eq.7)}$$

$$c_3(d_1, d_2, d_3^*) \leqslant c_3(d_1, d_2, d_3) \text{ by } d_k \in [0; 1] \ \forall k = \overline{1, 3}, \quad \text{(Eq.8)}$$

then situation $\{d_1^*, d_2^*, d_3^*\}$ is an equilibrium (by Nash). The equilibrium situation is explicitly determined by inequalities (*Eqs. 6–8*) represented in the form of inequalities

$$\begin{gathered} 1 + a(d_1^* d_2 + d_2 d_3 + d_1^* d_3) + (b - 3a) d_1^* d_2 d_3 - d_1^* \leqslant \\ \leqslant 1 + a(d_1 d_2 + d_2 d_3 + d_1 d_3) + (b - 3a) d_1 d_2 d_3 - d_1 \text{ by } d_k \in [0; 1] \ \forall k = \overline{1, 3}, \end{gathered} \quad \text{(Eq.9)}$$

$$\begin{gathered} 1 + a(d_1 d_2^* + d_2^* d_3 + d_1 d_3) + (b - 3a) d_1 d_2^* d_3 - d_2^* \leqslant \\ \leqslant 1 + a(d_1 d_2 + d_2 d_3 + d_1 d_3) + (b - 3a) d_1 d_2 d_3 - d_2 \text{ by } d_k \in [0; 1] \ \forall k = \overline{1, 3}, \end{gathered} \quad \text{(Eq.10)}$$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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$$1+a\left(d_1d_2+d_2d_3^*+d_1d_3^*\right)+\left(b-3a\right)d_1d_2d_3^*-d_3^*\leqslant \leqslant 1+a\left(d_1d_2+d_2d_3+d_1d_3\right)+\left(b-3a\right)d_1d_2d_3-d_3 \text{ by } d_k\in\left[0;1\right]\ \forall k=\overline{1,3}. \quad \text{(Eq.11)}$$

Inequalities (*Eq. 9–11*) are subsequently simplified:

$$d_1^*\left(a\left(d_2+d_3\right)+\left(b-3a\right)d_2d_3-1\right)\leqslant \leqslant d_1\left(a\left(d_2+d_3\right)+\left(b-3a\right)d_2d_3-1\right) \text{ by } d_k\in\left[0;1\right]\ \forall k=\overline{1,3}, \quad \text{(Eq.12)}$$

$$d_2^*\left(a\left(d_1+d_3\right)+\left(b-3a\right)d_1d_3-1\right)\leqslant \leqslant d_2\left(a\left(d_1+d_3\right)+\left(b-3a\right)d_1d_3-1\right) \text{ by } d_k\in\left[0;1\right]\ \forall k=\overline{1,3}, \quad \text{(Eq.13)}$$

$$d_3^*\left(a\left(d_1+d_2\right)+\left(b-3a\right)d_1d_2-1\right)\leqslant \leqslant d_3\left(a\left(d_1+d_2\right)+\left(b-3a\right)d_1d_2-1\right) \text{ by } d_k\in\left[0;1\right]\ \forall k=\overline{1,3}. \quad \text{(Eq.14)}$$

So, a situation with probabilities d_1^*, d_2^*, d_3^* becomes a Nash equilibrium if inequalities (*Eqs. 12–14*) hold. In such a situation, an environmental pollution subject cannot pay less acting on its own. If two or three subjects start searching for decreasing their costs, the equilibrium is ruined as subsequent costs for at least one party will be greater than previously. Obviously, equilibration is strongly dependent on what 2-fine and 3-fine are and how much they differ. The best situation is such that

$$d_1^*=d_2^*=d_3^*=d^*\leqslant 1/3 \quad \text{(Eq.15)}$$

for the stated problem.

A range of the 2-fine

The model claims that the polluted water recovers without treating it by investing 2 units. This is a situation when two of three environmental pollution subjects apply their water treatment systems. When only a single subject applies, a unit is lacked. Measurement of a water pollution rate cannot reveal which subject is "clean" and which subjects work "polluted". Therefore, every subject is fined. The sum of three 2-fines should be equal to 1 unit or greater. So, a minimally possible 2-fine is one third of the unit.

Now, a maximal 2-fine should be considered. As it is mentioned above, setting $a>1$ is unfavorable. In the case of $a=1$ or close to that, the "clean" subject potentially pays a doubled cost. This is a kind of demotivation for conscientious subjects. Besides, this may be a (negative) motivation for violators. The case of $a=0.75$ is more attractive but nonetheless the "clean" subject may eventually pay too much. In the case of $a=0.5$ the investment into the reservoir is 2.5 units, which is sufficient for maintaining the ecologically healthy water balance. Hence, the 2-fine can be studied within range $\left[0.34; 0.5\right]$ by a step of 0.01 (instead of unsuitable 0.33, the marginal endpoint 0.34 is included for closing the range). However, if no solution is found, the range must be widened.

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A range of the 3-fine

When no one treats wastewater, the sum of 3-fines should be far greater than that (2 units), which is sufficient for the ecologically healthy water balance. A reason is that worse pollution may require more investment for recovering. This is so because of a water recover function is probably not linear. Another reason is to demotivate violators. Such demotivation must be stronger than a minimal 2-fine. Hence, a suitable range for the 3-fine is from a up to $5a$. The step can be set at $a/10$, rougher than for the 2-fine as the latter is more influential. Note that such a range implies inequality $b \geqslant a$ although inequality $b \leqslant 1+a$ is possible. This means that the case with the totally senseless equilibrium of a situation, when no one treats wastewater, here is not excluded.

Sampling the continuous set of situations in mixed strategies

It is apparent that the dyadic 3-person game (see *Fig. 1*), by fixing some a and b from their ranges, will not have profitable symmetric equilibria in pure strategies. So, the best solution will be sought in mixed strategies. But, even if a profitable symmetric equilibrium in mixed strategies exists, it is likely to contain probabilities which are either irrational numbers or irreducible fractions with great denominators. Practical realization of such probabilities is impossible: relative frequencies (of both pure strategies) tend to be represented as much simpler fractions like $1/2$, $n/3$ by $n \in \{1, 2\}$, $n/4$ by $n \in \{\overline{1, 3}\}$, and so on, because switches from “clean” to “polluting” manufacturing and backwards cannot be too frequent. An exception could be if the work of those environmental pollution subjects was observed and controlled for a few years without any corrections by inflation, development, implementation of new technologies, etc. In other words, such exception is hardly plausible.

Therefore, the continuous set of situations in mixed strategies is sampled to obtain a finite lattice of situations. The lattice is

$$\mathscr{L} = \bigtimes_{k=1}^{3} \left\{ d_k^{\langle j \rangle} \right\}_{j=0}^{M} \text{ by } M \in \mathbb{N} \setminus \{1\}, \tag{Eq.16}$$

where $d_k^{\langle j \rangle} = j/M$ is a mixed strategy (being factually a probability) of the k-th subject. Lattice (*Eq. 16*) is regular as its step $1/M$ is a constant (*Fig. 2*).

Nevertheless, the game may not have a symmetric profitable equilibrium even over the sampled set of situations in mixed strategies. Thus, concessions in costs will be considered.

Results

Approximate equilibria by cost concessions

When an exact equilibrium does not exist, or condition (*Eq. 15*) does not hold, an approximate equilibrium is found by a concession β (for more detailed analysis, see it in Romanuke, 2016). Instead of inequalities (*Eq. 12–14*), if inequalities

$$d_1^{\langle\beta\rangle}\left(a\left(d_2+d_3\right)+\left(b-3a\right)d_2 d_3-1\right)-\beta \leqslant$$
$$\leqslant d_1\left(a\left(d_2+d_3\right)+\left(b-3a\right)d_2 d_3-1\right) \text{ by } d_k \in [0; 1] \;\; \forall\, k=\overline{1, 3},$$

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$$d_2^{\langle\beta\rangle}\left(a\left(d_1+d_3\right)+\left(b-3a\right)d_1d_3-1\right)-\beta\leqslant$$
$$\leqslant d_2\left(a\left(d_1+d_3\right)+\left(b-3a\right)d_1d_3-1\right) \text{ by } d_k\in\left[0;\,1\right]\ \forall\,k=\overline{1,\,3},$$

$$d_3^{\langle\beta\rangle}\left(a\left(d_1+d_2\right)+\left(b-3a\right)d_1d_2-1\right)-\beta\leqslant$$
$$\leqslant d_3\left(a\left(d_1+d_2\right)+\left(b-3a\right)d_1d_2-1\right) \text{ by } d_k\in\left[0;\,1\right]\ \forall\,k=\overline{1,\,3}$$

hold for some $\beta>0$, then $\left\{d_1^{\langle\beta\rangle},\, d_2^{\langle\beta\rangle},\, d_3^{\langle\beta\rangle}\right\}$ is the approximate equilibrium. The concession is a kind of re-achieving an equilibrium at a situation by small losses when the situation is not in equilibrium (by Nash). By an appropriate concession β, condition

$$d_1^{\langle\beta\rangle}=d_2^{\langle\beta\rangle}=d_3^{\langle\beta\rangle}=d^{\langle\beta\rangle}\leqslant 1/3 \tag{Eq.17}$$

defines the best approximate equilibrium. Clearly, the greater the concession is, the less stable the respective approximate equilibrium becomes.

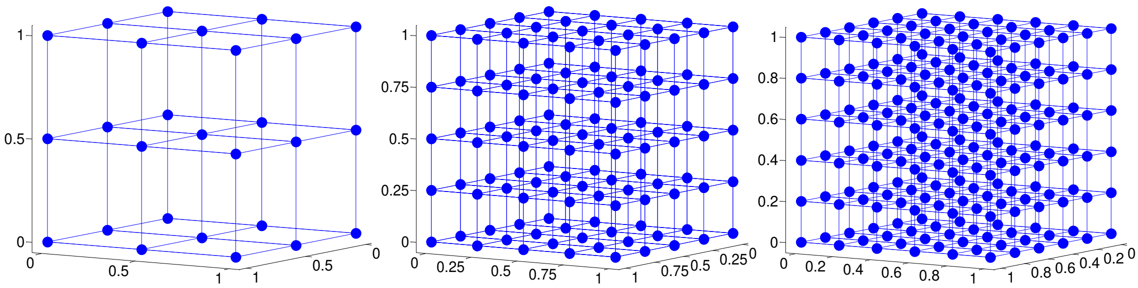

Figure 2. *The regular finite lattice of situations in the dyadic 3-person game by decreasing the sampling step (from the left to the right)*

In the game being considered, an initial concession can be set at between 0.01 and 0.11 with a step of 0.01 (the marginal endpoint 0.11 is included for closing the range). However, condition (*Eq. 17*) does not hold for $\beta\in\left[0.01;\,0.11\right]$ (see it by 2-fines along with concessions in *Fig. 3* and by 3-fines along with costs in *Fig. 4*). Subsequently, let the range of 2-fines be widened to $\left[0.34;\,1\right]$ and the range of concessions be widened to $\left[0.01;\,0.5\right]$. For these widened ranges, condition (*Eq. 17*) holds for $\beta\geqslant 0.21$ (*Fig. 5*), but sampling with a step of $1/16$ is canceled as it is practically unrealizable (although it is promising in bringing minimal and maximal fines, costs, and concessions closer).

The symmetric “cleanest” situation (see it in *Fig. 5* at $M=9$)

$$\left\{d_1^{\langle 0.5\rangle},\, d_2^{\langle 0.5\rangle},\, d_3^{\langle 0.5\rangle}\right\}=\left\{1/9,\, 1/9,\, 1/9\right\} \tag{Eq.18}$$

has pretty narrow gaps for 2-fines and costs (*Fig. 6*). The corresponding gap for 3-fines is the narrowest (*Fig. 7*). Nonetheless, situation (*Eq. 18*) considered as an approximate equilibrium is risky to be too unstable because of very great concessions (which are greater than maximal 2-fines). This is why situation (*Eq. 18*) is excluded from further consideration. On the other hand, this sampling has another two, less “clean”, situations

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DOI: http://dx.doi.org/10.15666/aeer/1702_14511474

$$\left\{d_1^{\langle\beta\rangle}, d_2^{\langle\beta\rangle}, d_3^{\langle\beta\rangle}\right\} = \{2/9, 2/9, 2/9\}$$

and

$$\left\{d_1^{\langle\beta\rangle}, d_2^{\langle\beta\rangle}, d_3^{\langle\beta\rangle}\right\} = \{1/3, 1/3, 1/3\} \quad \text{(Eq.19)}$$

which satisfy condition (*Eq. 17*). The concession for approximate equilibrium

$$\left\{d_1^{\langle 0.19\rangle}, d_2^{\langle 0.19\rangle}, d_3^{\langle 0.19\rangle}\right\} = \{1/3, 1/3, 1/3\} \quad \text{(Eq.20)}$$

is just 0.19, but the costs are higher than 0.9966 units.

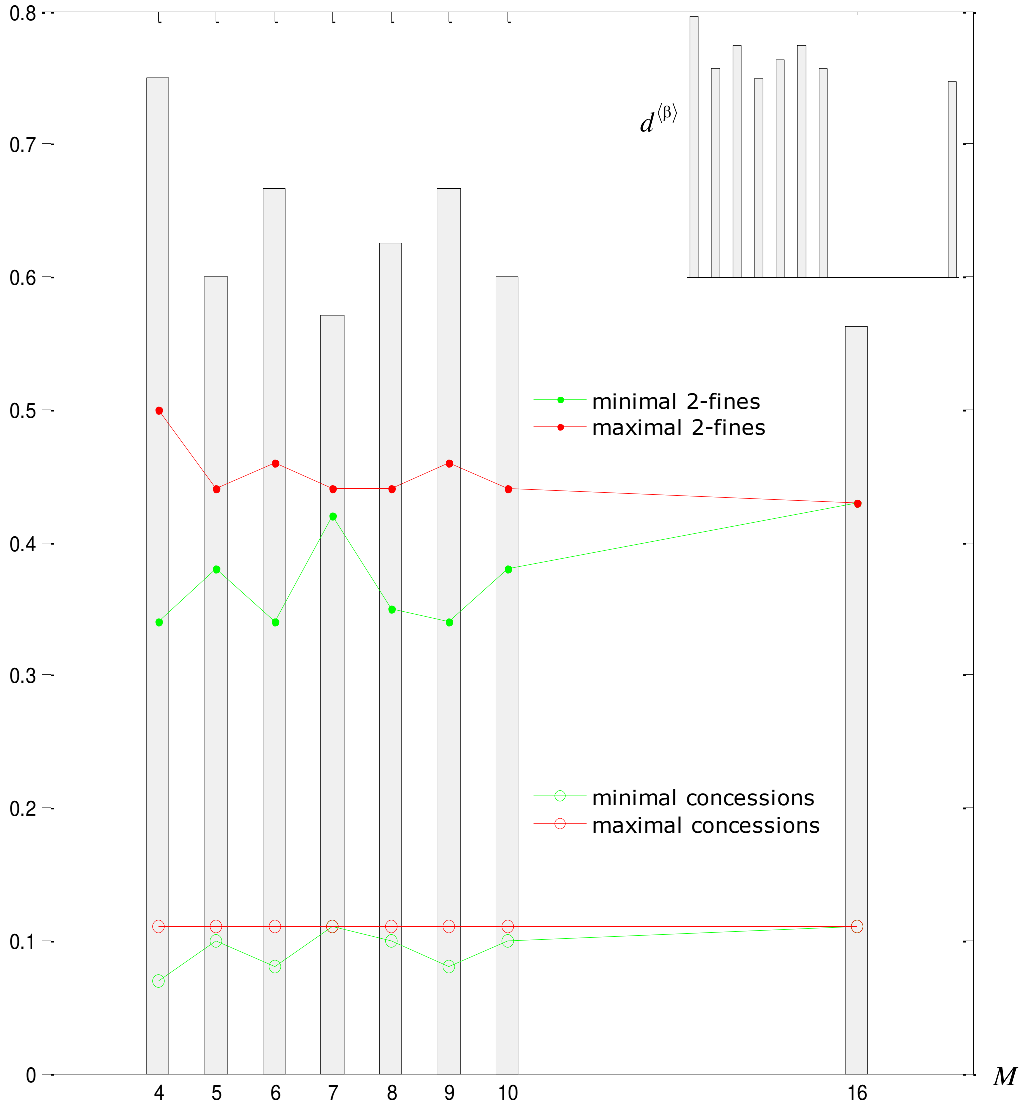

Figure 3. *The slowly decreasing minimal probability of discharging without treatment by the corresponding gap of 2-fines along with the corresponding gap of concessions for* $\beta \in [0.01; 0.11]$ *and* $a \in [0.34; 0.5]$

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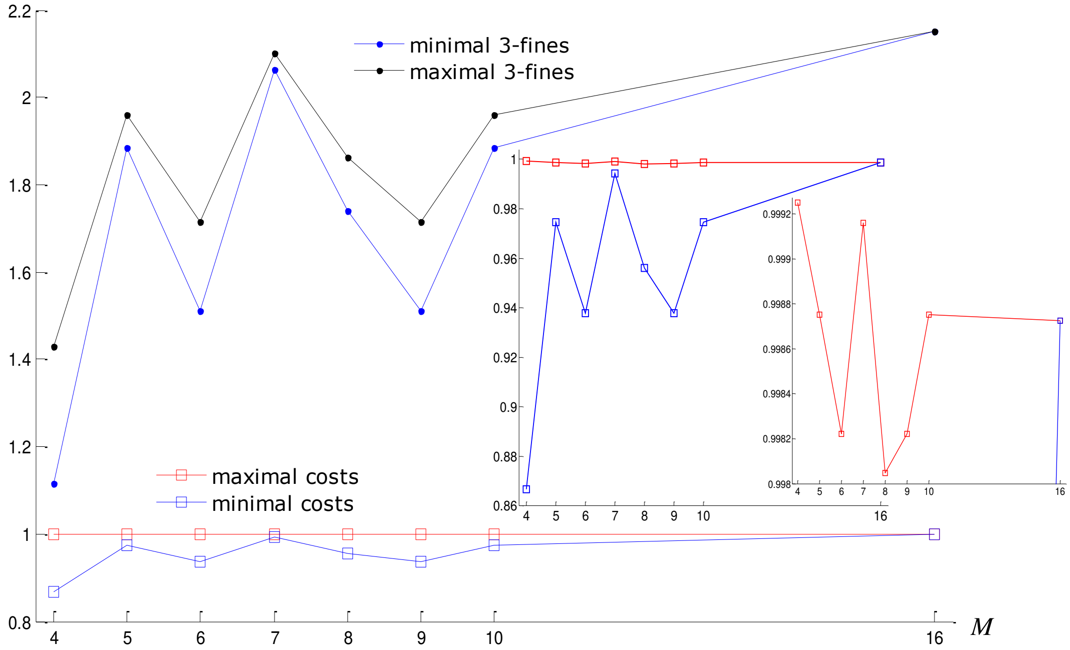

Figure 4. *The corresponding gap of increasing 3-fines along with the corresponding gap of costs (which, nonetheless, all are acceptable) for* $\beta \in [0.01; 0.11]$ *and* $a \in [0.34; 0.5]$

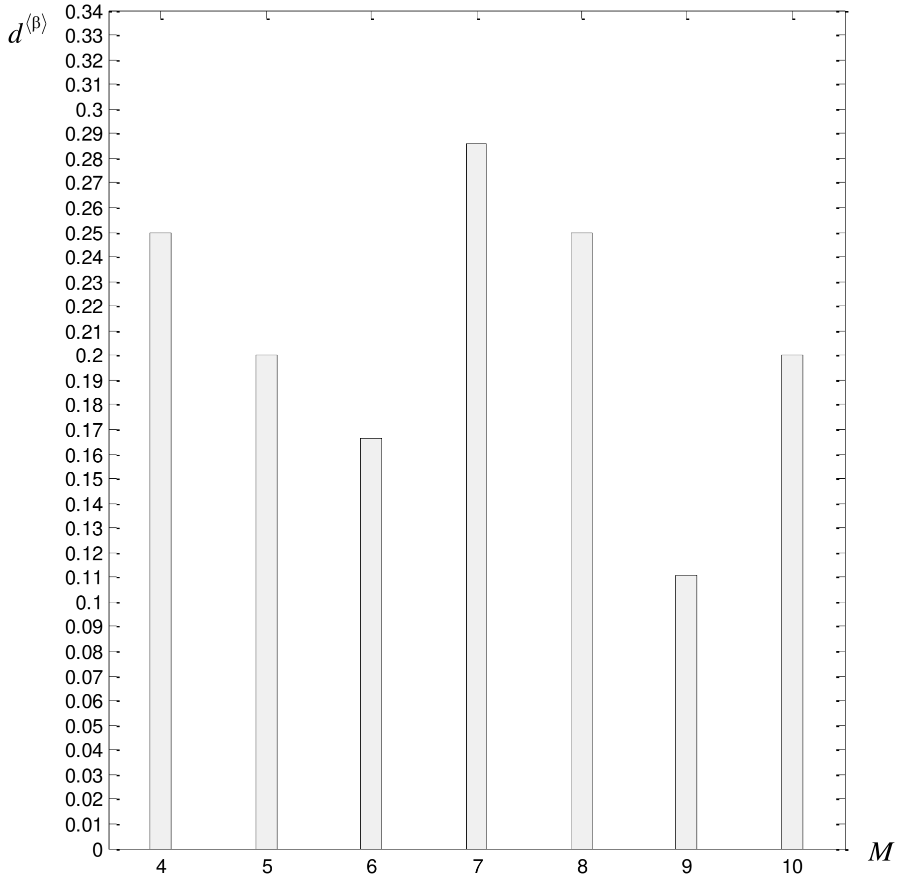

Figure 5. *The minimal probability of discharging without treatment for* $\beta \in [0.01; 0.5]$ *and* $a \in [0.34; 1]$*, at which the ecologically healthy water balance of the reservoir is maintained owing to recovering*

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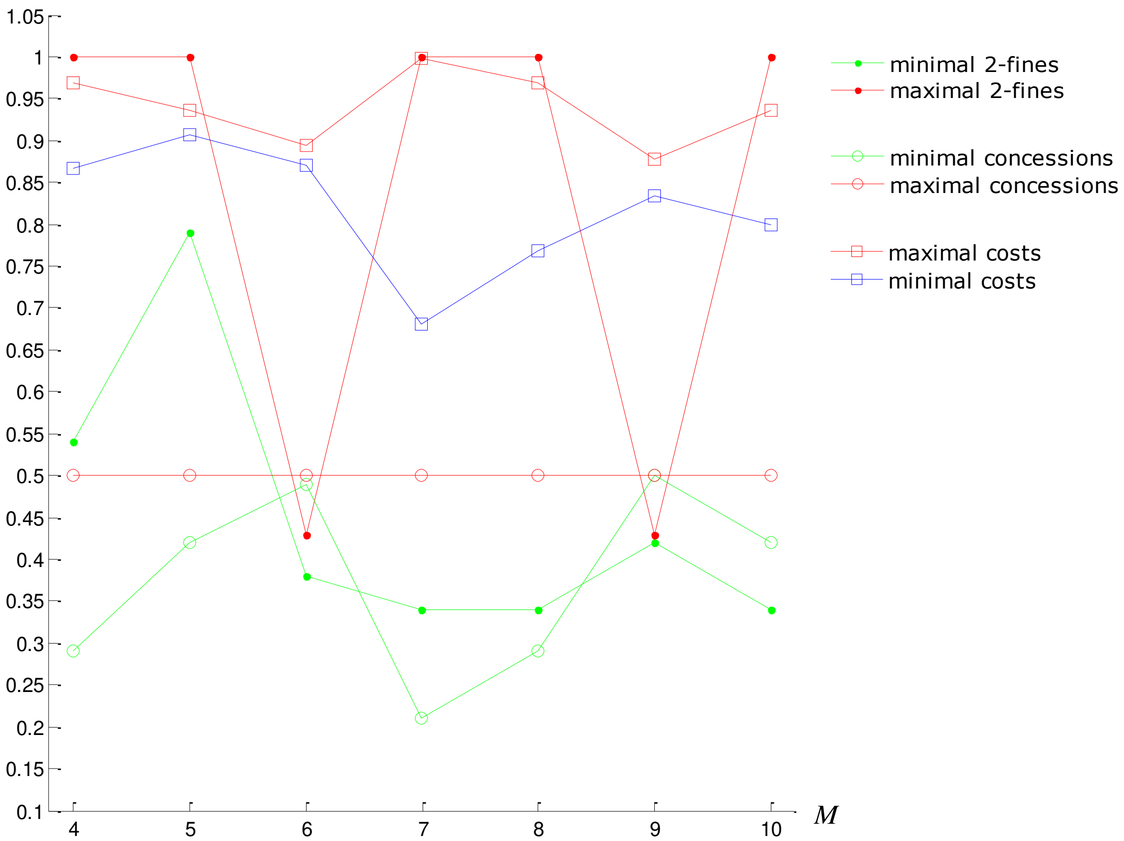

Figure 6. *The corresponding gaps of 2-fines, concessions, and costs for* $\beta \in [0.01; 0.5]$ *and* $a \in [0.34; 1]$*, at which the ecologically healthy water balance of the reservoir is maintained owing to recovering*

Sampling with a step of $1/4$ has wider gaps than those for $M = 5$. In its turn, sampling with a step of $1/5$ has wider gaps than those for $M = 6$. The widest gaps are at $M = 7$ but approximate equilibria

$$\left\{ d_1^{\langle\beta\rangle}, d_2^{\langle\beta\rangle}, d_3^{\langle\beta\rangle} \right\} = \{2/7, 2/7, 2/7\} \quad \text{(Eq.21)}$$

by such sampling are achieved by

$$0.21 \leqslant \beta \leqslant 0.5,$$
$$0.34 \leqslant a \leqslant 1,$$

and

$$0.62 \leqslant b \leqslant 4.85$$

which are very promising. In fact, situation (*Eq. 21*) is achievable at the smallest concession $\beta = 0.21$ (see the minimum in *Fig. 6*).

Despite "clean" situation (*Eq. 21*) implies the heaviest discharge without treatment, it is the most stable approximate equilibrium owing to the smallest concession. Thus, approximate equilibrium

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$$\left\{d_1^{\langle 0.21\rangle}, d_2^{\langle 0.21\rangle}, d_3^{\langle 0.21\rangle}\right\} = \{2/7, 2/7, 2/7\} \qquad \text{(Eq.22)}$$

is exactly achieved at $a = 0.97$ and $b = 4.85$. Eventually, however, situation (*Eq. 22*) forces every environmental pollution subject to pay about 0.9971 units. That makes this situation almost senseless under those fines. Besides, those fines are heavy and they seem discouraging.

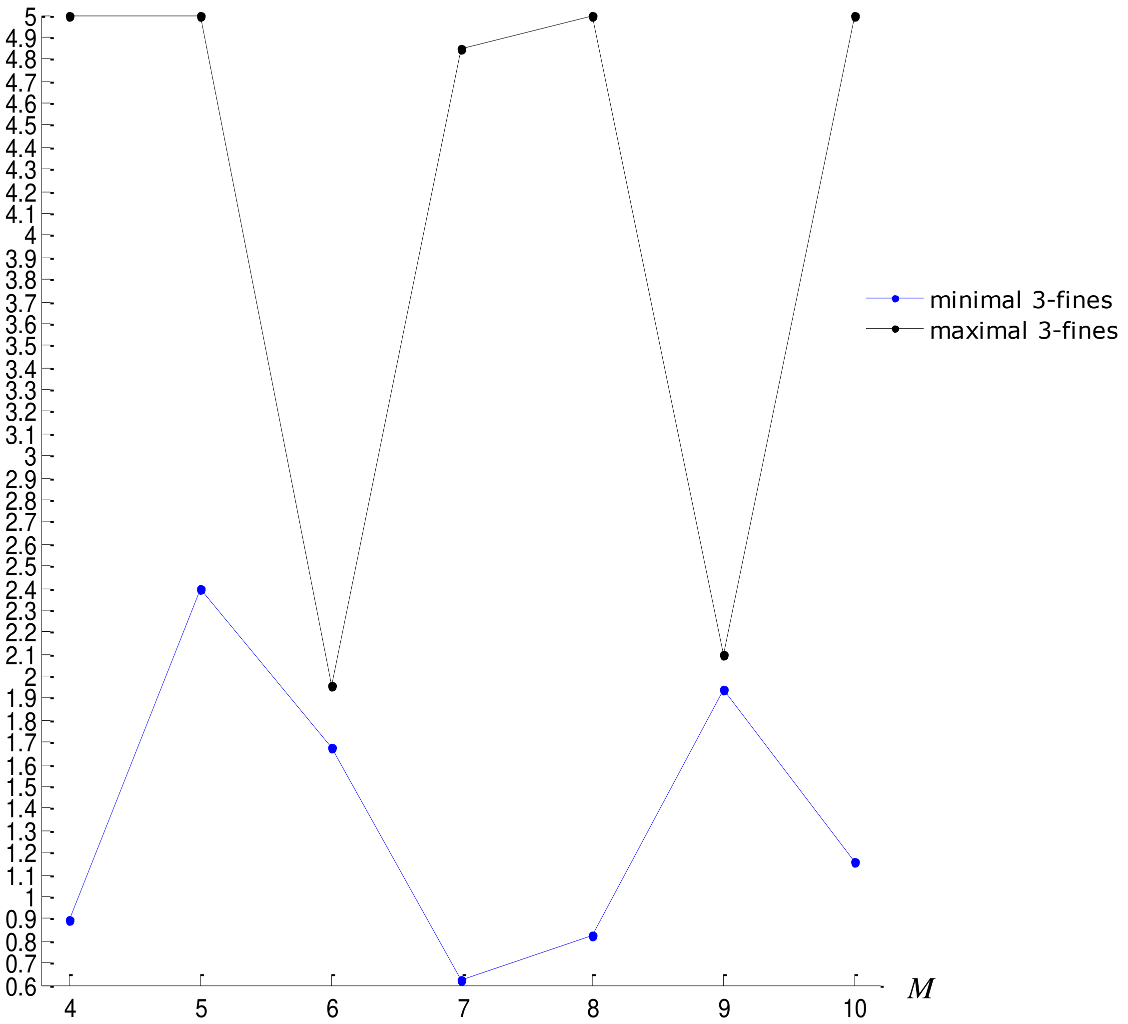

Figure 7. *The corresponding gaps of 3-fines for* $\beta \in [0.01; 0.5]$ *and* $a \in [0.34; 1]$*, at which the ecologically healthy water balance of the reservoir is maintained owing to recovering*

Approximate equilibrium

$$\left\{d_1^{\langle 0.29\rangle}, d_2^{\langle 0.29\rangle}, d_3^{\langle 0.29\rangle}\right\} = \{1/4, 1/4, 1/4\}$$

exactly achieved at $a = 0.99$ and $b = 4.95$ is rejected by similar reasoning: every subject will eventually pay 0.9666 units. Another relatively acceptable concession, possible at $M = 8$, does not seem better: approximate equilibrium

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$$\left\{d_1^{\langle 0.29\rangle}, d_2^{\langle 0.29\rangle}, d_3^{\langle 0.29\rangle}\right\} = \{1/8, 1/8, 1/8\}$$

is achieved at the same fines (plausibly, due to the doubled sampling) forcing the same cost. Meanwhile, a hardly acceptable concession, possible at $M = 6$, allows to achieve approximate equilibrium

$$\left\{d_1^{\langle 0.49\rangle}, d_2^{\langle 0.49\rangle}, d_3^{\langle 0.49\rangle}\right\} = \{1/6, 1/6, 1/6\} \qquad \text{(Eq.23)}$$

at $a = 0.4$ and $b = 1.92$, being quite acceptable fines, which costs only 0.89 units. Another dubious concession, possible at $M = 10$, will cost 0.8227 units if the subjects hold at approximate equilibrium

$$\left\{d_1^{\langle 0.42\rangle}, d_2^{\langle 0.42\rangle}, d_3^{\langle 0.42\rangle}\right\} = \{1/5, 1/5, 1/5\} \qquad \text{(Eq.24)}$$

by $a = 0.4$ and $b = 1.68$ (even more acceptable 3-fines for the subjects). Samplings at $M = 6$ and $M = 10$ have their second possible approximate equilibria: less "clean" situation (*Eq. 19*) at $M = 6$ and

$$\left\{d_1^{\langle \beta\rangle}, d_2^{\langle \beta\rangle}, d_3^{\langle \beta\rangle}\right\} = \{3/10, 3/10, 3/10\}$$

at $M = 10$. And again, the relatively small concession in approximate equilibrium (*Eq. 20*) stands against the same high cost as it has been in the case of $M = 9$. A relative stability of approximate equilibrium

$$\left\{d_1^{\langle 0.2\rangle}, d_2^{\langle 0.2\rangle}, d_3^{\langle 0.2\rangle}\right\} = \{3/10, 3/10, 3/10\}$$

at $M = 10$ is expensive: the costs are higher than 0.998 units.

So, it is apparent that struggling to minimize concessions within those gaps in *Figure 6* leads to increasing costs. Apart from democratizing fines, the next stage is to balance costs with concessions, starting off minimal concessions for each $M = \overline{4, 10}$. At that, concessions close to 0.5 (see *Fig. 6*) are unacceptable. For example, both approximate equilibria (*Eq. 23*) and (*Eq. 24*) are unstable, losing thus their practicability (in spite of their lower costs compared to more stable approximate equilibria).

Fines producing symmetric "clean" situations whose costs are the least

Henceforward, both costs and concessions are to be minimized. In order to do this, denote by $\gamma_i(M)$ the subject's cost at the i-th set of fines

$$\{a_i(M), b_i(M)\}$$

and the concession

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$$\beta_i(M) \text{ for } M=\overline{4,\,10} \text{ and } i=\overline{1,\,I_M}$$

for I_M sequences

$$\left\{\gamma_i(M),\beta_i(M),a_i(M),b_i(M)\right\}_{i=1}^{I_M}$$

at which $\gamma_i(M)<1$ and condition (*Eq. 17*) holds, i.e.

$$d_1^{\langle\beta_i(M)\rangle}=d_2^{\langle\beta_i(M)\rangle}=d_3^{\langle\beta_i(M)\rangle}=d^{\langle\beta_i(M)\rangle}\leqslant 1/3\,.$$

Having

$$\gamma_{\min}(M)=\min_{i=\overline{1,\,I_M}}\gamma_i(M)$$

and

$$\beta_{\min}(M)=\min_{i=\overline{1,\,I_M}}\beta_i(M),$$

the best couple of the cost and concession can be found by its index

$$i^*\in\arg\min_{i=\overline{1,\,I_M}}\sqrt{\left(\gamma_i(M)-\gamma_{\min}(M)\right)^2+\left(\beta_i(M)-\beta_{\min}(M)\right)^2} \qquad \text{(Eq.25)}$$

for the given M. With distance

$$\rho_i(M)=\sqrt{\left(\gamma_i(M)-\gamma_{\min}(M)\right)^2+\left(\beta_i(M)-\beta_{\min}(M)\right)^2} \qquad \text{(Eq.26)}$$

used in (*Eq. 25*), it is admissible to loose its minimum by 2%. Thus, a set of indices

$$L_*(M)=\left\{i\in\left\{\overline{1,\,I_M}\right\}:\rho_i(M)\leqslant 1.02\cdot\rho_{i^*}(M)\right\}\subset\left\{\overline{1,\,I_M}\right\}$$

will give the best couples

$$\left\{\gamma_{i^{**}}^*(M),\beta_{i^{**}}^*(M)\right\}_{i^{**}=1}^{|L_*(M)|}\subset\left\{\left\{\gamma_i(M),\beta_i(M)\right\}_{i=1}^{I_M}\right\}_{M=4}^{10} \qquad \text{(Eq.27)}$$

for the given M, where the superscript asterisk refers to re-indexation started from 1 again in the subscript. Couples

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$$\left\{\left\{\gamma_i(M), \beta_i(M)\right\}_{i=1}^{I_M}\right\}_{M=4}^{10}$$

are specifically shown in *Figures 8 — 14*, where thicker dots correspond to lower 2-fines, black dots correspond to that $b_i(M) < 2$, light dots correspond to that $b_i(M) \geqslant 3.5$, and dots are moderately darkened if

$$2 \leqslant b_i(M) < 3.5 .$$

Lines in those graphs link best couples (*Eq. 27*) to couple (drawn as a circled dot)

$$\left\{\gamma_{\min}(M), \beta_{\min}(M)\right\} \quad \text{(Eq.28)}$$

for every $M = \overline{4, 10}$. The thickest line in every graph links couple (*Eq. 28*) to couple

$$\left\{\gamma_{i^*}(M), \beta_{i^*}(M)\right\} \in \left\{\gamma^*_{i^{**}}(M), \beta^*_{i^{**}}(M)\right\}_{i^{**}=1}^{|L_*(M)|} \quad \text{(Eq.29)}$$

for the given M , which is the shortest in the graph.

Further below is a table (*Table 1*) with a list of subsets

$$\left\{\left\{\gamma^*_{i^{**}}(M), \beta^*_{i^{**}}(M), a^*_{i^{**}}(M), b^*_{i^{**}}(M)\right\}_{i^{**}=1}^{|L_*(M)|} \subset \right.$$
$$\left. \subset \left\{\gamma_i(M), \beta_i(M), a_i(M), b_i(M)\right\}_{i=1}^{I_M}\right\}_{M=4}^{10} \quad \text{(Eq.30)}$$

along with distances

$$\left\{\left\{\rho^*_{i^{**}}(M)\right\}_{i^{**}=1}^{|L_*(M)|}\right\}_{M=4}^{10} \quad \text{(Eq.31)}$$

by (*Eq. 26*), where the superscript asterisk refers to re-indexation started from 1 again in the subscript. Note that any two distances for different samplings are incomparable due couple (*Eq. 28*) changes as M increases (that is, sampling becomes more compact). Therefore, a generalized distance is introduced to a couple of the minimal cost

$$\gamma^*_{\min} = \min_{M=\overline{4,10}} \left(\min_{i=\overline{1,I_M}} \gamma_i(M) \right) \approx 0.6042$$

and the minimal concession

$$\beta^*_{\min} = \min_{M=\overline{4,10}} \left(\min_{i=\overline{1,I_M}} \beta_i(M) \right) = 0.19 .$$

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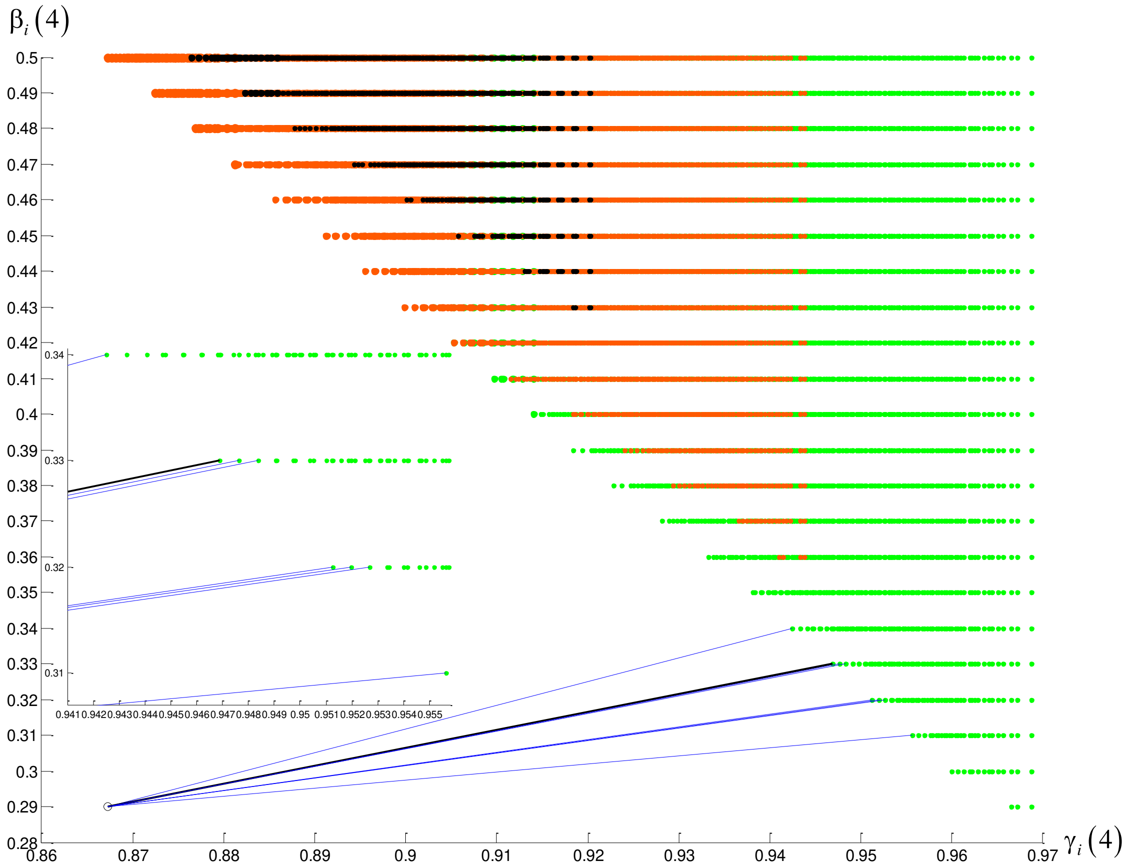

Figure 8. *The loosest sampling has 8 versions of best couples (Eq. 27) having concessions up to 0.34*

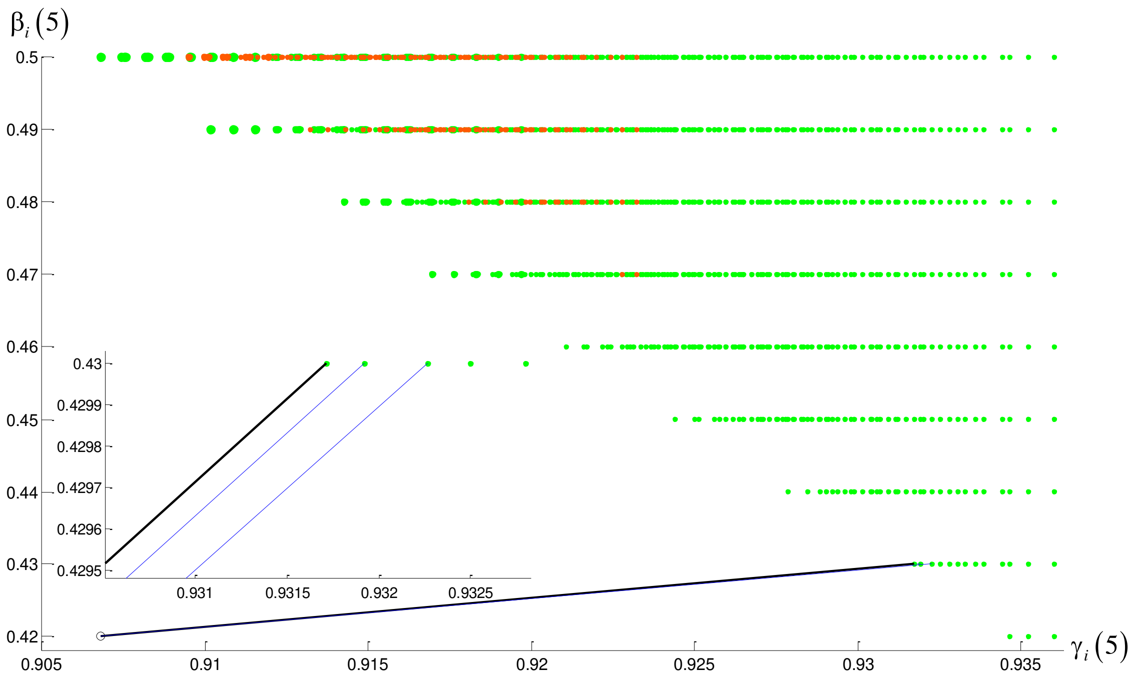

Figure 9. *Another 3 versions of best couples (Eq. 27) but the concession here is too great (and so are 3-fines throughout this sampling)*

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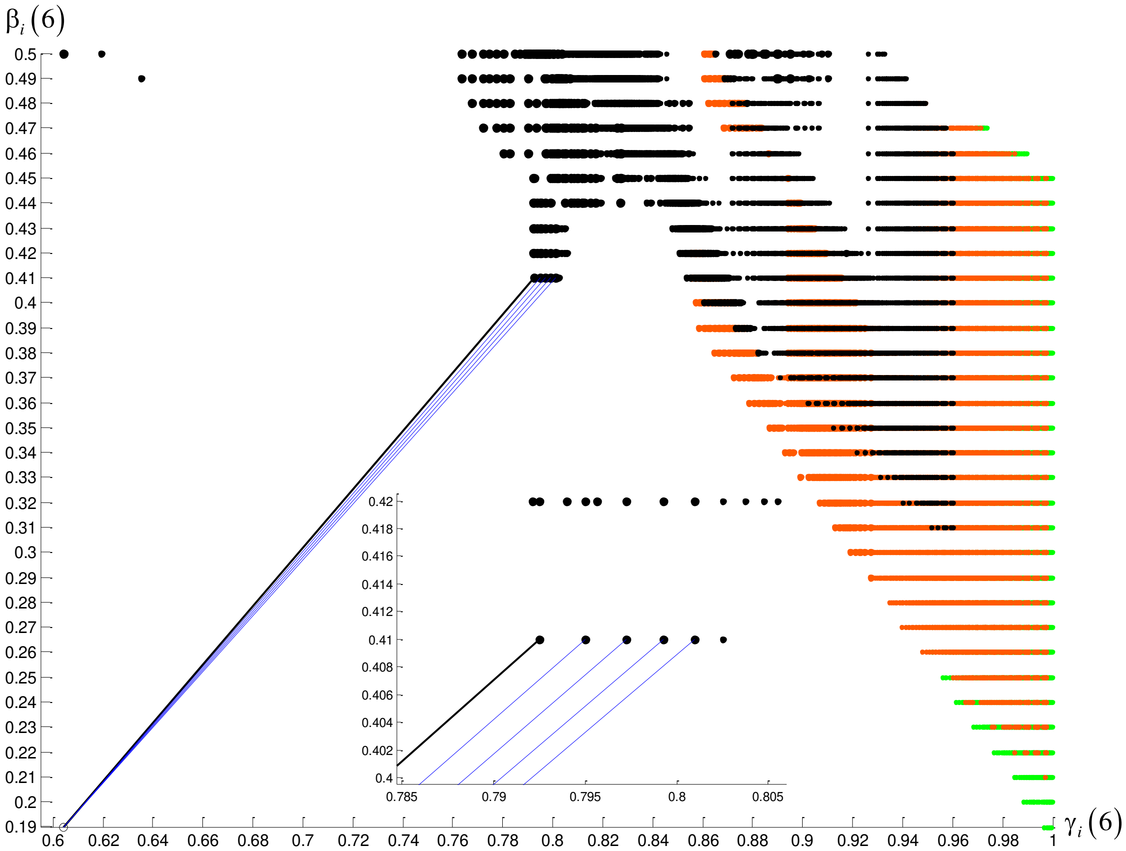

***Figure 10.** Great concessions but acceptable costs in these 5 versions of best couples (Eq. 27); the corresponding fines are light*

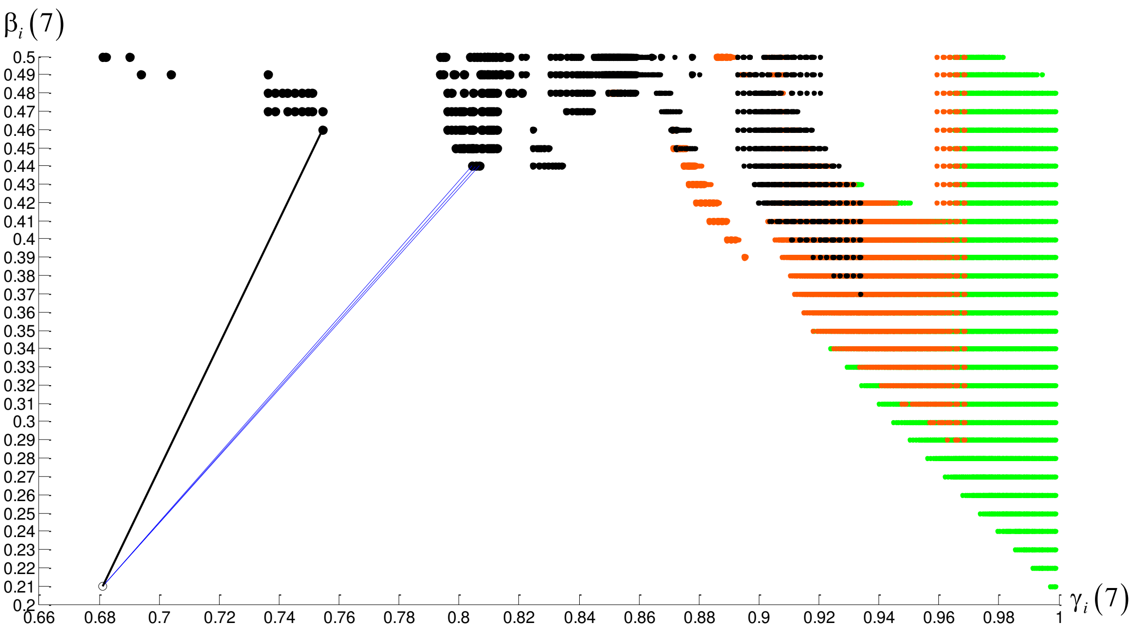

***Figure 11.** Great concessions but acceptable costs, especially for couple (Eq. 29), in these 4 versions of best couples (Eq. 27); the corresponding fines are light*

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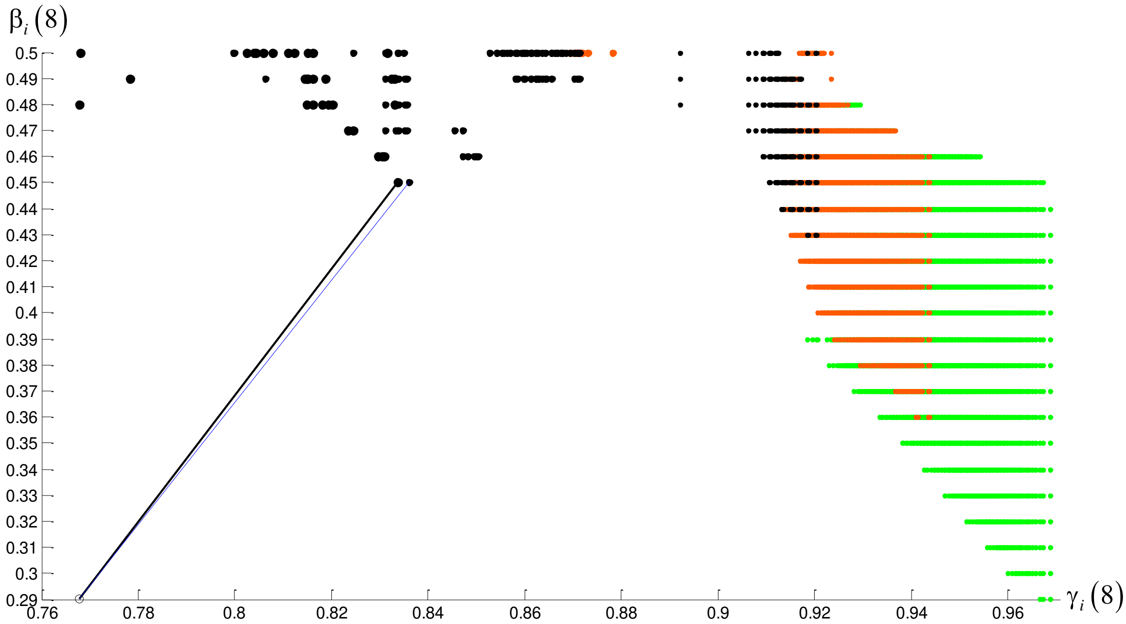

***Figure 12.** After the doubled initial sampling, here are only 2 versions of best couples (Eq. 27) including couple (Eq. 29) by unacceptable concessions; now the corresponding fines are a little bit heavier than in Figures 10 and 11*

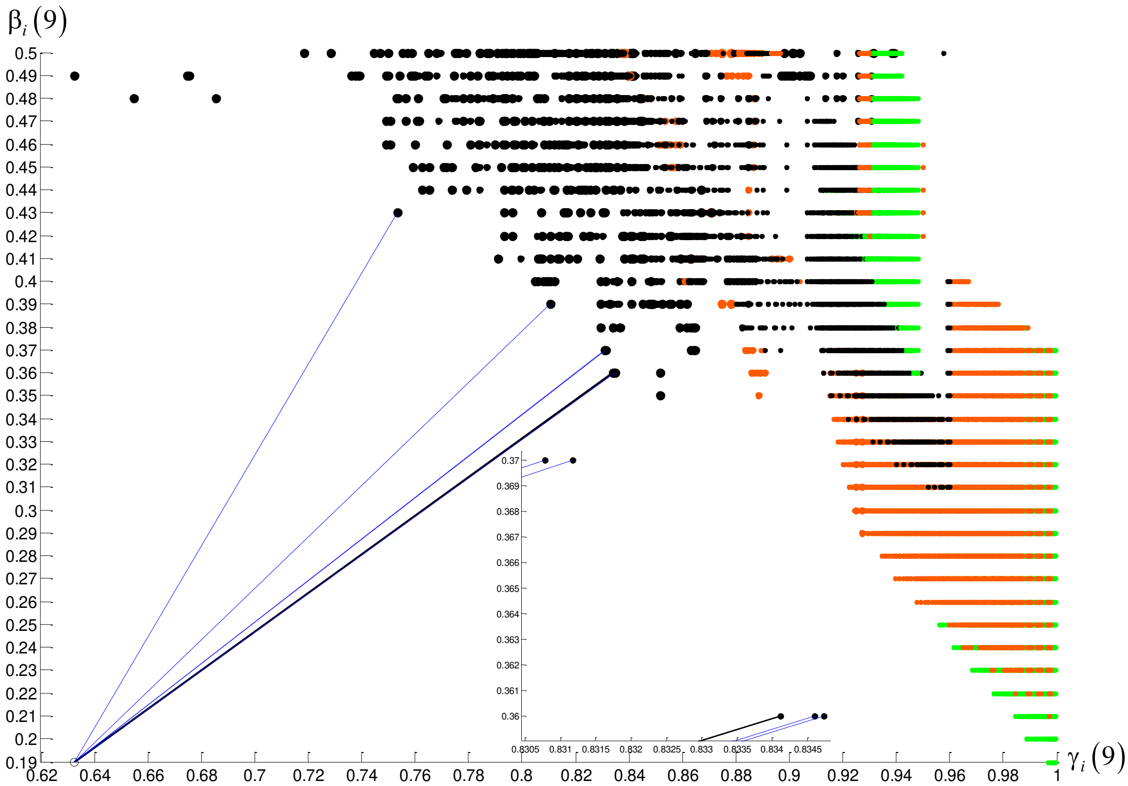

***Figure 13.** Acceptable costs and concessions among these 7 versions of best couples (Eq. 27), where just 2 versions (the 2 lines above) are excluded; the corresponding fines are light (there is a version wherein the 2-fine is 0.34 and the 3-fine is 0.986, i.e. even lesser than the cost of application of a water treatment system)*

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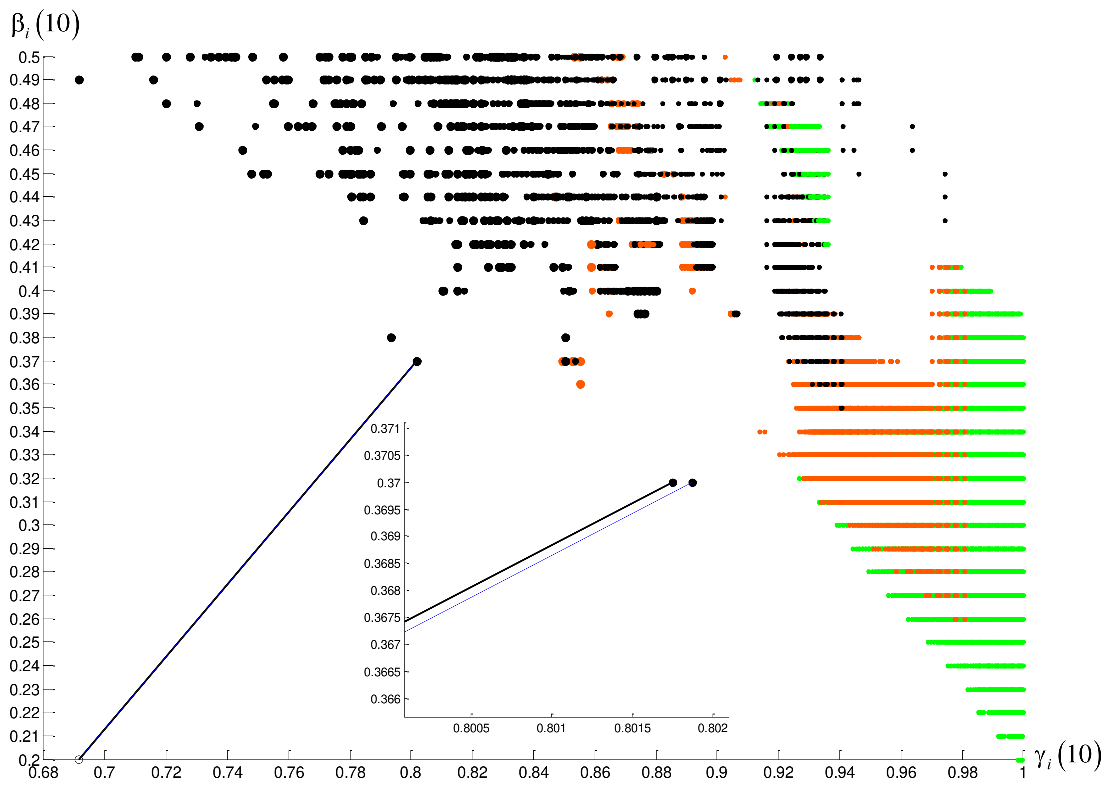

Figure 14. *After the tightest sampling, here are only 2 versions of best couples (Eq. 27) including couple (Eq. 29) by both acceptable costs and concessions; the corresponding fines are close to the relatively lightest*

The best couple of the cost and concession can be found by its index (a subset number in *Table 1*)

$$i^{***} \in \arg \min_{\left\{ i^{**} = \overline{1, \left| L_{*}(M) \right|} \right\}_{M=4}^{10}} \sqrt{\left(\gamma_{i^{**}}^{*}(M) - \gamma_{\min}^{*} \right)^2 + \left(\beta_{i^{**}}^{*}(M) - \beta_{\min}^{*} \right)^2}$$

independently of M, where distance

$$\rho_{i^{**}}^{\langle \min \rangle}(M) = \sqrt{\left(\gamma_{i^{**}}^{*}(M) - \gamma_{\min}^{*} \right)^2 + \left(\beta_{i^{**}}^{*}(M) - \beta_{\min}^{*} \right)^2} \tag{Eq.32}$$

similarly to (*Eq. 26*), for $i^{**} = \overline{1, \left| L_{*}(M) \right|}$ and $M = \overline{4, 10}$.

Distances (*Eq. 32*) are shown against the subset number (rows in *Table 1*) in *Figure 15*. It is roughly seen that as sampling becomes more compact (being tightened) both cost and concession tend to decrease. The best couple of the cost and concession has index $i^{**} = 1$ for $M = 10$ (row #30 in *Table 1*) whereas

$$\rho_{1}^{\langle \min \rangle}(10) \approx 0.2373 \tag{Eq.33}$$

but

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$$\rho_2^{\langle \min \rangle}(10) \approx 0.2374$$

for row #31 in *Table 1*. Therefore, the corresponding fines are $a_1^*(10) = 0.34$ and $b_1^*(10) = 1.394$, which allow every subject paying 0.8018 units by conceding 0.37 units to make situation

$$\left\{ d_1^{\langle 0.37 \rangle}, d_2^{\langle 0.37 \rangle}, d_3^{\langle 0.37 \rangle} \right\} = \{3/10, 3/10, 3/10\} \quad \text{(Eq.34)}$$

at $M = 10$ the best approximate equilibrium.

Table 1. *The list of subsets (Eq. 30) along with distances (Eq. 31) by (Eq. 26), where rows with best distances for its sampling are highlighted bold (all 31 rows correspond to the linking lines in Figs. 8–14)*

Subset #	M	Probability of discharging without treatment	Cost	Concession	2-fine	3-fine	Distance by (Eq. 26)
1	4	1/4	0.9425	0.3400	0.8800	4.4000	0.0903
2	**4**	**1/4**	**0.9469**	**0.3300**	**0.9000**	**4.5000**	**0.0891**
3	4	1/4	0.9476	0.3300	0.9100	4.4590	0.0898
4	4	1/4	0.9513	0.3200	0.9200	4.6000	0.0892
5	4	1/4	0.9484	0.3300	0.9200	4.4160	0.0904
6	4	1/4	0.9520	0.3200	0.9300	4.5570	0.0899
7	4	1/4	0.9556	0.3100	0.9400	4.7000	0.0906
8	4	1/4	0.9527	0.3200	0.9400	4.5120	0.0905
9	5	1/5	0.9319	0.4300	0.9700	4.8500	0.0270
10	**5**	**1/5**	**0.9317**	**0.4300**	**0.9800**	**4.7040**	**0.0268**
11	5	1/5	0.9323	0.4300	0.9900	4.6530	0.0273
12	**6**	**1/3**	**0.7925**	**0.4100**	**0.3900**	**1.1700**	**0.2896**
13	6	1/3	0.7950	0.4100	0.4000	1.1600	0.2912
14	6	1/3	0.7973	0.4100	0.4100	1.1480	0.2927
15	6	1/3	0.7993	0.4100	0.4200	1.1340	0.2940
16	6	1/3	0.8010	0.4100	0.4300	1.1180	0.2952
17	7	2/7	0.8043	0.4400	0.3400	1.5980	0.2609
18	7	2/7	0.8056	0.4400	0.3500	1.5750	0.2615
19	7	2/7	0.8066	0.4400	0.3600	1.5480	0.2620
20	**7**	2/7	**0.7544**	**0.4600**	**0.3600**	**0.9360**	**0.2605**
21	**8**	**1/4**	**0.8336**	**0.4500**	**0.4300**	**1.8060**	**0.1730**
22	8	1/4	0.8361	0.4500	0.4400	1.8040	0.1740
23	9	1/3	0.8108	0.3900	0.3400	1.6320	0.2679
24	9	1/3	0.7534	0.4300	0.3400	0.9860	0.2687
25	**9**	1/3	**0.8341**	**0.3600**	**0.3800**	**1.7480**	**0.2637**
26	9	1/3	0.8308	0.3700	0.3800	1.7100	0.2677
27	9	1/3	0.8346	0.3600	0.3900	1.7160	0.2640
28	9	1/3	0.8312	0.3700	0.3900	1.6770	0.2680
29	9	1.3	0.8347	0.3600	0.4000	1.6800	0.2641
30	**10**	**3/10**	**0.8018**	**0.3700**	**0.3400**	**1.3940**	**0.2026**
31	10	3/10	0.8019	0.3700	0.3500	1.3650	0.2026

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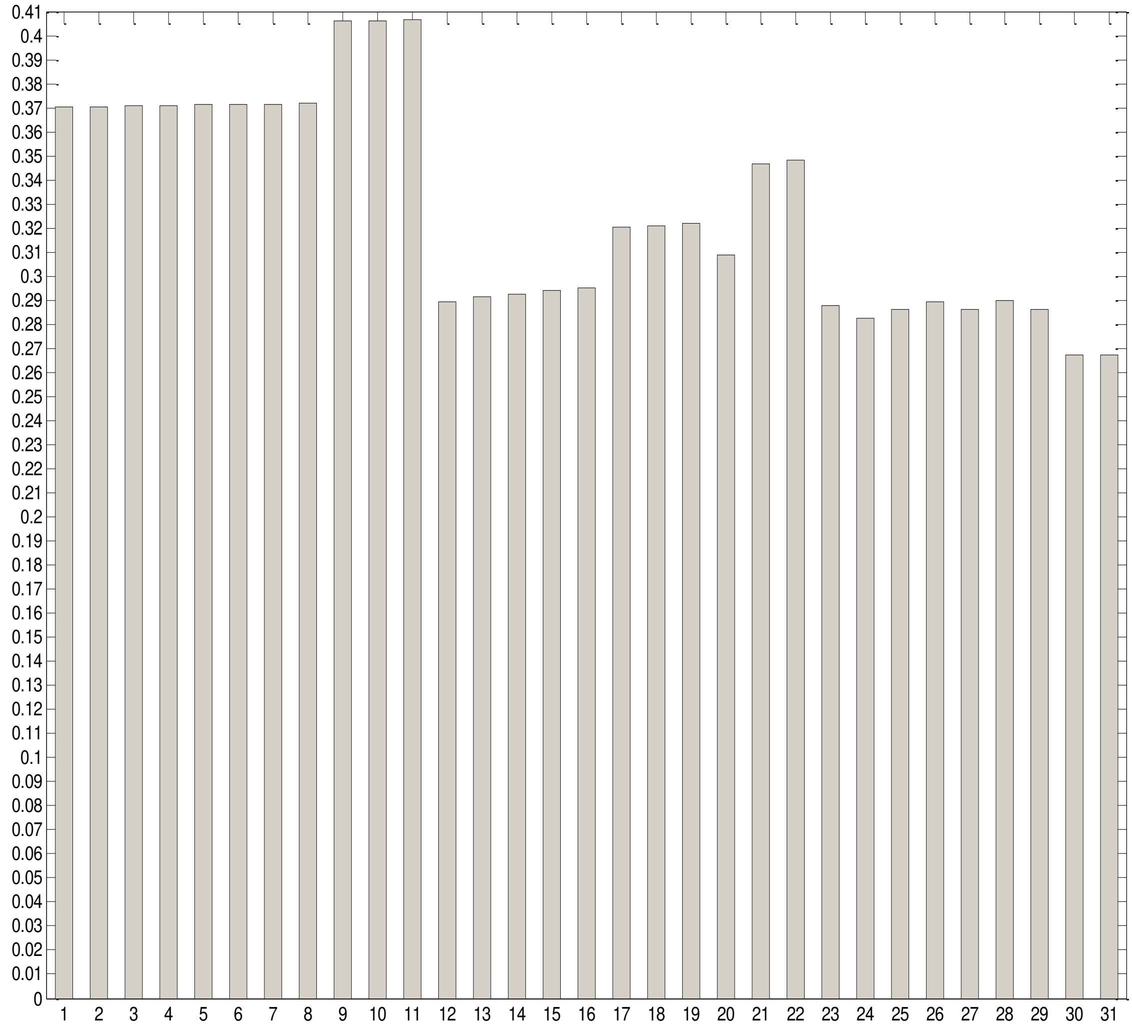

Figure 15. *Distances (Eq. 32) against the subset number (row) in Table 1*

Row #31 corresponds to approximate equilibrium (*Eq. 33*) also. The only difference is a slightly greater cost (0.8019 units). This is caused by a slight increment of the 2-fine (by 0.01 units). Amazingly enough, the 3-fine here is decreased more (by 0.029 units, which is 2.1%).

Discussion

First of all, it must be mentioned that subjects are not tied to any sampling, or to concessions. Moreover, the subjects may do not "know" the probability 0.3, not speaking about situation (*Eq. 34*). Hence, the sampling at $M = 10$, which has appeared to be the best according with distance (*Eq. 33*), should be imposed on the subjects. This implies that some conditions are to be established by which switching from "clean" to "polluting" manufacturing and backwards will be controlled exactly once per those 10 periods of time (10 days, weeks, or months), where the fastest switch is possible only after one period. Such controlling will allow seeing how many times each of the subjects switches per every 10 times. Once a recommendation of holding to the probability 0.3 is given (by which the water treatment system is turned off for 3 periods

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of 10), the environmental pollution subject is able to control it itself. Prior to that, the 2-fine is set at 0.34 units. This is the lowest rate of fining when only two subjects do simultaneously not treat wastewater. The 3-fine is set at 1.394 units, which is 4.1 times greater than the 2-fine, but it is not as heavy as it could have been. Nevertheless, this is enough preventive from bad pollution. Under threat of the heavy 3-fine, the subjects will definitely come to a convention of scheduling the water treatment systems. After every 10 periods of time, the grand total of 2-fines is 3.4 units per subject, and the grand total of 3-fines is 13.94 units per subject. Eventually, the cost of 8.018 units for a subject is just the expected spendings, whereas the least cost in 7 units is still possible. For instance, if a permanent schedule of water treatment is approved and followed (*Table 2*), then it costs the least for all.

Table 2. *An example of scheduled water treatment (1 – water treatment system is applied, 0 – water treatment system is turned off, * – obligatory control and fining, if there are violations)*

Period #	1*	2	3	4	5	6	7	8	9	10	1*	2	3	...
Subject #1	1	1	1	1	1	1	0	0	0	1	1	1	1	...
Subject #2	1	1	1	0	0	0	1	1	1	1	1	1	1	...
Subject #3	0	0	0	1	1	1	1	1	1	1	0	0	0	...

The suggested model along with approximate equilibrium (*Eq. 34*) can be useful especially for triples of industrial enterprises which are not situated in one country but use the same river water (such as the Danube, Tisza, Rhine, etc.). It is easy to implement this model across countries of the European Union using the same currency (the euro is the official currency in 19 of 28 member states of the European Union, as well as in some of the territories of the Union) and thus sharing the analogous background (Guo, 2018). Countries with different currencies are neighbors anyway, so discrepancies in exchange rate are not dramatic, and standardization to the conventional units is realizable (Wolf, 2009). Although a source of pollution could be determined easier for this case, it will always take additional costs from governments. Even if measurements of a water pollution rate along with some complex investigations could reveal which subject is "clean" and which subjects work "polluted", the process of eventual compensation might be badly lingered after suing (e.g. see the undecided tri-state water dispute described by Jordan and Wolf, 2006).

From the other side, a single enterprise may have its capacities allocated in various sites of the same reservoir. In such a case, those capacities can be grouped into three clusters, similar to each other, and the suggested model becomes applicable.

Conclusion

Generally, the environmental protection model in the form of the considered dyadic 3-person game is a basis for two balances. The first novelty is the ecologically healthy water balance maintained via a policy of approximate equilibrium (*Eq. 34*). The second novelty, but it is not less important, is that rationally substantiated fines allow environmental pollution subjects to function stably. Although concessions in (*Eq. 34*) may be thought as a destabilizing factor, they eventually mean that a seductive

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possibility to pay 3.7 units less (after the next 10 periods) will disappear as quickly as other subjects start violating. Therefore, the concessions are a deterrent factor rather than a seduction to decrease costs. Conceding is just a primary advantage, which will be destroyed once a control is executed. Thus, approximate equilibrium (*Eq. 34*) becomes really stable. This is realized by the balanced (economically more reasonable) fines, which also merge stable functioning of environmental pollution subjects with the ecologically healthy water balance.

In terms of legal theory, the suggested model is an environmental law based on rigorous inferences from stability, rationality, and non-biased profitability. It may serve as a substantiation as for small environmental conventions (within one country), as well as for international conventions (e.g. like the Kyoto Protocol and the mentioned Water Convention). This is quite tractable owing to that the model-based decisions are not only prescriptive and constraining but also are motivating to environmental protection.

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ROLE OF MULTIVARIATE APPROACHES IN FLORISTIC DIVERSITY OF MANOOR VALLEY (HIMALAYAN REGION), PAKISTAN

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(Received 9th Oct 2018; accepted 5th Dec 2018)

Abstract. The main source of botanical information of a particular area is its floristic checklists. Floristic study of any given area helps to evaluate the plant wealth and its potential values. To assess floristic diversity of an unexplored remote valley (Manoor Valley), frequent field visits were arranged in different growing seasons during 2015 to 2018. The life form classes and leaf spectra of all plant species were determined and further classified according to the Raunkiaer classification. Plant species were identified and deposited to the Herbarium at Hazara University, Mansehra. The floristic diversity consisted of 354 plant species belonging to 93 families. Herbaceous was the most representative growth form, with 259 species, followed by shrubs with 52 species, trees with 42 species and parasitic plant with one species. Asteraceae was the leading family with 36 species, followed by Lamiaceae species. Furthermore, results indicated the dominancy of Therophytic plants and Nanophyllous and Microphyllous leaves. July marked the peak of flowering period and September the peak of fruiting period. This study provided the first insight of the floristic inventory in relation to multivariate approaches in this unexplored area. This regional novel launched list may serve as a vital resource for all future endeavors in the field of phytosociological, pharmacological and conservational studies of natural resources.
Keywords: *floristic checklist, Asteraceae, principal components analysis, species response curve*

Introduction

Vegetation is an umbrella term that indicates plant life of a region (Rahman et al., 2018a) or, in other words, a group of plants growing together in a particular area and may be characterized by its component species (Malik, 1990). Flora is a priceless donation of nature upon which the mankind always relies (Khan et al., 2013). Flora comprises the total plant species of any specific geographic region, which are characteristic of a geological period or in habit a particular ecosystem (Durrani et al., 2005). According to the report of Walter and Hamiston (1993), approximately 422,000 flowering plants have been reported globally (Rahman et al., 2016a). Pakistan is blessed with diverse flora due to variability in climate (Rahman et al., 2018b) and presents about 6000 flowering plant species (Rahman et al., 2016a).

The main source of botanical information of a particular area is its floristic checklists (Safidkon et al., 2003). Floristic study of any given area helps to evaluate the plant wealth and its potential values (Shaheen et al., 2016). Local plant species documentation is very necessary to introduce specific floral species of the local area, their occurrence and finding new species (Ali, 2008). Many workers have contributed comprehensive floristic checklists of local flora in different regions (Qureshi, and Bhatti, 2008; Jabeen et al., 2009; Shaheen et al., 2011).

In these studies of floristic checklist, besides evaluating the species richness, it is also necessary to observe the plant life form, leaf size and phenology over the year (Rahman et al., 2018a). Life form is the indicator of micro and macroclimate and it is characterized by plant adaptation to certain ecological conditions (Shimwell, 1971). As the plants arranged by the Raunkiaer (1934) in order to form classes on the basis of their life form, five major classes were formed (Hussain and Perveen, 2009) which includes: Phanerophytes, Hemicryptophytes, Cryptophytes, Chamaephytes, and Therophytes. Plants are also classified on the basis of leaf sizes and this has been exceptionally helpful for association mapping of vegetation. The leaf size knowledge helps in understanding physiological processes of plants (Oosting, 1956). For instance, biotic agencies are the chief causes for changing the biological spectrum in a given floristic zone (Amjad, 2012).

Phenology demonstrates the relationship of plant development to seasonal variations as well as photoperiod to program their developmental stages and natural exercises appropriated with the normal seasonal conditions (Manske, 2006). Essentially, timings and interim of the intermittent natural occasions (biological events) among stages of plant species give a foundation of gathering and synthesizing quantitative data of plant communities, which are directly linked with phenology (Singh and Singh, 1992). There is a synchronization of phenological behavior of the plant species and the various elements of the environmental conditions that plants are discussed, the organic tickers (biological clocks). These are habitually controlled by external environmental stimuli (Zhang et al., 2006, Vilela et al., 2017). Leaf growth, leaf fall, flowering and fruiting of species occur in specific seasons of the year and the phenology of life forms varies and is associated with day length/ temperature (Rahman et al., 2018a). As reported by Ahmed (2017), the blossoming and fruiting could be connected with the climatic conditions for posterity survival. Cornejo-Tenoria and Ibarra-Manriquez (2007) recorded blossoming and fruiting behavior on month-to-month premise. Bhat and Muralli (2001) depicted the climatic factors, for example, precipitation, water accessibility, change in day length and temperature additionally triggers the phonological occasions and because of temperature contrast, there is huge variety among species in various climatic zones.

In this way, the unexplored remote valley Manoor Valley, Pakistan, has great potential for flourishing a rich plant biodiversity due to the presence of diverse microhabitats and topographic features. Since biodiversity is greatly affected by different environmental factors (Khan, 2012) and is facing serious challenges due to

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DOI: http://dx.doi.org/10.15666/aeer/1702_14751498

anthropogenic activities like deforestation and over grazing (Ijaz, 2014), it is fundamental to develop floristic checklist studies. Hence, the current study was designed to explore the biodiversity and document the floristic checklist of Manoor Valley. This study provides the updated insights into the floristic diversity of the area and it might also be very helpful for the future plant ecological, conservational and ecophysiological studies.

Materials and methods

Study area (Manoor Valley)

There are two sub-valleys in Kaghan Valley, i.e. Naran Valley and Manoor Valley. Naran Valley is located in upper Kaghan Valley, while Manoor Valley is reached from the main Kaghan Valley road at the junction 'Mahandri' (*Fig. 1*) and is about 50 Km north of Balakot (Rahman et al., 2016a, b). Floristically, the valley is very rich, diverse and unexplored. To date, very few references are available on floral studies of the area including few reports of medicinal studies (Rahman et al., 2016a, b, c; 2018c) and preliminary checklist (Rahman et al., 2018a).

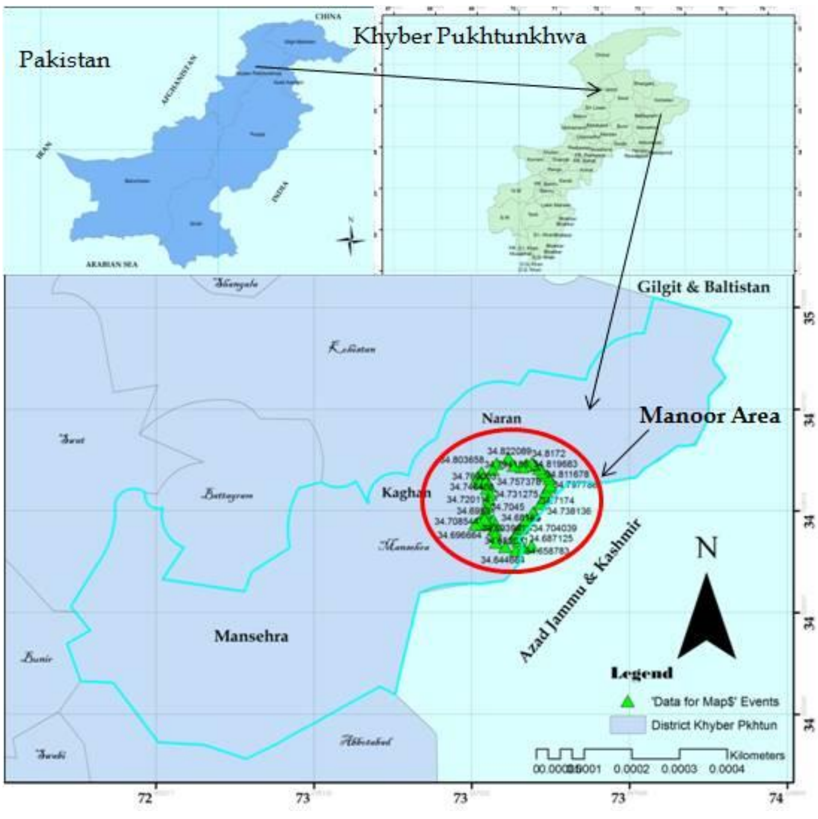

Figure 1. *Map of Mansehra area showing the Manoor Valley, Pakistan*

Field work, equipments, identification and preservation

From 2015 to 2018, field surveys were conducted to cover the understory area for collection of data regarding plant species. For this purpose, the study area was divided into 133 sampling sites or stations, where they were classified into life form and leaf

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sizes classes following Raunkiær (1934); Oosting (1956); Hussain (1989). Field notebook, pen, pencil, polythene bags, tags, trowel, scissors, camera, newspaper, plants presser and twig cutter were used during survey (Ijaz, 2014). The collected plants were properly dried and pressed by using newspaper for about 2-4 weeks at normal temperature. Then plants were treated or poisoned with chemical solution for preservation and mounted on standard herbarium sheets. Then data were shifted from field notebook on herbarium label of standard herbarium sheets. This herbarium label was pasted on right side of herbarium sheets (Ijaz, 2014; Ahmed, 2017). The size of herbarium sheet was standardized (11.5 x 16.5) (Ijaz, 2014). The specimens were identified by using the Flora of Pakistan (Nasir and Ali, 1971-1989; Ali and Nasir, 1989-1991; Ali and Qaiser, 1995-2017) and the identified specimens were deposited in the Herbarium of Hazara University, Mansehra, Pakistan (HUP).

Multivariate approaches

Recorded plant species data was analyzed through various multivariate approaches by using different statistical packages. CANOCO 5 version was used (Rokaya et al., 2012) for multivariate ordination analyses like 'dominance curve' (DC), 'principal components analysis' (PCA) (Rokaya et al., 2012, 1992) and species response curve (SRC). PCA was determined to examine the correlation between 354 plant species and 4 growth form categories. PC-ORD 5 was used for correlation and regression coefficient (Rahman et al., 2018c). The correlation and regression coefficient evaluates the variables behavior on different axes. Chord Diagram was made through R software using package 'circlize' (Gu et al., 2014).

Results

Floristic diversity

Flora of the study area consisted of 354 plant species belonging to 93 families. In this total, the leading plant habit was herbaceous having 259 spp., followed by shrubs with 52 spp., trees with 42 spp., and parasitic plant with one species, respectively (*Fig. 2*). Asteraceae was the leading families with 36 species, followed by Lamiaceae with 24 species, Rosaceae with 22 species. For a complete inventory see *Appendix*.

Based on biological spectrum, the flora was dominated by Therophytes (116 spp.) followed by Hemicryptophytes (90 spp.), Nanophanerophytes (48 spp.) and Chamaephytes (29 spp.) (*Fig. 3*). On the basis of leaf spectra, the study area was dominated by Nanophyll and Microphyll with 105 spp. and 100 spp., respectively, followed by Mesophyll (64 spp.), Leptophyll (58 spp.), and Megaphyll (23 spp.) (*Fig. 4*). Further, four species (*Cuscuta reflexa, Ephedra girardiana, Equisetum arvense* and *Periploca aphylla*) were found as Aphyllous. For a complete inventory of biological spectrum and leaf size see *Appendix*.

The flowering data showed that July marked the peak of flowering season where 90 plant species had flowers, followed by June with 80 species. In May, flowering was observed in 68 species, followed by April with 42 species. The fruiting data showed that September was the peak fruiting season for 82 plant species, followed by August for 79 species.

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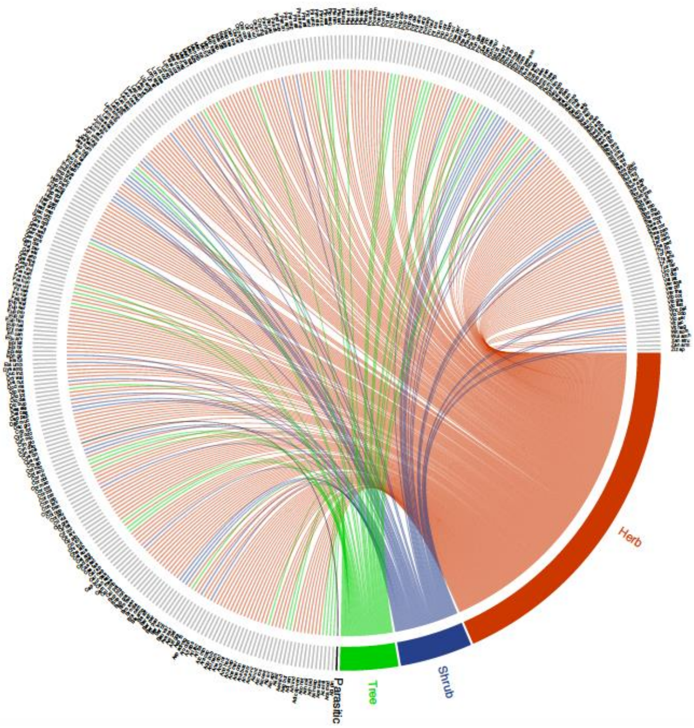

Figure 2. *Distribution of the 354 species found in Manoor Valley, Pakistan, among the growth plant habit: herb (red), shrub (blue), tree (green) and parasitic plant (black). Herbs are represented by 259 species, shrubs by 52 species, trees by 42 species and parasitic plants by 1 species. The full name of species is in Appendix*

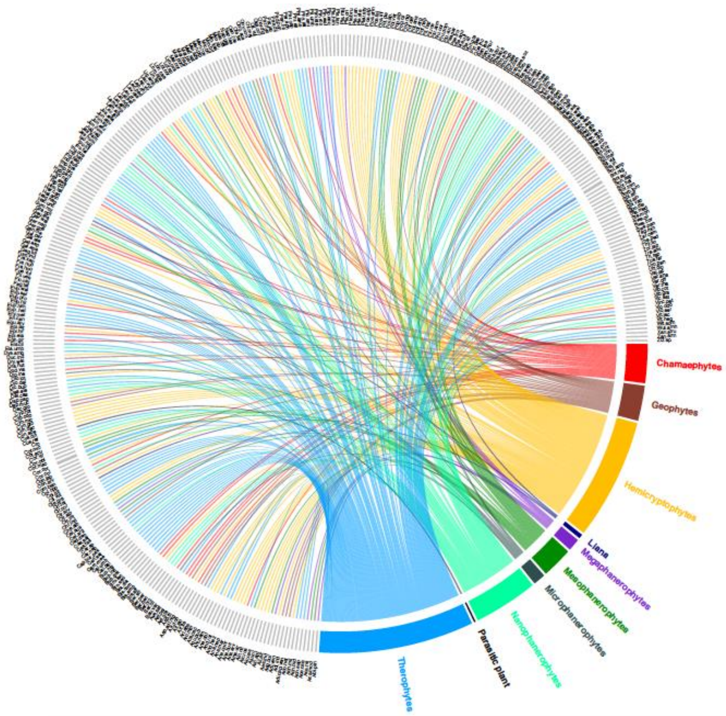

Figure 3. *Distribution of the 354 species found in Manoor Valley, Pakistan, among the life form classes. The full name of species is in Appendix*

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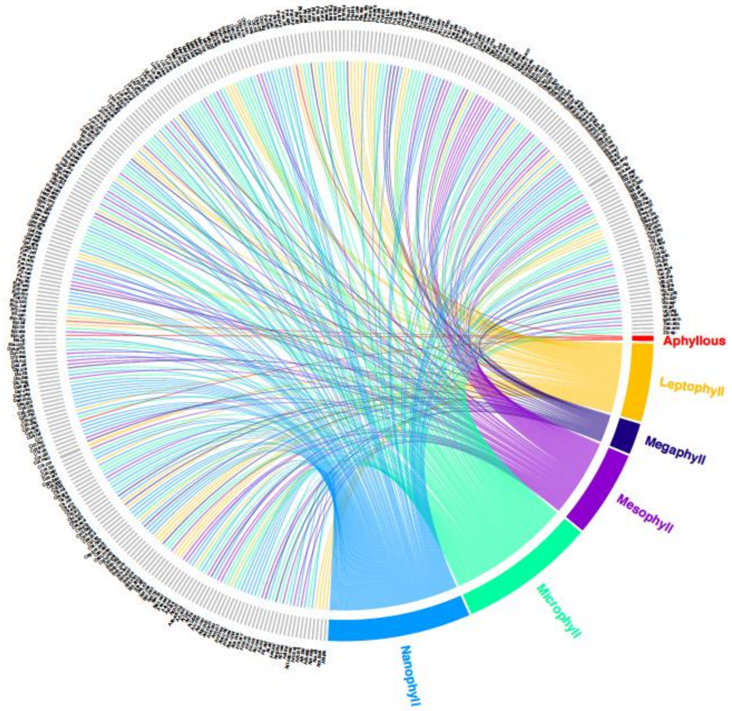

Figure 4. *Distribution of the 354 species found in Manoor Valley, Pakistan, among the leaf size classes. The full name of species is in Appendix*

Correlation and regression coefficient

The correlation and regression coefficient variables behaved differently on different axes. On axis 1, highly positive correlation (1.000) and highest positive tau value (0.957) was recorded for herbaceous plant habit, while on axis 2, positive correlation value was recorded (0.24) but negative tau value (-0.095) was logged for herbaceous growth form in comparison with all other growth forms (*Fig. 5A*). Moreover, on axis 1, highly negative correlation (-0.705) and highly negative tau value (-0.766) was recorded for shrubby plant habit, while on axis 2, highly positive correlation value (0.706) and highest positive tau value (0.766) was logged (*Fig. 5B*).

The correlation and regression results of tree growth form showed negative correlation (-0.586) and positive tau value (-0.464) on axis 1 and highly negative correlation value (-0.807) and highest negative tau value (-0.696) was logged on axis 2 (*Fig. 5C*). Further, the parasitic growth form on axis 1 showed minimum negative correlation (-0.74) and minimum negative tau value (-0.055) and similarly, minimum negative correlation value (-0.014) and minimum positive tau value (-0.089) was logged on axis 2 in comparison with all other growth forms (*Fig. 5D*). Axis 1 was dominated by herbaceous growth form, while axis 2 by shrubby plant habit (*Fig. 5A-D*).

Principle components analysis (PCA)

The PCA results revealed that 259 plant species were most frequently cited positively and significantly correlated with herbaceous growth form (*Fig. 6*). Fifty-two plant species indicates positive influence towards the direction of shrubby growth form. Tree growth habit was most frequently cited and positively correlated with 40 plant

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species. *Cuscuta reflexa* was the only species assigned to parasitic growth form category (*Fig. 6*).

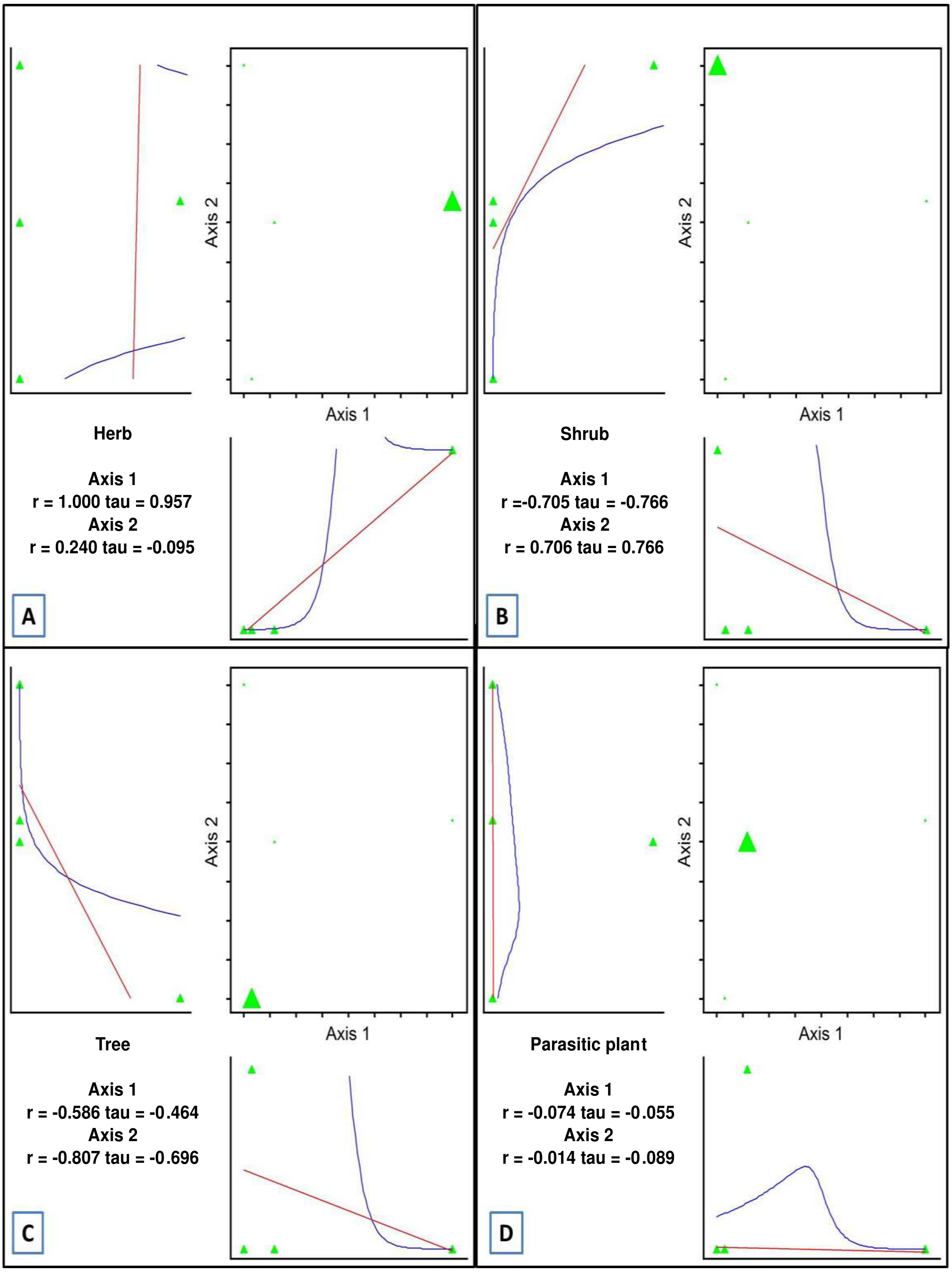

***Figure 5.** Correlation and regression coefficient of growth form on different axis: A) Herb, B) Shrub, C) Tree and D) Parasitic plant*

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Species response curve (SRC)

The analysis clearly indicates highly significant differences (F = 451, p < 0.00001; *Fig. 7*) for herb category in comparison with all other growth form categories due to maximum number of species (*Fig. 7A*). This growth form category also revealed highest response (99.3%). Nonetheless, shrub growth form also showed highly significance (F = 415, p < 0.00001) due to its number of species in comparison with tree and parasitic plant habit and presented a response percentage of 55.1%. Also tree growth form presented significant differences (F = 142, p < 0.00001) and response percentage (29.6%) in comparison with parasitic plant habit category. Parasitic plant showed non-significant differences (F = 0.81, p = 0.63206) and response percentage (0.2%) as given in the *Table 1*. Additionally, *Figure 7B* illustrates the flow of species cited within each growth form category from top to bottom as enlisted in *Table 1*.

Table 1. *Summary of fitted generalized linear models four response variables*

Response	Type	R^2[%]	F	p
Herb	linear	99.3	451	<0.00001
Shrub	linear	55.1	415	<0.00001
Tree	linear	29.6	142	<0.00001
Parasitic plant	linear	0.2	0.81	0.63347

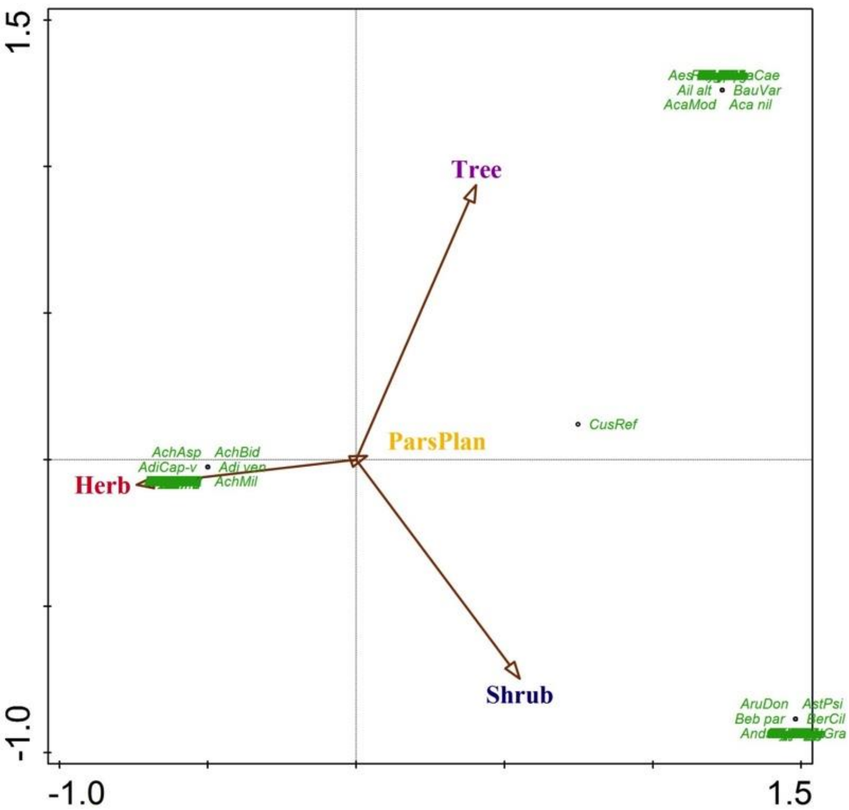

Figure 6. *Principle component analysis indicating the association of plant species with their growth forms*

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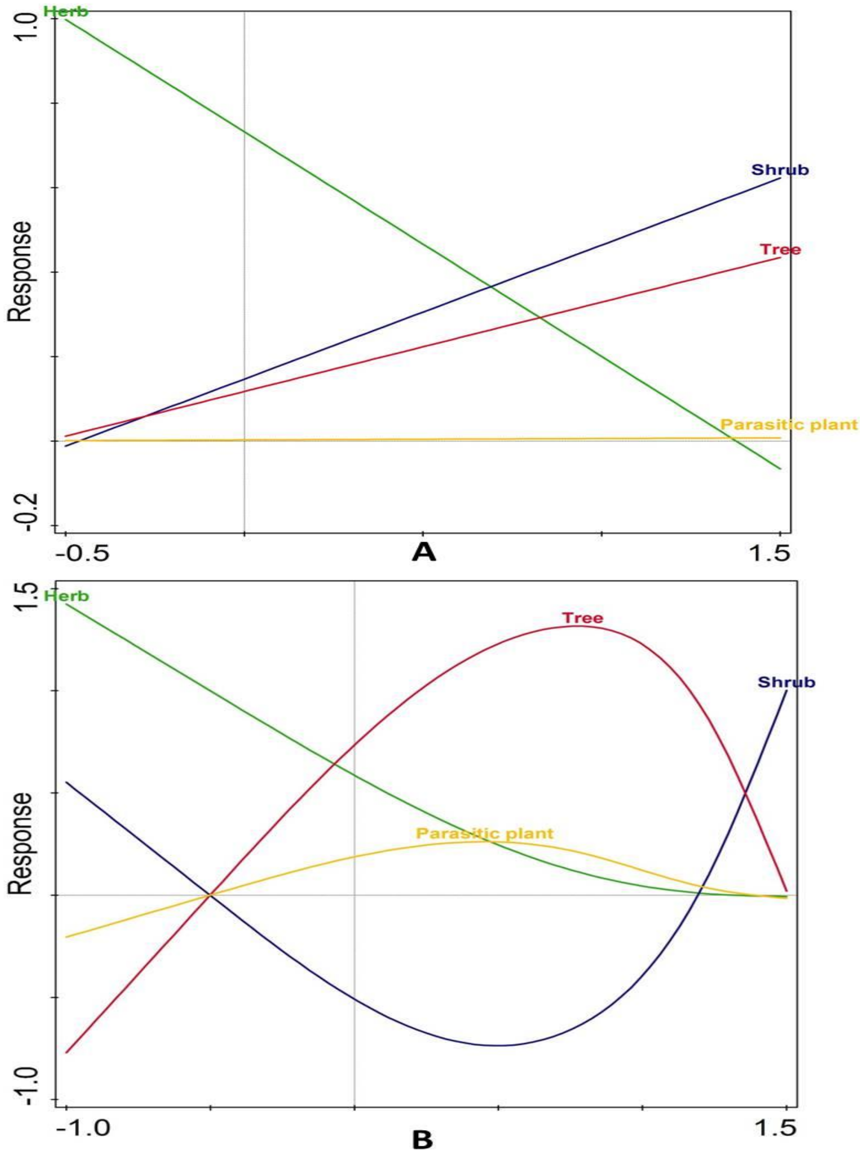

Figure 7. *A) Growth form categories response curve illustrating the significance level and B) flow of species cited within each growth form category*

Discussion

The results of this study showed that Manoor Valley, Pakistan, it is an area rich in plant species, presenting 354 species, being the herb habit most representative. Furthermore, it can be observed that the multivariate approaches showed significant differences, which supports the prediction and assertion that the herb habit is the principal plant habitat in this area.

Floristic structure is the main reflection of vegetation of any area (Rahman et al., 2018a). Plant species has its own ecological amplitude and interaction with its environment and also with other species (Giustiet al., 1995). The flora of Pakistan is diverse due to different ecological zones, diverse climatic and soil condition. In the present study, flora consisted of 354 species, where the dominating growth form was

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herbaceous with 259 species. In Pakistan, many researchers from different areas also mentioned herbaceous growth form as the leading one from their study areas (Ijaz et al., 2015; Khan et al., 2015a; Ijaz et al., 2016), which shows a predominance of herbaceous growth form in Pakistan.

Asteraceae was the leading familly with 36 species, followed by Lamiaceae with 24 species and Rosaceae with 22 species. Due to wide ecological amplitude, Asteraceae family are very diverse in habitat (Badshah et al., 2013). Khattak et al. (2015) found similar results in Karak, Pakistan and in addition Iqbal et al. (2015) also reported Asteraceae as the most predominant group in Malakand, Pakistan. On the other hand, Khan et al. (2015b) showed in a study made in Kabal (Swat), Pakistan, that Lamiaceae was the dominant family. Regardless of the family, Asteraceae and Lamiaceae appear to be the main plant families present in Pakistan's vegetation.

The flora of Manoor Valley was dominated by Therophytes, followed by Hemicryptophytes. Similarly, Badshah et al. (2013) observed Therophytes as the leading life form in Tank region, Pakistan. For various physiological processes of plants and plant communities leaf size plays a vital role (Oosting, 1956). On the basis of leaf spectra, Nanophyll and Microphyll were the most representatives. The species with Microphyllous leaves are rich due to ecological variation, which shows the percentage of different leaf form classes varied with rising altitudes and according to Cain and Castro (1959) Microphyllous species are the indication of steeps. Similar findings were observed by Saxina et al. (1987) who stated that the percentage of Microphyllous species was completely associated with the rising altitude.

Results showed that July had the flowering peak with 80 plant species, followed by June with 77 species (*Fig. 8*). These results are in agreement with those of Shrestha et al. (1998), where the authors noticed the blooming period from May to August in Kavrepalanchok, Nepal. As indicated by Marques et al. (2004), phenological period and atmosphere are associated with each other in terms of temperature, day length and precipitation or rainfall. Fruiting phase had peak in September and August (*Fig. 9*). Similar phenological scenario was reported by Morellato (1995) who reported that the blooming period begins toward the end of the dry season and at the starting of the wet season, thus fruiting takes place in dry season and that the next rainy period will offer appropriate conditions for seed germination (Morellato et al., 1989).

Conclusion

The present study indicated that the study area has rich plant biodiversity. Flora of Manoor Valley area consisted of 354 plant species, where the leading plant habit was herbaceous with 249 species and family was Asteraceae with 36 species. We can use these inventory lists as a vital resource for all future endeavors in the field of phytosociological, phytochemical, pharmacological and conservational studies of natural resources. Regarding the multivariate approaches, we can observe that these analyzes are extremely useful to show us significant differences in certain floristic survey studies, providing support and veracity in the discussions and conclusions found. Studies like this present are necessary to evaluate the plant richness and its potential value.

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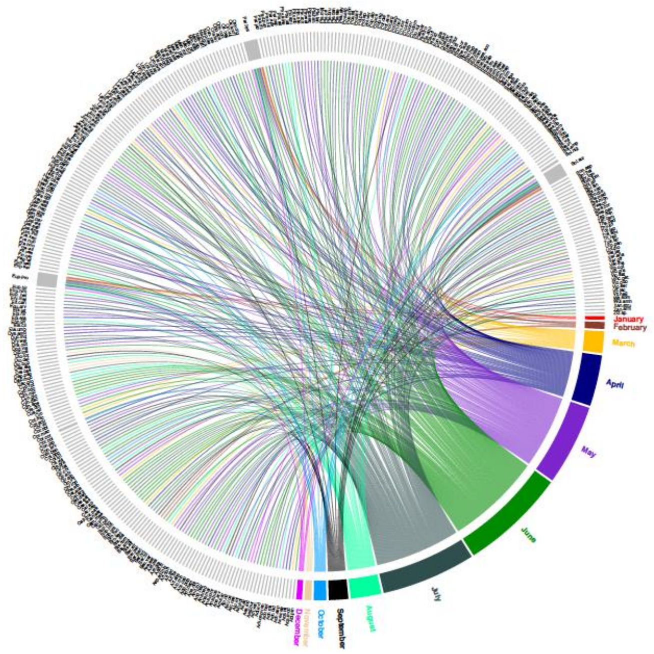

***Figure 8.** Distribution of the 354 species found in Manoor Valley, Pakistan, according to flowering period. The full name of species is in Appendix*

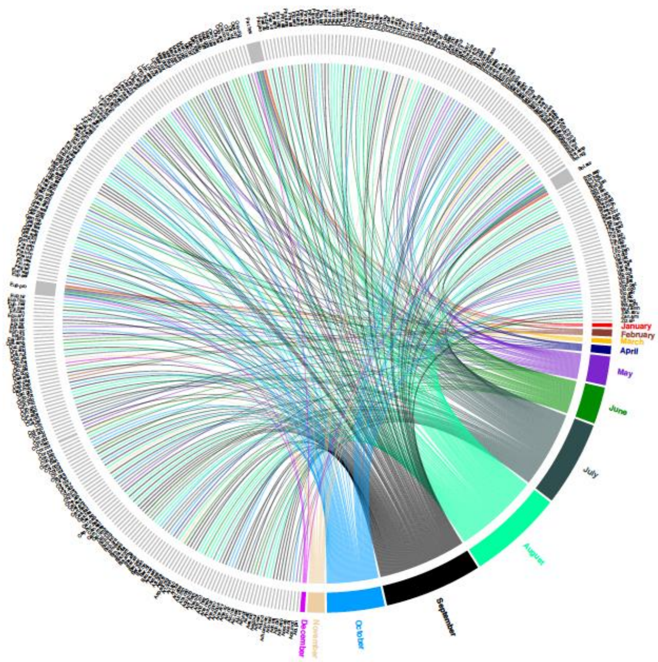

***Figure 9.** Distribution of the 354 species found in Manoor Valley, Pakistan, according to fruiting period. The full name of species is in Appendix*

Author's Contributions. IUR conducted the fieldwork, collected data and plant species, and designed the map, FI helped in the herbarium work. IUR drafted the manuscript and ESC helped in analysis of the data, NA helped in organizing the data. AA and ZI supervised the work. EFA, AA and ZI critically reviewed the manuscript. IUR, ESC, EFA and NA revised the manuscript, AAA, MSA, RK, MS and MI helped in revision. All the authors have read and approved the final manuscript.

Acknowledgements. First author would like to thank Higher Education Commission (HEC), Pakistan for granting scholarship under International Research Support Initiative Program (IRSIP) to conduct a research work at Missouri Botanical Garden, USA). The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding to the Research Group number (RG-1435-014).

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APPENDIX

Floristic composition, biological and leaf spectra, and phenological behaviour of the plant species of Manoor Valley, Pakistan

S. No	**Family name/** Scientific name	Habit	Biological spectrum		Phenology	
			Life form	**Leaf size**	**Flowering**	**Fruiting**
	Acanthaceae					
1	*Dicliptera bupleuroides* Nees	H	Th	N	Sept	Feb
2	*Justicia adhatoda* L.	S	NanP	Me	March	May
	Adiantaceae					
3	*Adiantum capillus-veneris* L.	H	G	N	July	Sept
4	*Adiantum indicum* J. Ghatak	H	G	Me	July	Aug
5	*Adiantum venustum* D. Don	H	G	N	June	Aug
6	*Asplenium adiantum-nigrum* L.	H	HemC	N	July	Sept
	Adoxaceae					
7	*Viburnum cotinifolium* D. Don	S	NanP	Ma	March	May
8	*Viburnum grandiflorum* Wall. ex DC.	S	NanP	Ma	March	May
	Amaranthaceae					
9	*Achyranthes aspera* L.	H	Th	N	May	July
10	*Achyranthes bidentata* Blume	H	Th	Mi	Aug	Sept
11	*Amaranthus viridis* L.	H	Th	Mi	Aug	Sept
12	*Celosia argentea* L.	H	Th	N	Aug	Sept
	Apiaceae					
13	*Aegopodium burttii* Nasir	H	HemC	Mi	June	July
14	*Anthriscus nemorosa* (M.Bieb.) Spreng.	H	Th	Mi	April	Aug
15	*Bupleurum nigrescens* E. Nasir	H	Th	N	June	Sept
16	*Bupleurum gracillimum* Klotzsch	H	Th	N	June	Sept
17	*Bupleurum longicaule* Wall. ex DC.	H	Th	N	June	Sept
18	*Foeniculum vulgare* Mill.	H	Th	N	July	Sept
19	*Heracleum candicans* Wall. ex DC.	H	HemC	L	April	June
20	*Pimpinella stewartii* (Dunn) Nasir	H	Th	N	July	Sept
21	*Pleurospermum brunonis* Benth. ex C.B.Clarke	H	HemC	L	June	Aug

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22	*Pleurospermum candollei* Benth. ex C.B.Clarke	H	HemC	L	July	Aug
23	*Pleurospermum stellatum* (D. Don) Benth. ex C.B. Clarke	H	HemC	L	July	Aug
24	*Pleurospermum stylosum* C.B. Clarke	H	HemC	L	July	Aug
25	*Sanicula elata* Buch.-Ham. ex D.Don	H	Th	Mi	May	July
26	*Seseli libanotis* (L.) W.D.J.Koch .	H	HemC	N	April	Aug
27	*Torilis japonica* (Houtt.) DC.	H	Th	Mi	June	July
28	*Trachyspermum amii* (L.) Sprague	H	Th	L	May	July
	Araceae					
29	*Arisaema flavum* (Forsk.) Schott	H	G	Ma	June	Sept
30	*Arisaema jacquemontii* Blume	H	G	Me	June	July
31	*Sauromatum venosum* (Dryand. ex Aiton) Kunth	H	G	Me	May	Aug
	Araliaceae					
32	*Aralia cachemirica* Decne.	H	HemC	Me	May	June
33	*Hedera nepalensis* K. Koch	H	L	Me	Oct	April
	Asclepiadaceae					
34	*Periploca aphylla* Decne.	S	NanP	Aph	May	July
35	*Vincetoxicum petrense* (Hemsl. & Lace) Rech. f.	H	Ch	Ma	June	July
	Asparagaceae					
36	*Asparagus fiicinus* Buch.-Ham. ex D. Don	H	G	Mi	May	June
	Asteraceae					
37	*Achillea millefolium* L.	H	Th	N	May	Sept
38	*Ainsliaea aptera* DC.	H	HemC	Ma	Dec	June
39	*Anaphalis margaritacea* (L.) Benth.	H	HemC	N	Aug	Sept
40	*Anaphalis busua* (Buch.-Ham.) DC.	H	HemC	N	Sept	Oct
41	*Anaphalis contorta* (D.Don) Hook.f.	H	HemC	N	Sept	Oct
42	*Anaphalis nepalensis* (Spreng.) Hand.-Mazz.	H	HemC	N	May	Sept
43	*Arctium minus* (Hill) Benh.	H	HemC	Me	June	Aug
44	*Artemisia absinthium* L.	H	Th	N	July	Aug
45	*Carpesium nepalense* Less.	H	Th	N	June	Sept
46	*Chrysanthemum indicum* L.	H	HemC	Mi	Oct	Nov
47	*Cichorium intybus* L.	H	Th	N	July	Aug
48	*Cirsium arvense* (L.) Scop.	H	Th	N	Aug	Oct
49	*Cirsium falconeri* (Hook.f.) Petr.	H	Th	N	July	Aug
50	*Conyza japonica* (Thunb.) Less. ex Less.	H	Th	Mi	July	Aug
51	*Cyanthillium cinereum* (L.) H.Rob.	H	HemC	Mi	June	July
52	*Erigeron canadensis* L.	H	Th	L	July	Aug
53	*Galinosoga parviflora* Cav.	H	Th	Mi	July	Sept
54	*Gerbera gossypina* (Royle) Beauverd	H	HemC	Mi	April	June
55	*Helianthus annuus* L.	H	Th	Ma	May	June
56	*Inula cuspidata* (Wall. ex DC.) C.B.Clarke	H	Th	Mi	June	Sept

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57	*Inula falconeri* Hook.f.	H	Th	Me	June	Oct
58	*Lactuca tatarica* (L.) C.A.Mey	H	Th	Mi	May	July
59	*Launaea procumbens* (Roxb.) Ramayya & Rajagopal	H	Ch	Mi	March	May
60	*Leucanthemum vulgare* Lam.	H	HemC	L	June	Sept
61	*Ligularia amplexicaulis* DC.	H	HemC	Ma	May	Aug
62	*Onopordum acanthium* L.	H	G	Me	April	June
63	*Parthenium hysterophorus* L.	H	HemC	N	All the year	All the year
64	*Saussurea sp.*	H	HemC	Me	March	May
65	*Senecio analogus* DC.	H	Th	Mi	Aug	Sept
66	*Senecio chrysanthemoides* DC.	H	Th	Me	Oct	Nov
67	*Silybum marianum* (L.) Gaertn.	H	Ch	Me	March	June
68	*Sonchus asper* (L.) Hill	H	Th	Mi	July	Aug
69	*Tagetes minuta* L.	H	Th	Mi	Sept	Oct
70	*Taraxacum campylodes* G.E.Haglund	H	HemC	Mi	May	Aug
71	*Tussilago farfara* L.	H	Th	Me	Feb	May
72	*Xanthium strumarium* L.	H	Th	Me	July	Sept
	Balsaminaceae					
73	*Impatiens bicolor* Royle.	H	Th	Me	July	Sept
74	*Impatiens brachycentra* Kar. & Kir.	H	Th	Mi	July	Sept
	Berberidaceae					
75	*Berberis lycium* Royle	S	NanP	L	June	July
76	*Berberis pachyacantha* Bien. ex Koehne	S	NanP	L	Feb	July
77	*Berberis parkeriana* C.K.Schneid.	S	NanP	L	Feb	July
78	*Epimedium elatum* C.Morren & Decne.	H	Th	Me	April	May
	Betulaceae					
79	*Alnus nitida* (Spach) Endl.	T	MesP	Me	July	Sept
80	*Corylus colurna* L.	T	MesP	Me	April	June
	Boraginaceae					
81	*Cynoglossum apenninum* L.	H	HemC	N	June	Aug
82	*Cynoglossum glochidiatum* Wall. ex Benth.	H	HemC	N	June	Aug
83	*Cynoglossum microglochin* Benth.	H	HemC	N	June	Aug
84	*Hackelia uncinata* (Benth.) C.E.C.Fisch.	H	G	Mi	June	Aug
85	*Lindelofia* sp.	H	Th	N	June	Aug
86	*Myosotis sp.*	H	HemC	Mi	May	July
87	*Pseudomertensia parviflorum* (Decne.) Riedl	H	HemC	N	May	July
88	*Pseudomertensia trollii* Stewart & Kazmi	H	HemC	N	May	Aug
	Brassicaceae					
89	*Brassica campestris* Dunn.	H	Th	Ma	June	July
90	*Capsella bursa-pastoris* (L.) Medik.	H	Th	N	June	Aug
91	*Erysimum melicentae* Dunn.	H	Th	Mi	July	Sept
92	*Sisymbrium irio* L.	H	Th	N	April	June
93	*Nasturtium officinale* R.Br.	H	G	Mi	May	July

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	Buxaceae					
94	*Sarcococca saligna* Mull.Arg.	S	NanP	Mi	Dec	March
	Caesalpiniaceae					
95	*Bauhinia variegata* L.	T	MicP	Me	March	April
	Cannabaceae					
96	*Cannabis sativa* L.	H	Th	Mi	July	Sept
97	*Celtis australis* L.	T	MesP	Ma	May	Aug
	Caprifoliaceae					
98	*Lonicera caerulea* L.	S	NanP	Mi	March	May
99	*Valeriana jatamansi* Jones	H	G	Me	May	July
	Caryophyllaceae					
100	*Minuartia biflora* L.	H	HemC	L	Aug	Sept
101	*Minuartia kashmirica* (Edgew.) Mattf.	H	Ch	L	July	Sept
102	*Silene conoidea* L.	H	Th	N	April	May
103	*Silene vulgaris* (Moench) Garcke	H	Th	N	July	Sept
104	*Stellaria media* (L.) Vill.	H	Th	N	Oct	Nov
105	*Stellaria monosperma* Buch.-Ham. ex D. Don	H	Th	Mi	Sept	Oct
	Celastraceae					
106	*Gymnosporia royleana* Wall. ex M.A.Lawson	S	NanP	Mi	March	May
	Chenopodiaceae					
107	*Chenopodium album* L.	H	HemC	N	June	Sept
108	*Dysphania ambrosioides* (L.) Mosyakin & Clemants	H	Th	Mi	May	June
	Clusiaceae					
109	*Hypericum perforatum* L.	H	Ch	N	July	Sept
	Colchicaceae					
110	*Colchicum luteum* Baker	H	G	N	July	Sept
	Commelinaceae					
111	*Commelina benghalensis* L.	H	Ch	N	Sept	Oct
	Convallariaceae					
112	*Polygonatum verticillatum* (L.) Allioni	H	Th	N	June	Aug
113	*Polygonatum sp.*	H	Th	Mi	May	July
	Convolvulaceae					
114	*Convolvulus arvensis* L.	H	HemC	N	May	July
115	*Ipomoea nil* (L.) Roth	H	Th	Mi	May	July
	Cornaceae					
116	*Cornus macrophylla* Wall.	T	MesP	Ma	March	May
117	*Cornus oblonga* Wall.	T	MesP	Me	June	Sept
	Crassulaceae					
118	*Sedum album* L.	H	Ch	N	July	Oct
119	*Hylotelephium ewersii* Ledeb.	H	Ch	Mi	Oct	Nov
120	*Sedum fischeri* Raym.-Hamet	H	Th	L	July	Sept
	Cucurbitaceae					
121	*Luffa sp.*	H	Th	Mi	June	July
122	*Solena amplexicaulis* (Lam.) Gandhi	H	Th	Mi	Aug	Oct

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	Cupressaceae					
123	*Juniperus communis* L.	S	NanP	L	June	July
124	*Juniperus squamata* Buch.-Ham. ex D.Don	S	NanP	L	June	Sept
125	*Juniperus excelsa* M.Bieb.	T	NanP	L	June	Oct
	Cuscutaceae					
126	*Cuscuta reflexa* Roxb.	Pp	Pp	Aph	Aug	Sept
	Cyperaceae					
127	*Carex sp.*	H	G	N	March	May
128	*Cyperus rotundus* L.	H	G	N	Aug	Oct
129	*Cyperus odoratus* L.	H	G	N	Aug	Sept
	Dioscoreaceae					
130	*Dioscorea deltoidea* Wall. ex Griseb.	H	HemC	Mi	Nov	Dec
	Dipsacaceae					
131	*Dipsacus inermis* Wall. in Roxb.	H	Ch	Me	May	July
	Dryopteridaceae					
132	*Dryopteris wallichiana* (Spreng.) Hyl.	H	G	Me	July	Aug
	Dennstaedtiaceae					
133	*Pteridium aquilinum* (L.) Kuhn	H	G	Ma	Aug	Nov
	Ebenaceae					
134	*Diospyros lotus* L.	T	MicP	Ma	June	Nov
	Eleagnaceae					
135	*Elaeagnus umbellata* Thunb.	S	NanP	Mi	April	May
	Ephedraceae					
136	*Ephedra girardiana* Wall. ex. Stapf	S	Ch	Aph	Aug	Oct
	Equisetaceae					
137	*Equisetum arvense* L.	H	G	Aph	April	May
	Ericaceae					
138	*Cassiope fastigiata* (Wall.) D.Don	H	Th	L	May	July
139	*Lyonia ovalifolia* (Wall.) Drude	S	NanP	Mi	May	Oct
140	*Rhododendron arboreum* Sm.	T	MicP	Me	April	May
	Rhododendron hypenanthum Balf. f.	S	NanP	Me	April	May
141	**Euphorbiaceae**					
142	*Euphorbia helioscopia* L.	H	Th	N	May	June
143	*Euphorbia prostrata* Ait.	H	Ch	N	All the year	All the year
144	*Euphorbia hirta* L.	H	Th	N	Aug	Oct
145	*Euphorbia serpens* Kunth	H	HemC	N	June	July
146	*Euphorbia wallichii* Hook. f.	H	Th	N	Aug	Oct
147	*Ricinus communis* L.	S	NanP	Me	June	Oct
	Fagaceae					
148	*Castanea sativa* Mill.	T	MesP	Ma	May	July
149	*Quercus incana* Bartram	T	MesP	Mi	May	July
	Fumaricaceae					
150	*Fumaria indica* (Hausskn) Pugsley	H	Th	N	April	June
	Gentianaceae					
151	*Gentianodes clarkei* (Kusn.) Omer	H	Th	N	Aug	Oct

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152	*Lomatogonium spathulatum* (A. Kern.) Fernald	H	Th	N	April	May
153	*Swertia paniculata* Wall.	H	Th	L	July	Aug
154	*Swertia ciliata* (D. Don ex G. Don) B.L. Burtt	H	Th	L	July	Aug
155	*Swertia cordata* (Wall. ex G. Don) C.B. Clarke	H	Th	L	July	Aug
	Geraniaceae					
156	*Geranium nepalense* Sweet.	H	Ch	Mi	July	Oct
157	*Geranium wallichianum* D.Don ex Sweet	H	Ch	Mi	July	Oct
	Hamamelidaceae					
158	*Parrotiopsis jacquemontiana* (Decne.) Rehder	S	NanP	Mi	June	Aug
	Hippocastanaceae					
159	*Aesculus indica* (Wall. ex Camb.) Hook.	T	MegP	Me	July	Aug
160	*Hippolytia dolichophylla* (Kitam.) K.Bremer & Humphries	H	HemC	Me	May	Oct
	Juglandaceae					
161	*Juglans regia* L.	T	MegP	Ma	July	Aug
	Juncaceae					
162	*Juncus* sp.	H	G	N	Sept	Nov
	Lamiaceae					
163	*Ajuga integrifolia* Buch.-Ham.	H	HemC	Mi	May	July
164	*Calamintha umbrosa* (M.Bieb.) Rchb.Benth.) Hedge	H	Th	N	July	Sept
165	*Clinopodium vulgare* L.	H	HemC	Mi	April	June
166	*Colebrookea oppositifolia* Sm.	S	NanP	L	July	Oct
167	*Dracocephalum nutans* L.	H	Th	N	July	Sept
168	*Elsholtzia ciliata* (Thunb.) Hyl.	H	Th	N	July	Sept
169	*Isodon rugosus* (Wall. ex Benth.) Codd	S	NanP	Mi	May	Oct
170	*Lamium album* L.	H	Th	Mi	July	Oct
171	*Lamium amplexicaule* L.	H	Th	Mi	July	Oct
172	*Mentha piperita* L.	H	HemC	N	June	Sept
173	*Mentha royleana* Wall. ex Benth.	H	HemC	Mi	June	Oct
174	*Micromeria biflora* (Ham.) Bth.	H	Ch	L	April	June
175	*Nepeta graciliflora* Benth.	H	Th	Mi	July	Sept
176	*Nepeta laevigata* (D. Don) Hand.- Mazz	H	Th	Mi	July	Sept
177	*Origanum majorana* L.	H	Th	N	June	Aug
178	*Origanum vulgare* L.	H	Th	N	July	Aug
179	*Prunella vulgaris* L.	H	HemC	N	Sept	Nov
180	*Rydingia limbata* (Benth.) Scheen & V.A. Albert	S	NanP	L	July	Aug
181	*Salvia lanata* Roxb.	H	Ch	Mi	April	July
182	*Salvia moorcroftiana* Wall. ex. Benth.	S	Th	Me	March	June
183	*Salvia nubicola* Wall. ex Sweet	H	Th	Me	June	Aug
184	*Thymus linearis* Benth.	H	HemC	N	July	Sept

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DOI: http://dx.doi.org/10.15666/aeer/1702_14751498

	Liliaceae					
185	*Gagea lutea* (L.) Ker Gawl.	H	G	N	Mar	May
	Linaceae					
186	*Reinwardtia trigyna* Planch.	H	Ch	L	June	July
	Lythraceae					
187	*Punica granatum* L.	S	NanP	Mi	May	July
	Malvaceae					
188	*Alcea rosea* L.	H	HemC	Ma	April	July
189	*Grewia optiva* J.R.Drumm. ex Burret	T	MesP	N	April	June
190	*Lavatera cachemiriana* Camb. in Jacq.	H	HemC	Me	May	July
191	*Malva parviflora* L.	H	HemC	Mi	April	June
192	*Malva neglecta* Wallr.	H	HemC	Mi	May	July
193	*Malvastrum coromandelianum* (L.) Garcke	H	HemC	N	April	Oct
194	*Sida cordata* (Burm.f.) Borss.Waalk.	H	HemC	N	May	Sept
	Meliaceae					
195	*Melia azedarach* L.	T	MesP	Me	April	July
	Mimosaceae					
196	*Acacia modesta* Wall.	T	MicP	L	March	May
197	*Acacia nilotica* (L.) Delile	T	MesP	L	April	Oct
	Moraceae					
198	*Ficus carica* L.	T	MicP	Me	May	Aug
	Oleaceae					
199	*Fraxinus hookeri* Wenz.	T	MicP	Me	April	Oct
200	*Fraxinus xanthoxyloides* (G. Don) DC	T	MicP	Me	May	Sept
201	*Jasminum humile* L.	S	NanP	N	June	Aug
202	*Jasminum sambac* (L.) Aiton	S	NanP	N	June	Aug
203	*Olea ferruginea* Wall. ex Aitch.	T	MesP	Mi	April	June
	Onagraceae					
204	*Circaea cordata* Royle.	H	Th	Me	June	Sept
205	*Circaea alpina* L.	H	Th	Mi	June	Sept
206	*Epilobium hirsutum* L.	H	Ch	Mi	May	July
207	*Epilobium latifolium* L.	H	HemC	L	July	Sept
208	*Oenothera rosea* L. Her ex Aiton	H	Th	N	June	Aug
	Orchidaceae					
209	*Spiranthes sinensis* (Pers.) Ames	H	G	N	June	Sept
	Orobanchaceae					
210	*Euphrasia himalayica* Wetts.	H	Th	L	July	Oct
211	*Pedicularis punctata* Decne.	H	HemC	N	July	Aug
	Oxalidaceae					
212	*Oxalis corniculata* L.	H	HemC	N	May	Sept
	Papaveraceae					
213	*Corydalis cornuta* Royle	H	Th	L	Oct	Nov
214	*Corydalis carinata* Lidén & Z.Y.Su	H	Th	L	July	Sept
215	*Corydalis virginea* Lidén & Z.Y.Su	H	HemC	N	March	April
	Papilionaceae					
216	*Astragalus grahamianus* Benth.	S	Ch	L	Sept	Nov

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217	*Astragalus psilocentros* Fisch.	H	Ch	N	May	Aug
218	*Campylotropis meeboldii* (Schindl.) Schindl.	S	NanP	L	June	Aug
219	*Crotalaria* sp.	H	Th	N	May	July
220	*Desmodium elegans* DC.	S	NanP	Me	July	Aug
221	*Indigofera heterantha* Brandis	S	NanP	L	July	Oct
222	*Indigofera australis* Willd.	S	NanP	N	May	Aug
223	*Indigofera hebepetala* Baker	S	NanP	N	May	Aug
224	*Lathyrus aphaca* L.	H	Th	N	April	June
225	*Lathyrus sativa* L.	H	Th	N	July	Sept
226	*Lathyrus odoratus* L.	H	Th	L	July	Sept
227	*Lotus corniculatus* L.	H	HemC	Mi	Aug	Sept
228	*Medicago sativa* L.	H	HemC	N	June	Sept
229	*Rhynchosia pseudo-cajan* Cambess.	S	NanP	Me	May	July
230	*Robinia pseudo-acacia* L.	T	MesP	Me	April	May
231	*Trifolium repens* L.	H	G	N	June	July
232	*Vicia sativa* L.	H	HemC	N	April	May
	Phyllanthaceae					
233	*Leptopus chinensis* (Bunge) Pojark. [Syn. *Andrachne cordifolia* (Decne.) Mull.Avg.]	S	NanP	Me	May	July
	Phytolaccaceae					
234	*Phytolacca americana* L.	H	Th	Mi	Aug	Sept
235	*Phytolacca latbenia* (Moq.) H. Walter	H	Ch	Ma	June	Aug
	Pinaceae					
236	*Abies pindrow* (Royle ex D.Don) Royle	T	MegP	L	June	July
237	*Cedrus deodara* (Roxb. ex Lamb.) G. Don	T	MegP	L	Sept	Oct
238	*Picea smithiana* (Wall.) Boiss.	T	MegP	L	May	July
239	*Pinus roxburghii* Sarg	T	MegP	L	May	July
240	*Pinus wallichiana* A.B.Jacks.	T	MegP	L	May	July
	Plantaginaceae					
241	*Plantago himalaica* Pilger.	H	HemC	Mi	July	Aug
242	*Plantago lanceolata* L.	H	HemC	Mi	April	July
243	*Plantago major* L.	H	HemC	Mi	July	Sept
244	*Veronica anagallis* L.	H	HemC	Mi	June	July
245	*Wulfeniopsis amherstiana* (Benth.) D.Y. Hong [Syn. *Wulfenia amherstiana* Benth.]	H	Th	N	April	July
	Platanaceae					
246	*Platanus orientalis* L.	T	MegP	Ma	June	July
	Poaceae					
247	*Arundo donax* L.	S	Ch	Mi	July	Sept
248	*Avena sativa* L.	H	Th	N	May	July
249	*Bromus diandrus* Roth.	H	Th	L	April	July
250	*Bromus secalinus* L.	H	Th	L	March	June
251	*Bromus tectorum* L.	H	Th	L	June	Sept
252	*Cynodon dactylon* (L.) Pers.	H	HemC	L	June	Sept

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253	*Dactylis glomerata* L.	H	HemC	N	July	Sept
254	*Paspalum dilatatun* Poir.	H	HemC	N	April	Sept
255	*Pennisetum orientale* Rich.	H	HemC	L	June	Sept
256	*Phragmites altissimus* (Benth.) Mabille	H	Th	Mi	Aug	July
257	*Piptatherum aequiglume* (Duthie ex Hook.f.) Roshev.	H	Th	L	May	Oct
258	*Poa alpina* L.	H	HemC	N	June	Sept
259	*Poa falconeri* Hook. f.	H	HemC	L	June	Sept
260	*Poa annua* L.	H	HemC	L	May	Aug
261	*Poa infirma* Kunth	H	HemC	L	April	June
262	*Saccharum spontaneum* L.	H	HemC	N	June	Aug
263	*Schismus arabicus* Nees.	H	HemC	L	June	July
264	*Sorghum halepense* (L.) Pers.	H	HemC	N	May	July
265	*Sporobolus diandrus* (Retz.) P.Beauv.	H	HemC	L	Sept	Sept
266	*Urochloa panicoides* P.Beauv.	H	HemC	N	July	June
	Polygonaceae					
267	*Bistorta affinis* (D.Don) Green	H	Ch	Mi	July	Aug
268	*Bistorta amplexicaulis* (D.Don) Greene	H	Ch	Mi	July	Aug
269	*Fagopyrum tataricum* (L.) Gaertn.	H	Th	Ma	July	Sept
270	*Oxyria digyna* (L.) Hill	H	Ch	Mi	June	July
271	*Persicaria capitata* (Buch.-Ham. ex D.Don) H.Gross	H	Th	L	June	Aug
272	*Polygonum plebeium* R.Br.	H	HemC	Mi	July	Oct
273	*Rheum australe* D. Don	H	Ch	Me	June	Aug
274	*Rumex dentatus* L.	H	Th	Me	Aug	Oct
275	*Rumex hastatus* D. Don	H	Th	N	June	Sept
276	*Rumex nepalensis* Sprenge	H	Th	Me	July	Oct
	Portulacaceae					
277	*Portulaca oleracea* L.	H	Th	N	May	July
	Primulaceae					
278	*Anagallis arvensis* L.	H	Ch	N	May	July
279	*Androsace hazarica* R.R. Stewart ex Y.Nasir	H	Th	Mi	May	July
280	*Androsace rotundifolia* Hardw.	H	Th	Mi	June	Aug
281	*Primula rosea* Y.J. Nasir	H	G	Mi	Oct	Nov
282	*Primula hazarica* Duthie	H	HemC	Mi	July	Aug
	Pteridaceae					
283	*Onychium contiguum* C.Hope	H	G	Me	June	June
284	*Pteris vittata* L.	H	G	Me	June	Aug
	Ranunculaceae					
285	*Aconitum heterophyllum* Wall. ex Royle	H	HemC	Me	June	Sept
286	*Anemone obtusiloba* D.Don	H	HemC	L	May	July
287	*Aquilegia pubiflora* Wall. ex Royle	H	HemC	Mi	June	Aug
288	*Caltha palustris* var. *alba* (Cambess) Hook.f. & Thomson	H	Th	Ma	July	Aug
289	*Clematis grata* Wall.	H	L	Mi	July	Sept
290	*Delphinium cashmerianum* Royle	H	Th	Mi	Aug	Sept

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DOI: http://dx.doi.org/10.15666/aeer/1702_14751498

291	*Ranunculus laetus* Wall. ex Hook. f. & J.W. Thompson	H	HemC	Me	June	Sept
292	*Ranunculus muricatus* L.	H	HemC	Ma	June	July
293	*Thalictrum pedunculatum* Edgew.	H	HemC	N	June	Aug
	Rhamnaceae					
294	*Rhamnus purpurea* Edgew.	T	NanP	Mi	May	July
295	*Ziziphus undulata* Reissek	S	NanP	Mi	June	Aug
	Rosaceae					
296	*Alchemilla cashmeriana* Rothum.	H	HemC	N	June	Sept
297	*Cotoneaster acuminatus* Wall. ex Lindl.	S	NanP	N	June	July
298	*Cotoneaster microphyllus* Wall. ex Lindl	S	NanP	N	May	July
299	*Duchesnea indica* (Andx) Fake.	H	HemC	N	April	June
300	*Filipendula vestita* (Wall. ex G. Don.) Maxim.	H	Th	Me	June	Aug
301	*Fragaria nubicola* (Hook. f.) Lindl. ex Lacaita	H	HemC	N	May	July
302	*Geum elatum* Wall. ex G.Don	H	HemC	N	Aug	Oct
303	*Malus domestica* Borkh.	T	MicP	Me	April	June
304	*Potentilla anserina* L.	H	HemC	L	June	Aug
305	*Potentilla argentea* L.	H	HemC	L	June	Sept
306	*Potentilla napalensis* Hook.	H	HemC	Mi	June	Sept
307	*Prunus cornuta* (Wall.ex Royle) Steud	T	MesP	Me	May	July
308	*Prunus armeniaca* L.	T	MesP	Mi	March	April
309	*Prunus domestica* L.	T	MesP	Me	May	Sept
310	*Pyrus pashia* Buch.-Ham. ex D.Don	T	MesP	Mi	April	May
311	*Rosa webbiana* Wall. ex. Royle	S	NanP	N	May	July
312	*Rosa brunonii* Lindl.	S	NanP	Mi	June	July
313	*Rubus fruticosus* Agg.	S	NanP	Mi	July	Sept
314	*Rubus sanctus* Schreber	S	NanP	Mi	July	Sept
315	*Sibbaldia procumbens* L.	H	HemC	N	Oct	Nov
316	*Sorbaria tomentosa* (Lindl.) Rehder	S	NanP	Me	June	Aug
317	*Sorbus tomentosa* Hedl.	S	NanP	Me	July	Aug
318	*Spiraea vaccinifolia* D.Don	S	NanP	Mi	April	July
319	*Spiraea affinis* R.Parker	S	NanP	Me	July	Aug
	Rubiaceae					
320	*Galium aparine* L.	H	Th	L	July	Oct
321	*Galium asparagifolium* Boiss. & Heldr.	H	Th	L	May	Aug
322	*Galium elagans* Wall.	H	Th	N	July	Aug
323	*Himalrandia tetrasperma* (Wall. ex Roxb.) T.Yamaz.	H	HemC	Mi	June	Aug
324	*Leptodermis virgata* Edgew. ex Hook.f.	H	Ch	N	May	June
	Rutaceae					
325	*Zanthoxylum armatum* DC.	S	MicP	Mi	July	Sept
	Salicaceae					
326	*Populus alba* L.	T	MesP	Ma	June	Aug
327	*Populus ciliata* Wall. ex Royle	T	MesP	Ma	May	June

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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328	*Populus nigra* L.	T	MesP	Ma	June	Aug
329	*Salix alba* L.	T	MesP	Mi	June	Oct
330	*Salix denticulata* subsp. *hazarica* (R. Parker) Ali	T	MesP	Mi	June	Oct
331	*Salix tetrasperma* Roxb.	T	MesP	Mi	June	Oct
	Sambucaceae					
332	*Sambucus wightiana* Wall. ex Wight & Arn	S	Th	Me	July	Sept
	Sapindaceae					
333	*Acer caesium* Wall. ex Brandis	T	MegP	L	April	July
334	*Dodonaea viscosa* (L.) Jacq.	S	NanP	Me	April	Aug
	Saxifragaceae					
335	*Bergenia ciliata* (Haw.) Sternb.	H	Ch	Me	Aug	Oct
336	*Bergenia stracheyi* Hook.f & thomes.	H	HemC	Me	Sept	Oct
	Scrophulariaceae					
337	*Verbascum thapsus* L.	H	Th	Me	July	Aug
	Simaroubaceae					
338	*Ailanthus altissima* (Mill.) Swingle	T	MesP	Mi	May	July
	Smilacaceae					
339	*Smilax glaucophylla* Koltzsch	H	L	Mi	June	Aug
	Solanaceae					
340	*Hyoscyamus niger* L.	H	Th	Me	July	Sept
341	*Solanum nigrum* L.	H	Th	Mi	July	Oct
342	*Solanum surattense* Burm F.	H	Th	Me	All the year	All the year
343	*Withania somnifera* (L.) Dunal	H	Ch	Me	July	Oct
	Thymelaeaceae					
344	*Daphne mucronata* Royle	S	NanP	N	Nov	Feb
345	*Daphne papyracea* Wall. ex G. Don	S	NanP	N	Nov	Feb
	Urticaceae					
346	*Lecanthus peduncularis* (Wall. ex Royle) Wedd. Weed.	H	HemC	Mi	July	Sept
347	*Pilea umbrosa* Blume	H	Th	Mi	April	June
348	*Urtica dioica* L.	H	Th	Mi	Sept	Oct
	Verbenaceae					
349	*Pteracanthus urticifolius* (Wall. ex Kuntze) Bremek.	H	Th	Me	June	Aug
350	*Verbena officinalis* L.	H	Th	Mi	May	July
	Violaceae					
351	*Viola odorata* L.	H	Th	Mi	Sept	Oct
352	*Viola serpens* Wall. Ex Ging	H	G	Mi	Aug	Oct
	Vitaceae					
353	*Vitex negundo* L.	S	NanP	Mi	April	Aug
354	*Vitis jacquemontii* R. Parker	S	NanP	Me	June	Aug

Abbreviations: H – Herb, Pp – Parasitic Plant, S – Shrub, T – Tree; Ch – Chamaephytes, G – Geophytes, HemC – Hemicryptophytes, L – Liana, MegP – Megaphanerophytes, MesP – Mesophanerophytes, MicP – Microphanerophytes, NanP – Nanophanerophytes, Pp – Parasitic plant, Th – Therophytes; Aph – Aphyllous, L – Leptophyll, Ma – Megaphyll, Me – Mesophyll, Mi – Microphyll, N – Nanophyll

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14751498

THE CORRELATION AND INTERACTION OF THE SUSTAINABILITY AND GLOBAL INNOVATION IN TRANSITION COUNTRIES

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(Received 14th Oct 2018; accepted 5th Dec 2018)

Abstract. In the last decade, the question of the current disproportion of quality of life and economic progress of global proportions is often raised, in contemporary socio-economic and political-economic conditions. We are witnessing a constant tendency toward targeted well-being that can be seen through human, economic and environmental benefits. The first two dimensions are goals that imply the complete sustainability of social protection of people and the protection of the environment. They are in a strong correlation with each other, since they can not be regarded as separate goals, but mutually equal because they depend on each other. On the other hand, in order to achieve sustainability of economic well-being, it is necessary to include multidimensional aspects of innovation, which will provide tools, through which adjustments of policies to promote long-term growth of production and increase of productivity, ie decrease of unemployment rate, will be adapted. Therefore, as part of the globalization process, it is necessary to review the possibilities and capacities of efficient sustainability of people's quality of life and environmental protection, with constant and balanced economic prosperity, not only countries in transition, but all factors in the world economy.
Keywords: *quality, continuial, novelty, worldwide, guide*

Introduction

If the "sustainability index" can be taken as a genetic term, then it would include two of the most important aspects of quality of life, or a sustainability indicator. These indicators are:

- Sustainable Society Index (SSI) and
- Environmental Performance Index (EPI).

Twenty-four indicators are taken into account when determining the index of a sustainable society. They are divided into seven categories, and those in three dimensions (living standard of the population, environmental protection and economic well-being). By summing up the data obtained through these dimensions, the SSI is calculated. This index is used to monitor the prosperity of the country's determination, the establishment and determination of sustainability priorities, the comparison of achieved results at the level of countries and regions, and for further research and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

development. What is characteristic of this type of indicator, and based on previous research, is that high-income countries per capita at an annual level have a high standard of living but do not achieve such good results when it comes to environmental protection. There is a trend of improving living standards and economic well-being on a global level, while environmental protection is accompanied by a negative index, despite all plans and signed interstate agreements (Van de Kerk et al., 2008).

Although it can be said that, in general, progress has been made, it is disturbing that it is not balanced between the three dimensions of society's sustainability, and that there are differences in development between high and low income countries. Particularly disappointing are the still low results of organic agriculture, renewable energy and energy savings.

With the UN's sustainable development goals in 2018, the emphasis on the actions of the governments of countries that are subject to this type of indexing is on explaining the achieved results through a series of pollution control and challenges to natural resource management in relation to quantitative indicators. Precisely obtained data and empirical approach to environmental issues provide the possibility for a certain type of security in identifying problems, monitoring trends, highlighting the results of the current policy action, identifying best practices, and optimizing the benefits of environmental investments (Yale Univeristy, 2018).

The aforementioned empirical approach to environmental issues works through several instruments, and one of them is the environmental efficiency index. The EPI is a joint project developed by the Yale Center for Environmental Law & Policy (YCELP) and the Center for International Earth Science Information Network (CIESIN) at Columbia University, in collaboration with the Samuel Family Foundation and the World Economic Forum (Hsu et al., 2014).

Based on the index value obtained, countries are ranked, and an assessment is made of the success of meeting the set objectives for the improvement of environmental protection for each of them. In this way, the best applied approach to problem-solving is considered, and guidance is provided to countries with a tendency towards further progress.

The EPI offers the possibility of bridging the two opposing sides - environmental health, which increases economic growth and prosperity, and the vitality of the ecosystem, under the pressure of industrialization and urbanization. Strategic management is a critical and essential factor necessary to establish a balance between these two dimensions of sustainability.

With the aim of a comprehensive approach to innovation in the economy and the provision of instruments that help achieve the desired economic goals, the Global Innovation Index contributes to the creation of an environment in which innovative factors are continually valued.

The core of the GII report consists of ranking innovative capabilities and innovation results in the economies of indexing countries. Over the last ten years, this index has been established in practice as a leading innovation reference. A more fundamental analysis of the human factor, which is behind innovation, is essential for the development of development policies. Recognizing the key role of innovation, as a driver of economic growth and prosperity, and the need for a broad horizontal vision of innovation, the Global Innovation Index includes indicators that go beyond traditional innovation measures, such as the level of research and development (Cornell University, 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

In order to determine the GII, as the type of output required by the state-level leadership for decision-making, there is a range of inputs that need to be processed in this regard:

1. Institutions.
2. Human capital and research.
3. Infrastructure.
4. Market sophistication and
5. Complexity of business.

The main characteristic for computing the GII is that the overall result is a simple average of the input and output subindices. Also, the Global Innovation Index collects data from more than 30 sources, covering a large spectrum of drivers of innovation and results. The standard framework of the resource is reviewed every year, in transparent workshops, to improve the way innovation is measured.

The primary goal of this research is to develop a tool that can serve as a parametar for underdeveloped countries and countries in transition when directing and implementing state policy, by presenting the correlation coefficients between the SSI, EPI and GII in the countries of the region that are in the transition process. That refers to improving the sustainability of the society and the ecological effects efficiency, where the development of innovations, the overall well-being and the balance of modern systems imposes itself as the ultimate goals.

Review of literature

Inclusive green economy or ’green growth’ primarily refers to environmentally susatainable economic production and consumption that also embrace equity in distribution of resources. Green growth is defined as – ’improved human wellbeing and social equity, while significantly reducing environmental risks and ecological scarcities’ (Kumar, 2017).

According to Zugic (2014), countries in the transition process are continually addressing two basic economic issues: macroeconomic stability and structural transformation of the market environment. Macroeconomic stabilization should contribute to the establishment of a distorted economic balance, and solving the problem of structural transformation should enable these countries to efficiently and rationally engage. This requires the formulation of a macroeconomic policy with more efficient measures, mechanisms and instruments for the realization of the chosen objectives and tasks.

The more the economy of a country is integrated into the globalized world economy, the more it has to compete with other countries by simultaneously transforming its economic structure towards a green economy. This competition pressure has the advantage that industry does not look towards the domestic market for customers for its new products but also tries to find new customers for its new green products on the world market. The country is not just reliant on the shielded home market and can initiate a co-evolution process of technological, organizational and institutional changes (Schlor, 2017).

Climate change is a global concern. Growth in energy-related CO_2 emissions has been identified as the main cause of climate change. The growing trend will persist if no sustantial efforts on constraining emissions are made (Wang et al., 2018).

Based on Vaninsky’s research (2018), data combined on energy consumption from nuclear, hydroelectric, and other renewable sources, are refered to as clean energy since

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

CO_2 emissions are zero, or very small as compared to fossil fuels. As follows from the data, there are three main GDP producers in the world: OECD (The Organization for Economic Co-operation and Development is a forum of 35 democracies comprising ecnomomies - website http://www.oecd.org provides details) Europe, China and USA, with their GDP shares equal to 18.88%, 16.43% and 15.94%, respectively. They are also the greatest polluters, contributing 12.08%, 27.08% and 16.42% of the world's CO_2 emissions, respectively. It should be mentioned that while Chinese and US economies' CO_2 emissions exceed their GDP shares, the emissions are much less in the OECD Europe's economies. The energy from clean sources is mostly consumed in OECD Europe (34.74%) and the US (30.61%), comprising 65.35% of the total.

At the very begining of their research, Djekic et al. (2018) state that in line with the increased global attention of environmental and sustainable impact of the food chain, transportation environmental impacts became polemic tools in various environmental and food policies, and from an enviromental point of view, some authors assume that transportation of trade goods enhances over 20% of total global CO_2 emissions.

Barnhart's et al. (2016) approach offers several advantages for informing conservation targeting decisions. Constructed from multi-input/multi-output productions models, the EPI can accommodate multiple agricultural outputs and types of pollution. The distance functions used to construct the EPI allow for empirical estimation of the production technology without imposing a functional form on the relationship between inputs, outputs and environmental effects. This is particulary useful for identifying and ranking low-performing sites for targeting decisions.

Frank et al. (2016) propose a new methodological framework which allows companies to preform their own benchmark analysis to understand how they are positioned regarding environmental performance in comparison with their competitors. They suggested framework that can help to improve environmental competitiveness within the industry and thereby contribute to environmental sustainbility of the sector.

Also, Zuo et al. (2017) use the environmental performance index to indicate the environmental scores of China's 30 provincial administrative regions, which were developed from the pilot Environmental Performance Index that was published in 2002. Their studies provide useful environmental sugestions for local government.

Sustainable development measuring initiatives have reached such volume that perhaps a sustainable development index revolution is now warranted. As with all rapidly growing academic and professional topics, theoretical and applied research can become so focused on improving accuracy of current practices that they often lose sight of practical application. In this regard, measuring progress toward sustainbility now resembles true cost accounting rather than rapidly employable tools useful for the developed and developing world alike (Shaker, 2018).

The SSI covers sustainability in its broadest sense, including social, environmental, ecological and institutional aspects, while most other indexes do so only partly. One of the main objections to the SSI is the aggregation of all indicators into one single figure for the index. Should one only consider this figure in isolation, the results may be misleading and can easily be misused (Van de Kerk et al., 2008).

Many rapidly growing economies reveal similar findings, with some exceptions. According to the Dual Citizen LLC (2016), India, Bangladesh and Senegal had GDP growth between 6-8% in 2016. On the other hand, these countries perform poorly on the new global green economy index, raising the question of what kind of growth these nations are realizing and how sustainable it is. Interestingly, Cambodia realized the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

highest improvement on the global green economy index in 2016 compared to 2014. Given that Cambodia is projected to grow at 7% in 2016, it represents a possible model for how green growth can be integrated in similar developing countries. These overall findings are just the beginning of the snapshot that provides on the state of the global green economy today and the progress countries are making within it.

Dasic (2014) in his research states that the energy security is essential for sustainable economic development and the stability of the country. The inability to access energy resources causes serious economic disturbances and political instability. In the conditions of the global economy, disruptions are easily transmitted and it is, therefore, essential to permanently provide energy resources and make an effort to reduce the so-called energy poverty most affecting countries in transition (the countries of Southeastern Europe) and third world countries. The problem can be solved by the introduction of alternative or renewable energy sources. This greatly alleviates the problem, with the positive effects of helping local economic development and reducing the greenhouse gas emissions. However, for a successful solution, there is a need for awareness of the existence of problems, political will and the ability to implement the solution. South-East European countries are characterized by a good geostrategic position, which should be used in the field of energy in order to achieve the strategic goals of regional development.

By looking at the previous research, the results of the correlation between the index of sustainability, green economy, innovations, etc. were tested, both in the economies in the transition process, and in the developed economies, which significantly helped this research process, on the question of knowledge, on the one hand, and on the other hand, the systematization of the information provided.

For instance, the results of Van de Kerk et al. (2008) research show that one of the main objections to the SSI is the aggregation of all indicators into one single figure for the index. Should one only consider this figure in isolation, the results may be misleading and can easily be misused. This objection is inextricably bound up with aggregation into one final figure. It stresses the importance of presenting all the results of the SSI - values of all indicators and categories - in a transparent and easily understandable way. Since the ultimate goal is to achieve a score of 10 - expressing full sustainability for each indicator - there can be no trade-off between the indicators or categories.

A study of 30 Western Hemisphere nations was presented in Shaker (2018) research shows that, its overall goal was to create the first mega-index of sustainable development (MISD), with the aim to improve humanity’s ability to calculate progress toward sustainability through an inductive approach. In doing so, 31 known indices were reduced into seven underlying dimensions of sustainable development, then normalized 0–100, and aggregated by their geometric mean. The seven orthogonal axes (latent dimensions) were subjectively articulated as: (1) socioeconomic well-being synergies; (2) economic freedom and democracy; (3) environmentally efficient happiness; (4) ecosystem well-being; (5) peace to economic vulnerability tradeoff; (6) natural resources protection; and (7) environmental stewardship and risk resilience. Overall, this study found that the underlying socioeconomic themes of sustainability dwarfed environmental themes, signifying a greater need for more simple, accurate, and scaleless (spatial and temporal) biogeophysical indicators. Using Pearson’s correlation and bivariate ordinary least squares (OLS) regression, 11 common development indicators were then explored regarding collinearity and explanatory power of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

sustainable development dimensions and MISD. In sum, winning countries were characterized by low population density, increased forestland, decreased urban, and larger country area. The presented evidence is sufficient to suggest that just a few common and freely available indicators could eventually capture all present dimensions of sustainable development.

The aim of Latif et al. (2017) research as to develop an interactive model to develop the sustainability index for small and medium scale manufacturing industry.

In addition, Almeida et al. (2016) claim that Composite Index of Environmental Performance and Environtmental Performance Index models have initial objectives that guide all following steps, and there are many available variables that can be used to construct an ecological composite index. Some methodological techniques are used in each phase and the choices of the best methods were made based on theoretical background. Based on the three cited factors, the Composite Index of Environmental Performance and Environtmental Performance Index models are decomposed and analyzed in order to determine possible differences and similarities. The Composite Index of Environmental Performance and Environtmental Performance Index indicators have similar essential goals, which are to measure and rank environmental performance by country, although the focuses are different due the methods used to aggregate single variables. The first tool focuses on five dimensions, and the second focuses on nine environmental policy categories and two objectives ecological issues. In other words, the information provided by one, can be expanded by the other index. On the other hand, it is only possible to compare both indexes at the final stage when they are finished, and it is not possible to compare within subcategories. For example: it is impossible observe pressure on the ecosystem using the EPI, and it is impossible to assess ecosystem vitality using the CIEP, because they are aggregated through different categories. Both composite indexes analyzed use a positive approach because they measure performance, and thus the higher the value, the better are the results.

Also, Zuo et al. (2017) use EPI to evaluate China's environmental performance at the provincial level and then identifies weaknesses inenvironmental management for the purpose of improving government efficiency and solving severe environmental problems.

Crespo et al. (2016) explain the use of GII in their research. Their study should stimulate new research projects that compare the performance of innovation systems that distinguish between different levels of economic development. For instance, an extension might keep the sample together or might split the country sample into more disaggregated groups in order to understand the alternative recipes that could lead countries to high innovation performance. Another possible development deals with asymmetric causality. This approach might lead to better understanding of the causal conditions that lead countries to low innovation performance.

Materials and methods

The methodology of this article is based on the implementation of the sustainability index, which consists of the SSI and the EPI, and their connection with the GII, or the establishment of mutual correlation.

The subject of correlation analysis is to examine the mutual strength of the relationship and the dependence between sustainability and innovation. The selected variables are the SSI, the EPI and the GII. The examination of the direction and strength

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

of the interaction of these three indexes is carried out on the basis of correlation analysis. It is also implemented on a sample of ten countries: Albania, Bosnia & Herzegovina (B&H), Bulgaria, Croatia, Hungary, Macedonia, Montenegro, Romania, Serbia and Slovenia (see *Figure 1*).

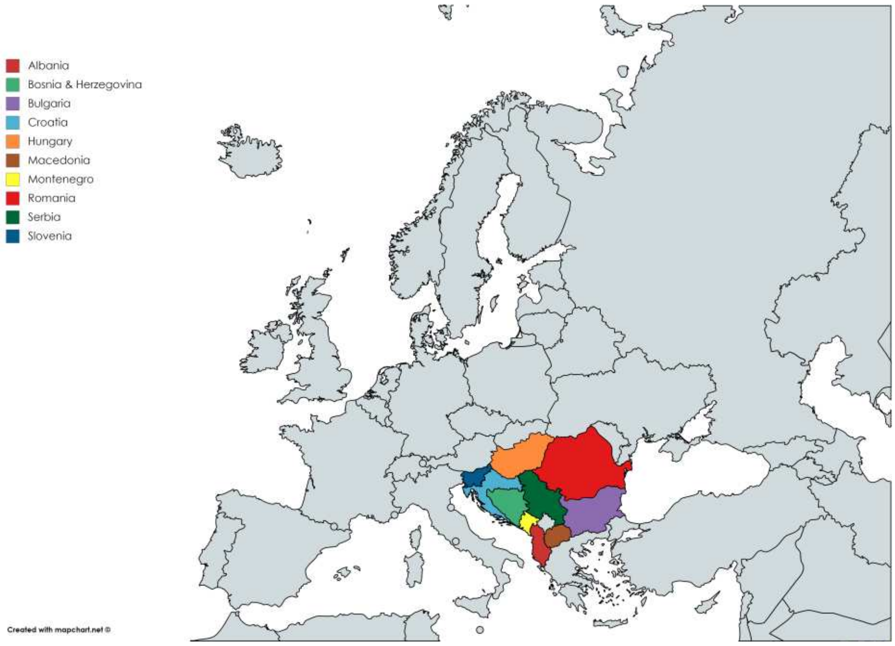

Figure 1. *The location of the countries involved in the presented study*

Classification (systematization) is the process of organizing information into categories or classes so that data can be more clearly analyzed or understood. Therefore, this research carried out the classification of the countries of Central and Eastern Europe, which are the subject of research, according to the following (*Table 1*).

Table 1. *Country classification according to two different criteria*

Country	Criterion	
	EU Membership Status	**Economy Development Status[a]**
Albania	Candidate	Economy in transition
Bosnia & Herzegovina	Potential candidate	Economy in transition
Bulgaria	Member	Developed economy
Croatia	Member	Developed economy
Hungary	Member	Developed economy
Macedonia	Candidate	Economy in transition
Montenegro	Candidate	Economy in transition
Romania	Member	Developed economy
Serbia	Candidate	Economy in transition
Slovenia	Member	Developed economy

[a] Information Source: Development Policy and Analysis Division of the Department of Economics and Social Affairs of the United Nations Secreteriat

Thus, the classification of countries in this survey will be based on the status of membership in the European Union, on the one hand, or on the basis of economic development, on the other.

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DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

The time interval to be tested is from 2010 to 2016. Testing the direction and strength of the interconnection will be expressed by the coefficient, using the Pearson linear method. It is a covariance of standardized variables X and Y. It is calculated using the following formula:

$$r = \frac{\sum_{i=1}^{n} x_i y_i - n\overline{x}\overline{y}}{n\sigma_x \sigma_y} \quad \text{(Eq.1)}$$

Or alternatively:

$$r = \frac{\sum_{i=1}^{n} x_i y_i - n\overline{x}\overline{y}}{\sqrt{\left(\sum_{i=1}^{n} x_i^2 - n\overline{x}^2\right)\left(\sum_{i=1}^{n} y_i^2 - n\overline{y}^2\right)}} \quad \text{(Eq.2)}$$

where:

- n is the sample size.
- X_i, Y_i are the sample points indexed with i.
- σ_x is standard deviation of X.
- σ_y is standard deviation of Y.

and the sample mean calculation would be

$$\overline{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \;, \quad \overline{y} = \frac{1}{n}\sum_{i=1}^{n} y_i \quad \text{(Eq.3)}$$

The coefficient takes values from a closed interval between -1 and 1. The zero value indicates that there is no linear correlation; the value plus one denotes a perfect positive fit, and minus one shows a perfect negative fit. The value of the coefficient is closer to 1, the linear bond is stronger. The lower value of the coefficient does not necessarily indicate a weak link between the variables, since there may be a very strong correlation between the variables, but the curvilinear, so the application of the linear coefficient of correlation is not appropriate in this case (Buturac and Ignjatijević, 2017).

Data from the Global Innovation Index Report (2010-2016), the Environmental Performance Index Report (2010-2016) and the results of the Sustainable Society Foundation research (2010-2016), are taken as information source, based on which this research was conducted.

Sustainable Society Index (SSI)

In the SSI, the parameters of human well-being and environmental performance are integrated. These are the goals that each country should strive for, since economic well-being, without the previous two, can not be sustainable.

For the SSI only public data sources that are current and available are used. However, data reliability still leaves room for arbitrariness. Especially in the production

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

of time series, processors are faced with many irregularities and obstacles in the creation of data. This problem can be reduced over time, as the significance of statistical data is now generally recognized.

Aggregation is an abstraction in which the connection between two objects or more object types is treated as an object at a higher level of abstraction. Because at the same time it is an object and a connection, aggregation is often referred to as a mixed type of object-connections. Aggregation objects are called aggregation components. For the aggregation of parameters of the SSI, a geometric average is used. The arithmetic average offers the possibility of compensation, where the low value of one indicator can be compensated by the high value of the other indicator. Since sustainability does not allow for compensation, it is best to use geometric compensation.

Van de Kerk et al. (2008) in their research demonstrated that the SSI is a simple instrument for assessing a country's sustainbility. The SSI, based on a solid definition, shows at a glance the present level of sustainability of a country and the distance to full sustainability. The SSI offers a country a practical tool for defining targets on its way to sustainability and for monitoring the progress over time.

The SSI calculation methodology

For lack of a scientific basis for the attribution of different weights to the indicators, every indicator has recieved the same weight for the aggregation into dimensions. In the data tables it is presented the averages per country. Thus one can notice the impact of weighting per person or per country. For each indicator the formula is shown, in which F(x) is the indicator score and X the value of the raw data (*Table 2*). In addition the range of validity is indicated.

Because of all of the above, the values of the three parameters are not aggregated into the total index, so that the researcher, could decide either for individual data, or for a summary result. Taking previously into consideration, the Sustainable Society Index data are trusted data, not the calculation of the authors, except the fact that we decided on the aggregate score of the index, which is presented in *Table 3*.

In the period from 2010 to 2016, the highest progress, according to the results of the conducted measurements (see *Table 3*), was achieved by Romania with a coefficient of 1.5, while the smallest progress was achieved by Albania with a coefficient of 0. The negative trend was achieved by the two countries, Montenegro and Slovenia (-0.4 and -0.2), however, this data should be taken with reserve, given that, according to the latest measurements from 2016, Slovenia is the second country in the region (coefficient of the Sustainable Society Index: 6.2), immediately behind the leading Romania (6.8).

Environmental Performance Index (EPI)

According to Almeida and Garcia-Sanchez (2016), EPI is one of the most known indexes, and it is used in several studies and frequently receives innovations and improvements. The EPI has published an annual report that ranks 180 countries, according to ecological performance comprised of twenty two four variables, combined into ten policy issue that are aggregated in two different groups (health of the environment and vitality of ecosystems).

These measures enable the quantitative expression of the effectiveness of the implementation of environmental performance policies at the national level. These results highlight advanced countries and those lagging behind in this segment, giving insights into best practices, and providing guidance for countries that are striving to be

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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leaders in environmental sustainability. The innovations in EPI data from 2018 and the methodology generated a new ranking based on modern approaches in natural science and analysis.

According to research of Yale University presented in EPI Report (2018), this index draws attention to the issues on which policy makers need to take further initiative. Although the EPI provides an analytical framework suitable for a more rigorous creation of an environmental policy, it also reveals and points to more serious failures through data. According to the Foundation for Environmental Protection, EPI projects that data collection, reporting and verification is necessary, through a series of ecological problems. Existing deficiencies are particularly evident in the areas of sustainable agriculture, water resources and waste management. Therefore, data systems are exceptional support for better management of sustainable development challenges.

Table 2. *Calculation formulas of the SSI*

Indicator number	Indicator Name	Formula
1	Sufficient food	F(X)=(100-X)/10 if 5≤X≤100 F(X)=10 if X<5
2	Sufficient to drink	F(X)=X/10 0≤X≤100
3	Safe Sanitation	F(X)=X/10 0≤X≤100
4	Education	F(X)=X/10 if 0≤X≤100 F(X)=10 if X>100
5	Healthy Life	F(X)=((X-20)/60)*10 20≤X≤80
6	Gender Equality	F(X)=X*10 0≤X≤1
7	Income Distribution	F(X)=EXP(-0,1*(X-4,5))*10 If 4,5≤X≤168, F(X)=0 if X>168
8	Population Growth	F(X)=-0,0067*X^2 -0,4333*X+8 if -5<X<15 F(X)=0 if X≥15, F(X)=10 if X<-5
9	Good Governance	F(X)=((X+15)/30)*10 -15≤X≤15
10	Biodiversity	F(X)=(F(X1)+F(X2))/2
11	Renewable Water Resources	F(X)=(100-X)/10 if 0≤X≤90 F(X)=0 if X>90
12	Consumption	F(X)=10-3*X*2/1,8 if 0≤X≤2,7 F(X)=0 if X>2,7
13	Energy Use	F(X)=-2*X+10 if X≤5 F(X)=0 if X>5
14	Energy Savings	F(X)=25*X+5 if -0,2≤X≤0,2 F(X)=0 if X<-0,2, F(X)=10 if X>0,2
15	Greenhouse Gases	F(X)=10-X if 0≤X≤10 F(X)=0 if X>10
16	Renewable Energy	F(X)=X/10 if 0≤X≤100 F(X)=10 if X>100
17	Oganic Farming	F(X)=9*(1-EXP(-0,25*X))+1
18	Genuine Savings	F(X)=10*ARCTAN(0,2*X)/π+5
19	Gross Domestic Product	F(X)=10*(1,01-EXP(-0,000065*X)) if 0≤X≤70000 F(X)=10 if X>70000
20	Employment	F(X)=EXP(-0,1*X)*10 if 0≤X≤60 F(X)=1 if X>60
21	Public Debt	F(X)=-3,8*ARCTAN(0,06*X-3,5)+5 if 2,5≤X≤117 F(X)=0 if X≥117, F(X)=10 if X<2,5

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DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

Table 3. *Sustainable Society Index of the selected countries in transition*

Country / Yr	2010	2012	2014	2016	Country / Yr	2010	2012	2014	2016
Albania	5.90	5.90	5.80	5.40	Macedonia	4.90	5.70	5.40	5.40
B&H	4.50	4.60	4.70	4.80	Montenegro	5.60	6.00	6.10	6.00
Bulgaria	5.70	6.00	5.90	6.10	Romania	6.10	6.40	6.50	6.80
Croatia	6.00	6.20	5.80	5.90	Serbia	5.40	5.30	5.20	5.30
Hungary	5.80	5.90	6.00	6.20	Slovenia	6.60	6.60	6.10	6.30

Source: http://www.ssfindex.com/data-all-countries/

The EPI calculation methodology

To create the EPI, raw datasets is transformed into comparable performance indicators, which requires standardizing raw values according to population, land area, gross domestic product, and other common units of measurement. Then statistical transformations are performed to normalize data distributions and ensure weights assigned in the aggregation phase affect data as intended.

The transformed data are used to calculate performance indicators. EPI indicators are developed using a 'proximity-to-target' methodology, which assesses how close each country is to an identified policy target. The targets are high performance benchmarks defined primarily by international or nation policy goals or established scientific thresholds.

A high performance benchmark can be determined through an analysis of the best-performing countries. Scores are then converted to a scale of 0 to 100 by simple arithmetic calculation, with 0 being the farthest from the target and 100 being the closest. In this way, scores convey analogous meaning across indicators, policy issues, and throught the EPI.

Each indicator is weighted within the issue categories to create a single issue category score. These weightings are generally set according to the quality of the underlying data, as well as an indicator's relevance or fit for assessing a given policy issue. If the underlying data for a particular indicator is less reliable or relevant than others in the same issue category, the indicator will be weighted less. Policy issues are weighted approximately equally within their objective. Contigent on the data strength in each category, slight adjustments to this weighting can be made (Yale University, 2018).

Countries only receive scores for issues that are 'material' or relevant to their environmental performance. The exclusion of certain issues for some countries proportionally increases the weight on other indicators within a policy issue and objective. The two objectives, Environmental Health and Ecosystem Vitality, are weighted equally to achieve a single value, the EPI score, for each country.

$$EPI = \sum_{i=1}^{n}(WiXi) \quad \text{(Eq.4)}$$

where

- i is total number of the indicator.
- n is total number of all indicators.
- Wi is weight of the i_{th} indicator.
- Xi is standard value of the i_{th} indicator (Zuo et al., 2017).

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It is interesting to compare the EPI trend in the countries of the region, taken as a sample in this survey. If we take into consideration the indicators from 2016, it can be established that most of the countries in the region are in front of Serbia, when index indices are considered (see *Table 4*). The three countries have an unfavorable outcome compared to the Republic of Serbia, which are Albania, B&H and Macedonia.

Table 4. *Environmental Performance Index of the selected countries in transition*

Country / Yr	2010	2012[a]	2014	2016	Country / Yr	2010	2012[b]	2014	2016
Albania	71.14	65.85	54.73	74.38	Macedonia	60.60	46.96	50.41	78.02
B&H	55.90	36.76	45.79	63.28	Montenegro[c]	/	/	78.89	78.89
Bulgaria	62.50	56.28	64.01	83.40	Romania	67.00	48.34	83.24	83.24
Croatia	68.70	64.16	62.23	86.98	Serbia	69.40	46.14	78.67	78.67
Hungary	69.10	57.12	70.28	84.60	Slovenia	65.00	62.25	76.43	88.98

Source: Environmental Performance Index Report (2010-2016). Publisher: Yale University: Center for Environmental Law & Policy
[a] Scores and rankings for the 2012 EPI cannot be compared with scores and rankings from eralier releases of the EPI owing to changes in data and methodology
[b] Ibid.
[c] No data available for Montenegro in the specified years

As it is presented in *Table 4*, between the countries of the region, which are highly ranked on the world ranking list, the difference in the index coefficient is negligible (88.98 - 83.24). According to these results, Slovenia is ahead, followed by Croatia, Hungary, Bulgaria and Romania.

A more sensitive difference in the value of the measured index was recorded in Montenegro (78.89), Serbia (78.67) and Macedonia (78.02). The following countries are followed by Albania (74.38) and B&H (63.28).

Data source for the EPI come from international organizations, research institutions, academia, and government agencies. These sources use a variety of techniques, including (Yale University, 2018):

- Remote sensing data collected and analyzed by research partners.
- Observations from monitoring stations.
- Surveys and questionnaires.
- Academic research.
- Estimates derived from both on-the-ground measurements and statistical models.
- Industry reports; and
- Government statistics, reported either individually or through international organizations, that may or may not be independently verified.

Taking this into consideration, the Environmental Performance Index data are trusted data, not the calculation of the authors.

The Global Innovation Index (GII)

The Global Innovation Index is important for achieving economic progress and competitiveness in the market, both in developed countries and in countries in transition. Many countries, when implementing their economic policies, include this index in the center of state strategies.

Also, one of the reasons for introducing this kind of measurement of the achieved results in terms of innovation in the economy of a particular country is to provide timely

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and accurate data to the state leadership, which is an important prerequisite for decision-making and improvement of innovation policies. This does not mean that the GII is merely the ranking of the economies of countries that are subject to indexing, based on certain parameters, but also in this way to assess the conditions that incite the potent trend of innovation, that is, to assess the outcome of the introduction of concrete improvements (Cornell University, 2017).

Although the final results are presented in several forms, in order to provide a better comparison of indicators, the GII is more focused on understanding and analyzing innovations, that is, identifying targeted policies, good practices and instruments that help improve innovation. Previously, comprehensive indexing is used at the level of the index, subindex or at the level of individual data used to monitor the performance over a specific time period, as well as to develop in relation to countries in the region.

According to the Cornell University's Global Innovation Index Report (2017) an innovation is the implementation of a new or significantly improved product (good or service), a new process, a new marketing method, or a new organizational method in business practices, workplace organization or external relations. The Global Innovation Index is not meant to be the ultimate and definitive ranking of economies with respect to innovation. Measuring innovation outputs and impacts remains difficult, hence great emphasis is placed on measuring the climate and infrastructure for innovation and on assessing related outcomes. Today innovation capability is seen more as the ability to exploit new technological combinations; it embraces the notion of incremental innovation and 'innovation without research.

The GII calculation methodology

The Global Innovation Index is an evolving project that builds on its previous editions while incorporating newly available data and that is inspired by the lateset research on the measurement of innovation. The GII relies on two sub-indicies – the Innovation Input Sub-Index, and the Innovation Output Sub-Index – each built around key pillars (*Figure 2*).

Five input pillars capture elements of the national economy that enable innovative activities: (1) Institutions, (2) Human capital and research, (3) Infrastructure, (4) Market sophistication and (5) Business sophistication. Two output pillars capture actual evidence of innovation outputs: (6) Knowledge and technology outputs and (7) Creative outputs (Cornell University, 2017).

Each pillar is divided into sub-pillars and each sub-pillar is composed of individual indicators (80 in total). Sub-pillar scores are calculated as the weighted average of individual indicators; pillar scores are calculated as the weighted average of sub-pillar scores. Four measures are then calculated:

- Innovation Input Sub-Index: is the simple average of the first five pillar scores.
- Innovation Output Sub-Index: is the simple average of the last two pillar scores.
- The overall GII score is the simple average of the Input and Output Sub-Indicies.
- The innovation Efficiency Ratio is the ratio of the Output Sub-Index over the Input Sub-Index.

Taking previously into consideration, the Global Innovation Index data are trusted data, not the calculation of the authors.

Expectedly, the values presented in the *Table 5* show that innovation in the EU Member States is more prevalent. The largest coefficient in 2016, from the countries that are the subject of consideration, has Slovenia (45.97) as a strong innovator, while the lowest coefficient is Albania (28.38). The countries who scored coefficient values among this range (28.38 - 45.97) also have their innovation status – moderate and modest innovators. It is interesting that in the period from 2010 to 2016, the GII registered a negative trend (Hungary, Serbia, Montenegro, B&H, and Albania), while in the period after this year, there was an increase. The status of innovators, in fact, follows the status of development and membership status in the European Union, which are previously set criteria for the classification of countries that are the subject of this research.

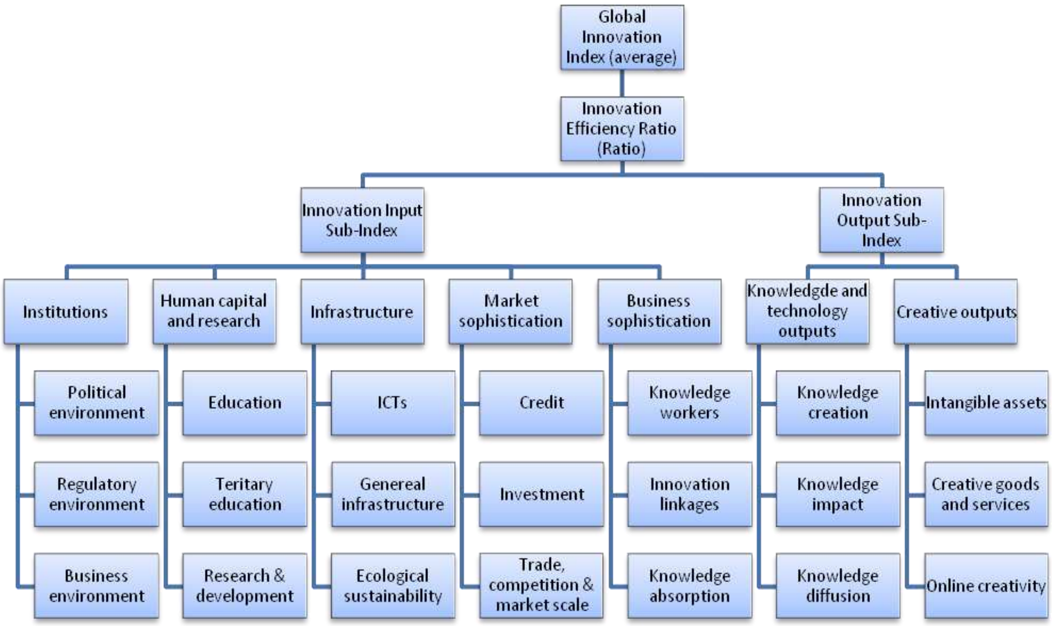

Figure 2. *The Global Innovation Index Framework*

Table 5. *Global Innovation Index of the selected countries in transition*

Country / Yr	2010[a]	2012	2014	2016	Country / Yr	2010[b]	2012	2014	2016
Albania	2.86	30.40	30.47	28.38	Macedonia	2.89	36.20	36.90	35.40
B&H	2.58	34.20	32.43	29.62	Montenegro	3.08	40.10	37.01	37.36
Bulgaria	3.26	40.70	40.74	41.42	Romania	3.22	37.80	38.08	37.90
Croatia	3.28	40.70	40.75	38.29	Serbia	2.68	40.00	35.89	33.75
Hungary	3.54	46.50	44.61	44.71	Slovenia	3.80	49.90	47.23	45.97

Source: https://www.globalinnovationindex.org/home
[a] Scores and rankings for the 2010 GII cannot be compared with scores and rankings from later releases of the GII owing to changes in data and methodology
[b] Ibid.

Results

Based on previously explained indicators of sustainable society, environmental performance and global innovations, in the first part of the research, the center was moved to determine and describe the coefficient of mutual correlation between the SSI and the GII.

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DOI: http://dx.doi.org/10.15666/aeer/1702_14991516

Since this is parametric statistics, where the coefficient of variation is less than 30 (in the concrete case SSI = 10.1100775091025, GII = 15.5680187865172), the Pearson method of correlation was applied, thus calculating the correlation coefficient of these two indices.

The correlation coefficient of the SSI and the GII is 0.749413095249505, that is, 0.75, which means that the correlation of these two parameters is above all: positive (> 0.50) and then strong.

In *Figure 3*, the correlation of these two indices is shown, along with the variation coefficients, also mentioned. Based on this, we conclude that a low SSI value (living standard of the population, environmental protection and economic well-being) also contributes to a low GII value. Thus, the underdevelopment of innovation is a result of low living standards and underdeveloped economies. This group of countries includes Albania and B&H.

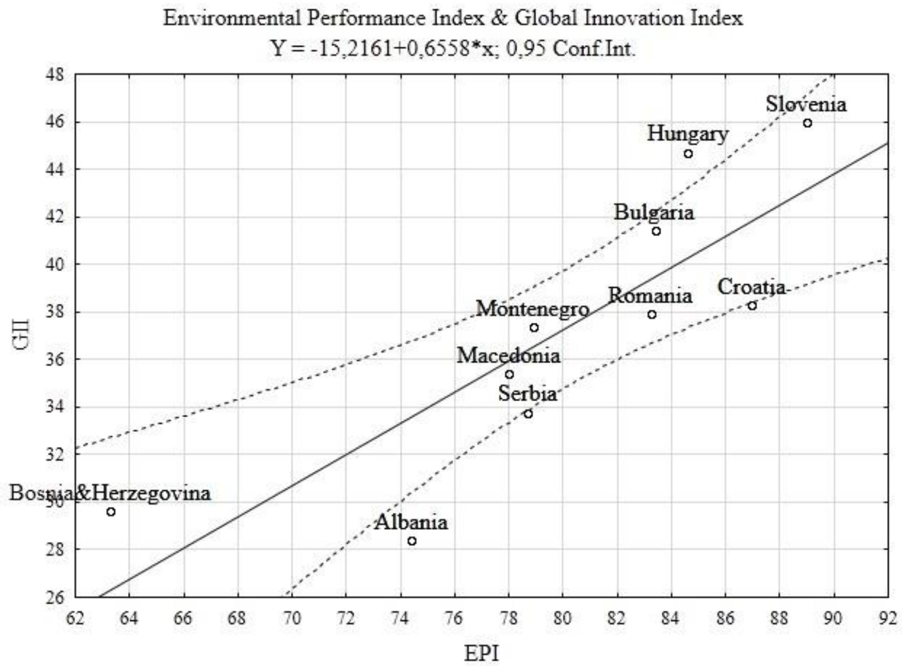

Figure 3. *Correlation between EPI & GII of the selected countries in transition*

The group of non-EU countries is led by Montenegro, followed by Macedonia and Serbia. Also, on the basis of an analysis of the mutual correlation of the index, we conclude that the countries of the regions that are members of the EU, have significantly better results than the rest of the considered sample. This group is led by Slovenia and Hungary, followed by Bulgaria, Croatia and Romania. The results of this group confirm that economic prosperity and high living standards of the population directly influence the level of innovation in the domestic economy.

Smilarly, comparing the Environmental Performance Index to the Global Innovation Index, we arrive at the results, and interpretations of the results close to the previous one.

Since in this case the coefficient of variation <30 (EPI = 9.22410037158007, GII = 15.5680187865172), the Pearson method of correlation was used. Correlation

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coefficient of the Environmental Performance Index and Global Innovation Index is 0.834337304281034. The correlation between these two parameters is positive, and more pronounced, than in the previous case.

The division of countries by groups is identical to the previous interpretation of the results. Furthermore, the low efficiency of environmental protection, that is, the low level of people's awareness of the need for environmental protection, directly affects the low level of innovation in the economies of these countries that are in transition.

In this index clash, the results are even more dramatically determined (see *Figure 4*). The worst results are recorded by Albania and B&H, which still have the status of underdeveloped transition countries. A group of non-EU countries that are on the right path to achieving the goals of the set strategies and policies is led by Montenegro, followed by Macedonia and Serbia. Far ahead are the EU Member States, led by Slovenia and Hungary, followed by Bulgaria, Croatia and Romania.

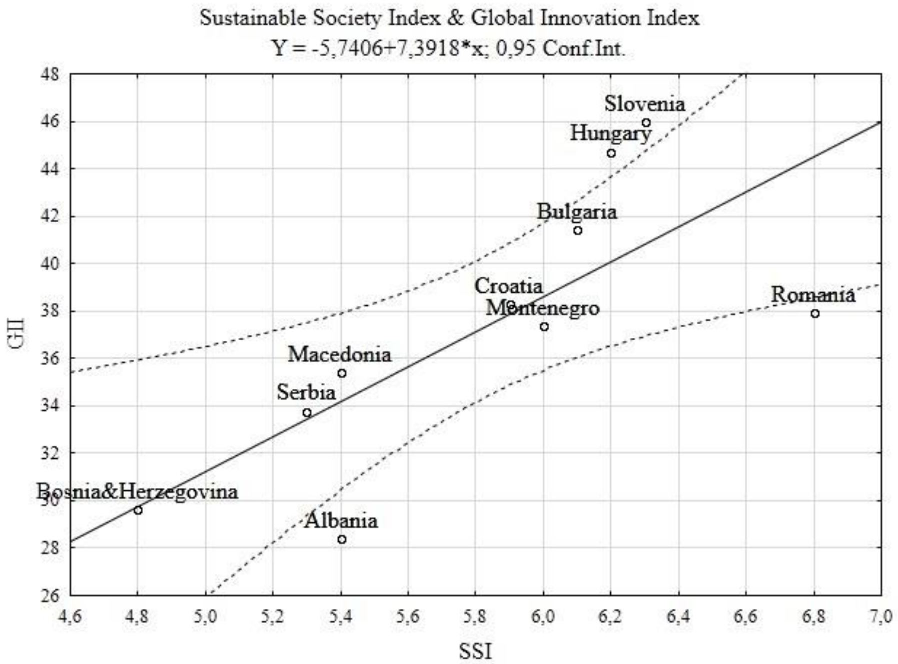

Figure 4. *Correlation between SSI & GII of the selected countries in transition*

The example of Slovenia, perhaps, is the best presentation of what each country in transition is tending to. The achieved high, desired results in the field of environmental protection directly influence the higher financial allocations and investments in innovations in the field of economy.

Discussion

Taking into account all the results obtained, the conclusion is that the paramteres in the subject of the conducted research are interconnected, as evidenced by the high coefficient of correlation. Looking at the individual results of countries in the region, they can be grouped according to the status of countries for EU membership.

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On a sample of ten countries in the region, which are related to a common transition, there is still a certain grouping, which is noticed in the graphic representation of the obtained results. Namely, the first group of countries are the countries that are in the status of a potential candidate for EU membership. This group belongs to B&H. However, according to unfavorable results in the considered areas of social activity, although in the status of EU candidate country, Albania joins B&H on the ranking list of the Sustainability and Innovation Index.

The next group of countries is a group of countries that are in the process of joining the EU. This group is led by Montenegro, followed by Serbia and Macedonia (although the country with the candidate status).

Expectedly, at the top of the list of countries in the region, Slovenia is the most developed country with the largest investments in innovation, which is the result of a favorable economic situation in the economy of this country, as well as high awareness of the need for environmental protection. Other EU member states are: Hungary, Bulgaria, Romania and Croatia.

On the other hand, a high degree of correlation of parameters, whose interdependence is researched, indicates interaction, and encouraging positive trends in the growth of innovation and improving the quality of life and living standards of the inhabitants of these countries. In order for the less developed countries in transition to reach the targeted level of sustainability of the society and the efficiency of environmental performance, it is necessary for the state leadership to direct and lead a more efficient policy towards the improvement of these areas. In this way, inevitably, as a consequence of this action, the development of innovations in the economies of these countries will be imposed, which, as the ultimate goal, represents the overall well-being and balance of modern systems.

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REMOVAL OF COD AND COLOR FROM REACTIVE RED 45 AZO DYE WASTEWATER USING FENTON AND FENTON-LIKE OXIDATION PROCESSES: KINETIC STUDIES

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(Received 21st Oct 2018; accepted 7th Jan 2019)

Abstract. The present study dealt with the removal of Reactive Red 45 (RR45) azo dye material through Fenton and Fenton-like oxidation processes. The effects of pH, Fe^{2+} and Fe^{3+} dosages, hydrogen peroxide (H_2O_2), concentration of colorant and temperature were evaluated. Removal of the colorant RR45 was monitored by considering color and Chemical Oxygen Demand (COD). Results showed that Fenton process is more efficient than Fenton-like processes in terms of color and COD removal efficiency. A removal efficiency of 91.1% of COD and 99.90% of color was obtained under optimum experimental conditions where initial solution pH was 3, the concentration of RR45 colorant was 200 mg L^{-1}, rates of Fe^{2+} and H_2O_2 were 75 mg L^{-1} and 400 mg L^{-1}, respectively, reaction temperature was 50°C and experiment time was 30 min. In addition, the first order, second order and Behnajady-Modirshahla-Ghanbery (BMG) models were applied and the best results were obtained by BMG model.
Keywords: *colorant, Fenton reaction, hydroxyl radical, H_2O_2, kinetic modelling*

Introduction

Today, industrialization is developing in line with the demand of increasing human population and consumption and several new industries are added to the existing industrial facilities depending on this increasing demand. Pollution parameters may vary depending on the types of industries and wastewater is produced with different characteristics.

The textile industry exhibits higher polluting characteristics in terms of wastewater volume and composition than other industrial sectors (Vandevivere et al., 1998). Water consumption in the textile industry ranges from 25 to 250 m^3 per ton of product (Chacón et al., 2006). Numerous different types of dyes are used in different industries for different purposes. Dyes contain different chemical structures. However, they are generally composed of two components, chromophore and function group. Chromophore is an important compound for the color of the dye. The functional group, on the other hand, allows dye to bind to cotton or wool yarn. Different types of functional groups are used for dyeing different types of textile materials (Kayıkooğlu and Debik, 2006). They are characterized in terms of their chemical structures as azo, triaryl methane, anthraquinone, heterocyclic and phthalocyanine dyes. They are also catagorised into six groups for their application methods as watt, reactive, direct, acidic, disperse and cationic dyes (Xu et al., 2004). In recent years, advanced wastewater treatment methods, known to be environmentally friendly in wastewater treatment, have been used since they can oxidize wastewaters which are hard to biodegrade to a more harmless form (e.g. carbon dioxide, water and inorganic salts) (Neyens and Baeyens,

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2003; Primo et al., 2008). Fenton process is one of these methods. In Fenton reaction, the hydroxyl radical ($OH^{\bullet}$) was shown to form after a series of reactions consisting of iron and iron salts and catalytic cracking of hydrogen peroxide (Harber and Weiss, 1934). This process is based on the formation of ($OH^{\bullet}$) under acidic conditions as a homogeneous catalytic oxidation process.

Fenton oxidation involves chemical mechanisms expressed by the following equations (*Eqs. 1-5*) (Sun et al., 2009a).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad \text{(Eq.1)}$$

$$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^{-} \quad \text{(Eq.2)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{-} + H^{+} \quad \text{(Eq.3)}$$

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + O_2 + H^{+} \quad \text{(Eq.4)}$$

$$H_2O_2 + OH^{\bullet} \rightarrow HO_2^{\bullet} + H_2O \quad \text{(Eq.5)}$$

As an overview, the Fenton process consists of four steps; pH adjustment, oxidation reaction, neutralization and precipitation reaction through coagulation. In this way, the organic materials are removed in two steps, oxidation and coagulation (Kang and Hwang, 2000). According to the studies conducted in literature, Fenton process is widely used in removing organic materials (Fan et al., 2013), reducing toxicity (Zhang et al., 2014), phenol removal (Mofrad et al., 2015), textile wastewater treatment (Latif et al., 2015), color removal (Bouasla et al., 2010; Ertugay and Acar, 2017) and removal of organic matter from cosmetic waste water (Bayhan and Değermenci, 2017). In this study, Fenton and Fenton-like process studies were evaluated for color and COD removal of wastewaters from synthetic textile products prepared with colorant called commercially Reactive Red 45 and used in textile dyeing. In the experimental studies carried out, the effects of pH, rate of Fe^{2+} or Fe^{3+} salts and H_2O_2, initial dye concentration and temperature parameters on removal efficiency were investigated. Obtained data of kinetic studies were applied to the kinetic models, first-order, second-order and BMG models.

Materials and Methods

RR45 colorant was obtained from a commercial company, Duraner Boya A.Ş. The molecular weight of azo colorant (RR45) 802.1 g mol^{-1}, whose molecular is $C_{27}H_{19}ClN_7Na_3O_{10}S_3$. RR45 exhibits a complex structure together with azo aromatic groups and is used commonly in textile industry (Swarnkar et al., 2015). As the result of spectrum scanning of colorant, maximum absorbance was found to be 243 nm, 283 nm and 541 nm. Molecular structure of RR45 colorant is given in *Fig. 1*.

The experimental device was constituted of a perfectly agitated reactor in which a volume (600 ml) of solution was studied. In experiments, pH adjustment of the dye prepared in the desired concentration was performed first by adding diluted H_2SO_4 and NaOH. Following this stage, $FeSO_4.7H_2O$ or $FeCl_3.6H_2O$ and H_2O_2 were added and stirred fast for 3 min at 150 rpm and then slowly for 27 min at 50 rpm and the resultant

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mixture was filtered through 0.45 μm membrane filter. Color and COD measurements were then performed. Both experiments and analysis were repeated twice at the same conditions to ensure the accuracy of data and the averages values were given. Color measurements were conducted and values were registered at 541 nm wave-length spectrophotometrically. COD analyses were also conducted spectrophotometrically at 600 nm, 148°C and Merck Spectroquant TR320 according to Standard Methods (APHA 1998). *Table 1* gives the average COD values for the initial colorant concentration. In Fenton and Fenton-like processes; the investigated parameters and ranges are given in *Table 2*.

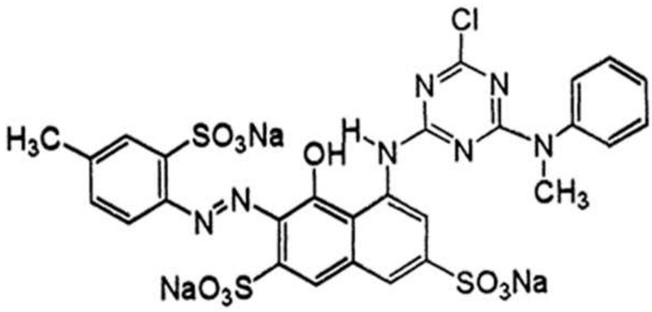

Figure 1. *Structure of RR45 dye*

Table 1. *Title of the table Average COD values for the initial colorant concentration*

Initial colorant concentration ($mg\ L^{-1}$)	**150**	**200**	**250**	**300**	**350**	**500**
Average COD value ($mg\ L^{-1}$)	115.08	135.10	186.99	219.44	264.87	326.52

Table 2. *Experimental parameters*

Process	**Parameters**	**Investigated parameters range**	**Fixed variables**
Fenton Process	**Fe^{2+} concentration ($mg\ L^{-1}$)**	25; 50; 75; 100; 125;150; 200	C_0=200 mg L^{-1}, H_2O_2=500 mg L^{-1}, pH=3, T=20^{0}C, t=30 min
	H_2O_2 concentration ($mg\ L^{-1}$)	100; 200; 300; 400; 500; 600	C_0=200 mg L^{-1}, Fe^{2+} =75mg L^{-1}, pH=3, T=20^{0}C, t=30 min
	pH	2.5; 3.0; 4.0; 5.0	C_0=200 mg L^{-1}, Fe^{2+} =75 mg L^{-1}, H_2O_2 is 400 mg L^{-1} T=20^{0}C, t=30 min
	Colorant concentration ($mg\ L^{-1}$)	150; 200; 250; 300; 350; 500	Fe^{2+}= 75 mg L^{-1}; H_2O_2= 400 mg L^{-1}; pH= 3; T= 20^{0}C; t= 30 min
	Temperature (^{0}C)	20; 30; 40; 50; 60	C_0=200 mg L^{-1}; Fe^{2+} =75 mg L^{-1}; H_2O_2 is 400 mg L^{-1}; t=30 min
Fenton-like process	**Fe^{3+} concentration ($mg\ L^{-1}$)**	25; 50; 75; 100; 125;150	C_0=200 mg L^{-1}, H_2O_2=500 mg L^{-1}, pH=3, T=20^{0}C, t=30 min
	H_2O_2 concentration ($mg\ L^{-1}$)	100; 200; 300; 400; 500; 600	C_0=200 mg L^{-1}, Fe^{3+} =75mg L^{-1}, pH=3, T=20^{0}C, t=30 min
	pH	2.5; 3.0 ; 4.0; 5.0	C_0=200 mg L^{-1}, Fe^{3+} =75 mg L^{-1}, H_2O_2 is 500 mg L^{-1} T=20^{0}C, t=30 min
	Colorant concentration ($mg\ L^{-1}$)	150; 200; 250; 300; 350; 500	Fe^{3+}= 75 mg L^{-1}; H_2O_2= 500 mg L^{-1}; pH= 3; T= 20^{0}C; t= 30 min
	Temperature (^{0}C)	20; 30; 40; 50; 60	C_0=200 mg L^{-1}; Fe^{3+} =75 mg L^{-1}; H_2O_2 is 500 mg L^{-1}; t=30 min

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Results and discussion

Effect of iron dosage

The dosage of the Fenton reactive in the Fenton and Fenton-like oxidation process plays a very important role in the degradation of organic substances and the determination of the total cost of the process. In general, as the dosage of Fe^{2+} and Fe^{3+} increases, degradation rate of organic materials increases. However, a very large increase in the Fe^{2+} and Fe^{3+} ions leads to an increase in the amount of total dissolved iron ions. Therefore, it is necessary to avoid the formation of excessive amount of sludge (Saini et al., 2017). Fe^{2+} and Fe^{3+} experiments were first carried out in the determination of color and COD removals in the Fenton and Fenton-like oxidation of RR45. For this aim, experiments were conducted by fixing samples prepared at a dye concentration of 200 mg L^{-1} to 500 mg L^{-1} H_2O_2 and adding Fe^{2+} and Fe^{3+} at the concentrations of 25, 50, 75, 100, 125, 150, 200 mg L^{-1}.

As the result of the experiments (*Fig. 2*), the largest removal yields were obtained at 100 mg L^{-1} Fe^{2+} concentration for Fenton oxidation and at 75 mg L^{-1} Fe^{3+} concentration for Fenton-like oxidation. In the Fenton process, when Fe $^{2+}$ concentration increased from 25 mg L^{-1} to 100 mg L^{-1} color removal yield continued at 98% level while COD removal yield increased from 55.2% to 80.31%. When Fe^{2+} concentration increases from 100 mg L^{-1} to 200 mg L^{-1}, COD removal yield decreases from 80.31% to 76.72% while color removal decreases from 98.69% to 82.13%. In the case of the Fenton-like oxidation, in all Fe^{3+} dosage experiments, color removal yield was obtained to be nearly 99% while in the same Fe^{3+} dosage range (25 mg L^{-1} to 75 mg L^{-1}) COD removal yield was between 63.80% and 74.10% and at 200 mg L^{-1} it decreased to 52.12%. Since no main factor is present to produce hydroxyl radicals in the absence of iron ion, an effective oxidation based on Fenton reaction cannot be achieved. As the Fe^{2+} and Fe^{3+} concentration increases, theoretically higher rate of $OH^{\bullet}$ radicals will be produced.

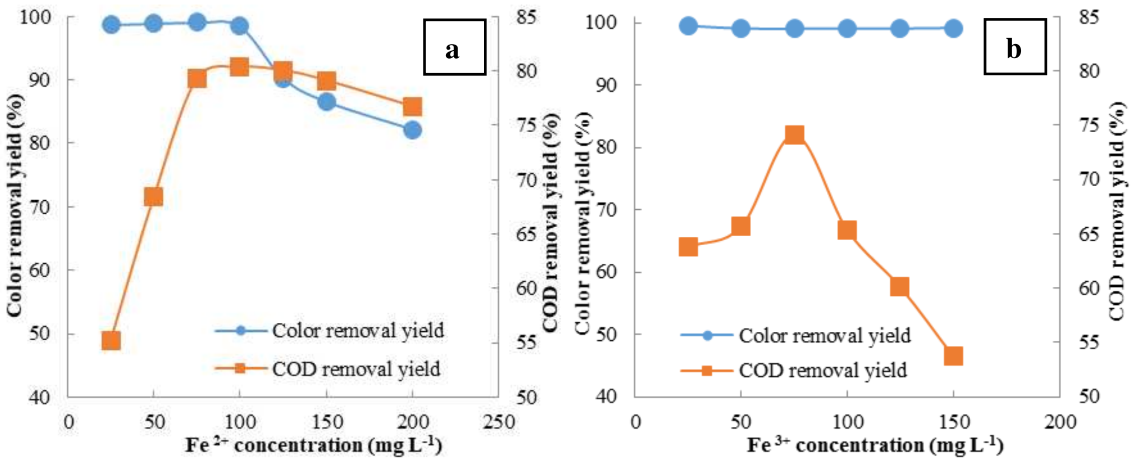

Figure 2. *Effect of iron doses on color and COD removal (a) Fenton and (b) Fenton-like processes (C_0=200 mg L^{-1}, H_2O_2=500 mg L^{-1}, pH=3, T=20°C, t=30 min)*

Such a situation is thought to affect unfavorably oxidation capacity of excessive Fe^{2+} concentration. It is known that in accordance with *Eq. 2*, higher concentrations of Fe^{2+}, can cause the consumption of $OH^{\bullet}$ radicals (radical scavenger effect) and thus

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decreasing removal yield (Tang and Chen, 1996; Lunar et al., 2000; Ntampegliotis et al., 2006; Pérez-Moya et al., 2007).

In addition, when solution media is acidic, higher Fe^{2+} concentrations can cause based on *Eq. 6*, and additional reaction resulting in the transformation of H_2O_2 into water (Tarr, 2003).

$$Fe^{2+} + H_2O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O \quad \text{(Eq.6)}$$

In Fenton process, as the result of experiments, at the concentrations of 75 mg L^{-1} and 100 mg L^{-1}, color and COD removal yields are nearly the same and therefore, optimum Fe^{2+} dosage was determined to be 75 mg L^{-1} for cost effectiveness. In a condition of fenton-like process, optimum Fe^{3+} dosage was determined to be 75 mg L^{-1} for the highest removal yields.

Effect of H_2O_2 concentration

H_2O_2 concentration is an important parameter in Fenton processes. Determination of the optimum H_2O_2 concentration value in Fenton processes is important for removal yield of the process and for its applicability based on the cost of H_2O_2. In Fenton reactions, hydrogen peroxide plays very important roles as the production source of hydroxyl radicals (Sun et al., 2009a).

In order to determine the most suitable H_2O_2 rate in fenton and Fenton-like process, doses were adjusted to 100 mg L^{-1} to 600 mg L^{-1} of H_2O_2 concentrations by fixing C_0= 200 mg L^{-1}, Fe^{2+} and Fe^{3+}= 75 mg L^{-1}, pH= 3 and T= 20°C. Experimental results are given in *Fig. 3*.

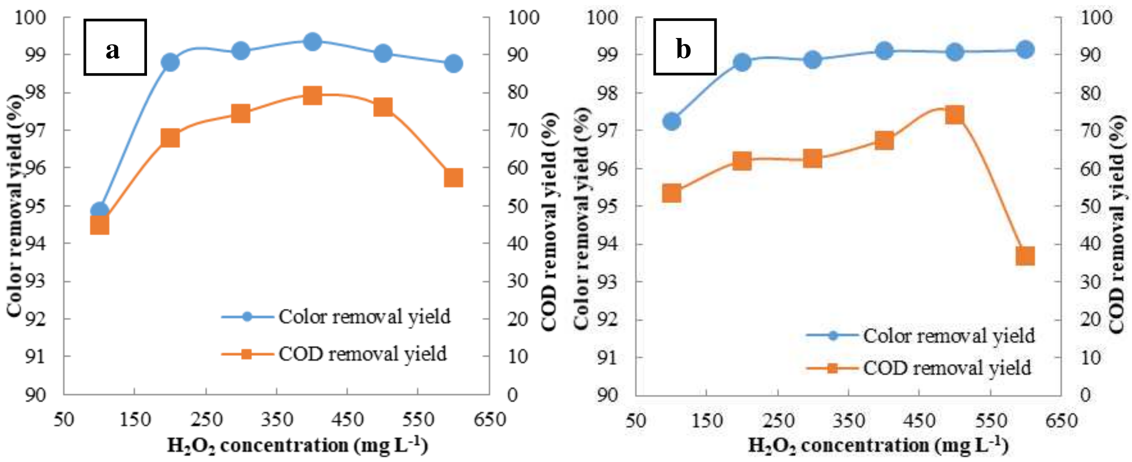

Figure 3. *Effect of H_2O_2 doses on color and COD removal (a) Fenton and (b) Fenton-like processes (C_0=200 mg L^{-1}, Fe^{2+} and Fe^{3+} =75mg L^{-1}, pH=3, T=20°C, t=30 min)*

When considered (*Fig. 3*), it is seen that at the end of a 30 – min reaction time, increase of H_2O_2 concentration from 100 mg L^{-1} to 400 mg L^{-1} causes increases in both color and COD removal yields. When H_2O_2 concentration increases from 100 mg L^{-1} to 400 mg L^{-1}, color removal yield rises from 94.87% to 99.35% while COD removal yield rises up from 44.66% to 79.25%. At the concentrations above 400 mg L^{-1}, H_2O_2 dosing

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causes decreases in color and COD removal yields. In case H_2O_2 concentration rises from 400 mg L^{-1} to 600 mg L^{-1}, color and COD removals were 98.77% and 57.38%. In the case of the Fenton-like oxidation, in all H_2O_2 concentration experiments, color removal yield was obtained to be nearly 98% while in the same H_2O_2 concentration range (100 mg L^{-1} to 500 mg L^{-1}) COD removal yield was between 53.40% and 74.10% and at 600 mg L^{-1} it decreased to 36.67%.

In general, increase in H_2O_2 dosage increases the degradation rates of pollutants (Lin and Lo, 1997; Lin et al., 1999; Kang and Hwang, 2000; Babuponnusami and Muthukumar, 2014). However, great care should be taken when deciding on H_2O_2 dosage in the studies. Unused part of H_2O_2 may cause COD in Fenton processes to increase and so at excessive concentration, H_2O_2 is not advisable (Lin and Lo, 1997). In the same way, H_2O_2 is harmful for several living organisms. Another unfavorable effect of H_2O_2 is the radical scavenger effect of ($OH^{\bullet}$) radicals (*Eq. 5*). Such a condition is seen at excessive concentrations and H_2O_2 (Tang and Chen, 1996; Kitis et al., 1999; Ntampegliotis et al., 2006; Sun et al., 2007). At the end of the experiments, optimum H_2O_2 concentration was found to be 400 mg L^{-1} for Fenton process and 500 mg L^{-1} for the Fenton-like process by considering both removal yield and the cost of H_2O_2.

Effect of pH

pH is a higly important parameter for producing $OH^{\bullet}$ radicals in Fenton and Fenton-like process (Singh and Tang, 2013; Mofrad et al., 2015). In the experiments in order to determine optimum pH in the treatment of RR45 colorant through Fenton and Fenton-like process, beakers were filled first with RR45 with the concentration of C_0= 200 mg L^{-1} taken from stocked solution and then, 75 mg L^{-1}'lik Fe^{2+} or Fe^{3+}, H_2O_2 is 400 mg L^{-1} (for Fenton process) or 500 mg L^{-1} (for Fenton-like process) found to be optimum were added and experiments were conducted between pH 2.5 and 5.0. Results obtained are showed graphically in *Fig. 4*. In the experiments, at the end of 30 – min experiment time, the highest color and COD removal yield for both fenton and Fenton-like processes were achieved at pH 3. In the experiments conducted with pH above and below 3, color and COD removal yields decreased.

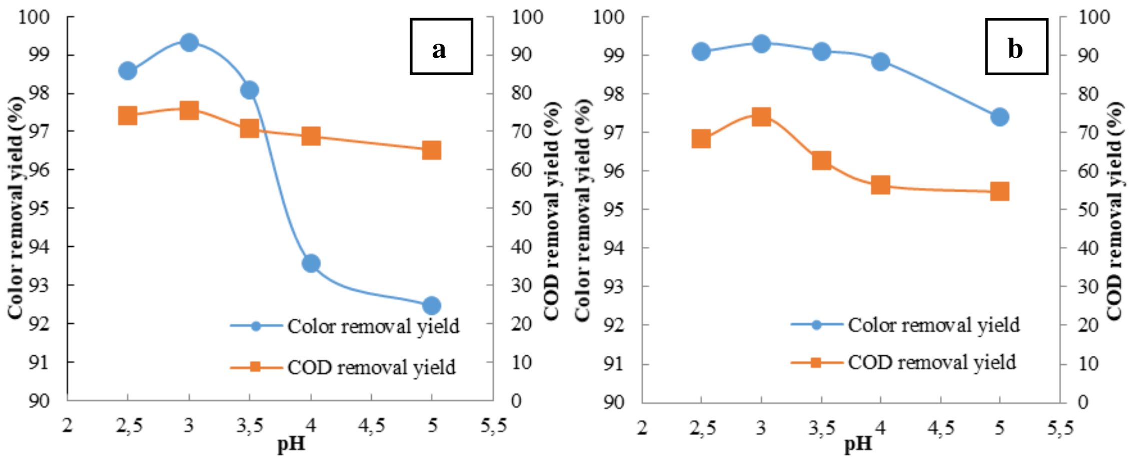

Figure 4. *Effect of pH on color and COD removal (a) Fenton and (b) Fenton-like processes (C_0=200 mg L^{-1}, Fe^{2+} and Fe^{3+} =75 mg L^{-1}, H_2O_2 is 400 mg L^{-1} (for Fenton process) or 500 mg L^{-1} (for Fenton-like process), T=20°C, t=30 min)*

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When pH is below 3, since complex iron types $[Fe(H_2O)_6]^{2+}$, $[Fe(H_2O)_6]^{3+}$, $[Fe(H_2O)_5OH]^{2+}$ reacting with H_2O_2 slower are formed, *reactions 2* and *7* also slow down and in that case oxidation removals also decrease.

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + OH^\bullet + H_2O \qquad \text{(Eq.7)}$$

In addition, under low pH conditions (pH<3), formation of oxonium ion $[H_3O_2]^+$ which increases the stability of H_2O_2 and limits the production of $OH^\bullet$ radical may cause the *reactions 3* and *8* to slow down (Bouasla et al., 2010; Singh and Tang, 2013).

$$R^\bullet + H_2O_2 \rightarrow ROH + OH^\bullet \qquad \text{(Eq.8)}$$

At pHs lower than 3, reason for the decrease in color and COD removal yields is that excessive amount of H^+ ions reacts with H_2O_2 and forms stable oxonium ion ($H_3O_2^+$) (*Eq. 9*).

$$H_2O_2 + H^+ \rightarrow H_3O_2 \qquad \text{(Eq.9)}$$

Production of $OH^\bullet$ radical decreases since precipitating $Fe(OH)_3$ is formed when pH is above 3 (*Eq. 10*).

$$Fe^{3+} + 3OH^- \rightarrow Fe(OH)_3 \qquad \text{(Eq.10)}$$

Following this precipitation, production of Fe^{2+} from Fe^{3+} stops and then the production of $OH^\bullet$ radical decreases (Chen and Pignatello, 1997; Bouasla et al., 2010). In addition, as pH increases, rate of self – degradation of H_2O_2 increases (*Eq. 11*).

$$H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{(Eq.11)}$$

The effect of initial colorant concentration

The effect of initial RR45 concentration on the removal yield in the Fenton and Fenton-like oxidation process was studied with dye concentrations of 150, 200, 250, 300, 350 and 500 mg L^{-1}. Experiments were conducted under the conditions, where pH is 3, H_2O_2 is 400 mg L^{-1} (for Fenton process) or 500 mg L^{-1} (for Fenton-like process), T is 30 min and Fe^{2+} is 75 mg L^{-1} or Fe^{3+} is 75 mg L^{-1}. Experimental results are shown graphically in *Fig. 5* and *Fig. 6*. As can be seen from *Fig. 5* and *Fig. 6*, as the initial colorant concentration increases both color and COD removal yields decreased. With the increase in initial colorant concentration from 150 mg L^{-1} to 500 mg L^{-1}, color removal yield decreased from 99.91% to 98.83% while COD removal yield from 77.93% to 55.13% in the Fenton oxidation and 73.80% to 46.70% in the Fenton-like oxidation process, respectively. Reason for this is that as the initial colorant concentration increases the number of dye molecules also increases. In this case, there was no change in the number of $OH^\bullet$ radicals since the concentrations of Fe^{2+} and H_2O_2 did not change in the medium. Reduced removal yields were observed since there would be less $OH^\bullet$ radicals matching with increasing number of dye molecules. Similar

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trend is seen in several studies in literature (Malik and Saha, 2003; Lucas and Peres, 2006; Sun et al., 2007; Bouasla et al., 2010; Harichandran and Prasad, 2016).

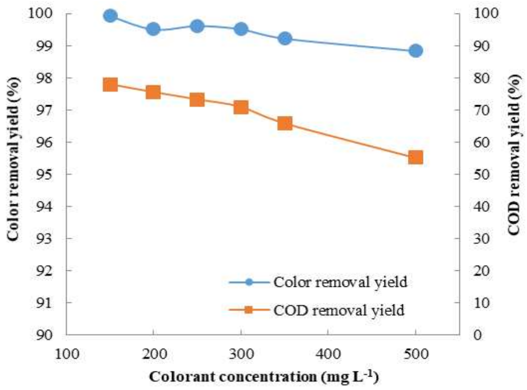

Figure 5. *Effect of initial colorant concentration in Fenton oxidation on color and COD removal (Fe^{2+}= 75 mg L^{-1}; H_2O_2= 400 mg L^{-1}; pH= 3; T= 20^0C; t= 30 min)*

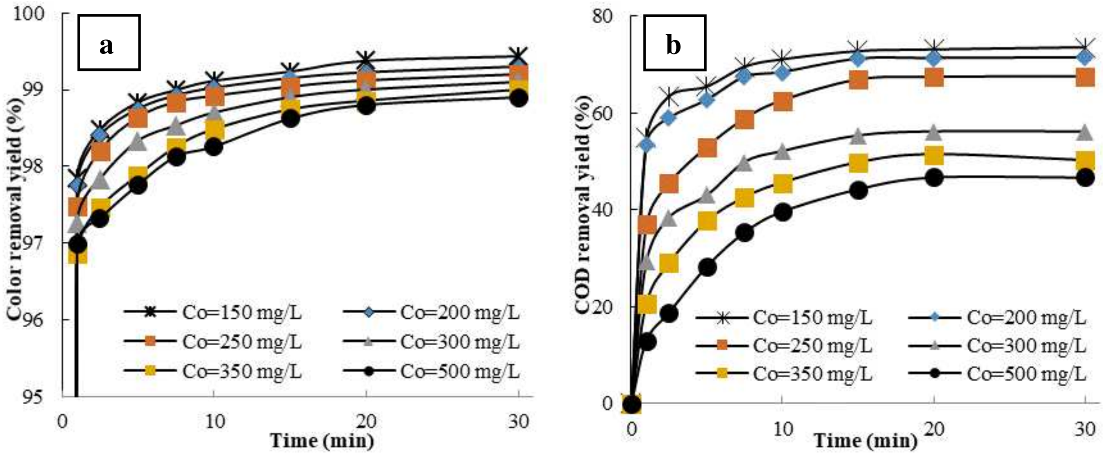

Figure 6. *Effect of initial colorant concentration in Fenton-like oxidation on (a) color and (b) COD removal (Fe^{3+}=75 mg L^{-1}; H_2O_2=500 mg L^{-1}; pH= 3; T= 20°C; t= 30 min)*

The Effect of Temperature

Temperature may have effects on the reactions between H_2O_2, Fe^{2+} and Fe^{3+}. Experiments were conducted at initial RR45 concentration of 200 mg L^{-1}, H_2O_2 concentration of 400 mg L^{-1} for fenton or 500 mg L^{-1} for fenton-like process, Fe^{2+} and Fe^{3+} concentration of 75 mg L^{-1}, pH=3, t=30 min and temperature between 20°C and 60°C in order to investigate the effect of RR45 on the temperature in the Fenton reactions.

Experiments were conducted in order to investigate the effect of temperature in the Fenton reactions of RR45. Results obtained are given graphically in *Fig. 7*. As can be seen from *Fig. 7*, in all temperature experiments, color removal yield was obtained to be nearly 100% while in the same temperature range (20°C to 50°C) COD removal yield was between 75.50% and 91.11% and at 60°C it decreased to 84.17% for Fenton process and COD removal yield was between 71.42% and 87.80% and at 60°C it decreased to

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DOI: http://dx.doi.org/10.15666/aeer/1702_15171529

75.09% for Fenton-like oxidation process. The reason for the increasing removal yield with increasing temperatures may be that the high temperatures can cause both higher Fenton, Fenton-like reaction rates and the formation of larger amount of $OH^{\bullet}$ radicals (Sun et al., 2009b). The reason for the decrease at 60°C may be the degradation of H_2O_2 at higher temperatures (Bouasla et al., 2010) and evaporation (Bayhan and Değermenci, 2017).

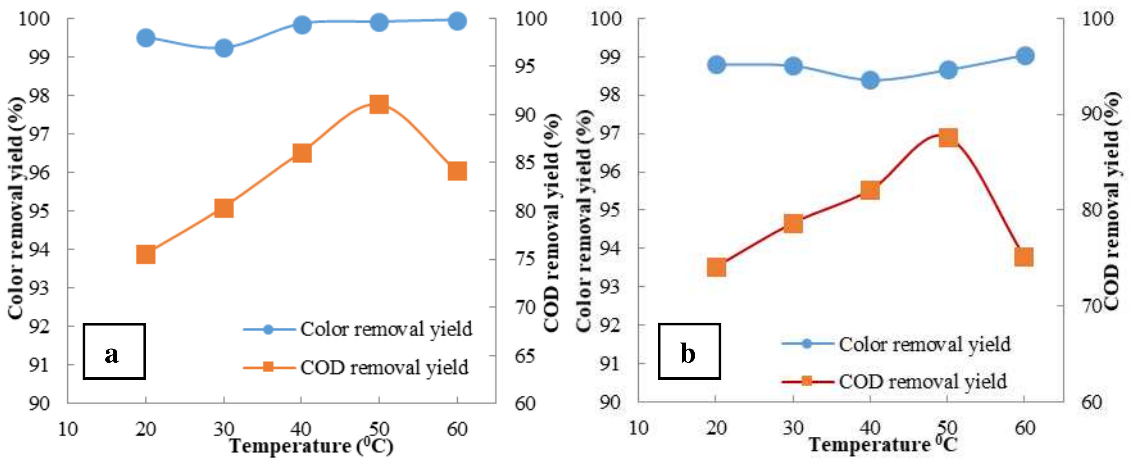

Figure 7. *Effect of temperature on color and COD removal (a) Fenton and (b) Fenton-like processes (C_0=200 mg L^{-1}; Fe^{2+} and Fe^{3+} =75 mg L^{-1}; H_2O_2 is 400 mg L^{-1} (for Fenton process) or 500 mg L^{-1} (for Fenton-like process); t=30 min)*

Kinetic experiments

In the present study, first-order, second-order and BMG reaction kinetics were used to study the removal COD kinetics of RR45 by the Fenton-like oxidation process. Kinetic studies were not performed in the Fenton process because color and COD removal yield have been obtained very fast. The kinetic parameters calculated for each reaction kinetics using the data obtained from experiments at different initial dye concentrations are given in *Table 3*. The results of kinetic analysis were shown in *Table 4*. The fitness of kinetic data to the first-order, second-order and BMG reaction kinetics model were given in *Figs. 8-9 and 10*.

Table 3. *The equations of kinetic models*

Kinetic model	Mathematical equations	Pilots	Equations	References
Pseudo-first-order	$\ln C_t = \ln C_0 - k_1 t$	$\ln(\frac{C_0}{C_t})$ *versus t*	(Eq.12)	Sun et al., 2009a; Ertugay and Acar 2017
Pseudo-second-order	$\frac{1}{C_t} = \frac{1}{C_0} + k_2 t$	$\frac{1}{C_t}$ *versus t*	(Eq.13)	Sun et al., 2009a; Ertugay and Acar 2017
Behnajady–Modirshahla–Ghanbery (BMG)	$\frac{C_t}{C_0} = 1 - \frac{t}{m+b}$ $\frac{t}{1-(\frac{C_t}{C_0})} = m + bt$	$\frac{t}{1-(\frac{C_t}{C_0})}$ *versus t*	(Eq.14) (Eq.15)	Behnajady et al., 2007; Ertugay and Acar 2017

where C_0 (mg L^{-1}) and C_t (mg L^{-1}) are the concentration of COD the concentration at initial and at any time, respectively; k_1 and k_2 are first-order and second-order reaction rate constant; t is the reaction time; b and m are two characteristic constants relating to the reaction kinetics and oxidation capacities

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Table 4. *The parameters of kinetic models and correlation coefficients (R^2) for the COD removal of RR45*

RR45 (mg L^{-1})	First-order kinetic model		Second-order kinetic model		BMG kinetic model		
	k_1 (min^{-1})	R_2	k_2 (L mg^{-1} min^{-1})	R_2	1/m	1/b	R^2
150	0.0280	0.4350	0.0060	0.5825	0.997	0.835	0.9980
200	0.0271	0.4554	0.0050	0.5987	1.678	0.724	0.9995
250	0.0299	0.6276	0.0003	0.7400	0.718	0.697	0.9977
300	0.0213	0.5918	0.0002	0.6788	0.590	0.581	0.9976
350	0.0198	0.6456	0.0001	0.7093	0.381	0.531	0.9951
500	0.0200	0.7555	0.00009	0.8047	0.185	0.512	0.9840

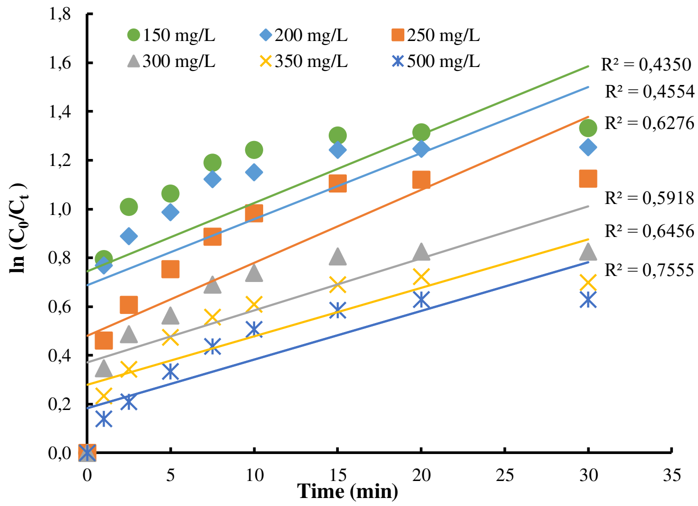

Figure 8. *First-order reaction kinetic for the COD removal of RR45 by Fenton-like oxidation (Fe^{3+} = 75 mg L^{-1}; H_2O_2 = 500 mg L^{-1}; pH= 3; T = 20°C; t = 30 min)*

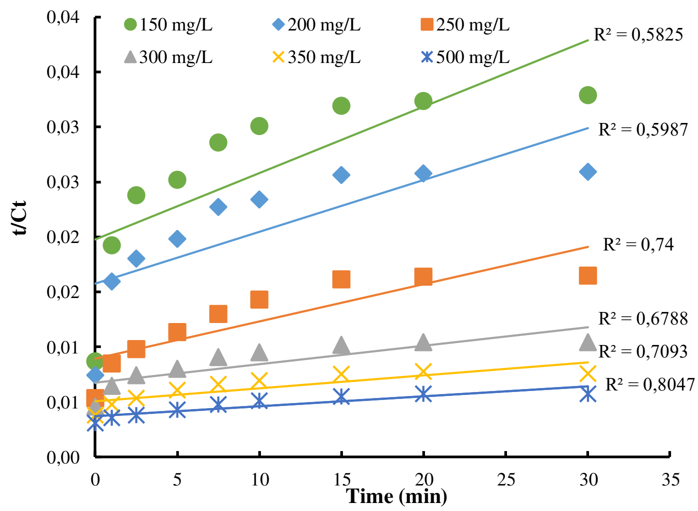

Figure 9. *Second-order reaction kinetic for the COD removal of RR45 by Fenton-like oxidation (Fe^{3+} = 75 mg L^{-1}; H_2O_2 = 500 mg L^{-1}; pH= 3; T = 20°C; t = 30 min)*

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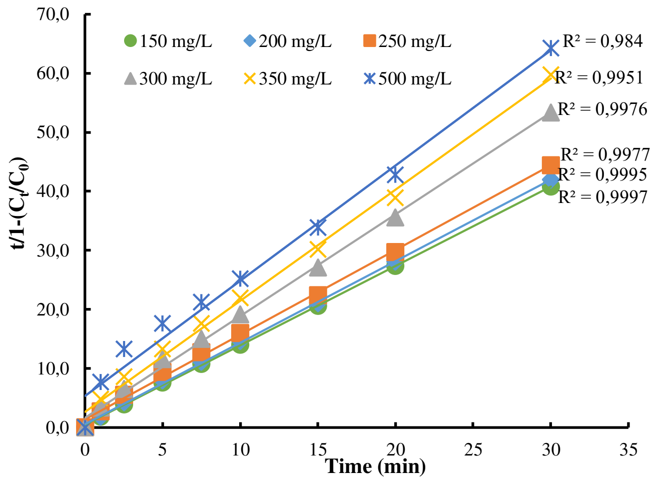

Figure 10. *BMG kinetic for the COD removal of RR45 by Fenton-like oxidation (Fe^{3+}= 75 mg L^{-1}; H_2O_2= 500 mg L^{-1}; pH= 3; T= 20°C; t= 30 min)*

As seen in *Table 4*, correlation coefficient for BMG model are higher than the first-order and the second-order models. The correlation coefficients for the the first order reaction kinetic equation have changed in the range of 0.435–0.755 for the second order reaction kinetic have changed in the range of 0.549–0.913 and for the BMG kinetic model have changed in the range of 0.9840–0.9995. These results have shown that the experimental data did not fit the first-order and second-order kinetic model.

Conclusion

The degradability of the reactive red 45 dye from azo colorants group through the fenton and fenton-like oxidation processes, was investigated in the present study. Degradability of RR45 was evaluated by considering color and COD parameters. Experiments were carried out for 30 minutes.

Effect of the rates of Fe^{2+} or Fe^{3+} (25, 50, 75, 100, 125, 150 and 200 mg L^{-1}), H_2O_2 (100, 200, 300, 400, 500 and 600 mg L^{-1}), pH (2.5; 3; 3.5; 4 and 5), the concentration of the dye (150, 200, 250, 300, 350 and 500 mg L^{-1}) and the temperature (20, 30, 40, 50 and 60°C) were investigated in the studies conducted on the degradation of RR45 colorant belonging to azo group through fenton and fenton-like oxidation processes. A removal efficiency of 91.1% of COD and 99.90% of color was obtained under optimum experimental conditions where initial solution pH is 3, the concentration of RR45 colorant is 200 mg L^{-1}, rates of Fe^{2+} and H_2O_2 are 75 mg L^{-1} and 400 mg L^{-1}, reaction temperature is 50°C and experiment time is 30 min. The experimental results obtained indicate clearly that the oxidation of RR45 by the Fenton and Fenton-like process is effective with a low concentration of H_2O_2, Fe^{2+} or Fe^{3+} and pH. High concentrations of iron and H_2O_2 concentrations cause consumption of hydroxyl radicals (radical scavenger effect). In addition, the increase in temperature from 20 to 50 increased the efficiency of removal in both processes. Kinetic studies were not performed in the fenton process because color and COD removal yield have been obtained very fast. In Fenton-like process, the BMG kinetic model provided the best correlation of the used experimental data compared to the pseudo-first and second-order kinetic models.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15171529

Acknowledgements. This study was supported by Ataturk University, Scientific Research Projects (BAP) unit under the project FYL-2018-6027.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15171529

THE EFFECTS OF AFRICAN CATFISH, *CLARIAS GARIEPINUS* POND FARM'S EFFLUENT ON WATER QUALITY OF KESANG RIVER IN MALACCA, MALAYSIA

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(Received 17th Oct 2018; accepted 7th Jan 2019)

Abstract. The aquaculture industry is increasing worldwide. However, many negative impacts have been associated with aquaculture effluent especially in the form of water pollution to the receiving water body. Hence, the aim of this study was to determine the effects of aquaculture effluents mainly from *Clarias gariepinus* (African Catfish) pond farm on the water quality of Kesang River in Malacca. Three sampling stations were selected at the fish farm. The parameters involved in the monitoring are dissolved oxygen (DO), pH, total dissolved solids (TDS), conductivity, biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS), ammonia-nitrogen (NH_3-N), nitrite, nitrate, total nitrogen and phosphate. The water quality index was calculated to monitor the quality of the river. Results showed that there were slight effects of aquaculture effluent to the river. It is suggested that the effects of aquaculture effluents are studied according to seasons and the variables are monitored continuously to determine the actual effects of the effluents.
Keywords: *aquaculture, sharptooth catfish, wastewater, surface water, physicochemical parameters*

Introduction

Aquaculture has been considered as an alternative to assist the world food security issue (Kobayashi et al., 2015) due to the increasing demand for seafood (Nadarajah and Flaaten, 2017). In 2014, the total global aquaculture production was 101.1 million tons, which was valued at US$ 165.8 billion where Asia dominates this production as much as 88.91% in volume (Nadarajah and Flaaten, 2017). Malaysia is also promoting aquaculture as an important engine in growing the national economy. Malaysia's aquaculture industry has been developed since 1920's involving three practices, which are freshwater, brackish-water and marine aquaculture (Hamdan et al., 2015).

Aquaculture in Malaysia includes freshwater and marine fishes, aquatic plants, mollusks and crustaceans (Dauda et al., 2018). This industry has increased consistently with the sum production at the end of 2015 was at 506,465.25 tonnes, which valued about RM 3,296,463 ($US 756,937) (Dauda et al., 2018). In Malaysian production, more than 30 finfish species are farmed, dominated by the African catfish and followed by red (hybrid) tilapia, sea bass, *Pangasius* sp., and red snapper (Department of Fisheries, 2016). African catfish, *Clarias gariepinus* is an exotic fish species in Malaysia. It was introduced from Thailand between 1986 and 1989 through aquaculture

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DOI: http://dx.doi.org/10.15666/aeer/1702_15311545

sector (Dauda et al., 2018). This non-native catfish is a fast grower fish, highly disease resistant and favored by the local people (Adan, 2000).

However, there are studies that report the aquaculture industry displays a variety of adverse impacts on the environment such as ecosystem degradation, land erosion, food chain pollution and water pollution (Ottinger et al., 2016). The adverse effects of aquaculture effluents are one of the significant impacts due to lack of enforcement on effluent discharge from aquaculture to the receiving river. According to Cao et al. (2007), effluents discharge without treatment into the receiving water is due to poor management of shrimp and fish farms, thus lead to environmental deterioration.

Rivers are the main source for raw water supply in Malaysia, supplying more than 90% of the country's water requirements (Hassan et al., 2018). The increasing population and expanding urban areas in Melaka State has caused a gradual rise in demand for water consumption (Daneshmand et al., 2011; Hazmi and Hanafiah, 2018). Melaka State is the third smallest state in Malaysia. Three dams and three reservoirs are the main water resources in Melaka (Tengku Azzlan et al., 2016). The Kesang River and Malacca River provide a total of 80 - 90% of the water supply in Melaka. The remaining 10 - 20% is imported from the Gerisik River of Johor (Malaysian Water Association, 2008). Kesang River is one of the main raw water supplies in Melaka State. There is a semi-commercial catfish pond farm located nearby the river, where the effluent is directly discharged to the Kesang River. The effluent discharged from fish ponds has a great impact on effective water treatment, which is due to the amount of organic material load, nutrients like nitrogen and phosphorus released to surface water (Teodorowicz, 2013). Water treatment in Malaysia mostly uses conventional water treatments which can only treat raw water that does not violate the National Water Quality Standards (NWQS). Raw water treatment at most water treatment plants is limited to coagulation, sedimentation, filtration and disinfection, which do not include treating the organic materials (Tengku Azzlan et al., 2016).

In 2010, a total of 1,055 water quality monitoring stations were monitored in Malaysia and it was found that 10% of these stations are polluted (Amneera et al., 2013). The water resource available may not be adequate to supply water to all consumers due to river pollution (Ashraf and Hanafiah, 2018; Harun and Hanafiah, 2018). Therefore, water quality monitoring is vital in Malaysia to identify the existing problem or emerging issues in the future. Monitoring the aquaculture effluents can be a valuable tool for guiding management actions to improve river conditions. Hence, this study was conducted to determine the impact of *Clarias gariepinus* pond farm effluent on physicochemical parameters of Kesang River in Malacca.

Materials and methods

Study area

The study was conducted in Sungai Kesang, Jasin, Malacca which is located in the west coast of Peninsular Malaysia between the latitude of 2°19'45.1"N and longitude of 102°23'54.7"E. Kesang River supplies 54 million liters of raw water daily to the state (Tengku Azzlan et al., 2016). The average annual runoff depth is about 500 - 600 mm (Shirazi et al., 2013). The length of the Kesang River is about 35 km, running through Jasin Town as a dividing line and a border between the southern states of Malacca and the state of Johor. Kesang River also passes through palm oil plantations. A large proportion of the land near the river is used for growing palm oil trees. Industrialization

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is restricted to around Jasin Town because Kesang River is gazetted as a catchment area. In this study, three sampling stations were selected along the river, first at the river before the farm, the second at the outlet of the farm, and finally is at the river after the farm. The sampling station is shown in *Figure 1*.

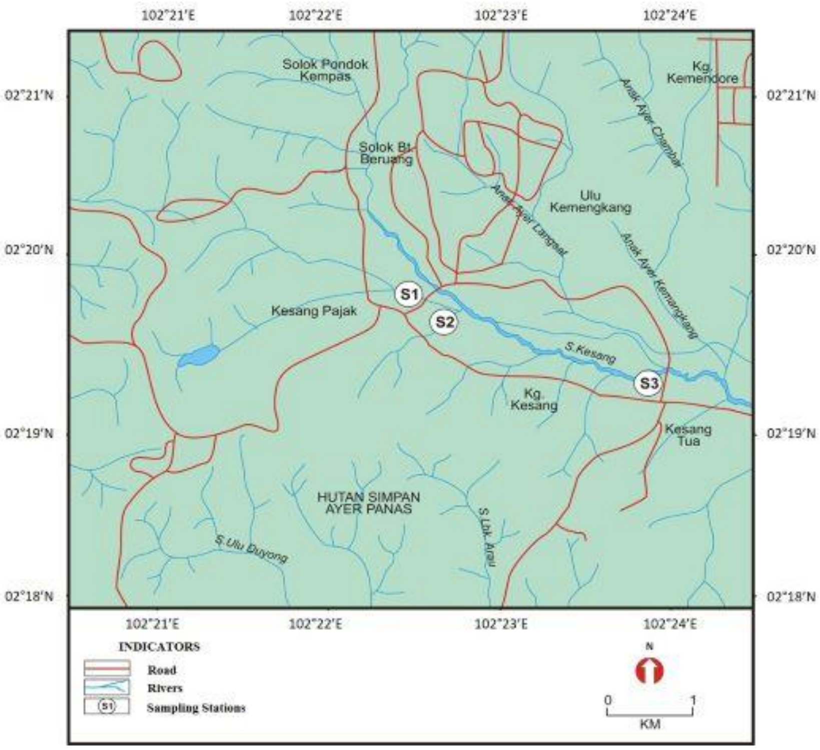

***Figure 1.** Sampling station map where S1 is Station 1, S2 is Station 2 and S3 is Station 3*

Physicochemical analysis

The data were obtained through sampling and laboratory work. The data obtained were collected six times from December 2017 to June 2018. The selected sampling stations were upstream of the river before the farm (Station 1), catfish pond farm effluent outlet (Station 2) and downstream of the river after the effluent outlet (Station 3). The selected water quality parameters used in this study include dissolved oxygen (DO), pH, total dissolved solids (TDS), conductivity, biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS), ammonia-nitrogen (NH_3-N), nitrite, nitrate, total nitrogen and phosphate. The WQI formula (Department of Environment, 2011) used to determine of the water quality at Kesang River is as follows (*Eq. 1*):

$$WQI = 0.22 \times SIDO + 0.19 \times SIBOD + 0.16 \times SICOD + 0.15 \times SIAN + 0.16 \times SISS + 0.12 \times SIpH \quad \text{(Eq.1)}$$

where WQI is water quality index, SIDO is dissolved oxygen subindex, SIBOD is biological oxygen demand subindex, SICOD is chemical oxygen demand subindex, SIAN is ammonia-nitrogen subindex, SISS is suspended solid subindex, and SIpH is pH value subindex. WQI classification is illustrated in *Table 1*. The method used for each parameter is summarized in *Table 2*.

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The water samples were kept in 1L polyethylene bottles and preserved with 65% nitric acid until the pH of the samples reached pH 2. Then, the water samples were kept in 4^{o}C until further analysis. The analysis was conducted within 24 hours after sampling.

All data were expressed in average ± standard deviation. Data were analyzed using Statistical Package for Social Science (SPSS, version 22) software. One-way ANOVA was conducted for comparing water quality parameter at different sampling stations. Tukey's HSD test was conducted for post hoc test. The significant value for the data analyzed was set at $p < 0.05$.

Table 1. *WQI classification*

Class	Uses
I	• Conservation of natural environment.
	• Water supply I: Practically no treatment required (except
	• by disinfection or boiling only).
	• Fishery I: Very sensitive aquatic species.
II A	• Water supply II – Conventional treatment required.
	• Fishery II - Sensitive aquatic species.
II B	• Recreational use with body contact.
III	• Extensive treatment required.
	• Fishery III: common of economic value and tolerant species; livestock drinking.
IV	• Irrigation.
V	• None of the above.

Source: Department of Environment, 2006

Table 2. *Methods used for each parameter*

Parameters	Methods	Instrument
DO		• YSI Pro DO Meter
pH		• pH meter HI8424
Temperature		• pH meter HI8424
Conductivity		• AZ8306 Conductivity / TDS / Salt meter RS232
TDS		• AZ8306 Conductivity / TDS / Salt meter RS232
BOD	BOD_5 (APHA 1999)	• DO Meter (YSI 5000)
COD	Method 5220D Closed Reflux Method; Colorimetric Method (APHA, 1992)	• HACH DR2800 Spectrophotometer • COD Reactor
NH_3-N	Direct Nesslerization (ASTM, 2008)	• HACH DR2800 Spectrophotometer
TSS	Photometric method (Krawczyk & Gonglewski, 1959)	• HACH DR2800 Spectrophotometer
Nitrite	Diazotization Method (Federal Register, 1979)	• HACH DR2800 Spectrophotometer
Nitrate	Method 4500-NO3 Cadmium Reduction Method (APHA, 1992)	• HACH DR2800 Spectrophotometer
Total Nitrogen	Method 4500-N Persulfate Method (APHA, 2005)	• HACH DR2800 Spectrophotometer • COD Reactor
Phosphate	Method 4500-P Ascorbic Acid Method (APHA, 2005)	• HACH DR2800 Spectrophotometer

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Results

All monitored physicochemical parameters were not diverse throughout the whole investigation period at all locations. The mean values of all studied parameters have been compared to the acceptable value of the Recommended Raw Water Quality Criteria which has been developed by the Ministry of Health Malaysia (Ministry of Health Malaysia, 2010). The classifications of water quality according to the Water Quality Index (WQI) were at class III for both river sampling stations, indicating that these stations need to be extensively treated but are suitable for irrigation and agriculture. The results of the study indicated that the effluent from the catfish pond farm have no significant impacts on water quality variations.

As shown in *Figure 2a*, the range of DO concentrations was from 32.5% at sampling Station 2 to 75.6% at sampling Station 1. The Ministry of Health Malaysia (2010) has no specific guide on DO. However, based on the National Water Quality Standards (NWQS) for Malaysia, DO values on sampling Station 1 and 3 are in class II, while DO values at sampling Station 2 is in class IV (EPA, 2008). ANOVA test showed that there was significant difference ($p < 0.05$) of DO concentrations between sampling stations. A post hoc Tukey's HSD test showed that Stations 1, 2 and 3 differed significantly at $p < 0.05$. However, there were no significant difference ($p > 0.05$) in the mean of DO levels between Station 1 and Station 3.

This study showed that the pH value ranged from 6.44 at sampling Station 3 to 7.02 at sampling Station 2 as shown in *Figure 2b*. The one-way analysis of variance (ANOVA) test showed that there was no significant difference ($p > 0.05$) for the pH values between all the sampling stations.

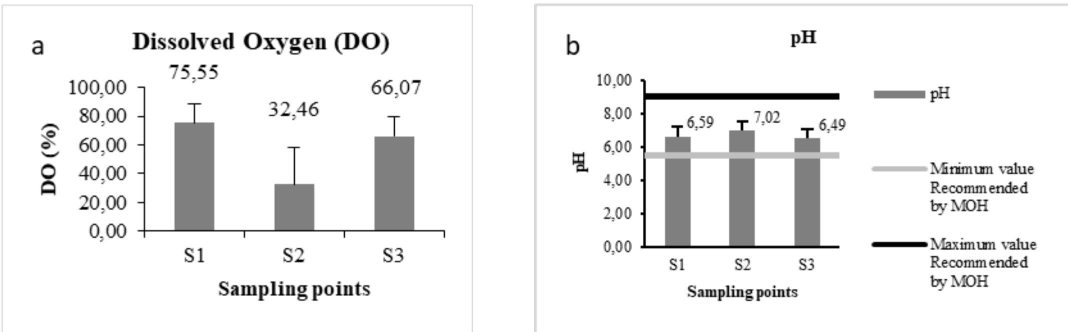

Figure 2. *(a) Average of DO concentrations for three sampling stations;(b) pH values for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3) Values in average ± standard deviation (n = 6) Standard from MOH is not available for DO parameter*

Figure 3a shows the mean value of temperature for all three sampling stations. The range of temperature value was from 27.1°C to 28.5°C. The highest value was recorded at Station 1 and the lowest value was found at Station 2. However, ANOVA test showed that there were no significant differences ($p > 0.05$) between each sampling station.

The results also showed that conductivity ranged from 87.41 μS/cm to 243.34 μS/cm (*Figure 3b*). The lowest value was recorded at Station 1, while the highest value has recorded at Station 2. The conductivity level lies within the acceptable value of the

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Recommended Raw Water Quality Criteria. The ANOVA test showed that there were no significant differences ($p > 0.05$) between each sampling station.

It was found that TDS values were ranged from 43.84 mg/L to 121.44 mg/L as shown in *Figure 4a*. The highest concentration of TDS was observed at Station 2, while the lowest concentration was obtained at Station 1. All the concentrations were still below the maximum value of the Recommended Raw Water Quality Criteria. The ANOVA test showed that there were no significant differences ($p > 0.05$) between all sampling stations.

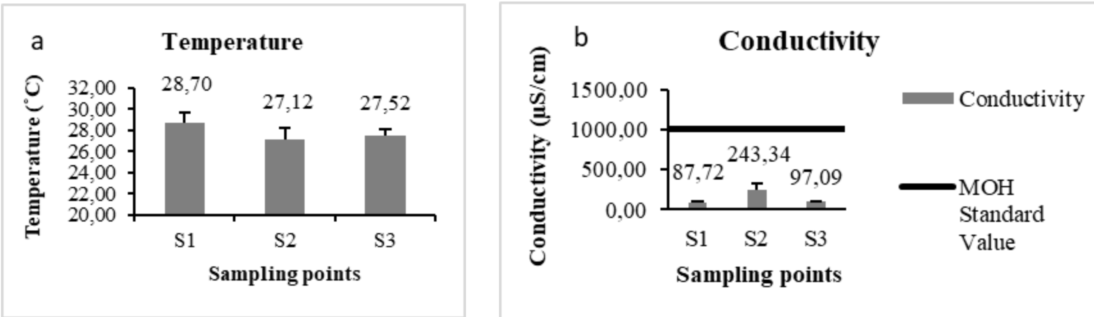

Figure 3. *(a) Temperature values for three sampling stations; (b) Conductivity values for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3) Values in average ± standard deviation (n = 6)*
Standard from MOH is not available for temperature parameter

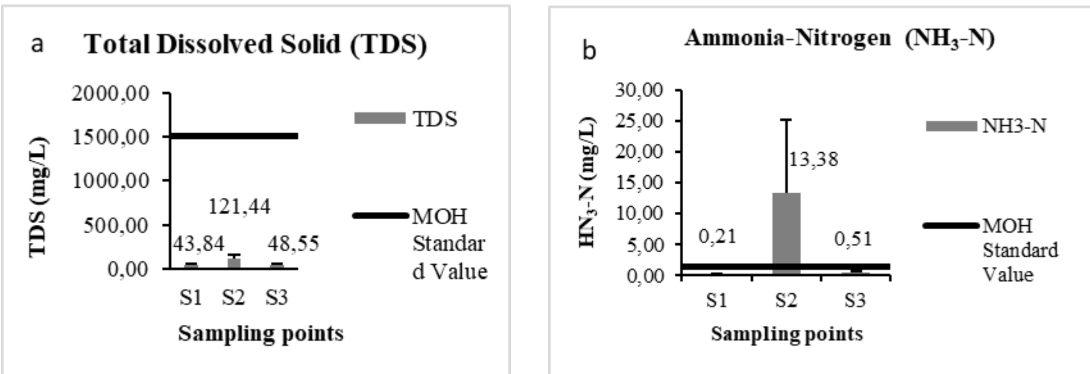

Figure 4. *(a) TDS concentrations for three sampling stations; (b) NH_3-N concentrations for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3), Values in average ± standard deviation (n = 6)*

The highest ammonia-nitrogen (NH_3-N) concentration was observed at 13.38 mg/L at Station 2. The lowest NH_3-N concentration was found at 0.32 mg/L at Station 1. *Figure 4b* showed that only Station 2 violates the Recommended Raw Water Quality Criteria acceptable value. The ANOVA test showed there were no significant differences ($p > 0.05$) between each sampling station.

This study also showed that the highest amount of BOD at 39.83 mg/L was observed at Station 2. The lowest BOD amount at 4.99 mg/L was recorded at Station 1. ANOVA

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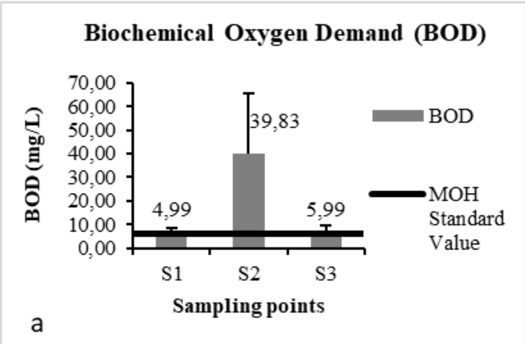
test showed that there was significant difference ($p < 0.05$) of BOD concentrations between sampling stations. A post hoc Tukey's HSD test showed that Stations 1, 2 and 3 differed significantly at $p < 0.05$. *Figure 5a* showed that only Station 2 had abused the acceptable value of the Recommended Raw Water Quality Criteria. BOD amount at Station 3 was on the border line of the Recommended Raw Water Quality Criteria maximum value.

COD ranged from 91.28 mg/L to 397.39 mg/L as shown in *Figure 5b*. The highest level of COD was recorded at Station 2 and the lowest at Station 1. All three sampling stations violated the Recommended Raw Water Quality Criteria acceptable value. ANOVA test showed that there were no significant differences ($p > 0.05$) between each sampling station.



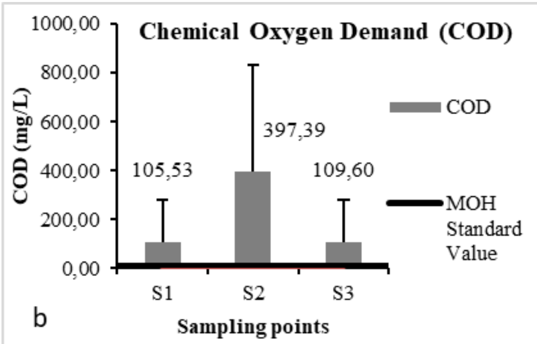


Figure 5. *(a) BOD concentrations for three sampling stations; (b) COD concentrations for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3), Values in average ± standard deviation (n = 6)*

This study observed that the range of TSS were between 37.44 mg/L to 468.17 mg/L. The highest concentration of TSS was recorded at Station 2, while the lowest concentration was recorded at Station 1 as shown in *Figure 6a*. Analysis using ANOVA test showed there were no significant differences ($p > 0.05$) between each sampling station. The Ministry of Health Malaysia (2010) has no specific guideline on TSS.

Figure 6b shows the mean value of phosphate for all three sampling stations. The range of phosphate concentrations were from 0.33 mg/L to 5.38 mg/L. The highest value was recorded at Station 2 and the lowest value was recorded at Station 1. Station 3 recorded a higher phosphate concentration than Station 1. ANOVA test showed that there was significant difference ($p < 0.05$) of phosphate concentrations between sampling stations. A post hoc Tukey's HSD test showed that Stations 1, 2 and 3 differed significantly at $p < 0.05$. There is no guideline set by the Ministry of Health Malaysia (2010) on maximum concentration of phosphate as well.

This study showed that the highest nitrite concentration was at 0.24 mg/L at Station 2. Meanwhile, the lowest concentration of nitrite was at 0.015 mg/L at Station 1. There were no significant differences ($p > 0.05$) between each sampling point based on ANOVA test. As seen in *Figure 7a*, even though the concentration of nitrite at Station 3 was higher than Station 1, the increase was very minimal. However, there is no guideline set by the Ministry of Health Malaysia (2010) on maximum nitrite concentration.

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Nitrate concentrations ranged from 90.76 mg/L to 142.89 mg/L. The highest and lowest concentrations have been observed at Station 2 and Station 1, respectively. The concentration of nitrate at Station 3 was slightly increased after passing through the fish pond farm. However, there were no significant differences (p > 0.05) between each sampling station using ANOVA test. *Figure 7b* shows that all three sampling points violated the Recommended Raw Water Quality Criteria acceptable value.

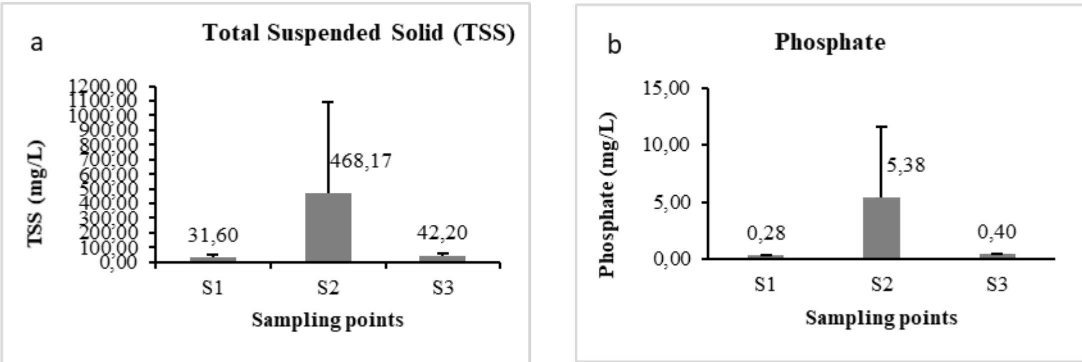

***Figure 6.** (a) TSS concentrations for three sampling stations; (b) Phosphate concentrations for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3), Values in average ± standard deviation (n = 6)*
Standard from MOH is not available for TSS and phosphate parameter

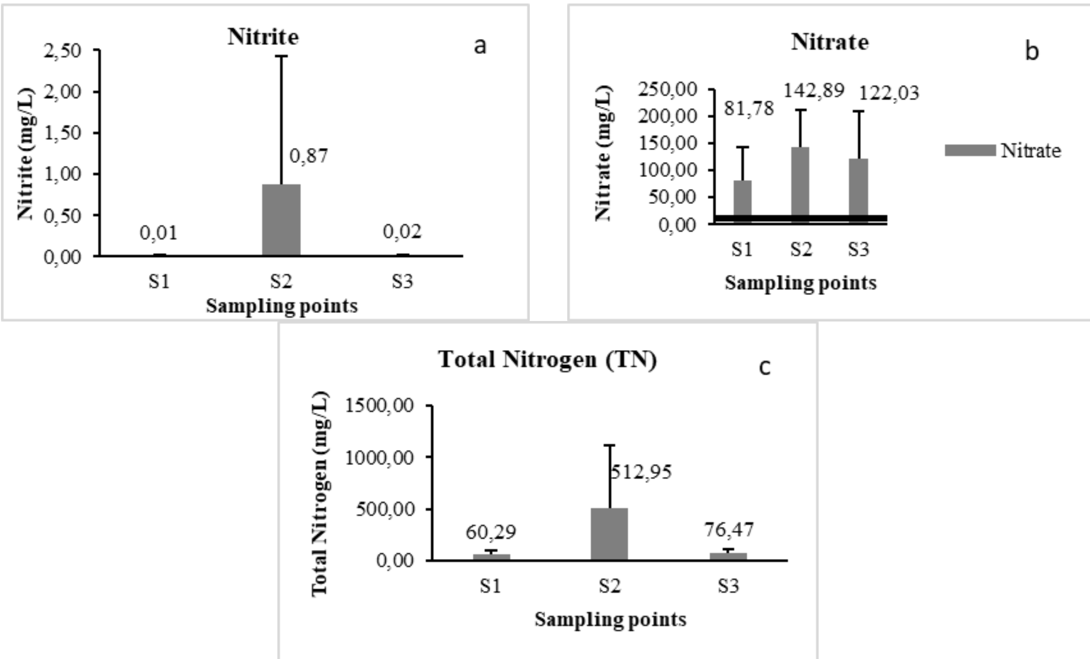

***Figure 7.** (a) Nitrite concentrations for three sampling stations; (b) Nitrate concentrations for three sampling stations; (c) Total nitrogen concentrations for three sampling stations, where; the upstream before African Catfish pond farm outlet (S1), African Catfish pond farm outlet (S2) and the downstream after African Catfish pond farm outlet (S3)*
Values in average ± standard deviation (n = 6)
Standard from MOH is not available for nitrite and total nitrogen parameter

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This study showed that the highest concentration of total nitrogen was at 512.95 mg/L at Station 2, while the lowest concentration at 60.29 mg/L at Station 1 as shown in *Figure 7c*. ANOVA test showed that there was significant difference ($p < 0.05$) of phosphate concentrations between sampling stations. A post hoc Tukey's HSD test showed that all sampling stations differed significantly at $p < 0.05$. There is no guideline set by the Ministry of Health Malaysia (2010) on maximum concentration of total nitrogen.

The WQI calculation of the water classification for Station 1 and Station 3 were reported to be in Class III. Class III is defined as moderately polluted where the water needs to be treated extensively.

Discussion

Dissolved oxygen (DO) is of uttermost importance in water quality, because it is essential for aerobic respiration (Boyde, 2015). Low DO concentrations at sampling Station 2 was due to the presence of high organic materials resulting from overfeeding of fish (US Environmental Protection Agency, 1974). DO concentration at sampling Station 3 was lower Station 1 due to the release of organic and inorganic pollutants as of biological matters from Station 2. However, the result of this research showed that the pollutants released from the fish farm had slightly decreased the DO levels of water that passed through it.

The pH of aquatic systems is an important criterion of the water quality in the watershed areas (Yisa and Jimoh, 2010). The value is used to determine the level of alkalinity or acidity of water (Hanafiah et al., 2018). The results were within the pH range of the Recommended Raw Water Quality Criteria (Ministry of Health Malaysia, 2010). The results in this study were in agreement to the study conducted by Teodorowicz (2013) where pH value had dropped after the water passed through the studied catfish pond farm. It was reported that the pH decreased due to the increase of feeding fish respiration rates, which increased the content of free CO_2. However, the pH values were still in the normal range of natural water pH that is 6 – 9 (Boyde, 2015) and is considered safe to aquatic organism (Teodorowicz, 2013). The extreme values of pH can cause destruction to the sensitive aquatic organism (Hassan et al., 2018).

The temperature of river water is an important characteristic for aquatic system because it could affect dissolved oxygen levels, species composition, chemical and biological activities (Shuhaimi-Othman et al., 2007; Hanafiah et al., 2018). This study showed that the temperature at the upstream sampling point is higher than downstream sampling point. This was not in line with the study conducted by Al-Badaii et al. (2013) in which the study found that temperature values increased from upstream to downstream. The concentrations of temperature recorded in this study were approximately comparable to the study reported for Malacca River (range 26°C–32°C) by Hua (2017). In general, weather conditions, sampling time and location are the factors that could give impact on the increase or reduction of temperature values (Al-Badaii et al., 2013). The weather conditions during the sampling time is shown in *Table 3*.

Conductivity is a substance's capability to carry an electrical current (Boyde, 2015). Conductivity in water was conveyed by inorganic dissolved solids such as calcium, chloride, aluminum cations, nitrate, sulfate, iron, magnesium and sodium (Al-Badaii, 2011; Boyde, 2015). It is also influenced by temperature and organic compounds such

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as oil, alcohol, phenol and sugar (Al-Badaii, 2011). According to Al-Badaii et al. (2013) and Laila et al. (2018), most freshwater conductivity ranges from 10 to 1000 μS/cm. Kesang River showed that the level of conductivity was lower than the study conducted in Malacca River (Hua, 2017) where the conductivity levels had ranged from 109.60 μS/cm to 1950.72 μS/cm. Harun et al. (2010) stated that the conductivity level in the water that receives pollution can exceed 1000 μS/cm.

Table 3. *Weather conditions during the sampling time*

Sampling months	Rainfal (mm)	Temperature (°C)
December 2017	0.0	26.4
January 2018	22.9	26.5
February 2018	0.0	28.0
March 2018	0.2	25.9
May 2018	0.0	28.5
June 2018	4.1	25.9

Source: Meteorology Department of Malaysia, 2018

Dissolved solids are tiny particles considered to be in true solution. Dissolved solids contain largely inorganic and some organic compounds (Boyde, 2015). A slight increase of TDS concentration was observed in Station 3 after the water had passed through the fish pond farm. TDS concentration in the river was due to the human activities along the river and suspended matter from overflow water (Al-Badaii et al., 2013). The TDS concentrations in Kesang River were much lower compared to TDS level observed in Malacca River (Hua, 2017) where the study reported that the TDS had ranged from 77.22 mg/L to 10444.61 mg/L. TDS concentration of less than 1000 mg/L in water can be used for drinking and irrigation (Oyem et al., 2014).

The presence of ammonia is due to the presence of decomposing urea, feces and organic matter since that is the initial product of the decomposition of nitrogenous organic wastes and respiration (Zweig, 1999). High NH_3-N concentration can be toxic to aquatic organisms, but it could provide nutrients for growth of algae in small quantities (Corwin et al., 1999). However, the ammonia toxicity is influenced by the increase in temperature, pH and decrease of DO (Zweig, 1999).

Biochemical oxygen demand (BOD) concentrations show the amount of dissolved oxygen used for the decomposition of organic matter by microorganisms (Boyd and Tucker, 2014). Typical BOD amount at pond aquaculture effluent is 10 – 30 mg/L (Suratman et al., 2015). BOD amount at Station 2 slightly exceeded the typical amount of BOD at pond aquaculture effluents. This is because there are many organic materials that can be broken down by microorganisms and this process requires dissolved oxygen (Asman et al., 2017). The sources of the organic matters are fertilizer, animal farm waste and septic system (Al-Badaii et al., 2013).

Chemical oxygen demand (COD) is the amount of dissolved oxygen required by the oxidizing agent of all the organic matter found in the body of water that produces CO_2 and ammonia as a product (Din et al., 2012). COD readings will be compared directly with the BOD reading to distinguish between the value of oxidized material in terms of biological and chemical oxidizing substances (Suratman et al., 2005). Usually, COD reading value is higher than BOD value. This is because it will go through the oxidation process and almost all organic matter will be oxidized (Vesilind et al., 1988). The concentration of COD in unpolluted water is usually less than 20 mg/L (Hassan et al.,

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2018). Thus, the high level of COD indicates the high level of water pollution in the study area (Waziri and Ogugbuaja, 2010) where there were occurrences of organic and inorganic matter oxidation (Hassan et al., 2018).

Total suspended solid (TSS) is defined as the total amount of organic and inorganic particles scattered in water (United States Environmental Protection Agency, 2003). According to Rosli et al. (2010), TSS level of less than 30.0 mg/L was considered low and TSS level of more than 50.0 mg/L was considered high value. The Department of Environment, Malaysia (2006) sets the maximum value of TSS in rivers as 150 mg/L. The river sampling points in this study did not violate the guideline set by Department of Environment (2006). High suspended solids concentrations can lower water quality by absorbing light and increasing the water temperature, making the ability of the water to hold oxygen needed for aquatic life to be reduced (Vesilind et al., 1988). TSS was also linked to nutrients, metals and chemicals transported by the river from anthropogenic activities (Rosli et al., 2010).

Phosphate in aquaculture effluents was continuously supplied by the decomposition of uneaten feed and feces (Boyd, 1998; Jegatheesan et al., 2011). Lin et al. (2002) reported that the range of phosphate concentrations in aquaculture effluent were 3.1 mg/L – 17.7 mg/L. The phosphate concentration at Station 2 was in line with the study reported by Lin et al. (2002). The high level of phosphate in water body is associated with the occurrence of eutrophication (WHO, 1998).

Nitrite is formed through a nitrification process primarily as an intermediary in converting ammonia to nitrate (Zweig, 1999). According to Boyd (2015) and Zweig (1999), high concentration of nitrite is uncommon in aquatic system because nitrite is quickly oxidized to nitrate. Usually, the presence of nitrite is below 0.05 mg/L in oxygenated water. However, the nitrite concentration at Station 2 was above 0.05 mg/L which might be due to low DO concentration (Boyd, 2015). Nitrite is toxic to aquatic animals when the concentration reaches 1 mg/L and above.

Nitrate is the final oxidation product of nitrogen which is very mobile in water (Al-Badaii et al., 2013; Hassan et al., 2018). Nitrate is much less toxic than other major inorganic nitrogen compounds (Zweig, 1999). However, nitrate concentration of 50 mg/L and above in ground and drinking water is considered as Nitrate Vulnerable Zone (Giammarino and Quatto, 2015). High concentration of nitrate in river water is potentially harmful to human and animal health. According to Ling et al. (2007), aquaculture is the main contributor of ecosystem pollution due to the discharging of aquaculture waste into the receiving water. The high concentration of nutrients in a fish pond farm is due to the accumulation of feed residue and fish excreta (Lin et al., 2002).

Total nitrogen is a combination of ammonia, ammonium, nitrite and nitrate (Boyd, 2015; Chen and Kang, 2016). Nitrogen can be found majorly as organic matter in bottom sediments, but small amounts of nitrogen can be found in the water to include nitrogen gas, nitrate, nitrite, ammonia, and nitrogen in dissolved and particulate organic matter (Boyd, 2015). The concentration of total nitrogen at Station 3 was slightly higher than Station 1. Jegatheesan et al. (2011) reported that 22% nitrogen still remains in the water of the catfish pond. About 7% – 32% of total nitrogen in the water was carried by suspended solid (Turcios and Papenbrock, 2014). Generally, the high concentration of nitrogen in rivers is due to the use of nitrogen-containing fertilizers, animal waste, septic system, industrial processes, atmospheric deposition from nitrogen oxide emission, irrigation and storm runoffs from farmlands which can affect the drinking water quality and aquatic ecosystem (Liu et al., 2018).

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Conclusion

In conclusion, we found the physicochemical characteristics of African Catfish, *Clarias gariepinus* pond farm's effluent affect the quality of water for certain parameters such as COD and nitrate that exceed the permissible limit of the Recommended Raw Water Quality Criteria. High concentration of COD and nitrate need to be further treated to reduce the accumulation of the pollutants to the receiving water body. Some parameters such as nitrate cannot be treated by conventional treatments. So, high concentration of nitrate could have an impact on human health if not treated properly. A regular monitoring is vital to determine the effects of catfish pond farm effluents to river.

Acknowledgements. The researchers wish to extend their appreciation to Universiti Kebangsaan Malaysia for funding this research through a research grant GUP-2017-044 and researchers also would like to express their gratitude to Malacca Water Regulatory Board (Badan Kawal Selia Air Melaka) for partial funding of this study.

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INFLUENCE OF PLUVIO-THERMAL CONDITIONS, GROWTH BIOSTIMULATORS AND HERBICIDE ON DRY MATTER CONTENT AND STARCH IN EDIBLE POTATO TUBERS

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(Received 22nd Oct 2018; accepted 7th Jan 2019)

Abstract. In the changing climate conditions, the knowledge of the impact of weather factors on the quality of crop plant yield is of great significance. The field research was conducted in 2015-2017 in the area of eastern Poland in Biała Podlaska commune, in Lublin Voivodship, on light, acidic soil. The purpose of the study was to assess the impact of meteorological conditions and cultivation methods with the application of growth biostimulators and herbicide on dry matter and starch content in tubers of three edible potato cultivars. The examined factors were: I. cultivars of edible potato – Bellarosa, Owacja, Vineta, II. methods of using growth biostimulators: GreenOk-Uniwersal Pro and Asahi SL, as well as their combination with herbicide Avatar 293 ZC. A close relationship between pluvio-thermal conditions, cultivation methods and the percentage content of dry matter and starch in edible potato tubers was proven as a result of the conducted study. The highest concentration of the discussed components was obtained in 2015, in which there was a shortages of precipitation. The methods of cultivation employed in the experiment had a positive impact on the rise in dry matter and starch content in potato tubers in comparison with the control object. The highest dry matter (on average 23.31%) and starch content on average 16.31% in potato tubers was obtained after the application of biostimulator GreenOk-Universal Pro and herbicide Avatar 293 ZC (object 4).
Keywords: *Solanum tuberosum L., weather conditions, Asahi SL, GreenOk-Universal, Avatar 293 ZC, nutrients*

Introduction

In the XXI century farming limitation of use of mineral fertilizers and chemical agents for plant protection, is strived for and more and more often preparations of natural origin are used (Sawicka et al., 2016). In plant production, pro-ecological cultivation methods are being researched. These trends are also visible in potato cultivation. Potato (*Solanum tuberosum* L.) is one of the main cultivated plants not only in Poland, but also worldwide. It is cultivated in over 130 countries and consumed every day by more than a billion people (Ezekiel et al., 2013; Bishwoyog and Swarnima, 2016).

Dry matter and starch content is one of the most important features determining the nutritional and technological value of potato tubers (Ramezani and Aminlari, 2004; Storey, 2007; Murnice et al., 2011).

Edible potato tubers contain about 75% of water and 17-25% of dry matter after harvest. Dry matter content is of great significance, as it determines nutritional value, taste value, consistency of raw and processed tubers, as well as resistance to mechanical damage (Wierzbicka, 2011). Depending on dry matter content, potato cultivars can be divided into 3 groups: with low <20%, medium 20-23% and high content >23% (Wierzbicka, 2012).

The main component of potato tubers' dry matter is starch, its content in dry matter equals from 60 to 80% (Lutaladio and Castaldi, 2009). Starch is a natural polymer

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

found in all plant organisms, further to being an indirect product of photosynthesis, hence it is called a renewable and biodegradable material. It more and more often replaces polymers of petrochemical origin in biotechnological processes and the chemical industry. Potato starch is found mainly in the form of grains with a diameter of 20 to 60 μm. Large grains are very useful in the processing of starch into glucose and dextrins, while small grains – in the production of starch-polyethylene concentrate (Leszczyński, 2004; Kołodziejczyk et al., 2013). Depending on the cultivar, edible potato tubers contain on average from 11.0 to 16.0% of starch, while starch potato tubers from 18.0 to 27.0%, respectively. Starch content is very significant on account of the usefulness of tubers in gastronomy and processing (Pobereżny and Wszelaczyńska, 2011). The impact of starch on the consistency of boiled tubers is connected with its swelling, which causes the destruction of cell structures (Lamberti et al., 2004). The caloric value of starch is the result of it being converted to glucose under the influence of enzymes, thus being quickly digested in the human organism (Leszczyński, 2012).

Dry matter and starch content in *Solanum touberosum L.* tubers is determined by the genetic features of cultivars which are the result of biological progress, the level of agrotechnology and habitat conditions, especially the course of pluvio-thermal conditions during plant growth. Currently, in climate change conditions, the knowledge of the impact of weather factors on the growth and development of crop plants and the quality of yield is of great significance. According to Tosheva and Alexandrova (2004), Shahbazi et al. (2009), Usowicz et al. (2014), Radzka and Rymuza (2015) agro-climatic factors, further to topographical and soil factors, define the kind of agricultural production and its efficiency. Meteorological factors can determine the yielding variability of potato in 40%, and with unfavourable weather conditions even in 50% (Kołodziejczyk, 2013, 2014b). Potato's water requirements in conditions of proper technology depend mainly on the duration of the growing season, the area of cultivation and the kind of soil (Kolbarczyk and Kolbarczyk, 2009). In Polish early potato cultivars have the highest yield with the precipitation of 250-300 mm (Nowak, 1989; Dzielżyc et al., 2012). The rise in air temperature of only 1-2°C increases crop plants' water requirements in Polish conditions on average by 6.3-14.5 mm on a monthly basis (Ziernicka, 2004).

Agriculture in the 21st century still needs modern means of production that will allow moderating the unfavourable effect of meteorological conditions on plant yielding and thus, stimulate plants to grow and develop more effectively (Matysiak et al., 2011; Sawicka et al., 2011). A solution can be the introduction of natural or synthetic preparations, classified as biostimulators or growth stimulators to plant production, (Paradikovic et al., 2011; Kołodziejczyk, 2014a). Growth biostimulators are defined as preparations supporting the natural vital processes of plants and increasing the resistance of plants to stressful conditions, are preparations friendly for the environment and consumer's (Sawicka et al., 2016; Pszczółkowski and Sawicka, 2018). However, the possibility of using these preparations in practice is not fully examined yet and the results of the research are often ambiguous and fragmentary.

Therefore, the purpose of the study was to assess the impact of pluvio-thermal conditions and cultivation methods with the application of growth biostimulators and herbicide on dry matter and starch content in tubers of three early edible potato cultivars.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

Materials and methods

Location of the experiment

The field experiment was conducted in 2015-2017 in eastern Poland, in the Biała Podlaska Commune (52°02'N; 23°07'E), located in the Lublin Voivodeship, on acidic light soil (*Figure 1*).

Figure 1. *Location of the experiment*

Experiment and plant material

A three-year experiment was performed in the split-plot system, in three replications. Two factors were examined in the experiment:

I. Factor – three early edible potato cultivars: Bellarosa, Owacja, Vineta.

II. Factor – methods of application of biostimulators and herbicide:

1. Standard object – mechanical treatment (without biostimulators and herbicide).
2. From sprouting of potato plants – mechanical treatment and after sprouting – GreenOK Universal–PRO bioactivator, three times to leaves: at a dose of 0.10 dm^3 ha^{-1} – peak–end of sprouting (phase BBCH 13-19) + 0.15 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55).
3. From sprouting of potato plants – mechanical treatment, and after sprouting – Asahi SL bioactivator, three times to leaves at a dose of 0.50 dm^3 ha^{-1} – peak–end of sprouting (phase BBCH 13-19) + 0.50 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.50 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55).
4. From sprouting – mechanical treatment, and after the final shaping of ridges and just before sprouting Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05). After sprouting – three applications of GreenOK Universal–PRO bioactivator at a dose of 0.10 dm^3 ha^{-1} – peak–end of sprouting (phase BBCH 13-19) + 0.15 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55).
5. From sprouting – mechanical treatment, and after the final shaping of ridges before sprouting of potato plants – Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05).

The biostimulator Asahi SL contains three active ingredients from the nitrophenol group: sodium para-nitrophenol – 0.3%, sodium ortho-nitrophenol – 0.2% and sodium

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DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

5-nitroguaiacol – 0.1%. These substances occur naturally in plant cells and participate in many metabolic processes. The preparation is known in the USA by its trade name as Chaperone, and in Europe as Atonik (Babuška, 2004). The preparation GreenOK Universal-PRO, according to the producer's information (Latvian Institute of Humic Substances), is a liquid organic fertilizer with biostimulating effect, consisting of humic substance concentrate (20 g dm^3) and elements NPK (0.13-0.09-0.7%). The herbicide Avatar 293 ZC applied in the experiment is a preparation in the form of capsule suspension. It is applied to soil, before sprouting of plants on moist soil. It contains clomazone (a substance from the isoxazolidinone group) – 60 g dm^3 (5.13%) and metribuzin (a substance from the triazinone group) – 233 g dm^3 (20.64%).

Each year in autumn, before establishment of the experiment, natural fertilization was used at a dose of 25 t ha^{-1}, as well as mineral fertilization with phosphorus 44.0 kg P ha^{-1} (triple superphosphate 46%) and potassium 124.5 kg K ha^{-1} (potassium salt 60%), and during spring – nitrogen fertilization (ammonium nitrate 34%) at a dose of 100 kg N per 1 ha. Potato tubers were planted in the second decade of April (in 2015 and 2016) and in the third decade of April (in 2017). Protection treatment against diseases and pests was used according to needs, in accordance with the plant protection recommendations. Harvest was performed in the phase of full technological maturity of tubers.

The content of dry matter in fresh tuber mass was determined by means of the oven-dry method, that of starch on Reimann's balance.

Data analysis

The results of the study were subjected to the statistical analysis by means of StatSoft Inc. STATISTICA v.10 using the method of the analysis of variance. Significance of variability sources was tested by means of the Fisher-Snedecor F test, and the assessment of significance of differences at the significance level $p = 0.05$ between the compared means using Tukey's range test was performed. In order to determine the relationship between pluvio-thermal conditions and dry matter and starch content in potato tubers, Pearson's r correlation coefficients were measured, and the significance at the confidence level 0.05 was tested with Student's *t* test. Statistical calculations were performed on the basis of average values from three years and means for three potato cultivars.

Weather conditions

Weather conditions in the growing seasons of 2015-2017 (years of research conduct) were characterized against the background of the years 1981-2010 on the basis of deviations of average monthly air temperature (°C) and the sum of precipitation (mm). The values of hydrothermal Sielianinov coefficient were measured (*Equation 1*), (*Table 1*) (Chereszkowicz, 1979; Skowera and Puła, 2004; Skowera et al., 2014).

$$K = \frac{P}{0.1\sum t} \qquad \text{(Eq.1)}$$

K – value of hydrothermal coefficient,
P – sum of monthly precipitation,
$\sum t$ – monthly sum of average daily air temperature >0°C.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

Values of air temperature and precipitation sum from the growing period of 2015-2017 years and the years between 1981-2010 came from Meteorological Station in Cicibór Duży (51°19'N; 22°16'E), located at a distance of 6 km from Biała Podlaska and belonging to the Experimental Station of the Central Research Facility of Varieties of Cultivated Plants (COBORU) in Słupia Wielka.

During the conduct of research diverse pluvio-thermal conditions occurred (*Table 1-2, Figures 2-3*). Growing season of 2015 was characterized by a high shortage of precipitation in the period from June to August (that is during flowering, growth of tubers and crop accumulation), and the month of August 2015 was extremely dry, deviations from the average multi-year air temperature in that month amounted to +3.7°C, while precipitation in comparison to the years 1981-2010 was lower by 60 mm, these months in 2017 were similar but the drought turned out to be milder. In 2016, in the period of the largest demand of potato for precipitation, thermal-precipitation conditions were more beneficial when compared to the remaining years of research (*Table 1-2, Figures 2-3*).

Table 1. *Hydrothermal conditions in potato's growing season (mean for years 2015-2017)*

Month/ Year	***Sielianinov hydrothermal coefficient *(K)***					
	2015		**2016**		**2017**	
IV	1.43	optimal	1.19	quite dry	2.68	very humid
V	2.28	humid	0.47	very dry	0.93	dry
VI	0.59	very dry	1.53	optimal	1.97	quite humid
VII	0.80	dry	1.97	quite humid	1.24	quite dry
VIII	0.11	extremely dry	0.48	very dry	0.66	very dry
IX	1.66	quite humid	0.26	extremely dry	2.37	extremely humid

*Sielianinov hydrothermal coefficient *(K)* – formula as in research methodology;
**Month's classification: extremely dry $K \leq 0.4$, very dry $0.4 < K \leq 0.7$, dry $0.7 < K \leq 1.0$, quite dry $1.0 < K \leq 1.3$, optimal $1.3 < K \leq 1.6$, quite humid $1.6 < K \leq 2.0$, humid $2.0 < K \leq 2.5$, very humid $2.5 < K \leq 3.0$, extremely humid
$K > 3.0$ (Chereszkowicz, 1979; Skowera and Puła, 2004; Skowera et al., 2014)

Table 2. *Mean air temperature (°C) and precipitation sum (mm) (in the years 1981-2010)*

Meteorological factor	**Months**					
	IV	**V**	**VI**	**VII**	**VIII**	**IX**
Air temperature (°C)	7.9	13.9	16.6	18.8	17.9	12.7
Precipitation sum (mm)	35	59	66	70	67	54

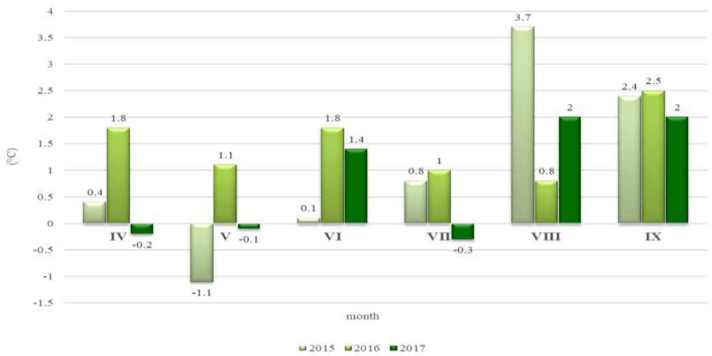

Figure 2. *Deviations of the atmospheric air temperature in April-September (2015-2017) from the long-term average value (1981-2010)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

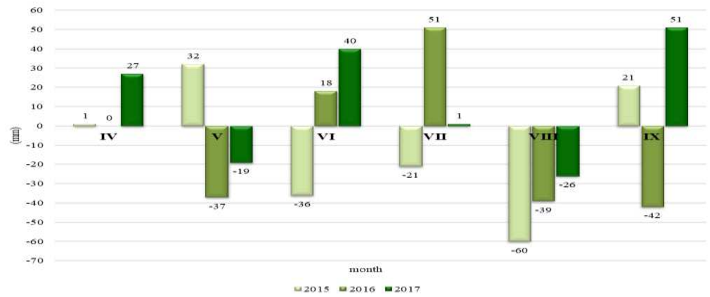

Figure 3. *Deviations of the sum of monthly precipitation in April-September (2015-2017) from the long-term average value (1981-2010)*

Results and discussion

The results of the study proved that there is a close relationship between pluvio-thermal conditions, cultivation methods and the percentage content of dry matter and starch in edible potato tubers.

The calculated Pearson's *r* correlation coefficients proved that there are significant negative relationships between the precipitation sum, Sielianinov hydrothermal coefficient, and dry matter and starch content in potato tubers (*Table 3*). A significant negative relationship between average precipitation sum in the years when the study was carried out and average dry matter and starch content in potato tubers was confirmed. In the case of dry matter content, the correlation coefficient was: r = -0.62 (strong correlation, substantial relationship), and in the case of starch content: r = -0.56 (moderate correlation, significant relationship). It means that the rise in precipitation sum had a significant impact on the drop in the percentage content of dry matter and starch in edible potato tubers.

Table 3. *The values of Pearson's r correlation coefficients between the percentage content of dry matter and starch in potato tubers and pluvio-thermal conditions (mean from three years and cultivars)*

Variable *X*	Variable *Y*	*r (X. Y)***	r^2	*t*	*p*
dry matter content (%)	air temperature (ºC)	0.20	0.04	1.37	0.1775
	precipitation (mm)	-0.62	0.39	-5.22	0.0000*
	Sielianinov hydrothermal coefficient *(K)*	-0.59	0.35	-4.78	0.0000*
starch content (%)	air temperature (ºC)	0.24	0.06	1.61	0.1154
	precipitation (mm)	-0.56	0.31	-4.42	0.0001*
	Sielianinov hydrothermal coefficient *(K)*	-0.54	0.29	-4.16	0.0002*

R (X. Y) – Pearson's *r* correlation coefficient, r^2 – coefficient of determination, *t* – the value of Student's t test, *p* – significance level, *significant differences at p<0.05, **the ranges of correlation according to Sobczyk (2011): below 0.2 – very weak correlation (no relationship), 0.2-0.4 – weak correlation (clear relationship) 0.4-0.6 – moderate correlation (significant relationship), 0.6-0.8 strong correlation (substantial relationship), 0.8-0.9 – very strong correlation (very substantial relationship), 0.9-1.0 – full relationship

A significant negative relationship between Sielianinov hydrothermal coefficient (K) and dry matter and starch content in tubers was proven as well (*Table 3*). In the case of

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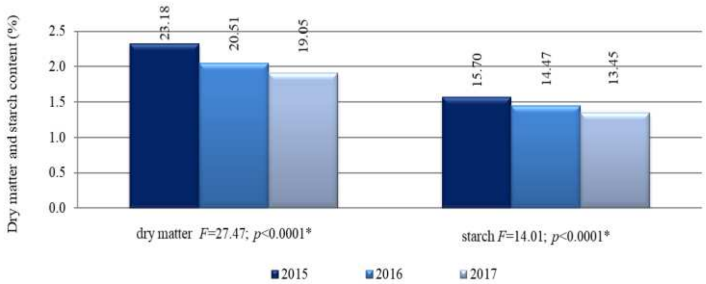
DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

these features, a linear negative correlation in the range of moderate correlation (significant relationship) was obtained.

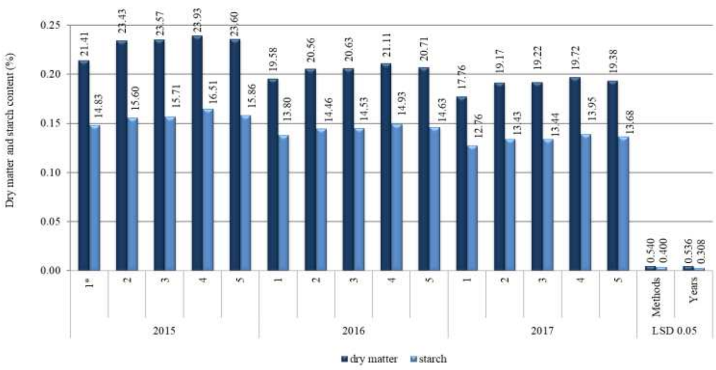
Analysing the impact of air temperature on dry matter and starch content, no significant statistical differences were proven, but instead, weak linear positive correlation (clear relationship) was found (*Table 3*). Other authors' studies also confirm the significant impact of pluvio-thermal conditions in particular periods of the growing season on dry matter and starch content in potato tubers. According to Skowera and Puła (2004), Wierzbicka (2011), Kołodziejczyk (2014b) substantial amounts of precipitation were a factor that significantly limited the storage of dry matter and starch in tubers. However, in the research conducted by Pszczółkowski et al. (2016) meteorological conditions with very humid May, June, July and dry August created favourable conditions for obtaining higher starch content in tubers. Wierzbicka (2012) and Kołodziejczyk (2014b) stated that air temperature, beside moisture conditions, has a significant impact on the storage of dry matter and starch in tubers, and especially warm and dry months late in the growing season create favourable conditions for the storage of dry matter and starch. Kalbarczyk and Kalbarczyk (2009) proved that the optimal air temperature allowing potato plants to grow and develop should be at the level of 15.2ºC.

Within own research significantly larger contents of dry matter and starch in edible potato tubers in dry growing season of 2015 than in the remaining years of research in which greater volumes of precipitation were recorded (*Figures 4-5*).



F - the value of *F* test in the analysis of variance, *– significant differences at $p<0.05$

Figure 4. *Average dry matter and starch content (%) in potato tubers in research years(2015-2017)*



* – labelling as in research methodology

Figure 5. *Average dry matter and starch content (%) in potato tubers depending on cultivation methods and research years*

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This study proved that the cultivation methods applied in the experiment with the use of growth biostimulators and herbicide had a significant impact on dry matter and starch content in potato tubers (objects 2.-5.) in comparison with only mechanical treatment (objects 1.) (*Figure 6*).

The highest content of dry matter as well as starch was found in the tubers harvested from objects 4. where mechanical treatment was used from sprouting of plants and Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} just before sprouting, and GreenOK Universal–PRO bioactivator at a dose of 0.10 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} three times after sprouting; and from objects 5. where mechanical treatment was used and then Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (*Figure 6*). The research of Sawicka (2003), Czeczko and Mikos-Bielak (2004), Sawicka and Krochmal-Marczak (2009), Kołodziejczyka (2016) also confirms the rise in dry matter and starch content in potato tubers in the conditions of using growth biostimulators. However, according to Maciejewski et al. (2007), the applied biostimulators Asahi SL and Atonik, did not have any impact on dry matter and starch content in potato tubers. The research of Gugała and Zarzecka (2010) indicated a positive impact of herbicides on the rise in dry matter and starch content in potato tubers. The mechanical and chemical treatment applied by these authors had a positive impact on the rise in dry matter and starch content in potato tubers in comparison with only mechanical treatment. However, in the study of Barabaś and Sawicka (2016), dry matter and starch content did not depend on potato cultivation method.

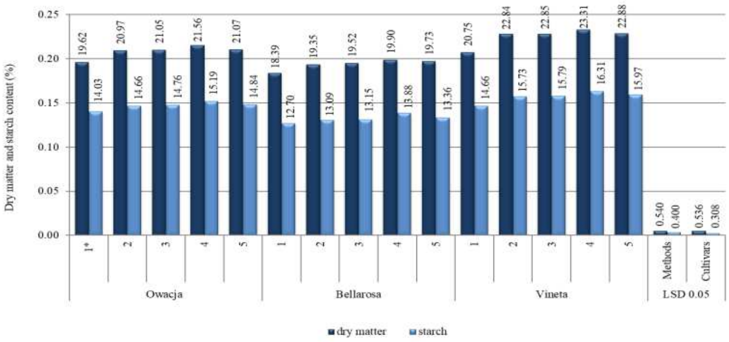

Figure 6. *Average Dry matter and starch content (%) in potato tubers depending on cultivation methods and planted potato cultivars (mean for years 2015-2017)*

The study proved that the cultivars' genetic features had a significant impact on dry matter and starch content in tubers (*Figure 6*). The highest average dry matter and starch content was stored in the Vineta cultivar (from 20.75% to 23.31% dry matter content and from 14.66% to 16.31% starch content), and the lowest in the Bellarosa cultivar (from 18.39% to 19.90% dry matter content and from 12.70% to 13.88% starch content) (*Figure 6*). According to other authors as well, the content of nutrients in potato tubers depended mainly on the cultivars' genetic features (Zarzyńska and Goliszewski, 2006; Abong et al., 2010; Krzysztofik and Skonieczny, 2010). However, Bombik et al. (2003) indicated that not only the genotype (cultivar) but also the environment and the genotype-environment interaction had a significant impact on most of the analysed quality features of potato tubers. Rytel (2004) and Lisińska (2006) stated that 20-22% content of dry matter and 15-17% content of starch determine the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

usefulness of cultivars to being processed into chips, and in the case of crisps, the values should be: 21-25% of dry matter and 16-20% of starch, respectively. The high content of dry matter in tubers causes lower fat absorption during technological processes, which makes the final product healthier for the consumer.

Conclusions

The content of dry mass and starch in edible potato tubers was determined by thermal-precipitation conditions. In the years characterized by a larger sum of precipitation in the growing period, potatoes gathered less dry mass and starch in comparison to the warm and dry growing season of 2015. Application in the experiment of the treatment methods with the use of GreenOk-Uniwersal Pro and Asahi SL biostimulators and their combination with Avatar 293 ZC herbicide positively impacted the increase of content of dry mass and starch in potato tubers in comparison to the tubers gathered from the control object. Moreover, genetic features of the cultivars had a significant impact on the content of dry mass and starch. The largest concentration of the discussed components was found in Vineta cultivar.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15471557

DETERMINING IRRIGATION SCHEDULING AND DIFFERENT MANURE SOURCES OF YIELD AND NUTRITION CONTENT ON MAIZE (*ZEA MAYS* L.) CULTIVATION

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(Received 8th Aug 2018; accepted 16th Jan 2019)

Abstract. The aim of this study was to determine the most suitable irrigation schedules and sources of manure for maize under Mardin ecological conditions. For this purpose, four sources of fertilizer and three irrigation schedules were applied in hybrid maize cultivar to find out their effects on the yield and yield factors in the years of 2014 and 2015. The study used a randomized complete block with split block design with 3 replications. In the study, significant differences were determined on traits at the level of 1% and 5%. The plant height, first cob height, cob length, number of seeds per cob, cob yield, 1000 seed weight, seed yield per unit, hectoliter, protein and oil ratio in seed, Mg, K, Fe, Ni, Cu, Zn, Mn, Na, Ca content of seeds were significantly influenced by irrigation schedules and sources of fertilizer for two years. The highest values were obtained from chicken manure with 9646 kg ha^{-1} in the first year and 11019 kg ha^{-1} in the second year, whereas the lowest values were obtained from control with 6363 and 6662 kg ha^{-1} for the first year and the second, respectively. But the difference between 180-80 kg N-P_2O_5-ha^{-1} and chicken manure was not statistically significant in the second year. According to the irrigation schedules, the grain yield ranged between 6386-10678 kg/ha in the first year and 7575-12133 kg ha^{-1} in the second year. The lowest grain yield per area was from the four-irrigation application, whereas the highest values were obtained from the six-irrigation application. The results of the study showed that grain yield and nutrient level of corn seeds increased, depending on irrigation time and number.
Keywords: *Zea mays, water application, organic and inorganic fertilizer, macro and micro element, yield*

Introduction

Maize is a widely cultivated grain cereal for food consumption on the global scale with 183 million hectares and 1.031 million tons of worldwide production. The area sown in Turkey is 6.390.000 hectares and it produces 5.900.000 tons. Average global yield per year is 563 kg ha^{-1}, 9230 kg ha^{-1} for Turkey (FAO, 2017). Maize is a major crop for both direct and indirect human consumption as it forms a major energy feed for livestock. It is a cheap source of raw material for various agricultural industries and extensively used for preparation of corn starch.

Appropriate development and growth of maize need favorable moisture in root zone. The amount of moisture in the soil decreases in time during dry seasons. Limited water supply during the growing season results in soil and plant water deficits, causing a decrease in maize yields (Gordon et al., 1995; Patel et al., 2006). Timing and supplemental irrigation is important in irrigation scheduling for the most effective use of available water in optimizing maize production. Water deficit affects timing of emergence, number of leaves per plant but delays tasseling initiation and silking,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

reduces plant height and vegetation growth of maize (Abrecht and Carberry, 1993; Singh et al., 2007).

The constant use of chemical fertilizers leads to hazards concerning health and environment (Pimentel et al., 1996). The importance of the use of organic manures under low input agricultural systems has been the subject of many studies. Majority of these studies aim at improving the quality and fertility of soil (DeJager et al., 2001; Palm et al., 2001). Manure can serve as a source of significant nutrient element which includes P and N (Gilley and Eghball, 2002). The application of manure to land give rise to similar results to those obtained when inorganic fertilizers are applied (Eghball and Power, 1999). The amount of organic matter in manure can significantly increase soil aggregation, structure and water holding capacity, infiltration, microbial activity, and can reduce erosion and soil compaction (Haynes and Naidu, 1998; Gilley and Risse, 2000). Poultry manure is relatively a cheap source of macro and micro nutrients (N, P, K, Ca, Mg, S, Cu, Fe, Mn, B). It has the potential to increase soil carbon and N content, soil porosity and enhance microbial activity. The hazards of chemical fertilizers, which occurred in heavy and unbalanced ways, led to the use of organic manures in intensively growing areas for sustainable production system. Therefore, in order to sustain the land and achieve potential crop production, careful use of organic manures has become important scientifically. It must be stressed that the importance of farm yard manure, vermicompost, chicken manure and green leaf manure in soil improvement depends on their nutrient content. It helps the improvement of soil structure and water holding capacity (Kale and Bano, 1986; Srivastava, 1998).

The aim of this research is to determine the effects of irrigation scheduling and different manure sources on yield and nutrition content in maize cultivation.

Materials and Methods

This trial was conducted to determine the most suitable irrigation schedules and sources of manure in a well-adapted maize (*Zea mays* L.) Farw. cultivar Limagrain Helen, in 2014 and 2015 in the fields of Kiziltepe Vocational High School of Mardin Artuklu University (longitude 40º57´59.01"E, latitude 37º07´34.74"N, and altitude 494.0 m) (*Figure 1*). Cultivar Limagrain Helen is intensively grown as a kind of grain maize in the region.

Figure 1. *The research was conducted in Kiziltepe/Mardin of Turkey*

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DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

In 2014 and 2015, precipitation throughout the season was 158.6 mm and 133.6 mm respectively, and the average over the long-term for the same period was 111.1 mm (*Table 1*).

Table 1. *Meteorological data for the growing seasons of 2014, 2015 and long-term averages in Mardin, Turkey (TSMS, 2016)*

	Precipitation (mm)			Average temperature (°C)				Relative humidity (%)	
Months	**2014**	**2015**	**LTA***	**2014**	**2015**	**LTA***	**2014**	**2015**	**LTA***
June	1.4	2.9	4.1	25.9	25.9	25.7	22.5	29.0	32.3
July	1.0	0.2	1.1	30.4	31.6	29.9	20.0	19.6	27.7
August	7.0	0.4	0.5	31.3	30.5	29.6	17.1	25.8	28.4
September	10.8	9.9	1.9	24.3	28.4	25.1	31.2	23.0	32.6
October	42.8	58.2	32.6	18.1	19.5	18.4	47.2	49.6	45.1
November	95.6	62.0	70.9	10.2	11.7	10.9	53.1	50.3	57.0
Total	158.6	133.6	111.1						
Average				23.4	24.6	23.3	31.9	32.9	37.2

*LTA = Long-term average (1960-2015)

Average temperature was 23.54°C in 2014 and 24.6°C in 2015. There was an increase in average temperature when it is compared to long term average of 23.3°C. Average relative humidity was 31.9% in the first and 32.9% in the second vegetation periods (TSMS, 2016) and 37.2% for the long term. The soil, in which this study carried out, is classified as entisols according to soil taxonomy (Soil Survey Staff, 1999). The results of calcareous soil analysis are as follows: sandy loam texture, very low organic matter and moderate in available phosphorus (*Table 2*).

The experiment was laid out in a randomized complete block design (RCBD) with split-plot arrangement keeping the irrigation schedules in main plots and integrated manure sources in sub plots. The experiment was replicated three times. The net plot size was 5m x 2.8m.

Table 2. *Some properties of the <2 mm fraction of the top 30 cm of soil used for site*

Soil properties	Site
Texture	sandy loam
pH[A]	7.59
Clay (%)[B]	29.6
$CaCO_3$ (%)[C]	18.9
Olsen soil test P (ppm)[D]	57.8
Total Salt (%)[E]	0.059
Organic matter (%)[F]	1.59

[A]1:2.5 soil:water, [B]Bouyoucos (1951), [C]lime by calcimetric methods, [D]Olsen et al. (1954), [E]Richard (1954), [F]Jackson (1962)

The treatments were three irrigation schedules i.e. I1: four irrigations (1st at three leaf stage, 2nd at ninth-leaf stage, 3rd at tasseling stage, 4th at milking stage), I2: five irrigations (1st at fourth leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at milking stage, 5th at dough stage) and I3: six irrigations (1st at third leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at blister stage, 5th at milking stage, 6th at dough stage) and four manure (M0: control, M1: 180-80 kg N-P_2O_5- ha^{-1}, M2: Chicken manure 15 t ha^{-1} and M3: Farmyard manure 15 t ha^{-1}). Manure and irrigation were applied

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DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

according to the treatments. All other agronomic practices were kept normal and uniform for all treatments. Observations on growth and yield parameters were recorded using standard procedures. All fertilization and irrigation applications were conducted as (Sharif et al., 2004; Hussaini et al., 2008; Rezvantalab et al., 2008). The concentrations of Zn, Fe, Mn and Cu in the digested solutions were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, OPTIMA 3300 DV, Perkin-Elmer, USA). IPE556 grain and IPE883 straw (Wageningen University, The Netherlands) were used as reference materials (Hussaini et al., 2008).

Three different irrigation methods were applied in the research, and the irrigation was the same in the both seasons of 2015 and 2016. Details of irrigation applications are given below (*Table 3*).

Table 3. *Details of different irrigation application have been shown*

Years	1.st irrigation application		2nd irrigation application		3rd irrigation application	
2015* 2016	1st at three leaf stage	125 mm	1st at three leaf stage	125 mm	1st at three leaf stage	125 mm
	2nd at ninth-leaf stage	125 mm	2nd at ninth-leaf stage	125 mm	2nd at ninth-leaf stage	125 mm
	3rd at tasseling stage	125 mm	3rd at tasseling stage	125 mm	3rd at tasseling stage	125 mm
	4th at milking stage	125 mm	4th at milking stage	125 mm	4th at milking stage	125 mm
			5th at dough stage	125 mm	5th at dough stage	125 mm
					6th at dough stage	125 mm
Total	500 mm		625 mm		750 mm	

* Irrıgation was used the same for both (2015 and 2016) seasons

The sowing was made 5 cm depth, the eight seeds counted for per square meter and the separate of between two rows calculated 70 cm and between two plant on bed 20 cm. The first-year sowing was down on 15th of June 2014 with trial plantation and harvest was made on 5 November 2014. The second-year sowing was made on 13 June 2015; the harvest was made on 10 November 2015. On the other hand, chemical application and hoeing were made against weeds during the growing season in the trial plots. Any chemical drugs were not used during these two years, because there was no shown disease in the trials.

Observations and measurements in each plot were calculated by taking the mean of 10 randomly selected plants. Observations and measurements were obtained as suggested by Turgut (2003).

The effect of treatments (Irrigation schedules and sources of manure) on maize were analyzed using the analysis of variance procedures for a randomized complete block design with the SAS (1998) statistical package. When the F-value of the ANOVA was significant at the $P<0.05$ level of probability. The means related with nutrient content, yield and yield components in maize were evaluated with Duncan's Multiple Range Test statistical analysis.

Results

Regarding maize grown under different fertilizer and irrigation treatments, results in *Table 4* indicated that all the studied characteristics were significantly affected by irrigation treatments over the two growing seasons.

The highest plant height, according to the sources of manure, were obtained from M1 with 226.9 cm and 215.7 cm for 2014 and 2015, respectively. But the difference with

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DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

M2 (chicken manure) was not statistically significant in both years. The lowest plant heights were obtained from control plots with 205.4 cm and 200.3 cm in 2014 and 2015, respectively. According to the irrigation schedules, the plant height ranged between 199.4 and 239.7 cm in the first year, and 190.5 and 239.9 cm in the second.

In terms of first cob height, the highest values were obtained from M1 with 89.7 cm and 91.8 cm for both years, whereas the lowest first cob height was obtained control plots (M0). On the other hand, the difference among M, M2 and M3 was found to be statistically insignificant in the first year. According to the irrigation schedules the first cob height ranged between 83.2 and 90.7 cm in 2014, and 80.2 and 90.4 cm in 2015. The lowest first cob heights were observed in the I1 application, whereas the highest values were obtained from the I3 application (*Table 4*).

Table 4. *Effect of irrigation schedules and sources of manure on yield and yield components of maize and Duncan groups (2014-2015)*

Characters	Years	Sources of manure				Irrigation schedules		
		M0	**M1**	**M2**	**M3**	**I1**	**I2**	**I3**
Plant height (cm)	2014	205.1 c	226.9 a	225.7 a	219.6 b	199.4 c	218.9 b	239.7 a
	2015	200.3 c	215.7 a	214.2 a	209.8 b	190.5 c	199. 5 b	239.9 a
First cob height (cm)	2014	83.4 b	89.7 a	88.9 a	88.3 a	83.2 c	87.7 b	90.7 a
	2015	84.9 c	91.8 a	87.9 bc	90.4 ab	80.2 b	85.7 a	90.4 a
Cob height (cm)	2014	17.7 d	19.1 c	20.9 a	20.3 b	17.2 c	19.3 b	22.1 a
	2015	18.1 c	19.5 b	21.3 a	19.9 b	17.8 c	18.8 b	22.6 a
No. of grains/cob (number)	2014	323.3 c	394.1 b	419.6 ab	431.3 a	289.3 c	347.6 b	546.1 a
	2015	375.3 c	390.2 c	468.7 a	448.1 b	371.7 c	399.8 b	490.2 a
Grain yield of cob (g)	2014	24.4 d	28.3 c	36.1 a	33.0 b	24.5 c	27.1 b	39.7 a
	2015	31.8 b	38.7 a	38.2 a	37.6 a	29.4 c	36.5 b	43.7 a
1000-seed weight (g)	2014	121.1 b	124.0 b	134.8 a	132.9 a	118.9 c	126.9 b	138.7 a
	2015	135.7 b	137.9 ab	143.7 ab	145.1 a	135.5 b	139.4 ab	148.9 a
Grain yield (kg ha^{-1})	2014	6362 d	7833 c	9646 a	8841 b	6386 c	7448 b	10678 a
	2015	Çö	11243 a	11019 a	9429 b	7575 b	9056 b	12133 a
Hectoliter (kg)	2014	69.8 c	71.1 b	73.9 a	72.1 b	69.4 b	73.1 a	72.6 a
	2015	69.9 d	71.4 c	72.8 a	72.1 b	70.4 c	71.7 b	72.7 a
Seed oil content (%)	2014	3.50 c	4.05 a	3.89 ab	3.84 b	3.60 b	3.73 b	4.13 a
	2015	3.57 d	4.32 a	3.94 b	3.72 c	3.58 c	3.91 b	4.17 a
Seed protein ratio (%)	2014	8.9 c	10.4 b	10.9 a	10.3 b	9.6 b	9.9 b	10.8 a
	2015	9.01 b	9.50 b	10.6 a	9.91 ab	9.30 b	9.46 b	10.51 a

I1: Four irrigations (1st at three leaf stage, 2nd at ninth-leaf stage, 3rd at tasseling stage, 4th at milking stage), **I2:** Five irrigations (1st at fourth leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at milking stage, 5th at dough stage) and **I3:**Six irrigations (1st at third leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at blister stage, 5th at milking stage, 6th at dough stage) and four manure (**M0:**control, **M1:** 180-80 kg N-P_2O_5- ha^{-1}, **M2**: Chicken manure 15 t ha^{-1} and **M3:** Farmyard manure 15 t ha^{-1})

The highest cob lengths according to the sources of manure were obtained from M2 with 20.9 cm and 21.3 cm for the first and second years, respectively. The lowest cob lengths were obtained from control plots (M0) with 17.7 and 18.1 cm for 2014 and 2015, respectively. The highest cob lengths for irrigation were obtained from I3 with 22.1 and 22.6 cm in the first year and the second, respectively, while the lowest values were obtained from I1 for both years.

The highest number of grains/cob, according to the sources of manure, was obtained from M3 with 431.3 grains/cob in 2015. The highest number of grains/cob was obtained from M2 with 468.7 grains/cob in 2015. The lowest grains/cob were obtained from

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control plots in both years but the difference between M0 and M1 was not statistically significant in the second year. In terms of irrigation schedule, the number of grains/cob ranged between 289.3 and 546.1 grains/cob in the first year, and 371.7 and 490.2 grains/cob in the second year.

In terms of grain yield of cob, while the highest value was collected from M2 with 36.1g, the lowest value was obtained from 24.4 g for the first year. Whereas, the highest value was obtained from M1 with 38.7 g but the difference among M1, M2 and M3 was found to be statistically insignificant in the second year. According to the irrigation schedules, grain yield of cob ranged between 24.5 and 39.7 g in 2014, and 29.4 and 43.7 g in 2015. The lowest values were collected from the I1 application, whereas the highest values were obtained from the I3 application (*Table 4*).

Significant results of both factors under study during both years of experimentation were found. According to the source of manure, the highest 1000 seed weight was obtained from M2 with 134.8 g in the first year, while the highest 1000 seed weight was collected M3 with 145.1 g in the second year. The lowest values were obtained from control plots in both years. In terms of irrigation schedule, the highest 1000 seed weights were obtained from I3 with 138.7 and 148.9 g for years, whereas the lowest values were obtained from the I1 application with 118.9 and 135.5 g for the first year and the second, respectively.

In terms of grain yield, the highest values were obtained from M2 with 9646 and 11019 kg ha^{-1} for 2014 and 2015, while the lowest values were obtained from M0 with 6363 and 6662 kg ha^{-1} for both years, respectively. But the difference between M1 and M2 was not statistically significant in the second year. These finding are also supported by (Seker and Ersoy, 2005). According to the irrigation schedules, the grain yield ranged from 6386 to 10678 hg/ha in the first year, and from 7575 to 12133 kg ha^{-1} in 2015. The lowest seed yield was collected from the I1 application, whereas the highest values were obtained from the I3 application (*Table 3*).

The highest hectoliter weight was obtained from M2 with 73.9 and 72.8 kg for the first year and the second, whereas the lowest hectoliter weight were observed from control plots both years. In terms of irrigation schedule, the highest hectoliter weights were obtained from I2 with 73.1 kg in the first year, I3 with 72.7 kg in 2015, the lowest values were obtained from I1 application with 69.4 and 70.4 kg for the first year and the second, respectively. But the difference between I2 and I3 was statistically insignificant in 2014.

The highest grain oil contents were reached from M1 with 4.05-4.32%, the lowest values were obtained from M0 application with 3.50-3.57% for 2014 and 2015, respectively. According to the irrigation schedules the seed oil content ranged between 3.60 and 4.13% in 2014, and 3.58 and 4.17% in 2015. The lowest seed oil contents were obtained from the I1 application, whereas the highest values were obtained from the I3 application (*Table 5*). In terms of seed protein ratio, the highest values were obtained from M2 with 10.9 and 10.6% for the first and the second years, the lowest values were obtained from control plots with 8.9 and 9.01% for both years, respectively. The highest seed protein ratio for irrigation schedule were obtained from I3 with 10.8 and 10.51% in 2014 and 2015, respectively, while the lowest values were obtained from I1 but the difference between I1 and I2 was statistically insignificant for both of the years.

The results on the nutrient contents of maize, based on the application of different manures and irrigation schedules, are showed in *Table 4*. When the effect of irrigation schedules and sources of manure on nutrient contents of maize seed, the highest K

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DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

values were obtained from the M2 application in both of the years but there among M1, M2 and M3 was not statistically significant in the first year. When potassium is examined in terms of irrigation schedule while the highest values obtained from I3, the lowest values obtained from I1, but the difference between I2 and I3 was statistically insignificant in the second year.

The highest Na, Mg and Ca values were obtained from M2 application, while the lowest values were obtained from M0 application in 2014 and 2015. According to the irrigation schedule, the I1application gave the lowest value while the I3 gave the highest value (*Table 5*).

Table 5. *Effect of irrigation schedules and sources of manure on nutrient contents of maize seed and Duncan groups (2014-2015)*

Characters	**Years**	**Sources of manure**				**Irrigation schedules**		
		M0	**M1**	**M2**	**M3**	**I1**	**I2**	**I3**
K (ppm)	2014	2468.7 b	2757.1 a	2926.5 a	2814.1 a	2121.2 c	2807.5 b	3296.0 a
	2015	2489.4 c	3018.3 b	3472.6 a	3084.5 b	2576.8 b	3136.7 a	3335.1 a
Na (ppm)	2014	18.31 c	27.72 b	33.74 a	27.85 b	13.30 c	27.18 b	40.23 a
	2015	27.02 c	32.86 b	36.46 a	32.40 b	24.70 c	30.80 b	41.06 a
Mg (ppm)	2014	667.7 b	675.5 b	717.7 a	663.2 b	664.0 c	686.3 b	712.2 a
	2015	713.8 c	740.1 b	763.1 a	742.3 b	725.6 b	734.5 b	759.3 a
Ca (ppm)	2014	47.55 b	56.68 ab	61.72 a	54.90 ab	46.10 b	56.64 ab	62.90 a
	2015	35.40 d	40.88 c	46.52 a	43.40 b	36.00 c	41.19 b	47.45 a
Fe (ppm)	2014	12.11 c	14.24 b	15.41 a	14.37 b	12.51 c	14.20 b	15.39 a
	2015	12.57 c	16.42 b	18.07 a	15.72 b	14.25 b	16.15 a	16.69 a
Cu (ppm)	2014	2.916 d	3.223 c	4.330 a	3.897 b	3.141 c	3.430 b	4.204 a
	2015	3.970 c	5.858 a	4.858 b	4.634 b	4.406 b	4.791 ab	5.293 a
Zn (ppm)	2014	13.45 d	14.70 c	17.37 a	16.05 b	14.80 b	15.07 b	16.30 a
	2015	14.11 b	14.88 b	16.72 a	14.50 b	13.10 c	15.37 b	16.83 a
Mn (ppm)	2014	5.484 c	5.755 b	6.258 a	6.191 b	5.524 c	5.74 b	6.369 a
	2015	2.955 c	3.455 b	4.123 a	3.615 b	3.383 b	3.426 b	3.802 a
Ni (ppm)	2014	0.316 b	0.394 ab	0.423 a	0.418 a	0.333ns	0.405ns	0.425ns
	2015	0.460 b	0.484 b	0.560 a	0.508 ab	0.511 a	0.466 b	0.531 a
Pb (ppm)	2014	0.367 ns	0.432 ns	0.350 ns	0.412 ns	0.319 b	0.372 b	0.480 a
	2015	0.405 c	0.577 b	0.638 a	0.546 b	0.430 c	0.571 b	0.657 a

I1: Four irrigations (1st at three leaf stage, 2nd at ninth-leaf stage, 3rd at tasseling stage, 4th at milking stage), **I2:** Five irrigations (1st at fourth leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at milking stage, 5th at dough stage) and **I3:** Six irrigations (1st at third leaf stage, 2nd at ninth leaf stage, 3rd at tasseling, 4th at blister stage, 5th at milking stage, 6th at dough stage) and four manure (**M0:** control, **M1:**180-80 kg N-P_2O_5- ha^{-1}, **M2:**Chicken manure 15 t ha^{-1} and **M3:**Farmyard manure 15 t ha^{-1})

In terms of Fe contents, the highest values were obtained from M2 application, the lowest values were obtained from the M0 application in both of the years. When Fe is examined in terms of irrigation schedule, the highest values obtained from I3, while the lowest values obtained from I1, but the difference between I2 and I3 was statistically insignificant in the second year.

When evaluated in terms of copper contents, the highest Cu content was obtained from M2 in the first year, M1 in the second. The lowest values were obtained from M0 in both years. In addition, the I3 application has the highest Cu content, whereas the I1 application has the lowest content both years. But the difference between I1 and I2 in 2014 and among I1, I2 and I3 in 2015 was not statistically significant.

The highest Zn, Mn, and Ni contents were obtained from M2 application, whereas the lowest values were obtained from the M0 application in 2014 and 2015. But the

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difference between M2 and M3 was statistically insignificant for Ni content in the first year. According to the irrigation schedule the highest values were obtained from I3, the lowest values were obtained from I1 except Ni content both years. Ni content was found to be insignificant in 2014.

According to the source of manure, Pb content was found to be insignificant in the first year, while the highest value was obtained from M2, the lowest value was obtained from M0 in the second year. In terms of irrigation schedule the highest values were obtained from the I3 application, the lowest values were obtained from the I1 application. But the difference between I1 and I2 was not statistically significant.

There was a significant interaction between irrigation schedules and different manure sources during both years. In *Table 6* I3 M2 (the first year: six irrigations and chicken manure) and I3 M1 (the second year: six irrigations and farmyard manure) interaction produced maximum grain yield (1348 kg ha^{-1} in 2014 and 1495 kg ha^{-1} in 2015).

Table 6. *Interaction between irrigation schedules and different manure sources affecting grain yield (kg ha^{-1}) during 2014 and 2015*

	Irrigation schedules	Sources of manure			
		M0	M1	M2	M3
2014	**I1**	5665 i	6309 h	6833 fg	6736 fgh
	I2	6402 gh	6857 fg	8618 d	7913 e
	I3	7020 f	1033 c	1348 a	1187 b
2015	**I1**	5674 g	8192 ef	8772 de	7664 ef
	I2	6286 fg	1057 cd	1082 c	8538 e
	I3	8025 ef	1495 a	1346 ab	1208 bc

Discussion

Each year was separately evaluated since there were significant differences between both years, as found from combined analysis. Maize yield and its components tend to be higher in 2015 growing season, compared with 2014 growing season. This could be attributed to favorable climatic conditions that were prevailing during the 2015 growing season. The lowest plant heights were obtained from the I1 application, whereas the highest values were obtained from the I3 application (*Table 4*). These results are similar to the findings of Randhawa et al. (2012), and El-Gizawi and Nasser (2005), who found taller plants by increasing irrigation interval. While the minimum plant height was obtained in control treatment. These results are inline as the results of Qasim et al. (2001), who reported an increase in plant height with farmyard manure application due to more availability and uptake of nutrients. Increasing scarcity and greater competition for use of freshwater resources will force irrigated agriculture to be more efficient in use of available supplies. Significant difference was found among the irrigation treatments and different manure sources on the production of first cob height and cob length. The highest values were obtained for the I3 irrigation application and the M2 fertilization. Similar effects were observed by Abera et al. (2013) and Majid et al. (2017). Rezvantalab et al. (2008) also reported higher number of grains cob-1 with application of farmyard manure and mineral fertilizers. While the highest values were obtained from I3, the lowest values were obtained from I1. Confirmatory results regarding number of grains cob-1 were given by El-Tantawy et al. (2007), who reported significant differences among different irrigation schedules. According to the irrigation schedules, grain yield of cob ranged between 24.5 and 39.7 g in 2014, and 29.4 and 43.7

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g in 2015. The lowest values were collected from the I1 application, whereas the highest values were obtained from the I3 application (*Table 4*). These data confirm that of El-Tantawy et al. (2007) and Majid et al. (2017), who reported significant response of supplementary irrigation on grain weight cob-1. The highest 1000 grain weight was produced with the I3 irrigation treatment whereas the minimum weight of 1000-grain was produced by the control treatment. Significant results of both factors under the study during both years of experimentation were found. In case of plant nutrition treatments, the highest 1000-grain weight was noted in treatment chicken manure 15 t ha^{-1} (M2) as compared to all other integrated plant nutrition treatments in 2014. The same trend was observed during 2015. The increase in 1000-grain weight might be due to integrated use of farmyard manure and I3. Reports supporting the present results have appeared in the literature by Sharif et al. (2004) and Kashiani et al. (2011). The levels of irrigation and different manure sources remarkably influenced the grain yield in cultivar Limagrain Helen. Minimum grain yield counted from the treatment I1 M0, and it differed statistically from other treatments applications. The water stress (deficit water) remarkably influenced productivity and quality in maize (Barutcular et al., 2016; El Sabagh et al., 2017). However, water availability is usually the most important crop production factor limiting yield and yield traits of maize. These facts are comparable with the study of (Zhang et al., 2007) who obtained higher yield with the increase in number of irrigations. These results are similar to the findings of (Randhawa et al., 2012). The highest grain oil contents were reached from M1 with 4.05-4.32%, the lowest values were obtained from the M0 application with 3.50-3.57% for 2014 and 2015, respectively. According to the irrigation schedules, the seed oil content ranged between 3.60-4.13% in 2014 and 3.58-4.17% in the second year. The lowest seed oil contents were obtained from the I1 application, whereas the highest values were obtained from the I3 application. These results were in agreement with what was obtained by Yalçın et al. (2006). Grain analysis has been used to reveal the deficiency, adequacy or excessiveness status of various nutrient elements in a soil-plant system. Chicken manure application significantly increased the concentrations of K, Ca, Cu, Fe, Mn, Zn, Ni and Mg in maize seed. Application of chicken dunk organic fertilizer not only capable to increase sweet corn production, but also had positive impacts in term of improvement of soil physical, chemical and biological properties. Improvement of soil physical properties is achieved because organic matter (organic fertilizer) functions as adhesive of loose soil particles or aggregate stabilizer compound (Gonzales et al., 2002; Margaretha et al., 2014). The highest Na, Mg and Ca values were obtained from the M2 application. The lowest values were obtained from the M0 application in the first year and the second. According to the irrigation schedule, the I1 application gave the lowest value while I3 gave the highest. Similar effects were observed by Hussaini et al. (2008).

Conclusions

The effects on all the characteristics researched for different sources of manure and irrigation schedules were statistically significant for 2014 and 2015. The results of our work indicated that the highest plant yield for maize planted in both growing seasons of 2014 and 2015 was obtained when the plants were irrigated six times. On the basis of this two-year study, it is concluded that in order to obtain higher grain yield of hybrid maize under agro ecological conditions of Mardin/Turkey, the crop should be irrigated six times and fertilized with organic manures (15 t chicken manure ha^{-1}). While the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

highest grain yield per area was obtained from the third irrigation schedule and chicken manure in 2014, the highest value was obtained from the third irrigation schedule and standard fertilizer with 14959 kg ha^{-1} and the third irrigation schedule and chicken manure with 13463 kg ha^{-1} in 2015. Chicken manure application significantly increased the concentrations of K, Ca, Cu, Fe, Mn, Zn, Ni and Mg in maize seed. The results revealed that increase in soil moisture and mineral contents leads to increase in plant growth promotion and biomass production.

Acknowledgements. This study is a research artcile and supported as a project number MAU-BAP-2015-MYO-15 by M.A. University Research Fund.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15591570

LIQUID CHROMATOGRAPHY-UV DETERMINATION OF HEAVY METAL IONS IN ENVIRONMENTAL SAMPLES USING DISPERSIVE LIQUID-LIQUID MICROEXTRACTION COUPLED WITH MAGNETIC NANOPARTICLES

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(Received 25th Aug 2018; accepted 6th Nov 2018)

Abstract. A novel, rapid low-density solvent based dispersive liquid-liquid microextraction combined with magnetic nanoparticles has been developed for extraction and preconcentration of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} in environmental samples prior to liquid chromatography with UV analysis. In the proposed method, the Schiff base was used as a complexing agent and the environmental samples were treated with acetonitrile/water (60:40, v/v) which was then utilized as disperser solvent in the dispersive liquid-liquid microextraction process along with using 1-octanol as extractant. In magnetic solid phase extraction approach, hydrophobic magnetic nanoparticles were used to retrieve and separate the 1-octanol in the dispersive liquid-liquid microextraction step. In fact the target of magnetic solid phase extraction was the 1-octanol rather than the metal ions directly. Several parameters including the volume of extractant, the pH of the sample, the concentration of complexing agent, the volume of magnetic nanoparticles were optimized. Under the optimized conditions, there coveries obtained for each metal ion ranged from 86 to 106% with relative standard deviations lower than 5.6%. The limit of detection were 0.05 μg L^{-1} for Cr^{2+}, 0.05 μg L^{-1} for Ni^{+}, 0.06 μg L^{-1} for Co^{2+}, 0.05 μg L^{-1} for Hg^{+}, respectively. The preconcentration factors were 205, 200, 195 and 190 for Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+}, respectively. The presented method has been successfully used for the determination of metal ions in real water samples.
Keywords: *preconcentration, 1-octanol, DLLME, hydrophobic magnetic nanoparticles, LC-UV*

Introduction

Heavy and transition metal ions are distributed widely in biological systems and the environment, and play important roles in many biological and environmental processes (Trautwein, 1997). Excess amount of these ions is, however, toxic. Lead poisoning can cause a variety of diseases, such as muscle paralysis, memory loss, irritability, and anemia (Strużyńska et al., 1997; Rifai et al., 1993; Rezende et al., 2011). The World Health Organization (WHO) has released the guide-lines for drinking water quality containing the guide line value of 10 μg L^{-1} for lead (WHO, 2006). Cadmium is not regarded as essential to human life and is known to damage organs including the kidneys, liver and lungs (Pourreza et al., 2010; Zhang et al., 2010). Methods for detecting the presence of one or several of these ions are thus urgently needed, and if the detection method could identify different component of the analytes, it would be an excellent improvement compared with other sensing methods. Although many methods for their analysis have been well developed, existing methods require complex equipments and sophisticated operations.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

In many cases the determination of trace metal ions in various samples such as natural and waste water, biological and alloy samples, the direct determination with various instrumental methods is not possible owing to matrix effect and low concentration of metal ions in a sample (Andruch et al., 2012a). Thus separation and preconcentration procedures are required for elimination or minimization of matrix effects and concomitants, lowering the detection limit of many metals with different techniques and enhancing the delectability for many metals. A variety of procedures for preconcentration of metals, such as solid phase extraction (SPE) (Andruch et al., 2012b; Baig et al., 2009), liquid–liquid extraction (LLE) (Duran et al., 2007; Karami et al., 2008; Fritz et al., 2000), coprecipitation, ion-exchange (Kocúrová et al., 2011), flotation (Li et al., 2009), and cloud point extraction (CPE) have been developed (Mohammad et al., 2013; Nascentes et al., 2003; Parham et al., 2009; Rezende et al., 2011; Tuzen et al., 2006). SPE is one of the most frequently employed methods, as it is surface-dependent processes and their efficiency directly depends on the particle size and the surface area of the sorbent (Taylor et al., 1996; Tang et al., 2004). Dispersive liquid-liquid microextraction (DLLME) has been brought up as an exciting microextraction technique in 2006 (Yuet al., 2014; El-Shahawi et al., 2013; Rezaee et al., 2006).

In this procedure, a mixture of high density organic solvent (serving as extraction solvent) and water miscible polar dispersive solvent (dispersive solvent) is rapidly injected into an aqueous sample to form an emulsion consisting of fine droplets of the extraction solvent, dispersive solvent, and water. Due to the extraction solvent being highly dispersed in the aqueous phase, the surface area between extraction solvent and sample solution is infinitely large, thus speeding up the extraction. While, the main disadvantage of DLLME is that the extraction solvent is generally limited to solvents with higher density than water in order to be sedimented by centrifugation, typically chlorinated solvents such as chlorobenzene, chloroform, and tetrachloromethane, all of them are potentially toxic to humans and the environment.

Then, another microextraction technique based on magnetic nanoparticles (MNPs) has gained research interest since the MNPs can be easily isolated from matrix by using an external magnetic field without retaining residual magnetization after its removal (Giannoulis et al., 2013; Shih et al., 2014; Gómez-Pastora et al., 2014; Zou et al., 2013). Also MNPs offer a significantly higher surface area-to-volume ratio and a shorter diffusion route than conventional sorbents, resulting in high extraction capacity, rapid extraction dynamics and high extraction efficiencies (Faraji et al., 2010; Chen et al., 2011; Román et al., 2011).

By combining the merits of the dispersive liquid-liquid microextraction (DLLME) and magnetic solid phase extraction (MSPE) techniques, a new sample preparation method was proposed to trace metal ions of environmental samples. The applications of magnetic nanoparticles in biotechnology and environmental, and so on, has attracted considerable attention in recent years, because of their rapid adsorption ability, surface modification, easy to separation and good biocompatibility etc. The quick separation and excellent adsorption performance of magnetic nanoparticles have gained increasing application in sample pretreatment. The magnetic nanoparticles with suitable surface modification are capable of binding molecules selectively. Magnetic nanoparticles and the magnetic separations, magnetic detections have advantages of rapidity, convenience and high selectivity.

This is the first report regarding preconcentration of Cr^{2+}, Ni^{2+}, Co^{2+}, Hg^{+} by DLLME-MSPE prior to liquid chromatography-UV analysis. A novel, rapid low-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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density solvent based dispersive liquid-liquid microextraction combined with magnetic nanoparticles has been developed for extraction and preconcentration of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} in environmental samples prior to liquid chromatography with UV analysis.

Materials and methods

Chemicals and materials

The standards of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} (1000 mg L^{-1}) were purchased from National standard material network (Beijing, China). Working standard solutions were freshly prepared by diluting the stock solutions with high-purity water. 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol and decanoic acid were obtained from the Aladdin Reagent Corporation (Shanghai, China). As a complexing agent, the Schiff base was from our own laboratory. $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, ammonium nitrate and the other used chemicals were supplied by Tianjinzhiyuan (Tianjin, China).

Instrumentation

High-purity deionized and doubly distilled water (UPS-I40L, Ulupure, Shanghai) was used during this study. A pH-meter (PHS-3E, China) was used for pH measurements. An ultrasonic homogenizer (LTB-300, China) equipped was used as a dispersing agent. A centrifuge (TDZ4-WS, China), with a centrifugation speed in the range from 500 to 6000 rpm, was used for phases separation after the microextraction procedure. A High performance liquid chromatography system (Agileng 1260, Agilent, USA), which consisted of an UV detector, was used for the analysis.

Real sample preparation

Environmental water samples were from local river water and lake water and were analysed by DLLME-MSPE/LC-UV procedure. All water samples were stored at 4 °C.

Synthesis of magnetite nanoparticles (MNPs)

Preparation of magnetic nano materials Fe_3O_4 by hydrothermal reduction method. The $FeCl_3$ was reduced to Fe_3O_4 by using ethylene glycol. The addition of anhydrous sodium acetate and surfactant polyethylene glycol was added to prevent the agglomeration of particles. The specific reaction condition, 1.35g$FeCl_3.6H_2O$ dissolved in 40ml of glycol, adding 3.6g anhydrous sodium acetate and 1.0g polyethylene glycol under magnetic stirring, at 60°C under vigorous stirring until completely dissolved, then the mixed solution into the reaction kettle in a 50mL, under 200°C reacted 12h and then cooled to room temperature. The synthesis of Fe_3O_4 nano particles with water and ethanol alternate cleaning, vacuum drying at 80°C.

Extraction procedure

DLLME step

A 15 mL aliquot of the sample solution containing Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} was transferred into a 50 mL glass test tube. The pH of the sample was adjusted to 5.0 with the acetate buffer. Then 500 μL 10% w/v Schiff base (complexing agent) and 80 μL of 1-octanol (as extraction solvent of DLLME) and the acetonitrile/water (80:20, v/v)

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extract (used as disperser solvent) were added and the mixture was diluted to 40 mL by deionized water. Then, the tube was sealed and swirled on a vortex agitator at 2000 rpm for 30 s to accelerate the formation of the fine droplets of the extraction solvent and to enhance the transfer of the analytes.

MSPE step

50 mg of the synthetic magnetic nanoparticles were quickly added into the upper tube and the mixture was vigorously blended using a vortex agitator for 60s at 5000 rpm. The Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} (complexes of Schiff base)-containing 1-octanol was successfully partitioned on the surface of MNPs. Subsequently, a magnet was held next to the outer wall of the test tube to attract and isolate the MNPs, and the sample solution was discarded by decanting it. After then, an aliquot of water was added into the vial to rinse the residue. Ultimately, 200 µL of methanol was used to desorb the 1-octanol as well as the Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} from the MNPs by swirling for 30 s and 10 µL of the eluent was used for LC-UV analysis.

Liquid chromatography

A 10 µL aliquot of the extractant phase was injected into the liquid chromatography with UV detector. An Agilent C18-AR column (250 × 4.6 mm, 5 µm particle size) was used as the analytical column. The mobile phase consisted of methanol/water at a ratio of 65/35 (v/v). The flow rate of the mobile phase was at 1.0 mL min^{-1} and the detection wavelength was set at 256 nm.

Results and discussion

Characterization of decanoic acid@Fe_3O_4 nanoparticles

The size and morphological features of decanoic acid@Fe_3O_4 (DA@Fe_3O_4) MNPs were visualized with Transmission electron microscope (TEM). As can be seen in *Figure 1,* the MNPs have an average size of about 10 nm. The prepared DA@Fe_3O_4 NPs were stabilized against agglomeration by a monolayer of decanoic acid. And the edge morphology of DA@Fe_3O_4 NPs became blurred because the particles' surface was encapsulated with 10 carbons of acid.

The functional groups of DA-MNPs were identified by Fourier Transform Infrared Spectrometer (FT-IR). The FT-IR spectra of DA-MNPs and 1-octanol-DA-MNPs are shown in *Figure 2*. The spectrum of DA-NMPs exhibited strong bands at 621 and 3434 cm^{-1} due to Fe-O-Fe and O-H stretching vibrations of magnetic nanoparticles. The peaks at 2924 and 2849 cm^{-1} were attributed to the C-H stretching vibrations of decanoic acid and the peak at 1599 cm^{-1} contributed to the C=O stretch band of the carboxyl group. All these adsorption peaks confirmed the successful coating of Fe_3O_4 nanoparticles by DA.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

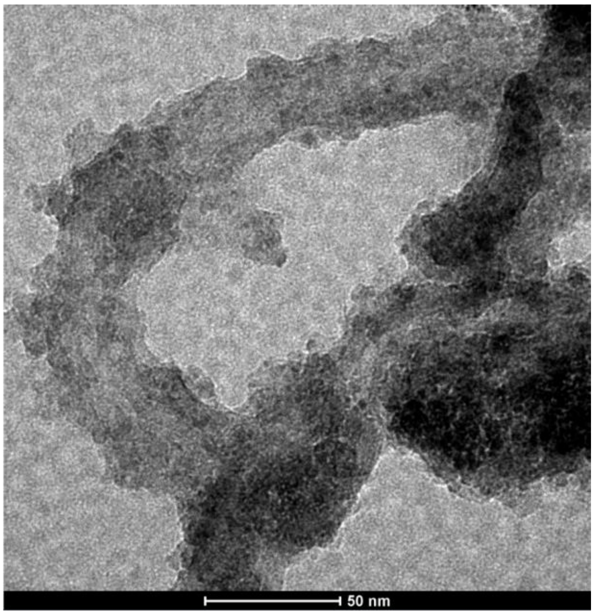

Figure 1. *Transmission electron microscope images of synthesized Fe_3O_4@MNPs*

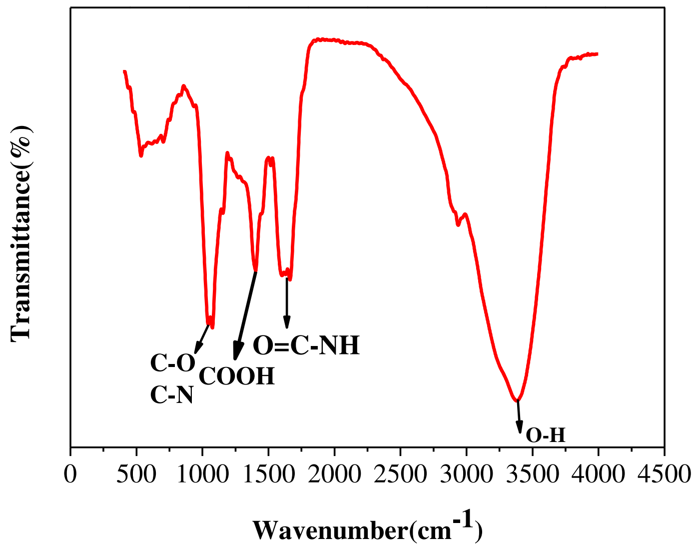

Figure 2. *Fourier transform infrared spectras of Fe_3O_4 magnetic nanoparticles*

Optimization of the DLLME-MSPE method

During the two-step extraction procedure, several parameters affect the extraction performance and the recoveries. Several parameters, including sample pH, the concentration of complexing agent, the type and volume of extraction solvent, equilibration time, amount of MNPs were optimized by investigation a single parameter when the other parameters remained unchanged.

Selection of the disperser solvent

The applicability of several organic solvents including MeOH, MeCN, EtOH, MeOH 80% and MeCN 80% was investigated in the preliminary experiments taking into

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DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

account that it should used to primary extract of the analytes from real water samples must then act as disperser solvent in DLLME process. *Figure 3* shows the recoveries of the whole procedure (extraction + DLLME + MSPE) of all extraction solvents tested. The results revealed that the maximum extraction efficiency was achieved by MeCN/water (80:20 v/v).

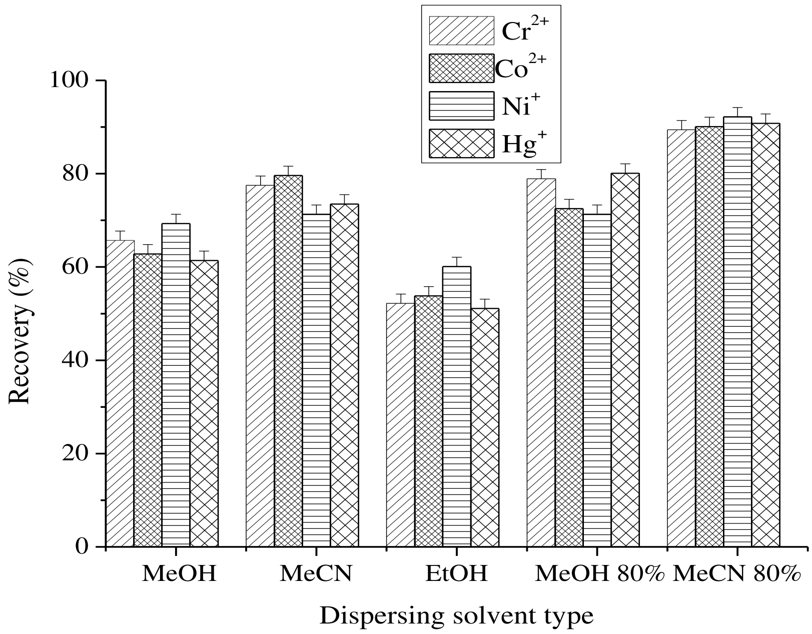

Figure 3. *Effect of dispersing solvent*

To study the volume effect of disperser solvent, it was varied from 4 to12 mL in the interval of 2 mL while extraction solvent was kept at 80 µL. The obtained results showed that the extraction efficiency increased with increasing the volume of MeCN 80% up to 10 mL and then dropped down slightly above 10 mL due to decreasing the distribution ratio.

Therefore 10 mL MeCN 80% was selected as both the extraction solvent of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} from water samples and as disperser solvent in DLLME for subsequent experiments.

Effect of solution pH

The effect of sample pH was investigated in the range of 2.0–9.0. Adjustment was done using hydrochloric acid, sodium hydroxide and acetate buffer. The achieved areas of the liquid chromatographic peaks were the highest at pH = 5.0 and these conditions were used for further experiments.

Selection of extraction solvent

Selection of appropriate extraction solvent is of great importance in microextraction technique in order to obtain efficient extraction. For the DLLME, the extraction solvent should meet the following requirements: (1) be immiscible with aqueous solution (2) able to extract the analytes, and (3) possess low toxicity. To avoid the use of more highly toxic solvents such as trichloromethane, tetrachloromethane, tetrachloroethylene,

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DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

chlorobenzene etc., five low density organic alcohol including 1-hexanol (density, d = 0.819 g mL^{-1}), 1-heptanol (d = 0.820 g mL^{-1}), 1-octanol (d = 0.821 g mL^{-1}), 1-nonanol (d = 0.827 g mL^{-1}), and 1-decanol (d = 0.828 g mL^{-1}) were evaluated as extractants. Among them a stable cloudy solution and good extraction recovery were observed with 1-octanol. The fine droplets of 1-octanol afford larger surface area to contact with the water sample and can be completely absorbed by MNPs after magnetic separation, so that higher extraction performance can be attained. As can be seen in *Figure 4*, 1-octanol has higher extraction recovery than those obtained by other extraction solvents for the Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+}. The volume of 80 µL of 1-octanol was selected as an optimum solvent volume for further studies.

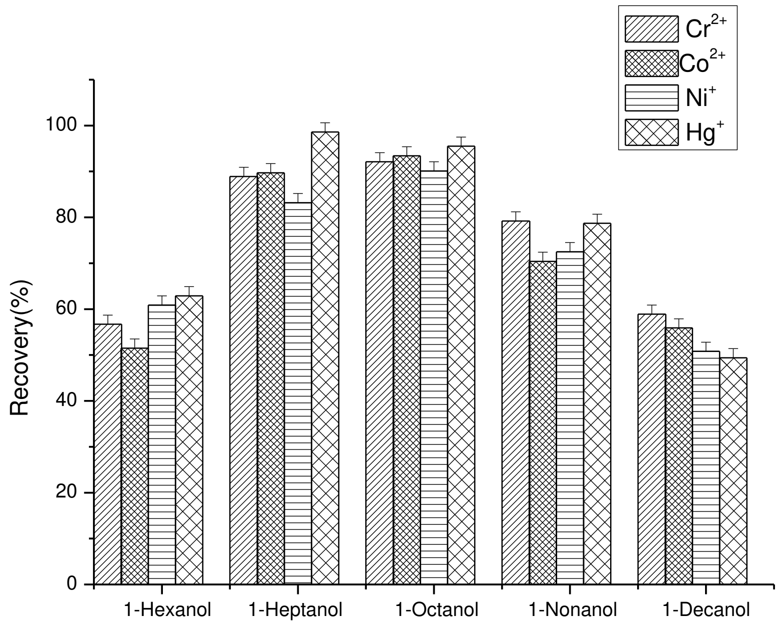

Figure 4. *Selection of extraction solvent*

Influence of sonication time

In DLLME extraction, sonication time is defined as an interval time from the occurrence of cloudy solution and before addition of magnetic adsorbents. The effect of extraction time on the adsorption was investigated for 0-120 s. The result showed that sonication time has no influence on extraction efficiency. It is well-known that vortex process is an effective way to enhance mass transfer from aqueous phase to the extraction phase. Consequently, short time was required for extraction. Generally, 30 s of extraction time was enough to achieve high extraction recovery, and prolonged extraction time did not contribute significantly to an increase in extraction recovery. Therefore, vortex time of 30 s was selected.

Influence of concentration of Schiff base

Schiff base has the ability to form metal-ligand complexes (1:2) with divalent metal ions. The effect of Schiff base concentration on the formation of metal complexes was studied in the range from 0.05 to 0.50 (%, w/v). The results show that the extraction

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DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

efficiency of metal ions enhances up to 0.20 (%, w/v). Hence, 0.30 (%, w/v) of Schiff base was selected for preconcentration of the metal ions for further experiments.

Influence of amount of MNPs

After the DLLME procedure, the magnetic sorbents were dispersed in sample solution to rebind 1-octanol. In order to guarantees the quantitative separation of metals-containing1-octanol from DLLME step, the effect of the amount of DA@Fe_3O_4 MNPs in the solution was investigated in the range from 20 to 80 mg. As shown in *Figure 5,* the extraction recovery for the analytes increased rapidly when the DA@Fe_3O_4 MNPs amount was increased from 20 to 60 mg and then remained almost constant when the amount of the adsorbent was above 60 mg. High surface to volume ratio of nanoparticles causes the quantitative extraction could perform using very low amount of adsorbent. Based on the above results, the addition of 60 mg DA@Fe_3O_4 MNPs to samples was optimal for magnetic retrieval.

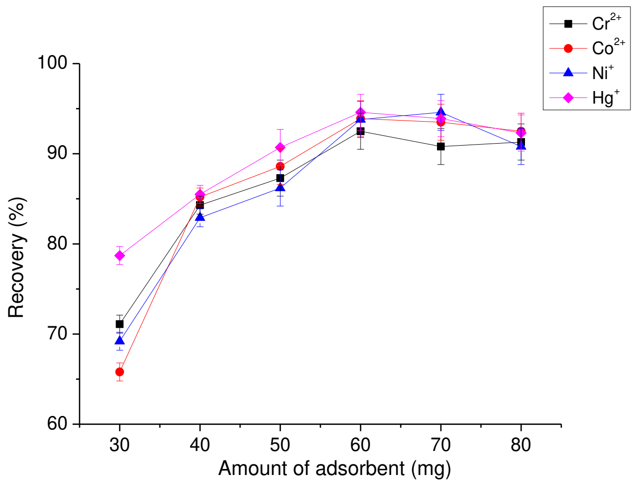

Figure 5. *Effect of amount of magnetic nanoparticles*

Analytical figures of merit of the DLLME-MSPE method

The linearity ranges, correlation coefficients, limits of detection and enrichment factors of the Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} were evaluated under the optimized DLLME-MSPE-LC/UV conditions. As listed in *Table 1*, the linearity of the method was examined using a mixed working solution of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} with a concentration range of 0.2-100 μg L^{-1} in environmental water sample solutions. All of the analytes exhibited good linearity, with coefficients that ranged from 0.9985 to 0.9990. The limit of detection (LOD) was calculated on the basis of signal to noise (S/N) ratio. The S/N = 5 was used for calculation of LOD. The LODs were 0.05 μg L^{-1} for Cr^{2+}, 0.05 μg L^{-1} for Ni^{+}, 0.06 μg L^{-1} for Co^{2+}, 0.05 μg L^{-1} for Hg^{+}, respectively. The relative standard deviation (RSD) for five replicate measurements of 1.0 μg L^{-1} in standard solution was 5%. Preconcentration factors calculated as the ratio of the calibration curves slopes for analytes before and after the preconcentration step, were 205, 200, 195 and 190 for Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+}, respectively. The precision study was carried out in five parallel experiments and provided relative standard deviations below 5.6%.

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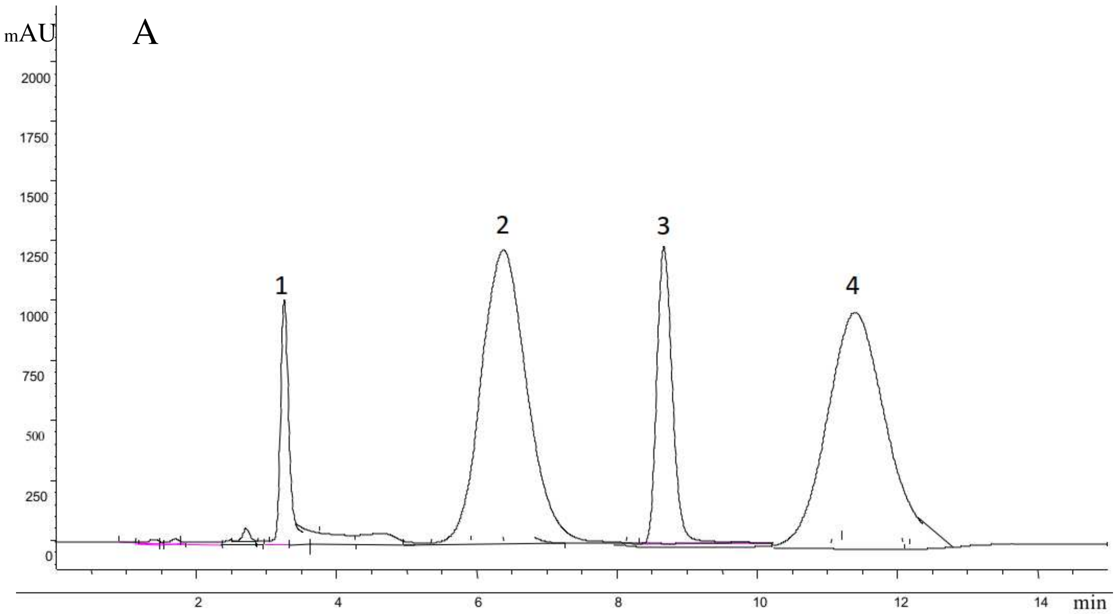
***Table 1**. Figures of merit for the dispersive liquid-liquid microextraction coupled with magnetic solid phase extraction of Cr^{2+}, Ni^{+}, Co^{2+}, Hg^{+} (n = 5)*

Metal ions	**LR/ (μg L^{-1})**	**R**	**LOD/ (μg L^{-1})**	**EF**	**RSD / %**	**Recovery/%**
Cr^{2+}	0.2-100	0.9989	0.05	205	2.7	90
Co^{2+}	0.2-100	0.9985	0.06	195	2.8	102
Ni^{+}	0.2-100	0.9987	0.05	200	3.9	95
Hg^{+}	0.2-100	0.9990	0.05	190	4.2	99

Real sample analysis

To test the applicability of the proposed method, it was applied to the determination of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} in river and lake water samples. The river water samples were obtained from Liucangqiao River (Bijie, China), the lake water samples from the Jinhai lake wetland park (Bijie, China). Under the recommended experimental conditions, the developed method is employed to determine Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} in water samples. And all the water samples are spiked with Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} standard solutions at different concentration levels to assess the matrix effects. Non-spiked samples are also analyzed. The results are shown in *Table 2*. It shows the developed method applied to the determination of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} in water samples. Also, *Figure 6A* shows the typical chromatogram of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} at 100 μg L^{-1} without preconcentration, and *Figure 6B* shows the chromatogram from water sample solution spiked at 100 μg L^{-1} of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} after DLLME-MSPE. Comparison of the chromatograms and acceptable recoveries demonstrated that the interferences from the substances in the real sample had no effects on the performance of the presented method and this method had higher sensitivity for determination of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+}.



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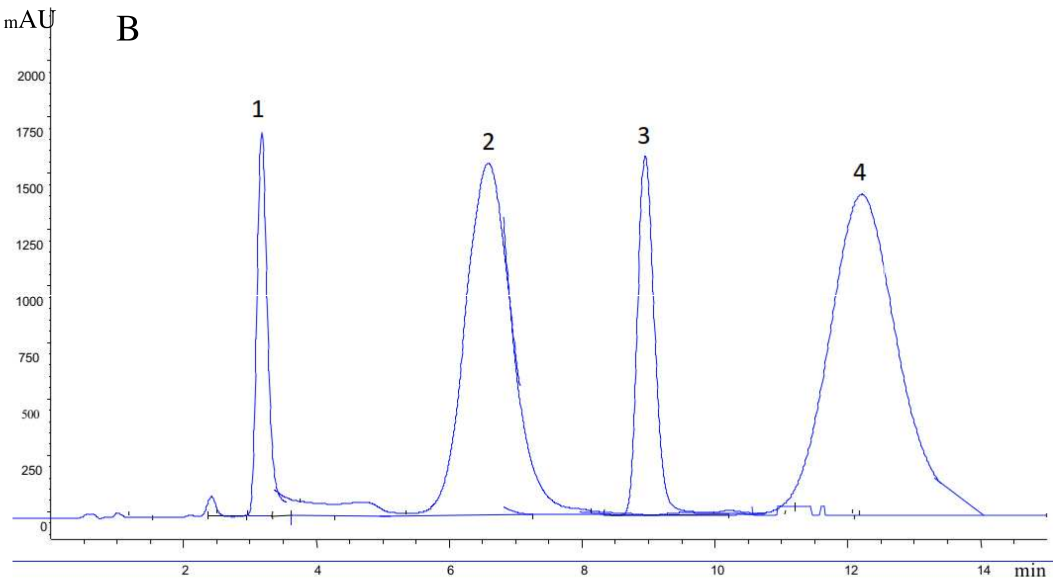

Figure 6. *Typical chromatograms: (A) water sample spiked with Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} without dispersive liquid-liquid microextraction-magnetic solid phase extraction and (B) after dispersive liquid-liquid microextraction-magnetic solid phase extraction. 1, Cr^{2+}; 2, Ni^{+}; 3, Co^{2+}; 4, Hg^{+}*

Table 2. *Determination of Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} and recoveries for water samples*

Water sample	Element	Added (μg L^{-1})	Found (μg L^{-1})	Recovery (%) (n = 5)
Lake water	Cr^{2+}	0	1.12±0.07	
		5	4.98±0.12	99
		10	10.26±0.09	102
	Co^{2+}	0	0.33±0.11	
		5	5.31±0.05	106
		10	10.07±0.03	100
	Ni^{+}	0	0.72±0.06	
		5	5.22±0.15	90
		10	10.52±0.04	98
	Hg^{+}	0	5.71±0.56	
		5	10.95±0.07	104
		10	14.32±0.15	86
River water	Cr^{2+}	0	0.57±0.02	
		10	10.79±0.05	102
		20	19.89±0.04	96
	Co^{2+}	0	0.37±0.09	
		10	9.98±0.05	96
		20	18.08±0.04	89
	Ni^{+}	0	1.58±0.03	
		10	11.02±0.07	94
		20	22.58±0.09	105
	Hg^{+}	0	1.74±0.05	
		10	11.50±0.02	98
		20	19.85±0.05	90

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Comparison with other methods reported

Comparisons of the mentioned method with other methods reported for extraction and determination of target metal Cr, Ni, Co and Hg ions were summarized in *Table 3* in details. According to the comparison results obtained, the proposed method showed the recovery comparable with most of the previously reported methods, whereas the detection limit (LOD) for the proposed method were obviously lower than those of the reported methods. Moreover, the extraction time was also shorter than for the other methods. It demonstrated that the developed technique was a powerful alternative for rapidly analyzing target metal Cr, Ni, Co and Hg ions in water samples.

Table 3. *Comparisons of analytical methods for the analysis of* Cr^{2+}, Ni^{+}, Co^{2+} *and* Hg^{+}

Method	LODs (μg/L)	Extraction time (min)	Recovery (%)	RSD (%)	Reference
SPE-FAAS[1]	24.9	-	>95%	<9.0	Pytlakowska (2016)
CPE-FAAS[2]	1.0	30	98-105	0.5	Magda et al. (2016)
MSPE-FAAS[3]	0.37	6	96.8-102.5	2.1	Tahmasebi et al. (2014)
DLLME-FAAS[4]	90	>5	107.5-125.2	0.7	Özzeybek et al. (2017)
DLLME-MSPE-LC/UV[5]	0.05	5	90-102	2.7	This work

[1]SPE-FAAS: solid phase extraction- flame atomic absorption spectrometry
[2]CPE-FAAS: cloud point extraction- flame atomic absorption spectrometry
[3]MSPE-FAAS: magnetic solid phase extraction- flame atomic absorption spectrometry
[4]DLLME-FAAS: dispersive liquid-liquid microextraction- flame atomic absorption spectrometry
[5]DLLME-MSPE-LC/UV: dispersive liquid-liquid microextraction-magnetic solid phase extraction-liquid chromatography with UV analysis

Conclusion

In the present study, a new two-step microextraction technique, based on dispersive liquid-liquid microextraction (DLLME) and magnetic solid phase extraction (MSPE), was developed for determining Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+} in water samples. A low density solvent (1-octanol) was successfully used in conjunction with magnetic nanoparticles (MNPs) that allowed convenient operation of the procedure. The extraction could be achieved within 2 min and good extraction efficiencies were obtained. Such facile separation is essential to improve the operation efficiency which avoiding time-consuming centrifugation or freezing or manual collection of extractant. Coupled with LC-UV analysis, the proposed method exhibited good linearity and acceptable repeatability, and the limits of detection were as low as in the nanogram per milliliter range for Cr^{2+}, Ni^{+}, Co^{2+} and Hg^{+}. In general, this methodology can be considered as a promising procedure for environmental samples or other samples.

Acknowledgements. This research was supported by the Youth Science and technology talent development project of Guizhou Province, Bijie City and Guizhou University of Engineering Science (KY[2018]402 and KY[2018]390), Key Disciplines of Guizhou Province(ZDXK[2016]13), Key Laboratory of Medicinal Plants for Bijie City([2017]05),the Engineering Research Center of Guizhou Province(KY[2018]009)supported this work. The authors are grateful for the referee's help on the manuscript.

Conflict of interests. The authors declared that there is no conflict of interests regarding the publication of this paper.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15711584

EFFECT OF WASTE WATER TREATMENT ON THE GROWTH OF SELECTED LEAFY VEGETABLE PLANTS

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(Received 12th Sep 2018; accepted 29th Nov 2018)

Abstract. Quetta, the capital city of Baluchistan province which was once called as “little London” has been affected by severe drought since 1998. This situation led to fresh water shortage which compelled the local population to irrigate their crops by waste water. In this way, heavy metals started to accumulate in the soil and as well as in our food chain. The main objectives of this research were to elucidate the effect of waste water on leafy vegetables growth. For this purpose, three leafy vegetables were selected i.e. Coriander (*Coriandrum sativum),* Purslane *(Portulaca oleracea)* and Lettuce (*Lectuca sativa).* The research is also aimed to know the influence of waste water treated plants on human health. The experiments were performed in the botanical garden, Botany Department, University of Baluchistan. Waste water was collected from waste water channels of the Quetta city. From March 2016-14 June, 2016, after an extensive survey, different sites of the municipal were selected for the collection of waste water used as sample collected from the Agriculture Department of Quetta city. Vegetative growth parameters such as height, number of leaves, weight of fresh, dry leaves and rate of germination of seed were measured to know the impact of waste water treatment. Waste water treatment had found a significant effect on the growth of leafy plants. All the growth parameters were measured in the three selected leafy vegetable plant species i.e. Coriander (*Corianderum sativum),* Purslane *(Portulaca oleracae)* and Lactuca *(Lactuca sativa).* Comparatively, better results were found by treating the plant with waste water rather than fresh water. The usage of municipal wastewater for irrigation can be an abundant resource of the nutrients essential for plant’s growth. However, keeping in view the health hazards, edible crops irrigated with this untreated waste water should not be grown in spite of its positive impact on the physical parameters of some plants.
Keywords: *wastewater utilization, land irrigation, leafy vegetables plants growth, nutrients, human health*

Introduction

The use of waste water in the agricultural sector to overcome the severe drought is becoming a substitute throughout the world, particularly in China. In developing countries, wastewater is used in 70% of irrigations. Approximately, 20 million ha areas in the world is cultivated by using waste water. Waste water is composed of several minerals which are essential for plants growth. Similarly, using clean water for irrigation has itself some drawbacks. Its increases mainly the scarcity of drinking water. For that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

reason, in Mexico, peasants using dirty water can let their land at three times the price of those who use clean water, but these waters also contain risks of disease and pollution. Research studies revealed that Mexican children under four years old living in areas where wastewater is being used for irrigation of plants, have found sixteen times more intestinal problems than the other areas (Achakzai et al., 2011; Adenle et al., 2011).

The World Health Organization (WHO) reveals in one of its laboratory manuals titled "Analysis of waste water for use in agriculture" that the use of waste water for crop irrigation is becoming more common. As wastewaters contain mineral nutrients which are good for the development of plants growth, the yield is superior. However, there is a risk that irrigation with waste water facilitates may enable the transmission of diseases related to intestinal nematodes and fecal bacteria to consumers and farmers (WHO, 2006; ATSDR, 1993; Chang et al., 2002; Mushtaq et al., 2018).

Like in many other counties, untreated waste water is used for irrigation in over 80% of all Pakistan societies with a population of over 10,000 inhabitants. More specifically, considering the case of Quetta, which is the capital city of Baluchistan, with an area of 2,653 km^2, surrounded by the Takato and Murdaar mountains, it has been seen that in Quetta, people have started to fulfil the food requirement by growing the daily use vegetation and vegetables using the waste water of the city (Khalil et al., 2011; Saidi, 2010; Shah, 2014; Ahmad et al., 2004; Amerasinghe, 2004; Siraj et al., 2018).

In Pakistan, there are predominantly agricultural societies, most of the fresh water is used for irrigation purpose and with increased competition for water by rapidly renewed interest the total estimated area directly irrigated by waste water is 32,500 ha, with 19,250 households depending on direct waste water use for their livings. Quetta is the capital city of Baluchistan. Its population is approximately 3 million. It has a rocky landscape. Mostly, its climate is semi-arid with a great variation in the summer and winter temperature (Ensink, 2004; Amerasinghe, 2004; Nidaa et al., 2017; Siraj et al., 2018).

The Quetta city was once very famous for its scenic beauty but has now turned into probably the most polluted city in the country, especially with respect to sanitation and hygiene conditions. The workers reported herewith that due to overpopulation, the basic needs of life have become inadequate for the people. Therefore, the people have started to fulfil the food requirement by irrigating their crops with the wastewater in the areas of Sabzal Road, Spini Road, Saryab Road and Hazar Gangi. Polluted water is composed of hospital, domestic and industrial waste. The pH of sewage water range from 7.24-9.21. The concentration of essential nutrients (macro+ micro) and poisonous metals varied greatly in samples between two selected locations (Kakar et al., 2010; Amerasinghe, 2004' Shah, 2014; Bazai et al., 2005; Zahid et al., 2011; Siraj et al., 2018).

The concentration of P and K^+ was high (1.18 ppm) in Habib nala than Ispini and Subzal road sewage drain. The concentration of micro nutrients (Fe^{++}, Mn, Zn, Cu) were within acceptable limits. In relation with toxic metals, Pb was generally greater (0.12 ppm) in Ispini road drain wastewater as compared with (0.05-0.08 ppm) other sewage drains. According to Kakar et al. (2006), the minimum and maximum (0.09 and 0.18 ppm) Ni was found in Ispini and Subzal road drain. Wastewater has a drastic impact on the seed germination also. The results revealed that the germination of seed, and roots, shoot elongation was significantly affected by two types of waste water (Kakar et al., 2010; Shah, 2014; Bazai et al., 2005; Abdul et al., 2013).

Black Niger *(Nigella sativa)* plant was highly affected in domestic waste water treatment and pharmaceutical waste water treatment followed by Coriander (*Coriandrum sativum).* Germination percentages of Mustered (*Brassica juncea)* in domestic and pharmaceutical

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

waste water usage were 18% and 22%, respectively. Fenugreek (*Trigonella foenum-graecum)* germination percentage was less affected by wastewaters as only 20% and 17% reduction was observed in the domestic and pharmaceutical industry waste water. Germination reduction in Barley (*Hordeumv ulgare)* showed 23% under domestic polluted water condition while its reduction under industrial waste water treatment was near to 35%. It has been determined that the germination and growth of seeds and seedlings of various species of plants are significantly affected by domestic and industrial waste water (Herpin, et al., 2012; Al-Jamal et al., 2002; Blumenthal et al., 2000).

The hospital waste of the city which comprises the awash of patient suffering from typhoid fever, malaria, hepatitis causes food borne illnesses. As the contaminants of waste water flow through the food chain of an ecosystem and affect each trophic level and disseminate the diseases in each level of food chain. The farmers of the developing countries prefer the use of waste water for irrigation just because it is economical and does not need organic fertilizers, but, the people are unaware of the consequences of using these vegetables. Waste water is a significant source of water and nutrients for irrigation in developing countries, particularly but not restricted to those located in arid and semi-arid areas. The use of waste water is extensive and represents around 10 percent of the total irrigated surface worldwide, although varying widely at local levels. While, the farmer gets benefits using the waste water, mainly related to their income level, but it has adverse effects on human health and the environment (Jimenez, 2006; Disciglio et al., 2015; Fonseca et al., 2007).

Materials and methods

Experimental research approach was chosen to conduct this research. As the experimental research approach is all about using manipulation and testing in a controlled manner to understand the casual processes, so, this is the reason this approach was best-suited considering the nature of the research. The study was based on mixed method approach; both descriptive and calculation analysis was done to describe the findings in an understanding way.

Geographical position of sampled plant species

Three types of leafy green vegetable plants i.e. Coriander (*Coriandrum Sativum)*, Lactuca (*Lactuca sativa)*, Purslane (*Portulaca oleracea)*, belonging to the family of Apiaceae, Asteraceae and Portulacaceae, respectively were selected for the experiments to assess the impact of waste water on their growth and phenotypic parameters and to examine the impact of plants irrigated with untreated wastewater on the health of the people. In Quetta city, the practice of growing these edible plants through municipal wastewater is common. Therefore, these types of plant seeds were selected for the investigation of impact of wastewater on these plants.

Seed sample collection

Fresh Certified Seeds of Coriander (*Coriandrum sativum),* Lactuca *(Lactuca sativa)*, and Purslane (*Portulaca oleracae)* were collected from the Federal Certification Department, Quetta, Balochistan, Pakistan.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

Soil sample collection

Soil samples were collected from the water and soil testing center Quetta field through random selection method by digging out the soil inside the ring. Then this soil was air dried and sieved through a 2 mm sieve.

Particle size determination

Particle size distribution is a vital parameter in soil classification. The texture of the soil was determined by calculating the percentage of clay, silt and sand present in the soil sample. The standard size of soil particles are presented in *Table 1* (Glendon, 2002; Lado et al., 2012; Keller et al., 2002; Zafar et al., 2010).

Experimental procedure

The experiments were conducted in the Botanical Garden, Botany Department, University of Baluchistan, Quetta, Pakistan. Waste water was collected from waste water channels of the known Quetta city. Various concentrations of waste water i.e. T_0 (containing 100% pure or distilled water), T_1 (containing 20% waste water), T_2 (having 40% waste water), T_3 (containing 60% waste water), T_4 (containing 80% waste water), T_5 (containing 100% waste water) were employed in the experiment to check their effect on the growth of the selected plant species using tap water as control. The selected plant species were grown in 54 pots, 18 pots for each type of plant. Each plant in a pot was irrigated with a specific prepared concentration of waste water. After irrigating, the three types of plants with different percentage of waste water, the effect of waste water on the germination rate of seed and phenotypic parameters of these selected plant species were recorded.

Data collection

Different sites of the municipal wastewater were selected for the collection of waste water after an extensive survey from March 2016 to Jun 2016. After selection of sewage effluent sites, waste water was taken for the various experiments of the study. From the recommended cultivars, the seeds of Coriander (*Coriandrum sativum),* Purslane *(Portulaca oleracea)* and Lactuca (*Lectuca sativa)* were screened for germination potential in the laboratory.

Detailed experiments were held in the Botanical Garden of Baluchistan University. In the meanwhile, the resources used in this research were many including books and scientific journals, periodicals, research articles, digital media and the materials of Human Resource Department (HRD), University of Balochistan, Pakistan.

Results and discussion

Wastewater utilization

The scarcity of water increasingly makes agriculture dependent on the reuse of water, which has led to a search of alternative sources of irrigation that justify the yield potential of crops and can also be used as a substitute to fresh water in plants. It has been forecasted that by 2050, due to the population explosion, a severe water crisis would occur for irrigation of these crops. Waste water irrigation is becoming an attractive practice for agriculture as its use not only supplies nitrogen and phosphorus

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

but also nitrogen to the plants and helps to recycle nutrients in wastewater to reduce and minimize the direct use of fertilizers. It is thus reducing significantly the pollution of receiving water bodies (Connor et al., 2017; Tarantino et al., 2009; Hein et al., 1992; Connor, 2015; Ensink et al., 2004).

The waste water of any city is mainly used for irrigating vegetables near the cities. Leafy vegetable plants such as cauliflower, cabbage, spinach, etc. grow by irrigating with waste water, while some vegetables such as radishes growth does not give positive response to wastewater. Vegetables irrigated with waste water contain many heavy metals that pose serious health risks for the community and animals. This problem is of particular importance, since untreated sewage is used for a long time for vegetable growing in urban areas (Mensah et al., 2002; Kakar et al., 2006; Borkin et al., 2000).

Soil management

Managing the productivity of soil is another problem that farmers face. The ability of soil to produce useful crops is called soil productivity. It produces valuable amounts of crop and is related to the physical and chemical properties of the soil as well as hydrological and atmospheric factors of the systems in which it is located. The loss of soil productivity has been a persistent problem worldwide. With regard to the application of waste water, its use increases the production of leafy vegetable plants in the short term. However, further use has serious consequences for the productivity of soil. The optimum parameters for soil management is given in the proposed *Table 1* (Gupta, 2008; Herpin et al., 2007; Leila et al., 2007).

Table 1. *Soil/particle parameters*

S/No.	Name particle diameter	Soil particle size
1	Clay below	0.002 mm
2	Silt	0.002 to 0.05 mm
3	Very fine sand	0.05 to 0.10 mm
4	Fine sand	0.10 to 0.25 mm
5	Medium sand	0.25 to 0.5 mm
6	Coarse sand	0.5 to 1.0 mm
7	Very coarse sand	1.0 to 2.0 mm
8	Grave	1 2.0 to 75.0 mm
9	Rock greater than	75.0 m (~2 inches)

Leafy vegetable plants

Vegetables have a significant role in meeting the food requirements of people in the world and are also a good source of various essential ingredients i.e. minerals, fibers and vitamins. Vegetables are also a rich source of essential nutrients like proteins, iron and calcium that have important health benefits. Beet is a great source of vitamins like vitamins A, vitamins B_6, vitamins C, vitamins E and folate, dietary fiber of copper, calcium and its consumption is very effective for preventing a wide range of health problems (Olaniran et al., 2013; Muchuweti et al., 2006; Adhikari et al., 1998; Hernandez et al., 1991).

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

Radish has also many benefits for health point of views and used to treat various diseases, including pertussis, stomach problems, cancer, cough, constipation, dyspepsia, liver disorders, arthritis, gallbladder problems, gallstones, intestinal parasites and kidney stones. If leafy vegetable plants are irrigated with wastewater containing toxic heavy metals such as nickel, chromium, copper, lead, arsenic, cadmium and zinc etc., it has an adverse impact on human health (Ahmad et al., 2013; Ehmann et al., 1996; Henry et al., 2013).

Aluminum and chromium often occur concurrently in agricultural soils and causes enormous damage to plant productivity. The interactive effects of aluminum and chromium emphasize the absorption of nutrients and metals in barley. In such a situation, vegetable plant species accumulate increased amounts of heavy metals as compared to the soil not affected. This is because they absorb nickel, chromium, copper, lead, arsenic, cadmium and zinc through their leaves (Amoah et al., 2005; Joseph et al., 2002; Latare et al., 2014).

In general, urban waste water is a potential source of metals but it is still used to grow vegetables in cities. Irrigating of vegetable plants by these waste water containing toxic metals can cause various physiological and biochemical disorders (Ahmad et al., 2013; Alia et al., 2013).

This waste water is a rich source of organic matter as well as other plant nutrients. Waste water from industries of textile and paper is released into the water body or directly on the surface of agricultural land. This waste water is usually used for the irrigation of vegetables and fodder plant species due to fresh water scarcity. For increasing the soil productivity of soil mainly for leafy vegetable plants, use of untreated waste water can lead to the accumulation of metals in plants in phytotoxic amounts. The consumption of these toxic metals in vegetable plants cause diseases such as typhoid, cholera, leukemia, brain damage, diarrhea, vomiting, gastroenteritis, lung cancer, high blood pressure, pregnancy toxicity, pigmentation of the fingers nails, impairment of growth, heart failure, low blood pressure, liver neurosis, skeletal abnormalities, myocardial infarction, dermatitis, alcopia tumor, hair loss, depression and parkinsonism etc. (Ahmad et al., 1994; Husain et al., 2010).

Effect on seeds germination

Many researchers predict that the high pressure of textiles, paper, marble, dairy and brewery as well as strong osmotic pressure decreased seeds germination. Mohammad and Khan (1985) found that beans and ladyfingers plant species in the presence of industrial effluents reduced the percentage of seeds germination. However, there was no hazardous effect in the presence of treated waste water. Strong reduction in germs and early growth of radish, turnip and brassica occurred due to the use of untreated textile waste water. It was more pronounced in turnip, than in radish and brassica. Panasker and his co-workers studied the effect of textile mill effluent on the seeds germination and growth of black grams. Yousaf et al., found that with a lower concentration of sewage, seeds germination and seedling growth were higher than the control, but at higher concentrations, seeds germination and growth decreased gradually (Norton et al., 2007; Pathak et al., 1999; Saruhan et al., 2012).

The best growth of seeds germination and seedlings were observed at 25% concentration. They concluded that textile waste water is present and can be used safely for irrigation after proper treatment and recommended the 25% dilution, accordingly. Dayama (1987) reports that even in the presence of highly diluted industrial waste (5%),

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

the germination of the seeds of Gram (*Cicer arietinum*) is diminished, whereas, Swaminanthan et al., found seed germination and chlorophyll improvement in the peanut plant (*Arachis hypogaea*) at 50% diluted waste. Mohammad et al., reported that 75-100% of concentrated waste water in the textile industry has harmful effects on seeds germination of Mung bean (*Phaseolus aureus)* and Okra (*Abelmoscus esculentus)*, whereas, a concentration up to 50% of the same effluent did not have any consequences (Dayama, 1987; Moreno et al., 2015).

Many researchers have documented that there are different concentrations of textile waste water and papers helping in the improvement of seedling lengths in various crops. However, it has been found that high levels of heavy metals in soils hinder the growth, metabolic processes and nutrient assimilation in plants. As per the given schedule, the rate of seeds germination were analyzed for all the plants species and checked proportionally by the waste water (Akbar et al., 2007; Dhanam, 2009).

It was clearly evident that increasing the percentage of waste water, the seeds germination were increased except in the Lactuca plant species. For clearer demonstration, the graphical representation rate of seed germination for every plant with every proportion of waste water is given in *Figure 1*. Lactuca is the plant with high effect on the growth of seed by the waste water. Increasing the waste water, its rate increased but in the other two plant species, the rate was partial. However, there is a decrease in germination of seed with waste water irrigation for coriander and purslane.

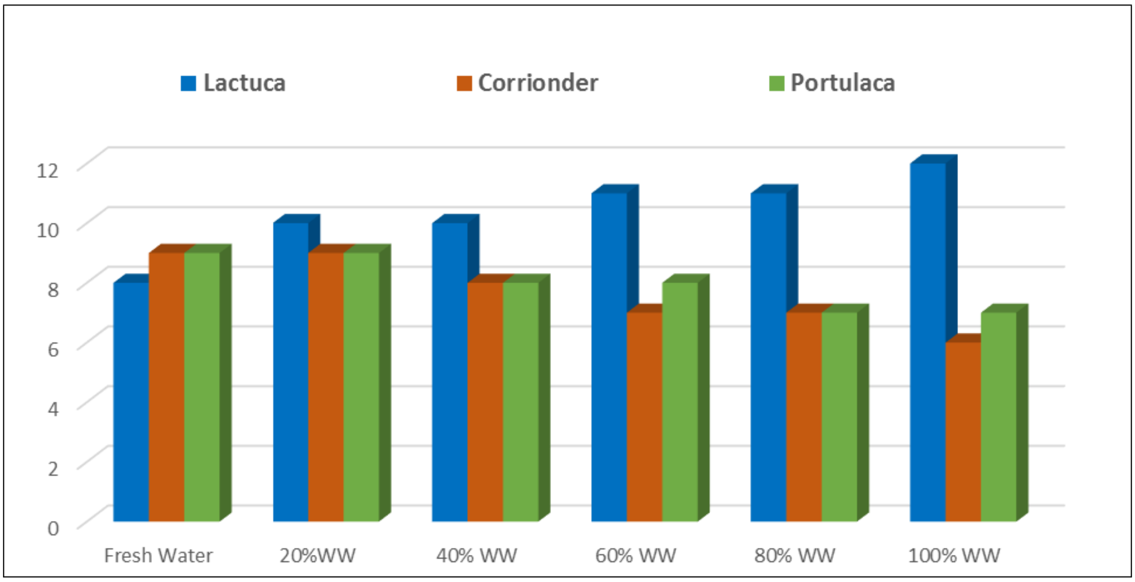

Figure 1. *Rate of seeds germination in different plant species*

It can be said that the rate of seed germination may or may not be influenced by the waste water irrigation depending on the environmental conditions. The next measuring parameter that was observed was height; the impact of waste water on the height of plants is shown in *Figure 2*. It is observed that with an increase in percentage of waste water, the height of plants was increased. An increase in height was measured in all the three plants but Purslane (*Portulaca oleracea)* has shown quicker response as compared to the other two plants.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

Effect on green leaves elongation

Examining the increase in number of green leaves, it was observed that the numbers of green leaves were increased with higher proportion in Purslane (*Portulaca oleracea)* and Coriander (*Corianderum sativum)* as compared to Lactuca (*Lactuca sativa).* The significant impact of waste water on the number of green leaves of the plants was reported as shown in *Figure 3*.

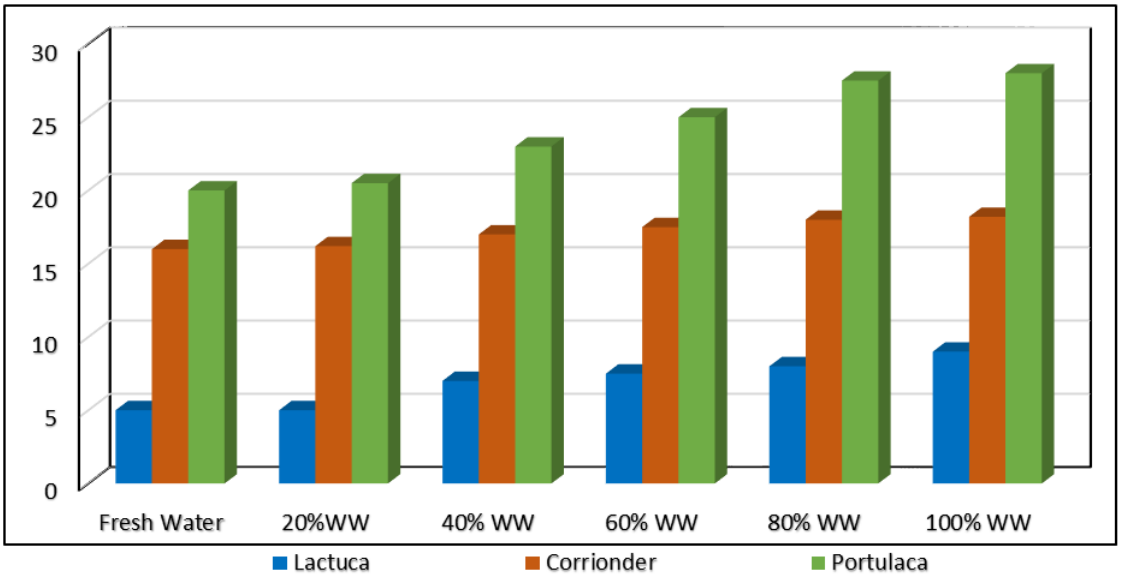

Figure 2. *Height status of different plants species (in cms)*

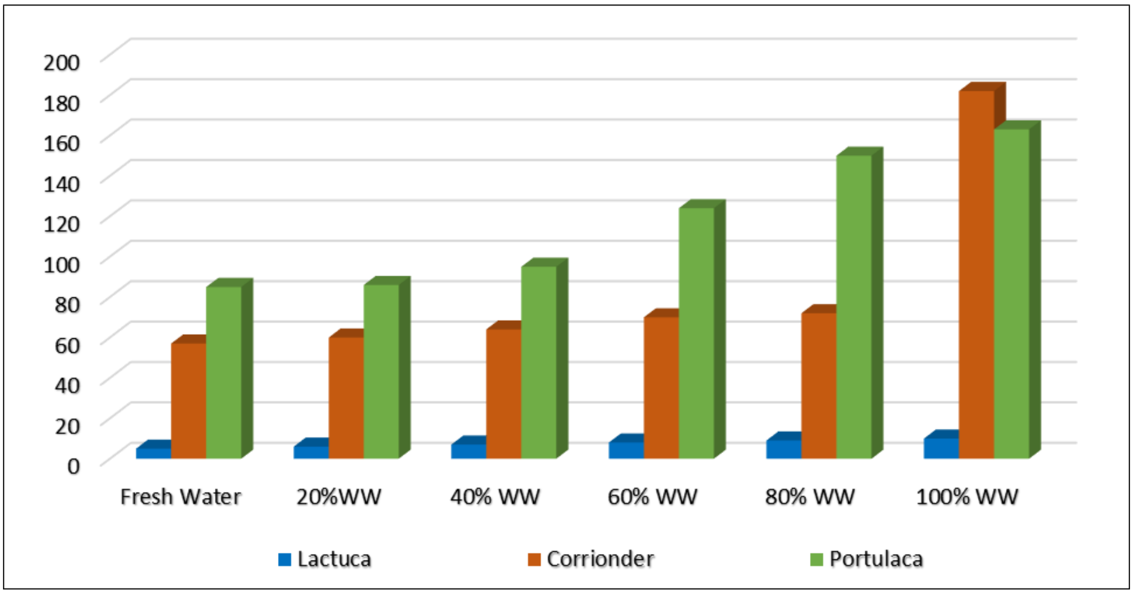

Figure 3. *Number of leaves per species in different plants*

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DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

Effect on biomass growth

By increasing the concentration of waste water, the vegetative growth of plants were increased as previously reported by other researchers (Khalil et al., 2011; Emongor et al., 2004). Waste water containing sludge increases the root length. Coriander stem length also increases with increasing waste water concentration. The sludge present in waste water also raises the biomass of coriander plant. By adding sludge to the soil a positive impact may be reached on the soil porosity, water retention by root, water-holding capacity of the soil mixture. By application of sludge to the soil, moisture content modified but the number of seed in each type of plants reduced by increasing the waste water concentration. Significant reduction in the number of seed by increasing the concentration of waste water was also reported.

Examining the increase in weight of leaves per plant, it was observed that the weight of both fresh and dry leaves were increased in Purslane (*Portulaca oleracea),* Coriander *(Corianderm sativum),* and Lactuca *(Lactuca sativa)*. It should be noted that the rate of increase in every attribute is dependent on the nature and characteristics of the plant itself. However, the increase in rate was measured for every plant but one of the plants showed much more increase in values as compared to the other two. Overall, the significant impact of waste water on the weight on fresh and dry leaves per plant was reported as shown in *Figure 4*.

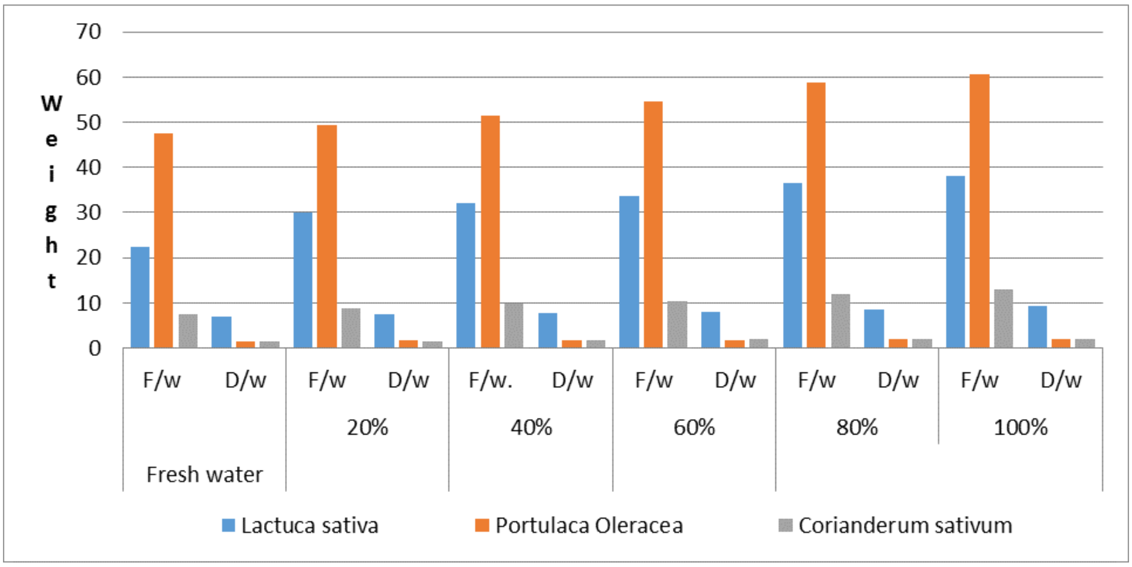

Figure 4. *Comparative weight of fresh and dry leaves in different plants species*

Conclusion

The sludge present in waste water showed a very positive impact on the growth of plants in general. By increasing the concentration of waste water, the concentration of sludge also increased and healthy vegetative growth had been observed in the waste water treated plant species. It is reported that application of sludge to the soil, moisture content was modified due to additional pores which are able to hold more water. Pore surface roughness, which enhance the water retention and reduction of micro-cracking of soil pores. Soils that hold water support more plant growth and are less susceptible to nutrient loss.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

Our study revealed that all growth parameters measured in the three selected leafy vegetable plants, i.e. Coriander *(Corianderum sativum),* Purslane *(Portulaca oleracae),* Lactuca *(Lactuca sativa)* were statistically greater in waste water irrigated plant species than in fresh water plants. The usage of municipal waste water for irrigation may be an abundant resource of nutrients and essential elements for plant's growth. In fact, waste water has high concentration of organic matter and nutrients as compared to fresh water. Therefore, the nutrient accumulation occurring in the soil and this high concentration of nutrients will make the plants easy to access these nutrients.

Acknowledgements. Authors would like to express their gratitude to the technical facilities provided by Botany Department, University of Baluchistan, Quetta and COMSTAS University Islamabad, Pakistan.

Author contributions. Fahmeda Ali and Niaz Muhammad Tareen, Tahira Bibi participated in the research project, experimental findings, data compilation and participated in the preparation of manuscript draft. Kifayat Ullah, Anwar Ullah and Sadullah Laghari participated in sample collection, identification, systematic studies and the species feasibility recommendation, taxonomic studies for the proposed project, and helped to draft the manuscript. Saeed Ur Rehman supervised the overall techniques and participated in the article preparation to draft the manuscript.

Conflict of interests. All the authors listed above in the article authorship line have contributed equally to the proposed research project, and all those authors who are qualified accordingly the proposal preparation, laboratory work and report compilation are listed. To the best of our knowledge, no conflict of interests here in this research work exists.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15851597

TECHNIQUES BASED ON THE POLYMORPHISM OF MICROSATELLITE DNA AS TOOLS FOR CONSERVATION OF ENDANGERED POPULATIONS

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(Received 18th Jul 2018; accepted 5th Dec 2018)

Abstract. The reduction of the size and range of populations, and the destruction of their breeding sites has led to situations in which the existence of a population depends on breeding performed by humans in controlled conditions. Such conservation and restoration work should be based on maintaining the ability of protected populations to adapt to environmental conditions. This ability is determined by genetic variation within populations as well as individual genetic characteristics specific to a given population. Managing and preserving the genetic variation of a conserved population is one of most important factors that determine the success of a conservation program. This paper reviews a method based on DNA microsatellite polymorphism and its associated techniques for detection, management and conservation of genetic variation. It covers the use of this method in detecting and monitoring changes in intrapopulation genetic variability. It also covers the usefulness of this technique in assembling breeding pairs based on their genetic profiles, evaluating genetic differences between populations, optimizing and managing genetic variation resources deposited in banks of cryopreserved gametes, and evaluating the effectiveness of programs for conservation and restoration of species. Finally, suggestions for further development of the method are included.
Keywords: *biodiversity, bioinformatic tools, conservation of endangered species, genetic variation, human dependent populations, molecular techniques*

Introduction

The rate of extinction of populations has been accelerating since the end of the 19th century, especially that of populations susceptible to environmental changes caused by human activity. Reduction of population ranges, destruction of breeding sites and reduction of population sizes have led to populations that depend on breeding performed by humans in controlled conditions (Koljonen et al., 2002). After being reared in controlled conditions, individuals are released into existing populations to extend the existence of those populations (Brown and Day, 2002).

Large numbers of progeny can be obtained from a few parents by using cryoconservation techniques and artificial breeding, but this method does not take into consideration the genetic differences between parents and their impact on population fitness. Although this method can be effective if environmental conditions are optimal, it leads to gradual reduction in the genetic variation of populations, which progresses with each succeeding generation (Fraser, 2008). This decrease in variation reduces the potential of the population to adapt and makes it vulnerable to changes in environmental conditions and invasion of pathogens, and thus could pose a threat to the existence of that population (Pastor et al., 2004; Zemanová et al., 2015).

Therefore, maintaining the ability of protected populations to adapt to environmental conditions should be the basis of conservation and restoration works. This ability is determined by genetic variation, which can be estimated with molecular techniques.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

This paper reviews a method of estimating genetic variability based on DNA microsatellite polymorphism and its associated techniques, which can be used to help conserve species endangered by extinction and to solve problems in conservation biology.

The paper should serve as useful compilation of up-to-date information about this method and its applications, as well as a source of suggestions for future developments of the method.

Microsatellite DNA

Microsatellite fragments of DNA constitute a substantial part of the genome of eukaryotic organisms (Charlesworth et al., 1994; Sawera et al., 2001). They are commonly used as a marker of genetic diversity in protected populations (Nielsen and Sage, 2001; Kim et al., 2004; Jurczyk, 2006; Zemanová et al., 2015). Microsatellite DNA is also used in monitoring the genetic fitness of conserved populations and in tracking their interaction with other populations (Wąs and Wenne, 2002).

Microsatellite DNA has a number of characteristics that make it useful for these purposes. First, changes within microsatellite sequences are inherited according to Mendel's laws. Second, because changes in microsatellite fragments are not subject to natural selection, it is likely that the individuals that are heterozygous in many of the microsatellite loci may also be heterozygous in loci encoding proteins responsible for their adaptability. Thus, the degree of polymorphism of microsatellite loci is a measure of the level of genetic variability in a stock or natural population (Guichoux et al., 2011). Third, microsatellite fragments can be used as universal markers and will not be affected by chromosomal mutations, because they are evenly distributed within chromosomes (Sawera et al., 2001). Fourth, these fragments are adjacent to evolutionally preserved flanking sequences (Santana et al., 2009). The presence of these flanking sequences enables the design of short oligonucleotides (primers) and the replication of mini-microsatellite sequences using the Polymerase Chain Reaction (PCR) (Sztuba-Solińska, 2005). Finally, microsatellite DNA can be used to detect very small genetic differences between organisms. This is because microsatellite fragments are some of the most polymorphic fragments of DNA, due to the presence alleles differing in length in a given chromosomal locus. The differences in length result from variations in the number of the repeat motifs that make up a microsatellite DNA fragment (O'Conell and Wright, 1997; Kelkar et al., 2010).

Advantages of Microsatellite DNA for Estimating Genetic Variability

Although the various techniques for estimating genetic variability have their advantages, the use of microsatellite DNA for this purpose has a number of advantages that make it a method of choice in a wide range of applications. For example, single nucleotide polymorphisms (SNP) can be used in studies on genetic variation as well. The SNP technique requires the analysis of many sites of point mutations, and therefore also requires detailed knowledge of the genome of the investigated species (Fernández et al., 2005). This can be a limitation of the SNP method because the genomes of many endangered species are mostly unknown. In contrast, the region flanking a microsatellite marker is evolutionary conserved, which allows these markers to be amplified when they are present in closely-related species and genomic knowledge about one of the species is lacking. Methods based on microsatellite DNA also offer other advantages over methods based on anonymous multilocus genomic markers (RFLP, AFLP, ISSR)

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DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

and on SNPs: the microsatellite technique is easily transferable between laboratories; it requires only limited equipment and computational facilities; it provides a high polymorphism information content (PIC) per locus; and it is highly cost efficient when only a small number of loci are needed (Bezault et al., 2012). For these reasons, microsatellites markers are likely to remain the optimal choice for a wide range of ecological and evolutionary studies (e.g., relatedness and parentage analysis, population diversity and demography assessment, noninvasive genetic analysis, and conservation) (Bezault et al., 2012).

Methodological Considerations

To properly assess genetic differences on the basis microsatellite DNA polymorphism, samples must be taken from an appropriate number of individuals (Ruzante, 1998; O'Connell and Wright, 1997). These samples need to be properly acquired and preserved. PCR primers and the PCR reaction must be properly designed, and the lengths of DNA fragments need to be measured.

Typically, the sample size for inferring the amount of genetic variation in the population is a few dozen individuals (Hart et al., 2014). The number of individuals in a sample is usually difficult to determine because it depends on the number of genetic markers used and their polymorphism (O'Connell and Wright, 1997). As the number of individuals and the number of microsatellite markers that are examined increase, the precision of the estimates of genetic differences within and between populations also increases (O'Connell and Wright, 1997). However, it can be difficult to increase the number of individuals in a study due to limited availability and increased costs. As a guideline, if at least 5 markers are used and their polymorphism varies between 5 to 10 alleles, a sample size of about 50 individuals should be used (O'Connell and Wright, 1997). In studies based on 10 polymorphic microsatellite markers, increasing the number of individuals from 50 to 100 had no significant effect in the level of genetic diversity estimated in the population (Ruzzante, 1998).

Samples for analysis of microsatellite DNA polymorphism can be collected from live individuals (Powell et al., 1996). This enables studies on commercially valuable individuals and endangered species, such as marine vertebrates (Thorpe et al., 2000) and many fish species (Kumar et al., 2007; Naik et al., 2018). If the results are used for optimizing the assemblage of individuals into breeding pairs, the individuals should be marked before sample collection, which can be done with microchip tags (Kaczmarczyk and Fopp-Bayat, 2012).

After sample collection, the tissue should be preserved until DNA extraction. Unfortunately, using 70% ethanol and then storing the tissue at 4°C does not always successfully preserve the DNA. A much more reliable method is drying samples in a laboratory drier, placing them in paper envelopes and storing them at about 20°C until DNA is isolated (Kaczor, 2013).

DNA can be extracted with a phenol/chloroform extraction protocol (Sambrook et al., 1989) or DNA extraction kits, which are designed for extracting DNA from tissues like blood, muscles, etc. (Żuchowska, 2011; Kaczor, 2013; Ocampo et al., 2018), allowing various tissues to be used as a source of DNA.

To amplify microsatellite fragments with the PCR technique, primers that are complementary to the regions of DNA that flank the microsatellite are needed. The primers’ sequences can be obtained from published reports on the genetic variation of a species, and from the National Center for Biotechnology Information database at

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www.ncbi.nlm.nih.gov. When studying a species with a little known genome, like the lake minnow, DNA sequencing is useful for identifying microsatellite loci (Furukawa et al., 2004; Grover and Sharma, 2016). Furthermore, because the sequences surrounding microsatellite DNA are often conserved, it is often possible to use a set of primers that have been developed for another species that is not too phylogenetically distant. For example, primer sets that were designed for use with the zebra fish (*Danio rerio*) or with other species of cyprinid fish such as *Campostoma anomallum* can be used in studies of the lake minnow (*Eupalasella percnurus*) (Kaczmarczyk and Żuchowska, 2011; Kaczmarczyk, 2013; Gadomski, 2013).

Microsatellites can be amplified separately, via singleplex PCR, or together in a single multiplex PCR reaction (Edwards and Gibs, 1994; Migliaro et al., 2013), which allows up to 21 genetic markers to be amplified in a single PCR reaction (Shewale et al., 2013). Multiplex PCR not only accelerates research and reduces the costs of analyses (Guichoux et al., 2011; Migliaro et al., 2013; Liu et al., 2017), but also combines well with the use of an automatic capillary DNA sequencer for detection of microsatellite DNA polymorphism. To assemble a group of genetic markers into a multiplex set, the primers need to be labelled with phosphoramide dyes at the 5' end so that the product of amplification of one locus can be distinguished from the product of another. Either the forward or the reverse primer should be marked in each set. If the PCR products of two or more microsatellites have a similar size range, each primer pair should be marked with a different type of phosphoramid dye (Liu et al., 2017).

Automated capillary electrophoresis is the primary method of detection of microsatellite DNA polymorphism. It can detect single nucleotide differences in DNA lengths (Butler et al., 2001), and it allows greater accuracy, speed, and reproducibility than previous methods of detecting polymorphism of microsatellite loci. Automatic capillary electrophoresis is carried out in a polyacrylamide gel that is optimized for use in a specific type of automatic DNA sequencer and for a specific method of measuring DNA fragment length (Mansor et al., 2015). It is combined with software that detects differences in the length of DNA fragments, stores the results, and can perform further analyses, such as genotyping (Dennis et al., 2010).

To meet the objectives of conservation biology, the genotyping results from the investigation of microsatellite DNA polymorphism can be subjected to mathematical and statistical analyses using various software packages. These packages can be divided into four groups. The first group comprises software that calculates indicators of genetic variation within populations and the genetic distance between populations, and compares those indicators across populations to track phylogenetic relationships. This group includes software such as ARLEQUIN (Excoffier et al., 2005; Excoffier and Lischer, 2010), MSA (Dieringer and Schlötterer, 2003), STRUCTURE (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009), GENEPOP (Rousset, 2007) and others. There is some overlap between the first group and the second, which comprises programs that can estimate the likelihood of events that affect genetic variation, such as migrations, and bottleneck and founder effects; these programs include ARLEQUIN (Excoffier et al., 2005; Excoffier and Lischer, 2010), BOTTLENECK (Piry et al., 1999), STRUCTURE (Pritchard et al., 2000; Falush et al., 2003, 2007; Hubisz et al., 2009). The third group comprises software for optimal assemblage of individuals into breeding pairs, based on their genetic profiles and indicators of the genetic variation expected in their progeny, such as GENEASSEMBLAGE 1.0 (Kaczmarczyk, 2015). The fourth group comprises programs that combine the use databases and genetic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

profiling to determine the origin of individuals or commercial products, for example SPAGeDi software (Hardy and Vekemans, 2002) and COLONY (JoneS and Wang, 2009).

Microsatellite DNA polymorphism for conservation of endangered populations

The uses of the analysis of DNA microsatellite polymorphism can be divided into two categories. First, genetic variation can be assessed at the intra- and interpopulation levels. This assists in identifying processes affecting genetic variation and assessing methods for maintaining and managing genetic resources, including the genetic variability of populations created in conservation programs and the restitution of extinct populations (Kim, et al., 2004; Hart et al., 2014). Second, the genetic profiles of individuals can serve a variety of other ends. For example, the survival of individuals obtained under controlled conditions can be compared to that of individuals produced via natural reproduction (ICES WGBAST, 2017). Individual genetic profiles can also be used to manage cryopreserved gamete banks, to optimize assemblage of individuals for breeding (Abdul-Muneer, 2014), and to identify interspecies hybrids (Cruz et al., 2014).

Detection and monitoring of changes in intrapopulation genetic variability

Breeding individuals from local populations under controlled conditions can increase the likelihood of breeding closely related individuals, and thus reducing the genetic variability of the population. When a small number of individuals whose relationship is unknown are used for breeding, the risk of inbreeding increases (Bentsen and Olesen, 2002). Inbreeding usually increases homozygosity (Yi et al., 2015), which may decrease allelic diversity, which may affect the adaptive ability of the population (Charlesworth and Charlesworth, 1987; Saccheri et al., 1996; Crnokrak and Roff, 1999; Hedrick and Kalinowski, 2000; Chapman et al., 2009; Nielsen et al., 2012) and may lead to expression of genetic defects encoded by recessive alleles (Charlesworth and Willis, 2009; Gao et al., 2015).

The harmful effect of inbreeding on viability and population fitness is more evident in populations than in commercial stocks (Crnokrak and Roff, 1999; Whitlock, 2004; Harrison et al., 2011) and may cause programs for conservation of endangered species to fail (FAO/UNEP, 1981; Olech, 2003; Hallerman, 2003). Estimating genetic variability based on microsatellite DNA polymorphism is thus a useful tool that can be used to determine the level of genetic diversity in a protected population. It can be used for comparisons with stable populations that do not show inbreeding depression (Kaczmarczyk and Żuchowska, 2011; Gadomski, 2013), and for detection of differences between populations that are important for their survival in their respective environments (Gadomski, 2013; Abdul-Muneer, 2014).

Based on the length of DNA fragments and genotyping results, indicators of genetic variation can be calculated. These indicators are the observed (H_o) and expected heterozygosity (H_e), genotypic and phenotypic diversity, the percentage of polymorphic loci (P), the average number of alleles in a locus (A) and allelic richness (R) (Min et al., 2015; Long et al., 2017). For assessing genetic variation and tracking its changes, the most important of these indicators are heterozygosity and allelic diversity (Hoban et al., 2014). Low values of (H_o), (H_e) and (A) indicate low genetic variation in the population (Szczecińska et al., 2016), and a statistically significant difference between (H_o) and (H_e) indicates a departure from Hardy-Weinberg equilibrium. This departure can reflect a disturbance in the genetic balance of the population, possibly connected with changes

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in population size or with non-random assemblage of individuals in reproductive pairs (Hansen et al., 2008). By comparing these indicators of genetic variation with historical data and data from other populations, the genetic diversity in a population can be assessed, and actions can be taken to counter diversity loss, if necessary (Kim et al., 2004). These indicators can be calculated with software packages such those mentioned above.

Detection of the consequences of reduction of genetic variability resulting from founder and bottleneck effects

A founder effect occurs when only a few individuals start a new population, causing the genetic variation of the new population to be only a fraction of that of the parent population (Whitlock and McCauley, 1990; Kusza et al., 2013; Rochet and Lise, 2016). A bottleneck effect might be caused by environmental changes or human activity, such as overfishing (Rochet and Lise, 2016). This temporary reduction in population size reduces the genetic variation in the population. A bottleneck effect may also result from reproduction under controlled conditions. Populations whose existence depends on reproduction in controlled conditions where a small number of parental individuals are used are especially vulnerable to genetic bottlenecks (Price and Hadfield, 2014).

The polymorphism of microsatellite DNA can be used to estimate the probability that a founder or bottleneck effect occurred, and the size of those effects (Knapen et al., 2003). To this end, microsatellite DNA fragments are used to track changes in the number of alleles, allelic frequencies and heterozygosity. Based on this data, the Garza-Williamson (G-W index) can be calculated. This index provides information about the size of the founder or genetic bottleneck effect that probably occurred in the history of the population. It is calculated on the basis of two numbers. The first is the number of alleles of a given microsatellite, and the second is the allelic range. This range is the difference in length between the longest and the shortest fragments that contain that microsatellite. The G-W index is based on the observation that, when population size decreases, the number of alleles at a microssatelite locus is more likely to be reduced than the allelic range. Thus, the index is calculated for polymorphic loci by dividing the number of alleles of a given microsatellite in a population by its allelic range plus one. In a population that did not experience a genetic bottleneck, the G-W index ranges from 0.8 to 1. Values below 0.8 indicate the possibility of reduction of genetic variability, while those below 0.6 indicate a significant reduction in genetic variability due to a genetic bottleneck effect (Garza and Williamson, 2001).

As an alternative, the expected heterozygosity (H_e) and the heterozygosity resulting from genetic drift and mutation (H_{eq}) can be compared, and the distribution of allelic frequency at microsatellite loci can be determined (Cristescu et al., 2010). In this procedure, one of three models can be used: the infinite alleles model (IAM), the stepwise mutation model (SMM), or a combination of these two hypotheses, the two phase model (TPM) (Di Rienzo et al., 1994). The two phase model is based on the observation that heterozygosity and allelic frequency reflect changes in population size (Rosa de Oliveira et al., 2009). If H_e is significantly higher than H_{eq} (typically at $p \leq 0.05$) and the distribution of alleles has changed from an L-shape to shifted, then the population is likely to have been affected by a bottleneck or founder effect in the recent past (Hart et al., 2014). These calculations can be performed with BOTTLENECK software (Piry et al., 1999; Cristescu et al., 2010).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

Assemblage of breeding pairs based on their genetic profiles

Using individuals for captive breeding without knowing their genetic characteristics can lead to a progressive decline in genetic variation, because the probability of breeding individuals that are genetically similar to each other is higher than that of breeding those that differ substantially (Kaczmarczyk, 2016; Aguiar et al., 2018). The resulting offspring will have lower heterozygosity and allelic diversity than their parents, and this process is likely to progress with each successive generation (Bentsen and Olsen, 2002; Fraser, 2008). For this reason, techniques for genetic management of broodstock should be applied (Blackie et al., 2011) genetic profiles should be prepared before individuals are selected for breeding and assembled into pairs. These profiles should include information such as the individual's sex, the population it came from, and a list of the alleles of microsatellite DNA that were detected across the investigated microsatellite loci (Kaczmarczyk, 2015). These profiles can be used to produce progeny that are as genetically diverse as possible because only the individuals that genetically differ the most will be selected and assembled into breeding pairs. To find the best pairs out of many possible combinations, software like Geneassemblage 1.0 (Kaczmarczyk, 2015) can be used, which is appropriate not only for diploid organisms but also for tetraploid or partially tetraploid organisms because it calculates the expected percentage of offspring with three alleles that are the same and a fourth that differs, i.e. "weak heterozygotes" (Fopp-Bayat et al., 2015).

Evaluation of genetic differences between populations

Allelic frequency can change in response to environmental conditions. These changes cause and maintain genetic differences between populations (Higgs and Derrida, 1992) that can be essential for their survival (Barraclough and Nee, 2001). Thus, preservation of genetic diversity is important for the success of conservation programs: for example, the maintenance of the unique genetic characteristics of cheetah, lion, panther and humpback whale populations is closely related to the success of conservation programs (O'Brien, 1994; Hedrick and Fredrickson, 2010).

Genetic distance is a measure of the difference in genetic characteristics between populations and species. Interpopulation genetic differences are manifested by the occurrence of private alleles and differences in allele frequencies (Avise, 2004). Private alleles are variants of microsatellite DNA fragments specific to a given species, population or group of populations. Identification of the private alleles in an individual's genotype enables determination of the population it came from and whether it is an interspecies hybrid (Van Dongen et al., 2012). In contrast to private alleles, common alleles are detected in all studied populations (Slatkin, 1981). As the phylogenetic distance between populations increases, the number of private alleles increases, the number of common alleles decreases, and the frequencies of alleles in the respective populations differ to a greater extent (Rosenberg, 2011).

To estimate genetic distance, gene flow between populations and interrelations between species, methods based on microsatellite polymorphism can be used (McManus et al., 2015). Although a description of all of these methods is beyond the scope of this paper, more than one method should be used, and their results should be compared (Balloux and Lugon-Moulin, 2002; Haris et al., 2013). Two methods are given below as examples.

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One of the most commonly used methods is based on the fixation index (F_{ST}). To determine the statistical significance of F_{ST}, a permutation test is done, and the *P*-value indicates the proportion of the permutations that give an F_{ST} greater than or equal to the observed F_{ST} (Reynolds et al., 1983; Slatkin, 1995). F_{ST} is proportional to the genetic differences between populations. Its value ranges from 0 to 1, where 0 indicates no genetic distance, and 1, the largest possible difference between populations. Typically, a value of 0.05 or less indicates a very small genetic difference; 0.05-0.1 a moderate; 0.1-0.25, a large; and above 0.25, a very large genetic distance (Balloux and Lugon-Moulin, 2002).

As an alternative to F_{ST}, the difference in mean size of alleles ($\delta\mu^2$) can be calculated, which compares the mean allele sizes of microsatellite segments in terms of repeat number (Goldstein et al., 1995). The method is largely unaffected by differences in sample sizes, although it shows bias with small sample sizes (Ruzzante, 1998). Unlike F_{ST}, there is no upper limit to the value indicating genetic distance that is calculated by this method. A value of $\delta\mu^2$ above 10 indicates a very large genetic distance between populations (Goldstein et al., 1995; Harris et al., 2013). $\delta\mu^2$ can be estimated using MSA 4.05 (Dieringer and Schlötterer, 2003).

The genetic distance between pairs of populations and the intrapopulation genetic diversity can be represented graphically (Waser and Strobeck, 1998). The graph is based on a genetic assignment test, which determines the probability of occurrence an individual with a specific genotype in its native population versus the probability of its occurrence in another population (Larson et al., 2014; Kaczmarczyk and Wolnicki 2016). Those calculations are performed by Arlequin software (Excoffier et al 2005; Excoffier and Lischer, 2010), and can be imported into an Excel spreadsheet (Microsoft, USA) and scaled as co-ordinates in an X–Y coordinate system. If a point representing an individual lies on the diagonal between the X and Y axes, the probability of its occurrence in its native population is the same as the probability of its occurrence in the alternative population. Points that are closer to one axis than to another have a greater probability of occurring in the population that corresponds to the nearer axis than in the other population, and the further the points are from the diagonal, the greater the probability of that individual occurring in the population that corresponds to the nearer axis. The spread of points within a cloud of points representing a group of individuals shows the degree of intrapopulational differences, and the distance between the points indicates the genetic differences between the individuals in that population (Kaczmarczyk and Wolnicki, 2016).

Optimization and management of genetic variation resources deposited in banks of cryopreserved gametes

Cryopreservation of gametes is a method for protecting the genetic resources of a population (FAO, 2012), and is used to safeguard the level of genetic variability (Yang and Tiersch, 2009) in the event of a catastrophe resulting in the extinction of the population. It is used for both endangered species and economically valuable breeds and breeding lines (Zhang, 2004; Cabrita et al., 2010).

The potential usefulness of cryopreserved gametes in protecting the genetic variability of a population depends on the genetic diversity of the individuals from which the gametes were collected (FAO, 2012). If gametes from a group of individuals are cryopreserved without taking into account the genetic differences between them, only a small part of the genetic variation in that population may be preserved. This

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

could lead to a strong founder effect, resulting in a restored population with too little genetic variation to ensure its survival. Increasing the size of the group from which gametes are collected is not an ideal solution because it not only increases the cost of creating a gamete bank, but also does not always safeguard the genetic diversity of the population. This is particularly a concern for species such as the lake minnow, whose populations are characterized by low levels of genetic diversity (Kaczmarczyk and Żuchowska, 2011; Kaczmarczyk and Wolnicki, 2016).

To address the potential problem of a lack of genetic diversity in preserved gametes, genetic profiles of gamete donors can be developed using microsatellite DNA polymorphism to indicate which individuals differ the most and are especially valuable for conservation of genetic diversity in that population. This technique can be used with genomes that are relatively little investigated, and it enables maximum conservation of genetic diversity with the minimum number of samples. The resulting information can be used to assemble breeding pairs (Danchin-Burge et al., 2009) and maintain the genetic diversity of the population (Fernández et al., 2005).

The polymorphism of microsatellite DNA as a tool for evaluation of the effectiveness programs for conservation and restoration of species

Maintaining the level of genetic diversity of protected populations is one of the criteria for assessing the effectiveness of conservation programs (Stem et al., 2005), and investigations of the polymorphism of the microsatellite DNA can be used for this purpose. The results of investigations of microsatellite DNA that are obtained from samples taken from the current population can be compared with those from archival material (Abdul-Muneer, 2014), which can be formalin or paraffin-treated tissues (Shi et al., 2004; Wu, 2005) and scales (Kumar et al., 2007), or bones (Piglionica et al., 2012). Alternatively, the genetic diversity of a conserved population can be compared with that of stable populations in which there is no decrease in viability (Gadomski, 2013). It is possible to check whether the genetic variability of the newly created population of conserved species is close to that of a population that has existed for many years (Kashiri et al., 2018). It is also possible to estimate the size the founder effect involved in the creation of a new population (Gadomski, 2013).

In human-dependent species, the survival rate of individuals obtained in controlled conditions and released into the environment also indicates the effectiveness of conservation programs. In this context, microsatellite markers are used to determine whether an individual was produced by natural reproduction or human-controlled reproduction (Martínez and Fernández, 2008). A database with the genetic profiles of individuals used in reproduction and the use of SPAGeDi (Hardy and Vekemans, 2002) or COLONY software (Jones and Wang, 2009) enable estimation of the probability that an individual was produced by artificial or natural reproduction. Estimating the relative amounts of individuals produced by natural reproduction and those produced in controlled conditions indicates the relative effectiveness of natural breeding and conservation or restoration programs. This technique has been used for example in evaluation of the effect of the release of brown trout (*Salmo trutta* L.) obtained under hatchery conditions on the genetic characteristics of the Danish population of this species (Hansen et al., 2000) and in monitoring the effectiveness of programs for the conservation of sea trout (*Salmo trutta morpha trutta)* and salmon (*Salmo salar*) (Bartel, 2000; Wąs and Wenne, 2002; Nilsson et al., 2008; Jones et al., 2010; ICES WGBAST, 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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One approach that is used in conservation programs is limited translocation, in which endangered populations are relocated to safer habitats. This method is currently used in the conservation of many species including the lake minnow (Kusznierz et al., 2006; Kaczmarczyk and Wolnicki, 2016). Because the effectiveness of this strategy depends on the degree of genetic diversity achieved by the population in the target location, the factors that decrease genetic variability, such as the founder effect, should be limited (Fraser, 2008). When the genetic characteristics of the source population and the genetic profiles of individuals are known, it is possible to minimize the size of the founder effect by choosing the optimal size and composition of the group that will be transferred (Gadomski, 2013). Furthermore, the information obtained about the source population will allow comparison of the genetic diversity of the source population with that of the newly established population, and estimation of the influence of the founder effect on the new population, which in turn allows assessment of the effectiveness of this method of species conservation.

To counteract changes in the environment due to human activities, species restoration can be employed. The main objective of these programs is to ensure that the genetic characteristics and functionality of the restored population are as close as possible to those of the original (extinct) population. To this end, individuals are selected from the available populations whose genetic characteristics are as similar as possible to those of the extinct population (Falk et al., 2001). The accuracy of this selection depends on the use of techniques such as the evaluation of genetic differences based on the polymorphism of the microsatellite DNA (Cibrian-Jaramillo et al., 2013). This technique was used, for example, in the program for restitution of Baltic salmon in Pomeranian rivers, in which evaluation of microsatellite polymorphism showed that the most similar existing population, and thus the most appropriate for use as stocking material, was the population spawning in the Daugava river (Bartel, 2001).

Future directions

Future developments in the application of microsatellites should include studies of the correlation between levels of genetic variation determined on basis of microsatellite DNA polymorphism and population fitnes. If we find that decreased genetic variation, as determined by this technique, correlates with reduced population fitness, then we will be able to use microsatellite DNA polymorphism to indicate which conservation projects should be given priority. This task will require genetic analysis of many individuals and populations, so the developement of multiplex PCR techniques for all relevant species is yet another task that should be undertaken. Finally, the future of investigations of microssatelite loci in conservation biology should involve combinations of the results of studies on intra- and interpopulation variation and genetic profiling with bioinformatic tools designed to manage genetic variation on both local and worldwide scales. Tools such as databases of genetic profiles of individuals and donors of gametes deposited in cryoconservation banks, combined with software designed to evaluate genetic similarity between individuals will be the future of conservation/restoration programs based on progeny produced in controlled conditions.

Summary

This paper has reviewed a molecular method based on analysis of the polymorphism of microsatellite DNA for use in the conservation and restoration of endangered

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

populations. This method is particularly useful when the conservation or restoration work involves supporting populations with individuals obtained in controlled breeding programs. Analysis of microsatellite DNA polymorphism can be used to estimate and monitor genetic variability, and to select individuals for breeding or transfer that will maintain a safe level of genetic variation in a population. Furthermore, this method can also be used to optimize the selection of gametes for cryopreservation. The analysis of microsatellite DNA polymorphism can help not only to ensure the lasting success of conservation and restoration projects, but also to reduce costs by indicating how many individuals should be included in a project.

Acknowledgements. This work was supported by the National Science Centre in Poland within project No. 2014/15/B/NZ9/05240 for 2015-2018.

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DOI: http://dx.doi.org/10.15666/aeer/1702_15991615

INVESTIGATIONS ON FARMERS' WILLINGNESS TO ASSOCIATE AND JOIN IN ENVIRONMENTAL RESPONSIBLE SHORT SUPPLY CHAIN IN ROMANIA

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(Received 4th Oct 2018; accepted 2nd Jan 2019)

Abstract. In contemporary agricultural based economies, smallholder farmers are encouraged to organize and reunite themselves into different forms of association, for overcoming major challenges and obstacles imposed by the market functionality and barriers, land grabbing and land fragmentations, ownership rights or reducing transaction costs. But farmers are often resistant to associate, due to the way in which cooperation was made in the previous regime in Romania. The objectives of this paper are to identify both the farmers' willingness to associate in any associative form, considering the current economic context in which cooperatives are established on voluntary bases, and also to highlight the determinants of their willingness to join an associative form from the broad perspective of a short supply chain. The research is specific targeted to for vegetables and fruits producers. The results provide an overview of the intensions of the farmers' willingness to associate and create better comprehension of this situation in the Romanian agriculture. Consequently, the paper points out, that the probability of farmers' willingness to adhere to a associative form or in a short supply chain environmental responsible is often hampering by factors as: age, education level, cultivated area, activity type, year of establishment, opportunities to access financial support which confirms the gap between economic necessity to associate and the traditional restrictions.
Keywords: *supply chain, environmental farming, ecologic products, agricultural policy, holdings*

Introduction

Emergent countries as Romania, with a significant share of the agricultural sector in the national economy provide a unique opportunity to study farmers' willingness to associate in any associative form and to adhere in environmental responsible short supply chain as land reforms have redistributed the land ownership rights and liberalized land exchange restrictions imposing the free market economy rules. While much studies has been written on land reforms (Cartwright, 2017), land ownership (Sabates-Wheeler, 2001; Vidican, 2009), land fragmentation (Thomas, 2006; Kuemmerle, 2009; Sikor, 2009) importance of agriculture in domestic economies (Aligica, 2003; Davidova et al., 2003; Mănescu et al., 2016; Aničić et al., 2018; Hu et al., 2018), farm restructuring and land grabbing (Van der Ploeg, 2015; Constantin,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16171639

2017) in transition countries, still few researches have concentrated on providing formal conceptual and economic analysis of the farmers' willingness to associate and their determinants to form agricultural cooperatives and other forms of joint exploitation of agricultural potential.

The central goal of stimulating the process of associating agricultural producers in agricultural cooperatives or other forms of specific agricultural association is to establish viable agricultural production structures in private farming sector. However, in Romanian agricultural sector, land privatization and numerous land reforms has led to a more problematic economic and social situation by dividing large land properties, previously existing and economically efficient, into numerous small privately and less economic viability holdings. The excessive land ownership fragmentation has caused significant problems in valuing agricultural potential and in creating agriculture that fulfills the demands of the market economy by increasing competitiveness and living standards in rural areas.

The evolution of the farmers' willingness to associate in any associative form has a long history in Romanian agriculture. The Romanian agricultural sector has experienced numerous and often unfinished land reforms during the process of implementing and achieveing a well and functional market economy imperatives. As (Dale and Baldwin, 2000; Hartvigsen, 2014) argues in their studies, numerous farm structures and holdings have emerged during the reforms which are now, incompatible with modern agricultural practice and tendencies. The contradictory evolution of farmers' willingness to associate is often due to the historical background of land ownership situation triggered by collectivization process and sharing agricultural benefits among members. From this perspective, achieving a competitive and highly functional agricultural sector impose agricultural cooperation as a functional and potential instrument in stimulating the capitalization of the national agricultural potential and developing the short supply chains. Taking into account the evolution of national agricultural sector, the term "cooperation" raises many contradictions and emotional reactions to Romanian farmers, since it is linked to the forced cooperation during the communist period. At that time, the cooperatives were not established on the traditional cooperative principles and values: voluntary and open membership, democratic member control, autonomy and independence (ICA, 2018), and this is the reason why farmers are still resistant to cooperation. Despite the fact, that nowadays, cooperation is made on voluntary membership and farmers' willingness to associate and has a more comprehensive approach which is centered on the principles of efficiency and competitiveness and less on considerations of social nature, belonging to a social community or social group in rural area.

As in literature (Nosenzo and Tufano, 2017), voluntary participation has a strong, positive effect on cooperation, as reported by Nosenzo and Tufano (2017). Thereby, the main objectives of the research are to find whether the farmers want to join an associative form, in the current economic context in which cooperatives are established on voluntary bases, and to identify the determinants of farmers' willingness to join a short supply chain and an associative form. In this context, a need to explore the determinants and economic impacts of membership in farmer cooperatives and food supply chains in Romania is necessary in explaining the reasons of farmers' willingness or resistance to membership.

The relevance of the topic emerged from the low level of farm efficiency in Romania, attributed to the small size of farms. There are 3,342,185 holdings and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16171639

12,502,535.5 ha of cultivated land, resulting an average farm size of 3.74 ha, as National Institute of Statistic of Romania reported for 2016 (NIS, 2018). Also, as it is reviled in some national studies (CRPE, 2016), in 2015, there were 743 agricultural cooperatives in Romania, most of them being found in the North-East region of the country, as reported by the National Trade Register Office. The Ministry of Agriculture and Rural Development presented that less than 1% of Romanian agricultural producers are part of associative structures, as compared to 34%, the European Union average.

For overcoming this problem, some authors (Narrod et al., 2009, Bernard et al., 2010) consider that the solutions could be either farmers' association in different forms (marketing, selling or processing cooperatives, producers' organizations or agricultural associations), known in literature as horizontal integration (Malassis, 1992), or farmers' integration into a supply chain, known in literature as vertical integration. Both horizontal and vertical forms of integration lead to higher performance in carrying out the agricultural activities (Ion, 2005).

The term supply chain is defined by Malassis et al. (1992: p. 94) as the itinerary of a product or group of products within the agro-food system and comprising all agents and operations that contribute to the formation and transfer of products to the final stage of use. In some cases, the chains are long and complex, as reported by Manole et al. (2005), implying numerous agents and operations. Since every agent within the chain asks for a certain profit considering the activities performed, increasing, finally, the prices that the consumer pays for products, the need to shorten the chain has emerged.

The attempts to shorten the supply chains are encouraged and financed by the Ministry of Agriculture and Rural Development of Romania, through the National Program for Rural Development. Short supply chain is defined as the configuration of the food chain that does not involve more than one intermediary between producers and consumers (MARD, 2016: p. 43). A short supply chain implies fewer agents, but the same number of operations, meaning that the activities of the chain are performed by the remaining two agents – farmer and retailer (Marin et al., 2017).

Since the solution agreed to overcome the low level of farm's efficiency seems to be the producers' association, the public agricultural policy in Romania aims to encourage farmers to associate, using subsidies, financial support or preferential financial leverage for associative forms. If referring to fruits and vegetables branch, the public policy grants financial support to producers' organizations (Romanian Government Decision no.740/2017). Integration, including association, is also stimulated through the National Program for Rural Development in Romania, where one specific measure (16.4) refers to financial support for horizontal and vertical cooperation between the actors in the supply chain (MARD, 2017).

Understanding farmers' motivations for cooperation, and the tension associated, is important for the expectation management and funding efficiency (Wynne-Jones, 2017), at a time when great funding and support is being channelled through the European Union Common Agricultural Policy to encourage cooperative and collaborative practices (Prager, 2015).

In this context, the research of the farmers' willingness to join a short supply chain or an associative form environmental responsible is needed for justifying the state's financial efforts to fund the farmers' projects aiming to create short supply chains and to encourage the establishment of cooperatives.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The questions arising are whether farmers want to join a supply chain or a cooperative or not, and what are the determinants of their membership. The answers to these issues are searched in this research.

Other additional objectives are considered within the study. Thus, research aims to find out the farmers' perceptions on different issues: the level of the state subsidies and other financial support, the public taxation in agriculture, the short supply chain functionality, and the association role within the short supply chain.

In achieving these issues, a survey of farmers across fifteen (15) counties in Romania has been conducted. The method used for data collection was the interview. The data were analyzed using SPSS. The hi2 test and coefficient of contingence were used to analyze the correlations between variables. The sample contains one hundred and forty (140) valid observations. Besides asking the farmers' willingness to join a short supply chain environmental responsible or a cooperative, the survey also asked the follow-up questions about the reasons why farmers do not want to join a short supply chain environmental responsible or an associative form of organization.

The relevance of the research lies in the economic, social and environmental benefits brought by collective actions in agro-food system. Short supply chains (vertical integration) and collective actions of farmers (horizontal integration) are studied in the light of multi-stakeholder cooperatives. When the two forms of integration are combined, they form multi-stakeholder cooperatives which bring together producers, processors and/or retailers, and consumers under one single enterprise. It has been demonstrated by Gonzales (2017) that multi-stakeholder cooperatives create more sustainable food flows between rural and urban areas and they overcome the limitations of conventional producers' cooperatives focused more on economic than social and environmental benefits.

The paper outcomes are expected to provide a specific framework for policy makers on economic, demographic and psychological factors that influence the farmers' willingness to join a short supply chain environmental responsible and an associative form of organization. Also, the main findings may contribute to develop policy initiatives to finance collective actions in food supply chains.

Following the introduction, the paper is structured into six parts, as follows. In Section 2, a holistic approach is taken to review the literature in the field of supply chains, integration and cooperation, with the final goal to establish the study hypotheses, in Section 3. The paper then proceeds to present the database and methodology, in Section 4, whereas, in Section 5, the main findings are discussed. Finally, the implications of the findings are considered and concluding remarks are drawn, in Section 6.

Literature review

There is abundant literature and numerous case studies and field research referring to agricultural integration, supply chains and farmers' associations. With respect to integration, Poole and Frece (2010: p. 7) recognized that it is a means of tackling poverty in rural areas, and that increasing vertically coordinated supply chains plays a significant role in linking smallholder farmers to more dynamic markets. Williamson (1985) showed that integration aim is to minimize transaction costs. Other authors (Neven and Reardon, 2004) argued that the supply chains need to be redesigned, advocating the development of new, smaller farmer markets closed to local residential

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areas that are able to facilitate an effective procurement system. This idea, linked to shortening the supply chain, meaning fewer agents, is sustained by Poole and Frece (2010: p. 100), as well. They found that one approach to the fair-trade sector consists on creating links between farmers and traders, importers or even retailers, thereby cutting out middlemen and maximizing profit margins for farmers.

Supply chains, described as all steps involved in production, manufacturing and distribution of food until its final consumption (Stone and Rahimifard, 2018), face vulnerabilities due to the limited shelf life of food, and variability in quality and availability of raw materials as organic products (Dani and Deep, 2010).

In respect to the process of collective collaboration in agriculture, many papers studied both its benefits and failure. The term "smallholder farmers' association" is referring to diverse types of groups who act collectively in order to benefit either as individuals or as a group (Poole and Frece, 2010: p. 17). The terminology used in the literature varies from terms such as cooperatives, farmer collectives, farmer associations, rural community enterprises, rural producer associations, community enterprises, and farmer organizations (Donovan et al., 2008). A cooperative is defined as "an autonomous association of persons united voluntarily to meet their common economic, social, and cultural needs and aspirations through a jointly owned and democratically-controlled enterprise" (ICA, 2018). Cooperatives are considered to play significant economic roles by reducing transaction costs and improving the bargaining power of individuals (Bonin et al., 1993), as a consequence, they are recognized to fight poverty in rural areas (FAO, 2005).

Some studies highlighted the socio-economic benefits of cooperatives. Fischer and Qaim (2012) reported the positive impact of cooperative membership on production and productivity in Ethiopia. Abate et al. (2014) claimed that improved technical efficiency of member of agricultural cooperatives is obtained due to better access to productive inputs and services, as compared to non-members. Marketing cooperatives allow small farmers to get better price by overcoming the powerful oligopolistic firms (Bontems and Fulton, 2009). Moreover, grace to marketing cooperatives, farmers have a better position for price negotiation and gain access to markets that they cannot access individually (Camanzi et al., 2011).

But cooperatives cannot be simply generalized as benefiting all their members and all locations (Mojo et al., 2017). Studies show that their performance varies across countries, regions and even sector (Bernard et al., 2010), and the skills of the managers and the administrative organization determine their success. Cooperative management seems to be the weak point of associative forms in Romania. Also, farmers' resistance to association is a characteristic of the Romanian agricultural system, as many authors observed in their works (Marin et al., 2016).

There are both practical and theoretical explanations of the failures of collective organization. Sound financial management, good organizational governance and the potential for free riding, remain problems for the more complex cooperatives (Poole and Frece, 2010: p. 46). But at the same time, there are fundamental reasons for collaborating: the potential for exploiting production and managerial economies of scale, overcoming market entry barriers, reducing transaction costs and cultivating supply chain relationships.

The term "new cooperative" has emerged (Cook and Chaddad, 2004) referring to the market-oriented producer organizations, and extending this commercial orientation further by linking ownership rights, investment and governance. Whereas traditional

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cooperative forms are described as being defensive, aiming to protect farmers and minimize risk, new generation cooperatives can be seen as 'offensive', able to cope with falling profit and income risks.

Not only horizontal integration creates new cooperatives, but also vertical integration advocates fundamental changes in supply chains in the advanced economies. A shift from competitive vertical relations towards the cooperative organization of the supply chain, or supply chain management has been identified by Poole and Frece (2010: p. 21).

Table 1 summarizes the main findings in the field of farmers' cooperation, supply chains and their economic implications, relevant for our research.

The links between supply chains and collective actions are also discussed in many papers. Lund (2012) studied the place of the modern multi-stakeholders' cooperatives in food and farming in the context of the emerging concept of value chains, which implies approaches beyond economics. The activities along value chains involve many cultural and social aspects such as taste, identity, connection with nature and community (Baggini, 2014). Bauwens and Kostakis (2014) go beyond multi-stakeholder cooperatives and value chains and call for "open coops", which combines multi-stakeholder ship and the co-production of the value chain by everyone affected by a provisioning service. The cooperatives are described as potential leading actors in shortened food chains, since they make possible economies of scope or synergy, versus simplistic economies of scale (Marsden et al., 2002).

Table 1. *Main findings in the field of farmers' cooperation, supply chains and their economic implications*

Issue	Explanation	Relevant studies
Vertical integration of activities within supply chains increases performance	Reduced transactions costs, smallholder farmers are closer to more dynamic markets, overcoming market entry barriers, increasing equity	Ion (2005), Lund (2012), Marin et al. (2016), Marin et al. (2017), New (1997: p. 19), Poole and Frece (2010), Williamson (1985)
Horizontal integration of activities within supply chains increases performance	Collective actions include greater accessibility of finance, technology and market information; improve farmer returns by lowering production costs, counterbalancing the negative economic impacts of market power and reducing producer income risks	Abate et al. (2014), Bonin et al. (1993), Bontems and Fulton (2009), Camanzi et al. (2011), Cook and Chaddad (2004), Markelova et al. (2009), Wynne-Jones (2017)
The length of the supply chain must be reduced	In longer chains, too many agents ask for profit, increasing, as such, the prices that the final consumer pays for food. In shorter chains, there are direct links, cutting out middlemen and maximizing profit margins for farmers	Chirwa et al. (2005), Manole et al. (2005), Marin et al. (2016), Poole and Frece (2010)
Multi-stakeholder cooperatives combine supply chains and collective actions (they bring together producers, processors and/or retailers, and consumers under one single enterprise)	Multi-stakeholder cooperatives create more sustainable food flows between rural and urban areas. Multi-stakeholder cooperatives have the potential to generate a diverse range of more-than-economic benefits: sectorial, legal, cultural, policy and public procurement, academic	Baggini (2014), Bauwens and Kostakis (2014), Gray and Stevenson (2008), Gonzales (2017), Lang et al. (2009), Lund (2012), Mooney (2004)

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Although there is abundant literature in the field of farmers' cooperation, supply chains environmental responsible and their economic implications, the need of analysing these phenomena in Romania arises from the agriculture's particularities in this country, especially by the existence of numerous and small agricultural holdings. The land reforms have determined extreme forms of land use fragmentation which has generated and maintained for a long time a small peasant property with a low degree of efficiency and poorly technically endowed. An opposite situation is drafted in Bulgaria, where according to (Lerman et al., 2004) more than 40% of land is being farmed in associations. Still, for Romania, the high fragmentation of agriculture structures creates premises for farmers' association as viable solution for overcoming market entry barriers, and increasing equity along the chain.

Romanian agriculture is facing a long and difficult process of land aggregation, marked often by the resistance of farmers to reunite in holdings. Despite the fact that, domestic agriculture is trying to diminish the contradictory effects of the dominance of the subsistence farming imposed by the peasant property, there are successful attempts in determining the farmers' association and achieving a comparative convergence level with the European agricultural model. The need for farmers' association and cooperation emerge as a stringent necessity imposed by real and determinant factors as, low productivity in agriculture, poor access to markets, low investment capacity, technical and technological capital shortage, and low levels of competitiveness.

This study conducted a survey to investigate the factors influencing the Romanian farmers' willingness to join a short supply chain environmental responsible and an associative form of organization. Surveys and behavioural approaches in agricultural studies are not new. They cover a broad range of studies that employ quantitative methodologies to the investigation of decision-making and farmers' willingness to adopt new technologies, instruments, services etc. Folorunso and Ogunseye (2008) explored the farmers' acceptance of a knowledge management system in agricultural extension services. Heyder et al. (2010) examined the major factors influencing the investment behaviour of agribusiness firms. Pappa et al. (2018) identified the drivers that determine the acceptance and use of electronic traceability systems in agro-food supply chains. Survey has been used before to understand consumers' preferences for vegetables and fruits (Ion, 2015), using as dependent variables consumer preferences for preserved vegetables and fruits and as independent variables gender, age, income. Adrian et al. (2005) investigated the perception and attitudinal characteristics of farmers who plan to adopt precision agriculture, using a survey to measure constructs of perception and attitudes. As such, our intention in this paper is to explore the changes in food chains and cooperation phenomenon in Romania, considering the financial efforts of the public agricultural policy to fund the creation of short supply chains environmental responsible and the establishment of associative forms of organization. Our main contribution is to add to the discussions and enrich the evidence supporting different factors which determine farmers' willingness to join short supply chains environmental responsible and cooperatives, including demographic and economic variables, economic impacts of membership, and farmers' perceptions on financial and fiscal issues in agriculture.

Research hypotheses

Considering the results of the previous studies described in Section 2, four hypotheses (H1 to H4) emerged and need to be studied subsequently, as:

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H1: Socioeconomic and demographic factors influence the farmers' willingness to join a short supply chain or an associative form of organization.

This hypothesis is based on the results of other studies (Marin et al., 2016; Marin et al., 2017), showing that membership in an associative form varies with the farmer's age and the date of establishment of the holding. Mojo et al. (2017) found that the probability of farmers' membership decision increases with age, education level, family size, social networks, land property and accessibility to cooperatives. Adrian et al. (2005) argued that perceptions of net benefit, farm size and farmer educational levels positively influenced the intention to adopt precision agriculture technologies.

H2: Membership in a collective form of organization and a short supply chain has a positive impact on farm income and assets.

This hypothesis is based on the assumptions mentioned in *Table 1* and it is sustained by many other studies (Abate et al., 2014, Bonin et al., 1993, Chirwa et al., 2005, Cook and Chaddad, 2004, Manole et al., 2005, Markelova et al., 2009, Marin et al., 2016). They show that both vertical and horizontal integration of chain activities increases performance.

H3: Farmers are resistant to join a short supply chain or an associative form.

This presumption is based on emotional connotations related to the communist period when association was not established on voluntary bases. One study regarding the problems encountered by the Romanian farmers reported that 62% of subjects do not intent to associate in the future (Marin et al., 2016: p. 19). The longstanding question raised in the literature – whether farmers are inherently disposed towards self-reliance and independent actions rather than collective and collaborative working (Wynne-Jones, 2017).

H4: Farmers' reasons for not joining short supply chains and associative forms of organizations are linked to the lack of trust in other members, the lack of legislation and to bureaucracy.

The assumption is made on people resistance to cooperation as experienced in the previous period and on the results of other research (Marin, 2017) showing that farmers do not want to associate because they do not trust other people, and that there are no legal support and preferences or incentives for cooperatives.

Poole and Frece (2010), Turek et al. (2007), Ion (2005) found that farmers act collectively to get accessibility of finance, technology and market information and to enter the final consumer market. By fostering long-term relationships rather than punctual commercial transactions, Lund (2012) argued that membership in a collective form can overcome the higher transactional costs that traditional economic theory would expect from the involvement of separate parts. Economic motivation is a determinant of the choice to become a cooperative member, as Wynne-Jones (2017) found in the research studying farmer cooperation drivers and effects.

Experimental section

The survey data were collected from randomly chosen farmers across 15 counties in Romania, from January to April 2018. The response rate is 92% and there are 140 valid observations out of 152 responses. The sampling method is based on voluntary selection of units, which is a non-probabilistic method, considering rational choices (Porojan, 1993: p. 205). Sample inclusion is based on the voluntary option of individuals to participate in the sample. The survey questions include farmers' willingness to

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participate in short supply chains and cooperatives, farms' characteristics, and farmers' socio-demographic characteristics.

In the questionnaire, the key questions involve two parts. The first question is phrased "If you are not part of an associative form, do you consider to associate in the near future?" (Q5). The respondents could answer yes, showing their willingness to associate, or no, showing their resistance to association. If no, the respondent is asked to provide reasons (Q6: What are the reasons why you did not associate?).

The second key question is phrased "Do you intend to integrate your exploitation into a short supply chain of vegetables/fruits?" (Q14). The respondents could answer yes, showing their willingness to join a short value chain, or no, showing their resistance to join a short value chain. Explanatory factors considered in this piece of research are: the year of farmer's establishment, the form of farmer's organization, holding surface, crops' structure, farm's revenues, the type and level of farmer's financial resources, occupation, education, studies in agriculture/horticulture, age, gender and nationality (*Table 2*).

Table 2. *Dependent variables used in the model and other studies which considered them relevant*

Variable	Explanation	Relevant studies
Farmer's characteristics: age, gender, nationality, residence, civil status, education, studies in agriculture/horticulture occupation	Demographic characteristic of the subjects may influence their decision to join a short supply chain or an associative form of organization. The age of the household head is positively correlated with participation in cooperatives. It is assumed that the farmers who have studies in the field of agriculture or horticulture make informed choices and decisions	Bernard (et al., 2013), Burton (2004), Hansson et al. (2012), Knowler and Bradshaw (2007), Marin et al. (2016), Montefrio (2016), Nosenzo and Tufano (2017), Prokopy et al. (2008), Zhong (2016)
Farm's characteristics: year of establishment (farming experience), type of activity, farm size, structure of crops, financial resources	It is assumed that farmer's experience influences its process of decision making, including the decision of joining a short supply chain or a cooperative. The level of financial resources influence the farmers' decisional autonomy and, as such, their willingness to make the decisions on joining short supply chains and collective forms of organization	Bernard (et al., 2013), Gonzales (2017), Knowler and Bradshaw (2007), Marin et al. (2016), Pappa et al. (2018), Prokopy et al. (2008), Zhong (2016)

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Findings

The key questions of the research are: Are you part of an associative form in agriculture? (Q3), Are you part of a short value chain for vegetables and fruits? (Q4), If you are not part of an associative form, do you consider association in the near future? (Q5), What are the reasons why you did not associate? (Q6) and Do you intend to integrate your exploitation into a short supply chain of vegetables/fruits? (Q14). The answers to these questions show that 81.4% of the respondents are not part of an associative form and 78.6% are not part of a short value chain for vegetables and fruits. 82.1% of the respondents want to join an associative form and 67.1% of the respondents want to integrate their business into a short supply chain, in the near

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future. The main reason why farmers do not cooperate is the lack of legislation to support small and medium-sized producers, since 34.3% of the farmers have chosen this answer. Another significant reason is the high level of taxation (29%).

To achieve the research goals, the determinants of association are analysed, based on the correlations between variables. The results of the survey have been analysed using the values of the hi^2 test and the values of the contingency coefficient calculated with SPSS (Analyse-Descriptive Statistics-Crosstabs-Statistics-Chi-square/Contingency coefficient). Correlations between variables exist when the value of the hi^2 test do not exceed .05. Depending on the value of the contingency coefficient, this link may be of weak, medium or strong intensity.

The first assumption is that the socioeconomic and demographic factors influence the farmers' willingness to join a short supply chain or an associative form of organization (H1). To test this hypothesis, the correlations between variables are analysed. The dependent variable is the farmers' affiliation to an associative form of organization (Q3), and the independent variables are the farm's characteristics: the year of starting the activity (Q1), the activity type (Q2), the holding surface (Q7), the structure of crops (Q8.1), the exploitation's dimension (Q8.2), the estimated revenues (Q11), the subsidies (Q22), the accessing of European funds (Q25), and the farmer's characteristics: occupation (Qa), education (Qb), studies in the field of agriculture (Qc), age (Qd), gender (Qe), nationality (Qf), marital status (Qg).

Table 3 and *Figures 1, 2* and *3* illustrate the results of the analysis. Medium correlations have been found between the farmers' affiliation to an associative form of organization and the holding surface, because the value of hi^2 test is .005, lower than .05, and the value of the contingent coefficient is .308, over .300. It was noticed that farmers holding agricultural areas ranging from 1 to 5 ha are not part of an associative form.

Weak correlations were found between the farmers' affiliation to an associative form of organization and the activity type, the estimated revenues, the subsidies, the farmer's occupation and education level, where the values of hi^2 test are lower than .05, and the value of the contingent coefficient are below .300. Most of the farmers who act as physical persons do not belong to an associative form of organization, but some of the farmers who act as legal persons do belong to associative forms. Farmers with high income are part of associative forms, compared to farmers with lower income. Persons who graduated higher education are likely to associate, as compared to people who graduated only secondary schools.

There are no significant correlations between farmers' affiliation to an associative form of organization and the year of starting the activity, the structure of crops, the exploitation's dimension, the accessing of European funds, studies in the field of agriculture, age, gender, nationality, marital status.

Linear and positive relationships have been found between associative membership and holding surface (*Fig. 1*), supply chain membership and activity type (*Fig. 2*) and supply chain membership and holding surface (*Fig. 3*).

Medium correlations were found between the farmers' affiliation to a short supply chain environmental responsible and the activity type and the size of the farm, because the values of the hi^2 test are lower than .05, and the values of the contingent coefficients are .319, respectively .308 (*Table 3*). Farmers who act as physical persons and who hold small agricultural areas belong to a short supply chain environmental responsible.

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***Table 3.** The model estimated results for the hypothesis "Socioeconomic and demographic factors influence the farmers' willingness to join a short supply chain or an associative form of organization" (H1)*

Independent variable	**Dependent variable**	
	Q3: Affiliation to an associative form of organization	**Q4: Affiliation to a short value chain**
Q1: year of starting the activity	.0369	.777
Q2: activity type	.040 .277*	.007 .319*
Q7: holding surface	.005 .308*	.007 .308*
Q8.1: structure of crops	.163	.965
Q8.2: exploitation dimension	.162	.131
Q11: estimated revenues	.009 .252*	.594
Q22: subsidies	.049 .167*	.544
Q 25: the accessing of European funds	.167	.504
Qa: occupation	.009 .251*	.715
Qb: education level	.002 .281*	.002 .285*
Qc: specialized studies in agriculture	.117	.616
Qd: age	.968	.683
Qe: gender	.315	.691
Qf: nationality	.330	.287
Qg: marital status	.125	.610

Source: results of the model. The results show the values of hi^2 test. The values symbolized with * represent the values of the contingent coefficients, calculated only for those variables for which the values of hi^2 test are lower than .05

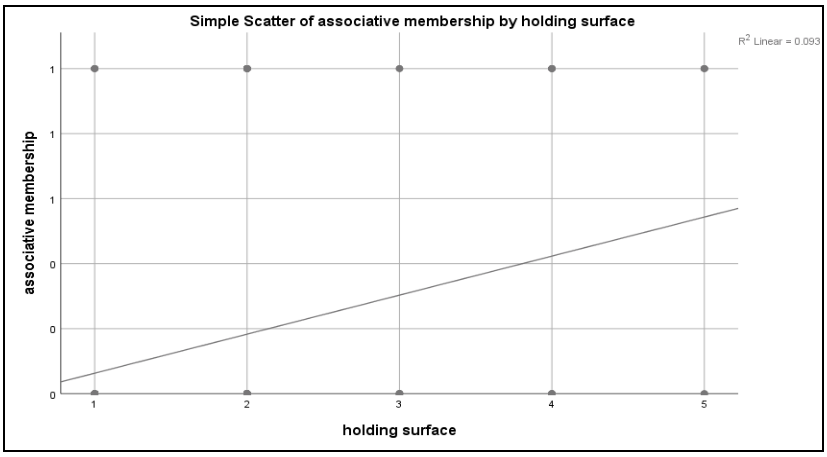

***Figure 1.** Correlations between the variables associative membership and holding surface. (Source: results of the model. The charts are built only for those variables in Table 3 for which the values of the hi^2 test are lower than .05 and the values of the contingent coefficients are higher than .300)*

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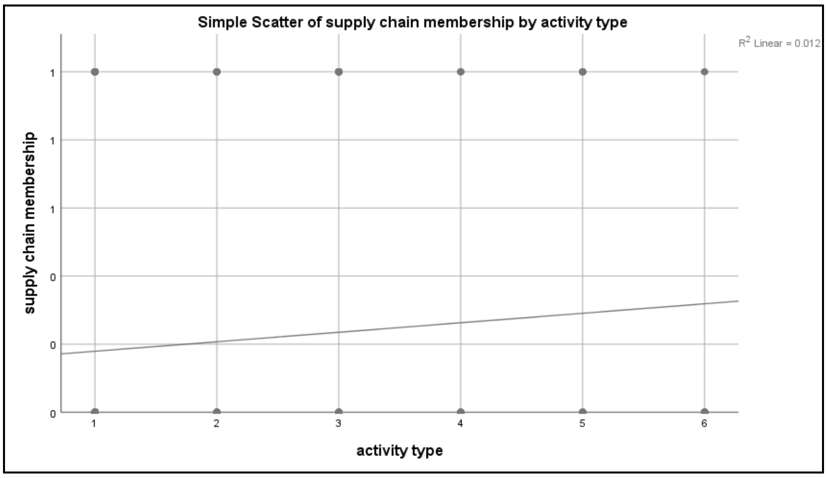

Figure 2. *Correlations between the variables supply chain membership and activity type. (Source: results of the model. The charts are built only for those variables in Table 3 for which the values of the hi² test are lower than .05 and the values of the contingent coefficients are higher than .300)*

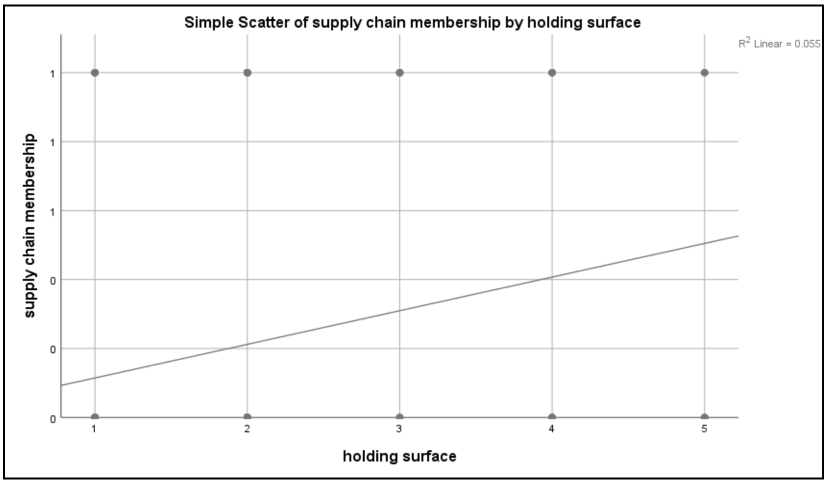

Figure 3. *Correlations between the variables supply chain membership and holding surface. (Source: results of the model. The charts are built only for those variables in Table 3 for which the values of the hi² test are lower than .05 and the values of the contingent coefficients are higher than .300)*

Weak correlation is found between the farmers' affiliation to a short supply chain and the farmer's education level, because the value of the contingent coefficient is .285. Persons who graduated higher education are likely to belong to a short supply chain environmental responsible, as compared to people who graduated only secondary schools. There are no significant correlations between farmers' affiliation to a short supply chain environmental responsible and the year of starting the activity, the structure of crops, the exploitation's dimension, estimated revenues, subsidies, the accessing of European funds, occupation, studies in the field of agriculture, age, gender, nationality, marital status. The second assumption is that "Membership in a collective form of organization and a short supply chain has a positive impact on farm income and assets" (H2). The dependent variable is farm's income and assets (Q11) and the

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independent ones are the affiliation to an associative form of organization (Q3) and the affiliation to a short supply chain (Q4). The results are presented in *Table 4*.

Table 4. *The model estimated results for the hypothesis "Membership in a collective form of organization and a short supply chain has a positive impact on farm income and assets" (H2)*

Independent variable	**Dependent variable** **Q11: Farm's income and assets**
Q3: Affiliation to an associative form of organization	.009 .252*
Q4: Affiliation to a short value chain	.594

Source: results of the model. The results show the values of hi^2 test. The value symbolized with * represents the value of the contingent coefficient, calculated only for those variables for which the values of hi^2 test are lower than .05

Weak correlation has been found between farm's income and assets and farmers' affiliation to an associative form of organization, because the value of the hi^2 test is below .05 and the contingent coefficient is .252, lower than .300. Farmers with higher levels of income are likely to belong to an associative form of organization. There are no significant correlations between the farm's income and assets and the farmers' affiliation to a short supply chain environmental responsible, because the value of the hi^2 test is over .05.

The third assumption is that "Farmers are resistant to join a short supply chain or an associative form" (H3). The answers to the questions: If you are not part of an associative form, do you consider association in the near future? (Q5) and Do you intend to integrate your exploitation into a short supply chain of vegetables/fruits? (Q14) show that most of the farmers (82.1%) want to join an associative form of organization and 67.1% of the respondents want to integrate their business into a short supply chain environmental responsible. The hypothesis is not validated.

For analysing the reasons why some farmers are resistant to association and integration, the determinants of this resistant are studied. The dependent variables are the farmers' willingness to associate (Q5) and their willingness to join a short supply chain (Q14). The independent variables are the farm's characteristics: the year of starting the activity (Q1), the activity type (Q2), holding surface (Q7), the structure of crops (Q8.1), the exploitation's dimension (Q8.2), the estimated revenues (Q11), the subsidies (Q22), the accessing of European funds (Q25), and the farmer's characteristics: occupation (Qa), education (Qb), studies in the field of agriculture (Qc), age (Qd), gender (Qe), nationality (Qf), marital status (Qg) (*Table 5*).

Linear and positive relationships have been found between the variables willingness to associate and year of starting the activity (*Fig. 4*), willingness to associate and holding surface (*Fig. 5*), willingness to join a short supply chain and year of starting the activity (*Fig. 6*).

Medium correlations have been found between the farmers' willingness to associate and the year of starting the activity and the surface of the farm, because the values of the hi^2 test are below .05 and the values of the contingent coefficients are over .300. Respondents who recently started the activity and who exploit smaller areas of land are willing to join an associative form of organization, as compared to others.

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Table 5. *The model estimated results for the hypothesis "Farmers are resistant to join a short supply chain or an associative form" (H3)*

Independent variable	**Dependent variable**	
	Q5: Willingness to associate	**Q14: Willingness to join a short supply chain**
Q1: year of starting the activity	.006 .321*	.001 .313*
Q2: activity type	.009 .252*	.080
Q7: holding surface	.002 .308*	.362
Q8.1: structure of crops	.530	.473
Q8.2: exploitation dimension	.507	.129
Q11: estimated revenues	.878	.375
Q22: subsidies	.020 .198*	.251
Q 25: the accessing of European funds	.480	.045 .203*
Qa: occupation	.809	.941
Qb: education level	.021 .229*	.761
Qc: specialized studies in agriculture	.861	.092
Qd: age	.124	.088
Qe: gender	.697	.540
Qf: nationality	.091	.071
Qg: marital status	.143	.537

Source: results of the model. The results show the values of hi^2 test. The values symbolized with * represent the values of the contingent coefficients, calculated only for those variables for which the values of hi^2 test are lower than .05

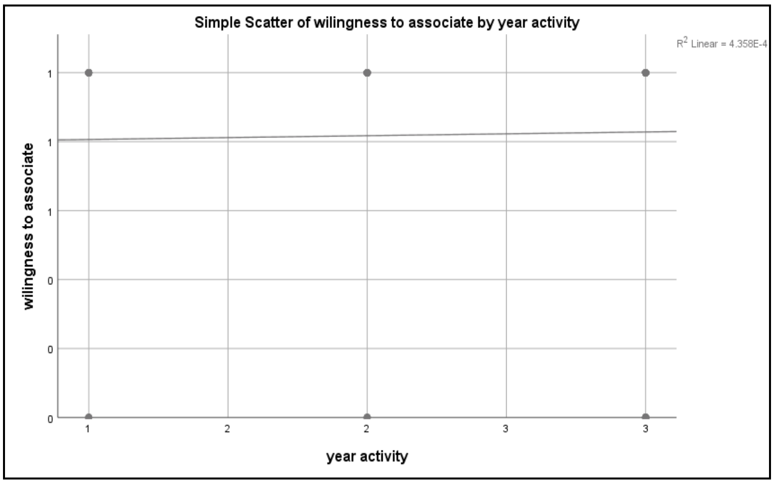

Figure 4. *Correlations between the variables willingness to associate and year of starting the activity. (Source: results of the model. The charts are built only for those variables in Table 5 for which the values of the hi^2 test are lower than .05 and the values of the contingent coefficients are higher than .300)*

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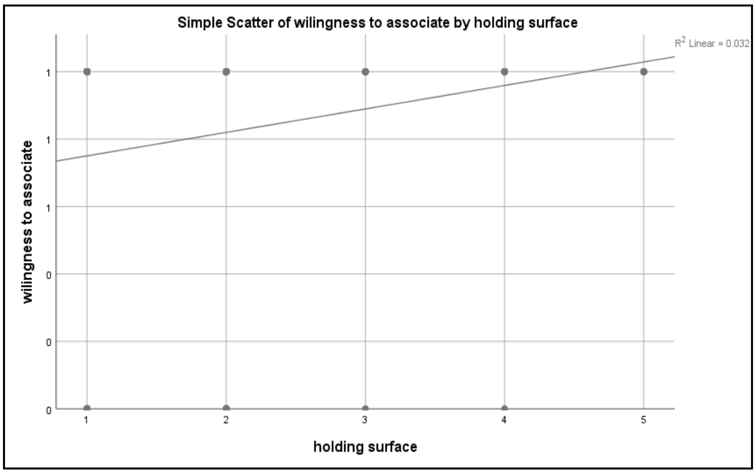

Figure 5. *Correlations between the variables willingness to associate and holding surface. (Source: results of the model. The charts are built only for those variables in Table 5 for which the values of the* hi^2 *test are lower than .05 and the values of the contingent coefficients are higher than .300)*

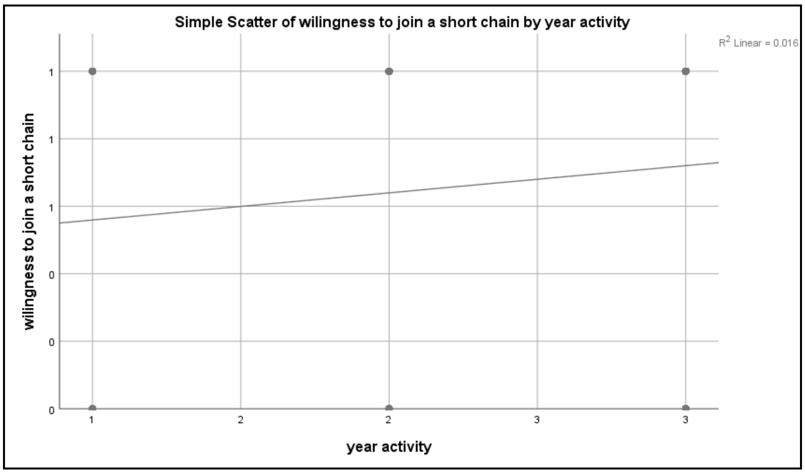

Figure 6. *Correlations between the variables willingness to join a short supply chain and year of starting the activity. (Source: results of the model. The charts are built only for those variables in Table 5 for which the values of the* hi^2 *test are lower than .05 and the values of the contingent coefficients are higher than .300)*

Weak correlations have been found between farmers' willingness to associate and the activity type, the access to subsidies and the farmer's education, because the values of the hi^2 test are below .05, but the values of the contingent coefficients are lower than .300. Physical persons who never accessed subsidies before and who have graduated higher education are more likely to associate, as compared to others.

No significant correlations have been found between the farmers' willingness to associate and the crops' structure, the farm's size, the estimated revenues, the access to European funds, occupation, specialized studies in agriculture, age, gender, nationality, and marital status.

Medium correlations are found between farmers' willingness to join a short supply chain and the year of starting the activity, because the value of the hi^2 test is below .05

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and the value of the contingent coefficient is .313, higher than .300. People who started an agricultural business after 2017 want to be part of a short chain more than others.

Weak correlations have been found between farmers' willingness to join a short supply chain and the possibility to access European funds, because the value of the hi^2 test is below .05, but the values of the contingent coefficient is .203, lower than .300. People wishing to access European funds want more than others to integrate into a short supply chain.

No significant correlations have been found between the farmers' willingness to join a short supply chain and the activity type, the holding surface, the structure of crops, the exploitation's dimension, the estimated revenues, the subsidies, and the farmer's characteristics: occupation, education, studies in the field of agriculture, age, gender, and nationality.

The fourth assumption is that "Farmers' reasons for not joining short supply chains environmental responsible and associative forms of organizations are linked to the lack of trust in other members, the lack of legislation and to bureaucracy" (H4). To test this hypothesis, the answers to the question "What are the reasons why you did not associate?" are presented in *Figure 7*.

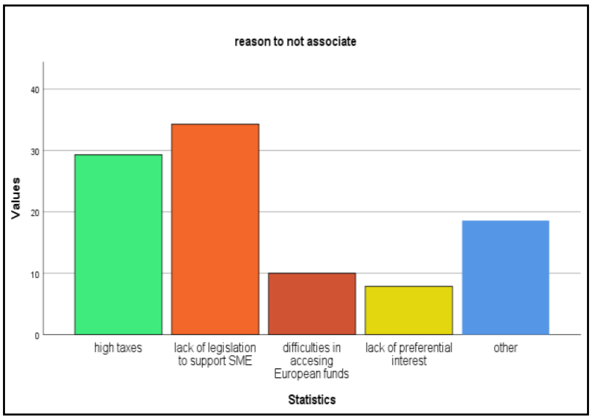

Figure 7. *Reasons why farmer do not want to associate. (Source: authors' own computations)*

Thus, the main reasons why the respondents did not associate are related to the legislation in force which is not supporting enough the associative forms, and the high level of taxes. For other reasons, the respondents mentioned the complex bureaucracy and the fact that they had no one to associate with, as well as the lack of trust. Hypothesis 4 is confirmed. Other objectives of the paper are to identify farmers' perceptions on the benefits of the short supply chain, and on the level of the state subsidies and taxes. The farmers' perceptions on the benefits of the short supply chain environmental responsible and association are presented in *Figures 8* and *9*.

Most people considered the short supply chain functional, since the average score is 3.08, on a scale from 1 to 5. Farmers consider that the association has a beneficial role in shortening the chain, since the most of the respondents (42.1) answered that farmers benefit from the association and 31.4 of them answered that the role of the association is very beneficial (*Fig. 9*). Moreover, high correlation has been found between the farmers' age and their perceptions on the role of the association in shortening the supply chain. People aged at first intervals (18-25 years old and 26-40 years old) consider that this role is beneficial to very beneficial.

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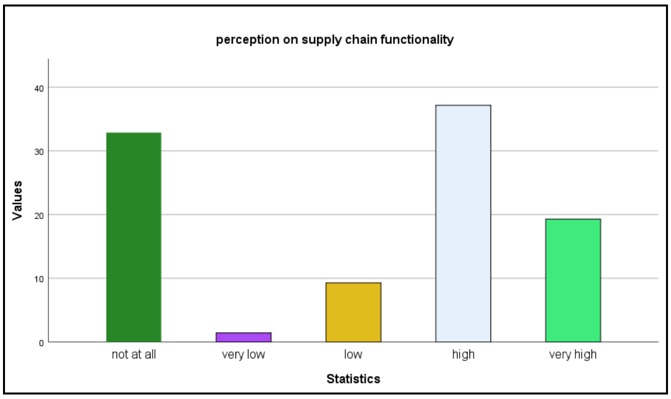

Figure 8. *Farmers' perceptions on supply chain functionality. (Source: authors' own computations)*

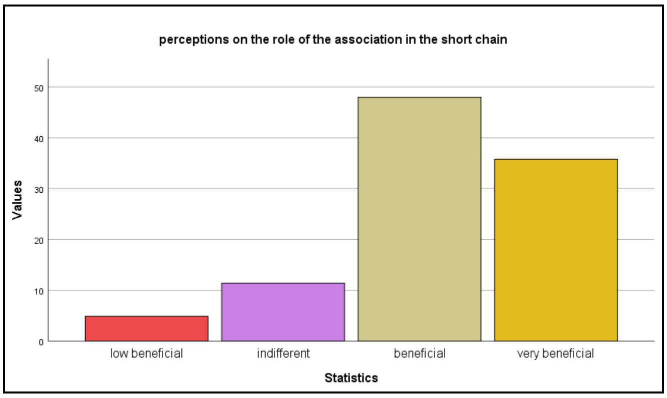

Figure 9. *Farmers' perceptions on the role of the association in the supply chain shortening. (Source: authors' own computations)*

The farmers' perceptions of the level of the state subsidies and taxation are presented in *Figures 10* and *11*. Over 70% of the respondents who answered the question "How do you appreciate state's financial support?" (128 out of 140) considered that the support provided by the state is insignificant and insufficient. Over 60% of farmers considered that the taxes and fees they owe the state are high and 32% consider them moderate.

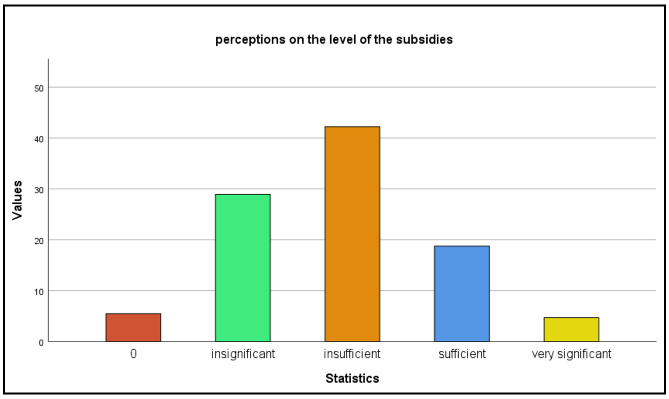

Figure 10*. Farmers' perceptions on state subsidies. (Source: authors' own computations)*

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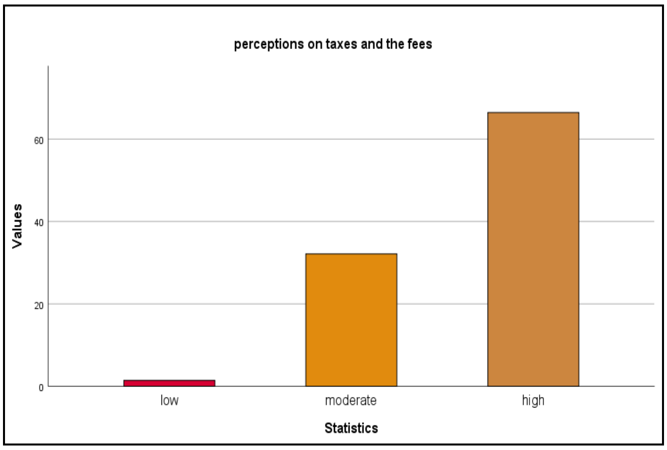

Figure 11. *Farmers' perceptions on taxation. (Source: authors' own computations)*

Conclusions

Farmers' willingness to associate and join in environmental responsible short supply chain in Romania is a complex and challenging research topic in Romanian agricultural economics, taking into consideration the impact of association on shaping agricultural production in modern agriculture. During the research it has been found that cooperation plays a significant role in shortened food chains and that association goes beyond simple farmers' cooperation to multi-stakeholder cooperatives. They become leading and powerful actors in food chains, since they generate a diverse range of benefits, besides economic ones: sectorial, legal, cultural, policy, academic.

The hi2 test and the contingent coefficients are used to analyze the correlations between variables. Findings indicate that the probability of farmers' membership in an associative form or in a short supply chain environmental responsible is determined by age, education level, cultivated area, activity type, year of establishment, opportunities to access financial support.

A final answer to the research question is that farmers want to involve their businesses into collective actions, since 67.1% of the farmers answered that they want to join a short supply chain, and 82.1% of them answered that they want to join an associative form of organization, in the near future. This result is opposite to those found in other research showing that 62% of subjects do not intent to associate in the future (Marin et al., 2016: p. 19).

But the association is poorly developed in Romania's agriculture, namely in the sector of vegetables and fruits, as long as only 18.6% of farmers declared that are part of an associative form of organization. Moreover, statistics show that only 1% of the Romanian agricultural producers are part of an associative form of organization.

The determinants of the farmers' affiliation to an associative form are: the type of activity, the size of the holding, the estimated revenues, access to state subsidies and farmers' occupation and education. The affiliation of farmers to a short supply chain is determined by the type of activity, the size of the holding and the level of education. As such, the hypothesis H1 is confirmed in the sense that socio-economic factors influence the farmers' willingness to join an associative form or a short supply chain with a weak to medium intensity. These results are similar to those found in the literature by Adrian et al. (2005), Marin et al. (2016), Marin et al. (2017) and Mojo et al. (2017).

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The hypothesis "Membership in a collective form of organization and a short supply chain has a positive impact on farm income and assets" (H2) is partly confirmed. The fact that farmers are part of an associative form influences their income, but not that they are part of a short supply chain.

The assumption that farmers are resistant to join a short supply chain or an associative form (H3) is not confirmed, since 82.1% of respondents want to join an associative and 67.1% of respondents want to integrate their business into a short supply chain.

The assumption that farmers' reasons for not joining short supply chains and associative forms of organizations are linked to the lack of trust in other members, the lack of legislation and to bureaucracy is confirmed (H4), and consistent to other findings (Marin, 2017).

Regarding the reasons for joining the short supply chains environmental responsible and associative forms of organizations, the determinants of the farmers' willingness to associate are: the year of starting the activity, the type of activity, the size of the holding, the access to subsidies and the level of education. The farmers' willingness to join a short supply chain is determined by the year of starting the activity and the opportunity to access European funds, with intensity from weak to moderate.

Most of the farmers considered the short supply chain functional and the association as positive in shortening the chain. Over 70% of the farmers who answered the question related to the perceptions on the level of the state subsidies considered that the support provided by the state is insignificant and insufficient.

To conclude, the profile of farmers who want to associate and/or to integrate the activity into a short supply chain can be drawn. The farmers willing to associate are young, under 40 years old, with higher education, legally organized as physical persons, exploiting smaller agricultural areas. They recently started their activity and they never accessed subsidies before. The farmers willing to join a short supply chain are organized as physical persons. They have higher education and exploit smaller agricultural areas. They recently started their activity and intend to access European funds for developing their businesses. The results may be used to develop policy initiatives to finance collective actions in food supply chains environmental responsible, because they provide insights into the socio-economic factors that influence the farmers' membership in short supply chains and associative forms of organization. The current study contributes to the literature by analyzing the determinants of association and short supply chain membership using the survey data of farms gathered from vegetables and fruits producers in Romania.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16171639

THE USE OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)'S EFFECT ON ESSENTIAL OIL RATE, ESSENTIAL OIL CONTENT, SOME MORPHOLOGICAL PARAMETERS AND NUTRIENT UPTAKE OF TURKISH OREGANO

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(Received 16th Oct 2018; accepted 2nd Jan 2019)

Abstract. The objective of this study was to evaluate the effects of mineral fertilizer (NP) and thirty-five N_2-fixing and/or P-solubilizing and different carbon sources utilizing-bacterial strains (7 isolates each of *Bacillıs megaterium, Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas putida* and *Pseudomonas fluorescens*) isolated from the acidic rhizospheric soil of native tea, grapevine and wild red raspberries, as bio-fertilizers on growth, yield and quality characteristics of the plant, on chlorophyll content, on macro- and micronutrient concentrations, on essential oil content and on major component of the essential of Turkish oregano (*Origanum onites* L.). The isolates were identified based on whole-cell fatty acid methyl ester (FAMEs) analysis using the MIDI system and BIOLOG assays. The study contains both applications NP-fertilizer and a control application without inoculation of bacteria and fertilizer application. This study was carried out in Greenhouse at Atatürk University, Faculty of Agriculture and Department of Agronomy in 2011-2012 growing season. As an average of years the treatments showed that the plant has 35.3-48.5 cm of length, 34.29-48.30 cm of canopy diameter, 36.56-47.19 SPAD units of chlorophyll concentration, 41.88-74.64 g/plant of green herb yield, 11.52-15.52 g/plant of drug herb yield, 6.53-12.18 g/plant of drug leaf yield and 1.85-2.78% of essential oil content rate. Increasing carbon source utilization rates significantly increased plant height, canopy diameter, green herb, drug herb and drug leaf yield. The main components of the oil were carvacrol (58.19-70.08%), followed by thymol (17.85-26.27%), linalool (1.64-8.13%), borneol (1.36-2.39%) and p-cymene (0.37-3.21%) which were the most abundant components. In addition, with PGPR inoculation, macronutrient concentrations (N, P and K,) of oregano leaves increased, main component of the essential oil also changed. In terms of growth, yield and quality criteria of Turkish oregano, in particular, the most effective N_2-fixing and P-solubilizing strains *P. fluorescens* (8/2, 58/3, 9/7, 53/6 and 51/2), *B. subtilis* (52/1, 6/3 and R 3/3), *B. megaterium* (21/3, K5E and 35/6), *P. polymyxa* (R2/2), and *P. putida* (55/2, 3/10 and 53/5) may be used instead of mineral fertilizer as bio-fertilizers in sustainable organic oregano cultivation.
Keywords: *medicinal and aromatic plants, Origanum onites, carvacrol, main component, nutrient uptake*

Introduction

Turkish oregano (*Origanum onites* L.), distributed in Western and Southern Anatolia is widely used as a spice and herbal tea in Turkey. In Turkey, *O. onites* is on the top of the list in case of its commercial *origanum* species which has a dominant position in the worldwide trade (Başer, 2002). Dried oregano is obtained both from wild populations in nature and from cultivated plants, its vegetative parts and biochemical essence are quite used in the food and spice industrial area, and also as a condiment herb for flavouring fish, soups, salads, olives, chicken, meat, vegetables, salad dressing, and wine (Tonk et al., 2010). As food preservation, natural antimicrobial and antioxidant, *O. onites* becoming increasingly popular (Stefanakis et al., 2013). Carvacrol, thymol, p-cymene, gamma-terpinene, borneol, linalool, and alpha-terpinene is main component of essential oil of *O. onites* (Bokov et al., 2015). Carvacrol is main component of essential oils of this species (Avcı and Bayram, 2013), which is an oxygenated monoterpene with multiple pharmacological actions (Baser, 2008), and an important impact compound of oregano aroma (Bansleben et al., 2009). It is also known for its antibacterial, antifungal, antioxidant, insecticidal, anti-angiogenic and anticarcinogenic activities, antispasmodic effects, lipid peroxidase inhibition, radical scavenging effect and cardiac depressant activity (Kotan et al., 2014).

In recent years, oregano cultivation has expanded rapidly because of its conservation of natural resources and production with high standard and quality. Consumed without further processing in medicinal and aromatic plant species is important because its components have not synthetic compounds in the harvested crop. Undoubtedly, excessive use of chemical fertilizer has adverse effects on plant and soil health. In view of environmental pollution and high costs of the production due to excessive use of fertilizers; plant growth- promoting rhizobacteria (PGPR) may be used in sustainable agricultural production (Cakmakçi et al., 2006). Plant growth with excrete of vitamins and phytohormones, decline of plant ethylene level, resistance to stress, contribution to food intake, solubilisation of inorganic phosphate, fixing N_2, and mineralization of organic phosphate can provide with applications of PGPR. As a new concept and alternative strategy emerging in plant growth and yield increase, PGPR can provide increase in agricultural productivity, decline in product cost and environmental protection against pollution. It is evident that rhizobacteria could possibly serve as eco-friendly, safe and sustainable alternative to the harmful synthetic fertilizers used for the nutrient management and sustainable production in medicinal and aromatic crops.

Knowledge of about the monoterpene accumulation and biosynthesis of secondary metabolites can provide new procedure for medical plant cultivation and other agricultural application without chemical input (Cappellari et al., 2015). Similarly to bio-fertilization, optimal and balanced mineral fertilization of aromatic plants, adjusted to their nutritional requirements and growing conditions, is an important cultivation factor determining the quantity and quality of essential oil (Nurzynska-Wierdak, 2013). Bacteria and plant related studies are usually focused on cereals and grassy plants and studies on medical plants are very few. In medicinal and aromatic plants, experiments with PGPR indicated yield and essential oil increases in Italian oregano and *Origanum majorana* L. (Banchio et al., 2008, 2010), rosemary (Leithy et al., 2006), *Pelargonium graveolens* (Mishra et al., 2010), sweet fennel (Rezvani Moghaddam et al., 2011), dill plant (Hellal et al., 2011), common basil (Ordookhani et al., 2011), coriander (Hassan et al., 2012), Mexican marigold (Cappellari et al., 2013), *Thymus daenensis* (Bahadori et al., 2013), summer savoury (Farahani et al., 2015). Although some experiments have addressed the role of PGPR added in medicinal plants (Santoro et al., 2011), the impact of PGPR on production of secondary metabolites is poorly known (Banchio et al., 2008). In general, physiological and morphological property of medicinal plants connected with rhizobacteria leftovers restricted and piecemeal (Cappellari et al., 2015).

In particular, the possibilities of medicinal and aromatic plants development, yield, volatile oil content and components to be increased or replaced have not been extensively investigated using multi-traits bacteria. However, there is no study on

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

agronomic factors such as application of bio-fertilizers as well as plant growth promoting rhizobacteria on biological soil properties, yield and essential oils of Oregano. In addition, there is a trend towards biological fertilizer applications and sustainable agricultural systems in the production of medical plants due to environmental impacts, which are caused by over application of chemical fertilizers, energies and expenses. Therefore, a study was conducted in order to investigate the effect of plant-associated beneficial multi-traits 35 bacteria on growth, yield and content and composition of essential oils of Turkish oregano.

Materials and methods

Bacterial strains

In this study, thirty-five different isolates of PGPR were selected from stock of 460 rizobacterial isolates obtained from the native grapevine, wild red raspberries and tea rhizosphere Selection criteria of these bacteria was made according to whether 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing, IAA-producing, N_2-fixing, P-solubilizing, and different carbon sources using ability. In the study, gram-positive *Bacillus megaterium*, *Bacillus subtilis* and *Paenibacillus polymyxa*, gram-negative *P. fluorescens* and *P. putida* species were used for at least seven different isolates from each species to be used in *Origanum onites*. The isolates were identified based on whole-cell fatty acid methyl ester (FAMEs) analysis using the Sherlock Microbial Identification System (Version 4.5) and Biolog microplate assays (Biolog Inc., Hayward, CA, USA). Also, characterized by using BIOLOG GN2 and GP2 MicroPlates, were used to determine the ability of bacterial strains to utilize 95 different carbon sources (Çakmakçı et al., 2010).

Acetylene reduction assay and phosphate solubilisation

Nitrogen fixation of the isolates was determined in a nitrogen free medium by the acetylene reduction assay (Hardy et al., 1968). Using a Hewlett Packard gas chromatograph, Ethylene production was measured (Model 6890, USA). Entire of the bacteria were tried in threefold for their phosphate solvent talent in sucrose-tricalcium phosphate agar media (Pikovskaya, 1948) by added 1 ml of 6-day-old culture (density 4×10^9) in 250-ml Erlenmeyer flasks containing 500-μg ml^{-1} of P as rock phosphate at 29-31°C (Çakmakçı et al., 2010).

Greenhouse experiment and growth conditions

This study was carried out in the Department of Agronomy at Atatürk University under greenhouse conditions in Erzurum, Turkey, in 2011-2012 growing season under two trial sets. Flowerpot were sterilized by 20% NaClO and replete with a sandy clay-loam which is virgin field soil with an organic matter content of 3.2 and 0.17% nitrogen, an available Olsen-P and changeable potassium calcium and magnesium content of 16.2, 448, 3420, and 472 mg kg^{-1}, respectively. Iron, manganese, zinc and copper contents were 5.9, 9.6, 1.2, and 1.8 ppm. Two set of experiment was conducted with the same treatments. The experiment was conducted in a completely randomized design with five replicates (each having five pots), having 37 treatments as 35 high N_2-fixing and/or P-solubilizing and different carbon sources utilizing-bacteria, NP fertilizer (200 mg N $plant^{-1}$ + 200 mg P $plant^{-1}$) applications as well as a control

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

treatment without inoculation and any fertilizer application. In our study, bacteria were developed in fifty percent tryptic soy broth. Bacteria in Rotary shaker was stayed three days (120 rpm; 25°C). Control applications were stayed 5 ml of diluted SPB without bacteria. Oregano seeds were germinated in a seed trays containing garden soil/peat/sandy (2:1:1 [v/v]). Our plants were grown conditions which 16 h day, and 8 h night conditions, at 19-29°C with about sixty percent humidity in greenhouse. Seedlings were removed from seed trays after one month. Uniform oregano seedlings were dipped and kept in bacteria solution for an hour, later three uniform 30-day-old inoculated seedlings were transferred into each pots containing virgin garden soil, and seedlings were thinned to one after two weeks. In all plants, irrigation was done every 4 days, keeping 70% water holding capacity. Weed control was done by hand when required. In the second year of bacterial inoculation, 5 ml bacterial suspension was injected into rhizosphere of each oregano plant. About three months after planting, the oregano plants were harvested twice approximately in mid-July and September in each year.

Essential oil extraction and GC–MS analysis

Plants were harvested at full flowering stage, were cut 5-6 cm above ground level and weighed to determine fresh herbage yield. Then, harvested the oregano herb was dried under natural conditions, in a dry, airy and shaded place. Essential oil of plants was obtained with using Clevenger apparatus. Dry herb was made hidrodistilation for 3 h v/w and essential oil of these plant was obtained. The essential oil' analyses was performed (Kordali et al., 2008). Oil components and RI (relative retention times) values detected according to Adams (1997).

Plant analysis

Before 2 g of leaf samples was grinded to be 1 mm, oven-dried at 68°C for 48 h. For determine total N the oregano leaves were used Kjeldahl method and a Vapodest 10 Rapid Kjeldahl Distillation Unit (Gerhardt, Konigswinter, Germany). After determined P and K with an inductively Coupled Plasma spectrophotometer (Perkin-Elmer, Optima 2100 DV, ICP/OES, Perkin-Elmer, Waltham MA, USA), extraction was made. For measuring chlorophyll contents of leaves which top fourth and fifth was used chlorophyll meter (SPAD-502, Minolta, Japan) which is used to measure leaf greenness of the plants. Firstly, it was measured at four locations on each leaf for each plant. After, this numbers were received for average.

Statistical analysis

The experiment was performed in a completely randomized design with five replicates. Each replicate consisted of five plants. The experiment was repeated twice. Our data were analyzed by SPSS 20 and the means were separated according to Duncan's Multiple Range Test.

Results

Plant growth parameters

Different rhizobacteria had variable effects (both negative and positive) on the measured plant growth parameters, the essential oil content and yield of *Origanum onites* (*Table 1*). In terms of fresh herbage yield in oregano, all application except *B.*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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megaterium 44, *B. megaterium* R2C, *B. subtilis* 36/10, *P. putida* 48/2, *P. fluorescens* 22B and *P. fluorescens* 8/6 were detected to be higher according to control. The maximum fresh herbage yield in oregano was obtained with the mineral NP fertilizer application, followed by *P. fluorescens* 8/2 inoculation. The maximum dry herbage yield in oregano was found after *P. polymyxa* R2/2 and *P. fluorescens* 58/3 inoculation, followed by *B. megaterium* 21/3, *B. subtilis* 52/1, and *P. fluorescens* 8/2, whereas the highest levels of dry leaf yield per plants were determined in treatments with *P. fluorescens* 58/3, followed by *B. subtilis* 52/1, *B. subtilis* 6/3, and *P. polymyxa* R2/2 (*Table 1*).

Table 1. *The effect of mineral fertilizer application and bacterial inoculations on plant growth parameter in Turkish oregano*

Treatments	Fresh herb yield (g/plant)*	Dry herb yield (g/plant)	Dry leaf yield (g/plant)	Plant height (cm)	Canopy diameter (cm)	Chlorophyll content (SPAD)	Essential oil yield (%)
Control	48.3 n-f	13.8 n-p	7.54 h-j	36.85 lm	34.92 op	37.38 kl	1.94 l-o
Mineral fertilizer (NP)	70.3 a	17.7 b-d	10.81 a-c	43.40 c-h	46.01 a	44.24 a-f	2.64 a-b
Bacillus megaterium K5E	59.4 g-k	16.9 c-g	9.78 e-h	39.90 h-l	38.62 ı-n	40.77 c-j	2.58 a-c
Bacillus megaterium 22D	52.1 m-o	16.0 f-ı	9.20 h-j	36.90 lm	38.38 ı-o	40.31 e-l	2.55 b-d
Bacillus megaterium 66/5	42.7 s	12.5 p-s	7.10 p-s	36.80 m	34.83 n-p	37.16 l	2.33e-h
Bacillus megaterium 21/3	65.7 b-d	18.5 ab	10.63 b-d	48.03 a	38.13 j-p	43.44 a-g	2.30 f-ı
Bacillus megaterium 44	46.0 p-s	14.5 j-n	8.27 l-n	38.20 ı-m	34.78 op	42.66 a-ı	1.86 o
Bacillus megaterium 35/6	66.8 b-c	17.1 c-f	9.81 e-h	40.57 g-l	44.00 a-f	44.42 a-e	2.10 ı-n
Bacillus megaterium R2C	43.0 s	12.0 rs	6.53 s	36.71 m	34.88 p	39.09 h-l	1.85 o
Bacillus subtilis R 3/3	62.6 d-g	17.7 a-d	10.07 d-g	40.38 g-l	39.89 g-k	45.02 a-c	1.98 l-o
Bacillus subtilis 2/8	48.9 n-p	12.9 o-r	7.07 p-s	41.05 f-k	36.96 k-p	37.18 kl	1.86 o
Bacillus subtilis 39/3	52.5 k-m	17.1 c-f	10.14 d-f	41.95 d-j	44.70 a-d	43.54 a-g	2.27 g-j
Bacillus subtilis 52/1	64.3 c-f	18.3 ab	11.23 ab	42.08 d-ı	45.95 ab	45.41 ab	2.71 a-b
Bacillus subtilis 6/3	61.9 d-h	17.8 a-d	11.22 ab	41.97 d-j	44.54 a-e	43.84 a-g	2.78 a
Bacillus subtilis 36/10	42.5 s	12.5 p-s	6.93 rs	36.85lm	34.79 op	38.83 ı-l	2.36 d-h
Bacillus subtilis 20D	57.9 h-l	15.9 f-ı	9.38 g-ı	43.28 c-h	38.46 ı-o	42.74 a-ı	2.53 b-e
Paenibacillus polymyxa R2/2	64.7 c-f	18.6 a	11.18 ab	45.28a-e	42.77 a-h	44.95 a-c	2.27 g-j
Paenibacillus polymyxa 2/.5	55.0 k-m	17.1 c-f	9.78 e-h	41.22 f-k	41.90 c-j	42.83 a-ı	2.01 l-o
Paenibacillus polymyxa 11/4	53.8 l-n	15.7 g-j	8.99 ı-k	41.80 d-j	40.01 g-k	44.65 a-d	1.93 m-o
Paenibacillus polymyxa 43/5	56.5 j-m	15.9 g-ı	9.44 g-ı	43.25 c-h	41.37 d-j	40.26 e-l	2.17g-l
Paenibacillus polymyxa 24/3	56.0 k-m	13.6 n-p	7.69 n-p	37.87 j-m	40.75 e-k	43.88 a-g	2.06 j-o
Paenibacillus polymyxa 31/5	56.1 k-m	16.5 e-h	9.54 f-ı	41.10 f-k	42.25 b-ı	41.77 b-ı	2.14 h-m
Paenibacillus polymyxa 56/4	57.6 ı-l	15.0 ı-l	8.48 k-m	40.31 g-l	40.50 f-k	40.92 c-j	2.09 ı-n
Pseudomonas putida 20c	55.0 k-m	15.3 h-k	8.50 k-m	41.46 e-k	40.41 f-k	40.02 f-l	1.92 n-o
Pseudomonas putida 3/10	61.2 e-ı	15.7 g-j	9.38 g-ı	44.77 a-f	46.01 a	42.68 a-ı	2.26 g-j
Pseudomonas putida 27/3	54.7 l-m	15.0 ı-l	8.26 l-n	45.90 a-d	39.54 g-l	42.56 a-ı	2.04 k-o
Pseudomonas putida 48/2	44.8 r-s	14.1 l-n	7.84 m-o	38.39 ı-m	36.00 l-p	40.23 e-l	2.24 g-k
Pseudomonas putida 53/5	52.4 m-o	14.2 k-n	8.52 j-l	41.30 e-k	42.74 a-h	43.42 a-g	2.58 a-c
Pseudomonas putida 55/2	60.7 f-j	17.4 b-e	10.54 c-e	44.70 b-g	43.13 a-g	43.93 a-g	2.59a-c
Pseudomonas putida 62/5	52.5 mn	14.9 ı-m	8.36 k-m	37.65k-m	38.45 ı-o	43.13 a-h	2.25 g-k
Pseudomonas fluorescens 22B	46.1 pr	11.9 s	6.57 s	39.06ı-m	35.14 m-o	39.76 g-l	2.17 g-l
Pseudomonas fluorescens 8/2	69.8 ab	18.0 a-c	10.85 a-c	47.61 ab	45.94 a-c	46.42 a	2.38c-g
Pseudomonas fluorescens 8/6	48.1 o-r	14.3 k-n	8.59 j-l	36.74 m	35.86 l-p	42.65 a-ı	2.29 f-ı
Pseudomonas fluorescens 9/7	57.0 ı-l	15.7 g-j	9.58 f-ı	39.87 h-l	40.41 f-k	43.44 a-g	2.52 b-e
Pseudomonas fluorescens 53/6	59.7 g-k	16.6 d-g	10.30 c-e	46.87 a-c	41.26 d-j	43.12 a-h	2.62 ab
Pseudomonas fluorescens 51/2	56.1 k-m	15.8 g-ı	9.43 g-ı	45.83 a-d	38.97 h-m	40.65 d-k	2.50 b-f
Pseudomonas fluorescens 58/3	65. 0 c-f	18.6 a	11.25 a	43.62 c-h	40.92 d-k	44.36 a-e	2.59 a-c
Mean	**55.9**	**15.7**	**9.15**	**41.34**	**40.11**	**42.20**	**2.27**

*Values followed by different lower-case letters in a column were significantly different (P ≤0.05) using Duncan's multiple range test; Values are the averages from the two experiments with five replications

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

In terms of dry herbage yield, 31 applications were found to be effective according to control. In terms of plant height, 5 applications were detected as taller according to mineral fertilizer. The maximum plant height in oregano was found after *B. megaterium* 21/3 (48.3 cm) inoculation, followed by *P. fluorescens* 8/2, whereas the highest levels of canopy diameter per plants were measured in treatments with mineral NP fertilizer and *P. putida* 3/10 (46.01 cm), followed by *B. subtilis* 52/1 and *P. fluorescens* 8/2. In terms of chlorophyll content (SPAD value), applications except *B. megaterium* 66/5, *B. megaterium* R2C and *B. subtilis* 36/10 were found as effective according to control. The maximum chlorophyll content measured as *P. fluorescens* 8/2 (*Table 1*). Control plants gave the lowest water content of air drying (71.4%) while NP application gave the highest water content (74.8%), bacterial inoculations having values generally higher than control but lower than NP applied plants.

Oil yield, content and chemoarray

According to two years results, in terms of essential oil yield, only 4 applications were found as less than control. All of *P. fluorescens* and *P. putida* applications were increased yield of essential oil when compared to control. The maximum essential oil yield in oregano was found as *B. subtilis* 6/3 inoculation, followed by *B. subtilis* 52/1, *P. fluorescens* 53/6, and mineral fertilizer (NP). Essential oil yields ranged from 1.85 to 2.78%. The main components of the oil were carvacrol (58.19-70.08%), followed by thymol (17.85-26.27%), linalool (1.64-8.13%), borneol (1.38-2.39%), and p-cymene (0.37-3.21%). Highest p-cymene (3.21%) in oil was determined in control application. Other components were changed with bacteria inoculations. The maximum linalool content of oregano oil was obtained with the inoculation of *P. polymyxa* 2/5 (8.13%), followed *P. polymyxa* 11/4 and *P. polymyxa* R2/2 (*Table 2*). Of the 37 treatments, the maximum borneol and thymol components of oregano oil were seen in *P. fluorescens* 51/2, *P. fluorescens* 9/7, *P. fluorescens* 53/6 and *P. fluorescens* 58/3 inoculations. Borneol and thymol components in all four applications were detected respectively as % 2.39 and 26.27. The maximum carvacrol content essential oil in oregano was obtained with the *P. putida* 20C and *P. putida* 3/10 inoculations, followed by *P. putida* 27/3 and *B. subtilis* 52/1 (*Table 2*).

Nutrient uptake

All applications were found to be higher according to control in leaf nitrogen content. Twenty-four, ten and eleven of the 35 PGPR strains test selectively increased nitrogen, phosphorus and potassium content leaf, and most of them improved the plant growth parameters and yield in oregano plants. In terms of leaf phosphorus and potassium content, all applications except *B. magaterium* 22D inoculation was detected more effective than control. While the highest leaf nitrogen contents were determined on *P. putida* 3/10 inoculated oregano leaves, followed by *P. putida* 53/5, 55/2 and 48/2; *P. polymyxa* 43/5 and *P. polymyxa* R2/2 inoculations (*Table 3*). The leaf nitrogen content of inoculated *P. putida* 3/10 was higher (16.3%) than mineral fertilization. In terms of leaf phosphorus content, *P. polymyxa* 43/5, *P. polymyxa* 31/5, *P. polymyxa* R2/2, *B. subtilis* 52/1 and *P. polymyxa* 24/3 applications were found effective. As in terms of leaf phosphorus content, *P. polymyxa* 43/5, *P. polymyxa* 31/5, *P. polymyxa* R2/2, *B. subtilis* 52/1 and *P. polymyxa* 24/3 applications were found effective, in terms of leaf potassium content, *B. subtilis* 6/3, *B. subtilis* 52/1 and *P. polymyxa* R2/2 applications were detected as effective (*Table 3*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

Table 2. *The effect of mineral fertilizer application and bacterial inoculations on basic essential oil components in Turkish oregano*

Treatments	p-cymene* (%)	Linalool (%)	Borneol (%)	Thymol (%)	Carvacrol (%)
RI[a]	1020	1095	1165	1289	1298
RT	14:66	18:32	22:05	27:75	29:39
Control	3.21 a	6.63 b	1.42 f	18.63 f	61.01h-j
Mineral fertilizer (NP)	1.21 bc	2.43 h-j	1.38 f	20.98 b-f	66.96 a-f
Bacillus megaterium K5E	0.54 ef	4.55 d-e	2.29 a-d	25.56 a	61.27 h-j
Bacillus megaterium 22D	0.67 ef	3.11 f-i	2.14 a-e	24.10 ab	63.86 e-i
Bacillus megaterium 66/5	0.39 f	3.26 f-h	2.34 ab	24.91 ab	63.37 f-i
Bacillus megaterium 21/3	0.73 c-f	5.12 cd	2.33 a-c	24.84 ab	60.68 ij
Bacillus megaterium 44	0.82 b-f	4.70 c-e	2.28 a-d	23.17 a-c	62.47 g-j
Bacillus megaterium 35/6	0.75 c-f	5.12 cd	2.33 a-c	24.68 ab	60.68 ij
Bacillus megaterium R2C	0.73 c-f	5.79 bc	2.22 a-e	24.43 ab	60.32 ij
Bacillus subtilis R 3/3	0.75 c-f	2.30 h-j	2.30 a-d	21.26 b-f	66.57 a-g
Bacillus subtilis 2/8	0.75 c-f	2.10 h-j	2.30 a-d	21.26 b-f	66.97 a-f
Bacillus subtilis 39/3	1.03 b-e	2.41 h-j	1.97 de	20.92 b-f	66.71 a-g
Bacillus subtilis 52/1	0.39 f	1.64 j	2.01 b-e	21.39 b-f	68.87 a-c
Bacillus subtilis 6/3	0.37 f	2.10 hj	1.88 e	21.09 b-f	68.47 a-d
Bacillus subtilis 36/10	0.49 f	1.92 i-j	2.01 b-e	21.13 b-f	68.63 a-d
Bacillus subtilis 20D	0.67 ef	2.10 hj	1.88 e	21.03 b-f	68.22 a-e
Paenibacillus polymyxa R2/2	0.47 f	7.69 a	2.00 b-e	23.33 ab	61.38 h-j
Paenibacillus polymyxa 2/5	0.43 f	8.13 a	1.98 c-e	23.93 ab	60.50 ij
Paenibacillus polymyxa 11/4	0.47 f	8.12 a	1.98 c-e	23.33 ab	60.98 h-j
Paenibacillus polymyxa 43/5	0.64 ef	4.28 d-f	1.98 c-e	23.38 ab	64.22 d-i
Paenibacillus polymyxa 24/3	0.83 b-f	3.21 f-h	1.91 e	22.67 a-e	65.29 b-h
Paenibacillus polymyxa 31/5	0.66 ef	4.63 de	2.02 b-e	24.00 ab	62.98 f-i
Paenibacillus polymyxa 56/4	0.64 ef	4.26 d-f	1.98 c-e	23.14 a-d	64.42 c-i
Pseudomonas putida 20c	0.60ef	2.61 g-j	2.05 a-e	18.59 f	70.09 a
Pseudomonas putida 3/10	0.60ef	2.41 h-j	2.05 a-e	18.89 ef	70.08 a
Pseudomonas putida 27/3	0.69 d-f	2.65 g-j	1.92 e	19.35 c-f	69.24 ab
Pseudomonas putida 48/2	1.19 b-d	2.38 h-j	2.29 a-d	18.41 f	68.40 a-e
Pseudomonas putida 53/5	1.19 b-d	2.38 h-j	2.29 a-d	18.35 f	68.50 a-d
Pseudomonas putida 55/2	1.19 b-d	2.18 h-j	2.29 a-d	18.35 f	68.70 a-d
Pseudomonas putida 62/5	1.03 b-e	2.16 h-j	2.21 a-e	19.23 d-f	68.39 a-e
Pseudomonas fluorescens 22B	1.26 b	3.63 e-h	2.15 a-e	17.85 f	68.05 a-e
Pseudomonas fluorescens 8/2	1.26 b	3.63 e-h	2.15 a-e	17.85 f	68.05 a-e
Pseudomonas fluorescens 8/6	1.26 b	3.63 e-h	2.15 a-e	17.85 f	68.05 a-e
Pseudomonas fluorescens 9/7	1.03 b-e	4.52 de	2.39 a	26.27 a	58.19 j
Pseudomonas fluorescens 53/6	1.03 b-e	4.53 de	2.39 a	26.26 a	58.20 j
Pseudomonas fluorescens 51/2	1.02 b-e	4.51 de	2.40 a	26.28 a	58.19 j
Pseudomonas fluorescens 58/3	1.03 b-e	4.52 de	2.39 a	26.27 a	58.19 j
Mean	**0.86**	**3.80**	**2.10**	**21.85**	**64.92**

*Values followed by different lower-case letters in a column were significantly different ($P \leq 0.01$) using Duncan's multiple range test. [a]Retention index relative to *n*-alkanes on SGE-BPX5 capillary column; GC: identification based on retention times of authentic compounds on SGE-BPX5 capillary column; MS, RI: tentatively identified based on computer matching of the mass spectra of peaks with Wiley 7N and TRLIB libraries and published data, and comparison of retention index of the compounds compared with published data (Adams, 2007)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

Table 3. *Effect of chemical fertilizer application and bacterial inoculations for nitrogen, phosphorus and potassium on contents leaf of Turkish oregano*

Treatments	N (%)*	P (g/kg)	K (g/kg)
Control	1.63 g	2.10 g	30.3 f
Mineral fertilizer (NP)	3.47 a-c	2.73 a-f	37.5 a-e
Bacillus megaterium K5E	1.85 e-g	2.08 g	30.3 f
Bacillus megaterium 22D	3.21 a-e	2.54 b-g	35.1 c-f
Bacillus megaterium 66/5	1.77 fg	2.08 g	37.8 e-f
Bacillus megaterium 21/3	2.46 b-g	2.41 d-g	33.8 c-f
Bacillus megaterium 44	2.10 d-g	2.27 fg	32.9 c-f
Bacillus megaterium 35/6	3.29 a-d	2.61 b-g	36.5 a-f
Bacillus megaterium R2C	1.77 fg	2.08 g	30.8 e-f
Bacillus subtilis R 3/3	3.29 a-d	2.78 a-f	39.2 a-d
Bacillus subtilis 2/8	2.10 d-g	2.36 e-g	35.1 c-f
Bacillus subtilis 39/3	3.01 a-f	2.65 b-g	36.1 a-f
Bacillus subtilis 52/1	3.41 a-d	2.98 a-d	42.3 ab
Bacillus subtilis 6/3	3.49 a-c	2.96 a-e	42.8 a
Bacillus subtilis 36/10	2.30 c-g	2.53 b-g	38.5 a-d
Bacillus subtilis 20D	2.80 c-g	2.61 b-g	36.9 a-f
Paenibacillus polymyxa R2/2	3.59 a-c	3.02 a-c	39.9 a-c
Paenibacillus polymyxa 2/.5	3.33 a-d	2.83 a-f	37.5 a-e
Paenibacillus polymyxa 11/4	3.33 a-d	2.83 a-f	37.5 a-e
Paenibacillus polymyxa 43/5	3.73 ab	3.27 a	38.5 a-d
Paenibacillus polymyxa 24/3	3.55 a-c	2.97 a-d	35.9 b-f
Paenibacillus polymyxa 31/5	3.48 a-c	3.11 a-b	37.5 a-e
Paenibacillus polymyxa 56/4	2.97 a-f	2.66 b-g	35.6 c-f
Pseudomonas putida 20c	3.55 a-c	2.34 fg	34.2 c-f
Pseudomonas putida 3/10	4.07 a	2.58 b-g	36.5 a-f
Pseudomonas putida 27/3	3.45 a-d	2.27 fg	32.5 d-f
Pseudomonas putida 48/2	3.75 ab	2.50 c-g	37.1 a-e
Pseudomonas putida 53/5	3.93 a	2.63 b-g	36.9 a-f
Pseudomonas putida 55/2	3.93 a	2.74 a-f	38.03 a-d
Pseudomonas putida 62/5	3.31 a-d	2.30 fg	33.9 c-f
Pseudomonas fluorescens 22B	1.92 e-g	2.27 fg	33.7 c-f
Pseudomonas fluorescens 8/2	1.92 e-g	2.27 fg	33.7 c-f
Pseudomonas fluorescens 8/6	2.77 a-g	2.5 c-g	34.4 c-f
Pseudomonas fluorescens 9/7	3.43 a-d	2.52 c-g	35.4 c-f
Pseudomonas fluorescens 53/6	3.43 a-d	2.52 c-g	35.4 c-f
Pseudomonas fluorescens 51/2	3.43 a-d	2.52 c-g	35.4 c-f
Pseudomonas fluorescens 58/3	3.54 a-c	2.60 b-g	36.5 a-f
Mean	**3.04**	**2.57**	**36.00**

*Values followed by different lower-case letters in a column were significantly different ($P \leq 0.05$) using Duncan's multiple range test

Discussion

Inoculation with multi-traits bacteria increased fresh and dry herbage yield, dry leaf yield, plant height, canopy diameter, chlorophyll content (SPAD) and essential oil yield in oregano compared with the control. The responses to inoculation, compared to uninoculated control plants, were: -13.7% to +34.8% for dry herbage yield per plant, -13.4% to +49.2% for dry leaf yield, -0.4% to +30.3% for plant height, -0.6% to +24.2% for chlorophyll content, and -4.1% to +43.3% for essential oil yield. Plant growth responses were variable and dependent on the inoculant strain used, as well as on the growth parameter being evaluated. Increased plant dry weight and the oil content and biosynthesis of terpenes provided the increases in total essential oil yield by PGPRs inoculation. Using PGPR in the study was promoted the growth, yields and essential oil

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

content of oregano and chemical fertilizer application was equal or lower according to bacterial inoculations. Several authors have indicated that PGPR inoculation increased in shoot and root biomass, leaf area, and stomatal density, and marked qualitative and quantitative changes in monoterpene content in different medicinal and aromatic crops (Banchio et al., 2009; Banchio et al., 2010; Cappellari et al., 2013; Çakmakçı, 2016).

According to our data, aromatic structure, content and essential composition of oregano oil changed positively with multi-traits PGPR inoculation. In our study, major components of oregano oil have been carvacrol, thymol and borneol. Turkish oregano' essential oil was described as high content of phenolic compounds enclosed thymol and carvacrol (Bokov et al., 2015) and they have various biologic and pharmacological role (Dundar et al., 2008), antioxidant activity and they are important effect for component of oregano aroma (Bansleben et al., 2009). In addition, essential oil content and its composition are the most significant of quality criterion for oregano in all purposes (Baydar et al., 2004; Yaldiz et al., 2005; Baser, 2008; Bansleben et al., 2009; Ekren et al., 2013). Total essential oil yield, chemical composition and biosynthesis of major essential oil components of medicinal and aromatic plants were significantly affected by inoculation with PGPR (Banchio et al., 2008, 2009, 2010; Santoro et al., 2011, 2015; Prasad et al., 2012; Bahadori et al., 2013; Cappellari et al., 2013). However, there are not enough studies on the effect of inoculation with PGPR on plant growth or on production of secondary metabolites in important aromatic plants.

Effective multi-traits PGPR species, such as *P. polymyxa* R2/2, *P. putida* 3/10, *P. fluorescens* 8/2 and *P. fluorescens* 58/3 improved the N, P and K, nutrition in oregano, and therefore encouraged plant growth, essential oil quantity and quality. When particularly effective strains are used, it is possible that without using of chemical fertilizers to grow Turkish oregano organically without any loss in yield and quality. Beneficial role of these PGPR in growth, yield, oil content and compositions of oregano plants can have related to IAA-production, N_2-fixation, P-solubilisation, ACC deaminase activity utilization of variety and high rate of carbon sources and metabolize root exudates by the effective strains may be possible to afford a competitive advantage and play an important role in adapting to plants and soil. Previous studies suggested that carbon sources which were differentially utilized by the strains tested and many carbon sources were preferred by PGPR strain, which was one of the best bio fertilizers strains (Çakmakçı et al., 2010). Nutrients such as N, P, K, S, Ca, Mg and microelements can change of essential oil yield and composition according to reported similar studies (Nurzynska-Wierdak, 2013). In addition, for essential oils synthesized by plants, phosphorus is an important source. Therefore, PGPR can stimulate essential oil synthesis in medicinal plants when increased P uptake (Lichtenthaler, 2009). Moreover, according to researchers, leaves of PGPR inoculated plants are more contain to N, P and K than un-inoculated plants. Therefore, PGPR can provide increase in growth characters of medicinal plants (Saharan and Nehra, 2011). According to result of this study, versatile PGPR can be effective for improving growth and nutrient uptake of aromatic oregano crop. Also, more uptakes of nutrients that involved in chlorophyll formation can provide increasing for total chlorophyll due to beneficial effect of PGPR uptake of mineral elements in tea plants by application of PGPR was provide increased the chlorophyll content in leaves (Çakmakçı, 2016).

The better nutrient status were positively correlated with growth rates and yield of essential oils (Trivino and Johnson, 2000) and resulted in increased assimilation and translocation of photosynthates (Singh et al., 2016). While, N strongly affected not only

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

herb yield, but also its essential oil content and major oil constituents (Ozguven et al., 2006), an increase in the amount of phosphorous of the plants resulted in the enhanced accumulation of essential oil (Khalid, 2014) and affects the primary and secondary metabolites (Pal et al., 2016). While an adequate nutrient supply results in larger biomass production and consequently higher oil yield, changes in the essential oil composition may be related to better nutrition. On the other hand, mineral element composition of medicinal and aromatic plants has gained an interest and essential macronutrients may have nutritive, preventive and curative role in human.

Rate of carbon assimilation and photosynthetic activity can have provided by higher chlorophyll content in inoculated plants and for evaluation of plant photosynthetic efficiency, chlorophyll is an important parameter (Cappellari et al., 2015). For replace the use of chemical fertilizers, Microbial strategy is an attractive way for herbal plants, but little is known about their potential effect and ability of PGPR to increase plant secondary metabolites. At this point, limited knowledge is available in respect to effects of inoculation with PGPR in aromatic and medicinal plants (Banchio et al., 2008). For investigate the possible mechanisms by which bacteria increase phytochemical constituents in medicinal plants at the tissue, cell, or molecular level are need with more studies (Egamberdieva and da Silva, 2015).

Conclusion

PGPR have clear potential for improving the productivity of aromatic plants, and may significantly increase plant growth and reduce the amount of fertilizers required for economically sustainable crop production. Bacterial inoculants may be an efficient biotechnological tool for stimulating secondary metabolism in oregano plants, and studies of their activities will increase our understanding of processes that affect the accumulation of monoterpenes and phenolic compounds for a variety of applications in food and cosmetic industries, and poorly understood at present. In particular, *P. fluorescens* (8/2, 58/3, 9/7, 53/6 and 51/2), *B. subtilis* (52/1, 6/3 and R3/3), *B. megaterium* (21/3, K5E and 35/6), *P. polymyxa* (R2/2), and *P. putida* (55/2, 3/10 and 53/5) stimulated overall plant growth, including plant height, canopy diameter, fresh and dry herbage and leaf yield; improving macro- and micronutrient uptake, chlorophyll and essential oil content, and oil yield of oregano. Responses were variable and dependent on the inoculants strain and parameter evaluated. In addition, these bacteria can be used instead of mineral fertilizer as bio-fertilizer in sustainable organic oregano cultivation. Beneficial plant-associated bacteria exhibiting plant growth promoting traits can play a key role in supporting and/or enhancing plant growth, dry herb yield, and essential oil content, yield and its components. The action of PGPR on the essential oil and their components in medicinal aromatic plants remains a focus area for future research. Additional field trials are required to confirm the effects of PGPR strains on the growth characters, yield, essential oil and its constituents and nutrient contents, in this and so on plant species under different conditions. Future researches will also need to focus on elucidating the exact mechanisms by which PGPR have their growth-promoting effect and biosynthesis of secondary metabolites and monoterpene accumulation in medicinal and aromatic plants.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16411653

ARSENIC CONTAMINATED GROUNDWATER IN CHINA AND ITS TREATMENT OPTIONS, A REVIEW

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(Received 19th Oct 2018; accepted 2nd Jan 2019)

Abstract. A high concentration of arsenic in groundwater is a worldwide problem, high concentration of arsenic in groundwater has been documented as a major health issue around the globe. According to WHO standard, the maximum contamination level for total arsenic in water is 10 μg/L. China is one of the most affected country facing health issues because of arsenic contamination in groundwater that is often greater than established limits for human health. Although arsenic is present in several geographical regions in mainland China, Northern China has been identified as high-risk area. It has been estimated that 19.6 million people are at risk of being exposed to arsenic contaminated groundwater — especially rural areas, as those communities use groundwater for drinking water and household chores. To overcome the aforementioned problem, it is important to know the actual concentration level of arsenic in China and introduce a strategy to remove arsenic contamination. This paper provides a comprehensive overview on arsenic contamination status, sources and exposure pathways in China. It also aims to review the arsenic removal technologies which are easily available. Furthermore, it can be a useful resource for researchers as well as policy makers to identify and investigate useful treatment options.
Keywords: *water pollution, arsenic poisoning, human health, water treatment, remedy, China*

Introduction

The term water pollution usually refers to the introduction of physical, chemical, or biological materials in a body of water which affects its usage in the future. Water pollution has different types of contaminants, from which arsenic is a more poisonous and harmful one. Arsenic is the 12th most common element in nature and it usually appears in three allotropic forms including black, yellow and gray. If heated, it rapidly oxidizes to arsenic trioxide (As_2O_3) and has a garlic odor (Fendorf et al., 2010a). Arsenic is also known as the "King of Poisons". It is classified as carcinogen, mutagens and teratogen. Generally, in groundwater, natural occurrences of high arsenic levels were reported in aquifers — especially unconsolidated sediment aquifers throughout the world and have been connected to several adverse health effects. Arsenic content in water can harm the immune system, kidney, lungs and liver. In addition to this, it causes bladder cancers. Due to these major health risks, World Health Organization (WHO, 2004) has established a standard value (10 μg/L) which states that the maximum arsenic contaminant level in water for drinking water should not exceed 10 μg/L. Arsenic originates from different sources particularly geological surroundings such as a polluted environment, polluted bodies containing manganese oxides, irons, and arsenic-rich

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

minerals. There are extensive scientific research-based investigations in high arsenic level in groundwater all over the world (Sanjrani et al., 2017).

Arsenic contamination has been found in more than 70 countries but, the countries which are at the top of list for arsenic poisoning in groundwater are: India, Cambodia, Vietnam, Chile, Argentina, America and China. In other parts of the world, high concentration of arsenic has also been found in the bodies of water, including parts of the USA, Chile, Mexico, Argentina, Hungary, Japan, Canada, Poland, Ghana, Bangladesh, India, China, Afghanistan, Pakistan, Myanmar, Taiwan, are also reported to have arsenic contaminated water (WHO, 1999; Nickson et al., 2000; Niedzielski et al., 2001; Smedley and Kinniburgh, 2002; Guo and Wang, 2005; Marchiset-Ferlay et al., 2012; Ahmad et al., 2013; Horner and Beauchemin, 2013; Oguri et al., 2014; Qurat-ul-Ain et al., 2017; Sanjrani et al., 2018; Zheng, 2018). In 2004, World health organization (WHO, 2004) issued a list of countries with arsenic issues in water. The list is shown in *Table 1*.

Table 1. *List of countries affected by arsenic contamination*

Americas	United States of America, Alaska, Chile, Peru, Dominica, Honduras, Nicaragua, Argentina, El Salvador, Mexico
Africa	South Africa, Ghana, Zimbabwe
Asia	India, Bangladesh, Japan, China, Cambodia, Iran, Pakistan, Myanmar, Vietnam, Nepal, Thailand
Europe	United Kingdom, Austria, Finland, Romania, France, Greece, Italy, Hungary, Russia, Croatia, Serbia, Germany
Pacific	New Zealand, Australia

Among the countries that produce arsenic, China is the top producer of white-arsenic who produces almost 50% world-share, followed by other countries such as Peru, Chile and Morocco. Studies have identified some areas of high-risk for arsenic contamination belong to northern areas of China, including the Xinjiang province, the Shanxi province, the Inner-Mongolia province, the Henan province, the Shandong province and the Jiangsu province. All of the provinces contain naturally occurring arsenic with a concentration exceeding 10 μg/L (Nickson et al., 1998; Smith et al., 2000; Sun et al., 2003; Li, 2005; Xie et al., 2008; He and Charlet, 2013). In recent decades, it estimated that more than 19.6 million people are likely to be at risk of waterborne diseases. Moreover their lives are in danger due to arsenic contamination in groundwater (Rodríguez-Lado et al., 2013). There is collaboration between Switzerland and China, a special team is conducting research on ground-breaking studies, using geological maps and geological data to pin-point areas more likely to be put at high-risk due to the poison (SWI, 2012). The conducted study shows the increasing health-threat in China, including those areas which were not previously at risk. Each year, approximately 19.0 million Chinese people get sick because of polluted water from either industrial or agricultural activities, which cause approximately 60,000 premature deaths (Sun et al., 2011; IWR, 2011). There is a particular health threat in northern China because communities who belong to this area mostly depend on groundwater for their lives. China is a big country with more than 10 million drinking wells which are difficult to screen. It is expected that recent advanced research will help policy makers and authorities in China manage their well-screening programs as contaminated wells need to be treated. (Chan and Griffiths, 2010; WHO, 2011; CR, 2013). The situation needs be

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

quickly placed under control and there is an urgent need to arrange drinking water free of arsenic to communities who belongs to rural areas. In this regard, many different approaches may be applied for different bodies of water. Treatment options are available for arsenic contaminated water. These treatment options include pond water, surface water and harvesting rainwater. There are also other treatment possibilities such as to create a safe environment while also providing low-cost and small level fitted systems for removal of arsenic from groundwater for removal of arsenic from groundwater. In rural areas, it may not be feasible to install such a system for many families; however with the government supports rainwater harvesting could be possible option to acquire safe water for more households (Cheng et al., 2004; Sarkar et al., 2010).

Around the world, arsenic is being removed in many areas. With the introduction of many arsenic removal technologies, it is easy to treat the water. These technologies helps in arsenic remediation, including activated alumina, ion exchange, membrane filtration, reverse osmosis, modified coagulation-filtration, and also enhanced lime-softening. However, affected rural areas or developing areas need ideal and effective technology that should be versatile, low-cost, transferable and adoptable for both community application and household units (USEPA, 2000; Berg et al., 2006; Visoottiviseth and Ahmed, 2008).

While considering the serious issues of arsenic pollution in groundwater of several regions of China and it treatment technologies, this paper aims to review recent status of arsenic contamination in several regions of China which are highly affected exceeding WHO standard level 10 ug/L. This paper also aims to review about its sources and reasons for mobilization, exposure pathways as well as suitable technologies for arsenic removal which can be applied for treatment. This paper will prove to be a helpful and useful resource for readers and researchers or policy makers in regards to the identification and investigation for the better solutions.

Contamination level of arsenic and affected areas in China

The distribution of arsenic concentration drawn (as shown by contour lines) in groundwater of different provinces in China and is presented in *Figure 1*. Investigations have identified several basin areas around China where groundwater frequently contains >10μg/L arsenic, which exceeds the current drinking water standard established by the Chinese Environmental Protection Agency for Public Water Supply. Every province has different arsenic range; after reviewing several studies, a map has been drawn to this review paper to help one understand the situation of arsenic in every province clearly. In the map, the most affected regions with arsenic contaminated aquifers indicated by the color red. Arsenic pollution occurs in many provinces (Beijing, Guangdng, Gansu, Henan, Hunan, Heilongjiang, Inner Mongolia, Jilin, Jiangsu, Liaoning, Ningxia, Qinghai, Sichuan, Shandong, Shanxi, Taiwan, Xinjiang, Yunnan and Zhejiang) (*Table 2*). It is important to take note of the method in which arsenic contamination has been detected in water samples. Many different organizations (government and non-government groups) have employed both field-kits and in-laboratory research to identify arsenic contaminated waters. These studies have yielded accurate results. These findings are based on the data from (Tseng et al., 1968; Huang et al., 1985; Wang et al., 1993; Luo, 1993; Jin et al., 2003; Chen et al., 2003; Pang et al., 2003; Liu et al., 2003; Deng et al., 2004; Guo and Wang, 2005; Pei et al., 2005; Shen et al., 2005; Li et al., 2006; Luo et al., 2006; Yu et al., 2007; Yang et al., 2008; Guo et al., 2008; Xie et al.,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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2008; Han et al., 2009; Zhu et al., 2009; Liu et al., 2009; Qin and Xu, 2010; Huang et al., 2010; Li et al., 2010; Jiang et al., 2010; Hao and Xing, 2010; Bo and Luo, 2010; He et al., 2010; Shi et al., 2010; Tang et al., 2010; Zhang et al., 2010; Han et al., 2010; Wang et al., 2010; Yang et al., 2011; Xie et al., 2011al., 2011b; Bian et al., 2012; Chen et al., 2012; Wen et al., 2013; Gao et al., 2013; Guo, 2013; Guo, 2014) previously published literatures. Most affected areas to date, rounded about 3998 individuals with arsenicosis, including children most who have been identified living in rural areas of the Shanxi province, in Northern, China (Wang et al., 2007; Sun et al., 2011).

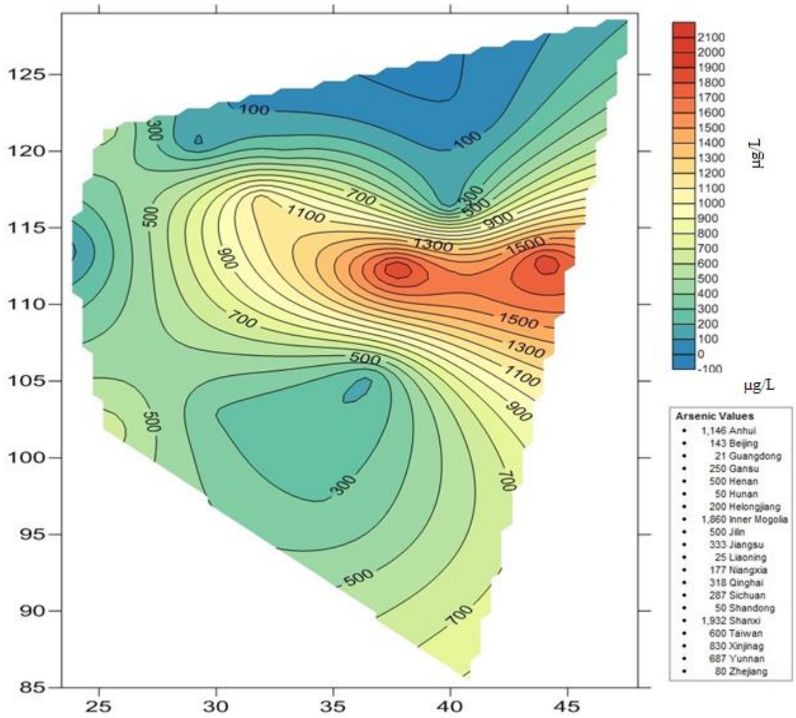

Figure 1. *Contamination level of arsenic*

The arsenic problem in China was first documented in the late 1970's and early 1980's; arsenic-specific changes were diagnosed in the Kuitun area of the Xinjiang province. Later in 1989, Inner Mongolia was reported as another serious arsenicosis area, followed by Shanxi province around 1994 (SWI, 2012, Unicef). Researchers are still interested in some areas because the arsenic value may vary every year especially in Shanxi province of China. Moreover, the Shanxi province has been recognized as a famous area with significant exposure in terms of high arsenic concentration (up to 1932 µg/L) level (*Figure 2*). According to new recent research the area of China with the highest arsenic content is Datong Basin. Its maximum arsenic content (2610.9 µg/L) was detected at the depth of 26.8 m (Sun, 2004; Xie et al., 2013). The Shanxi province —especially Datong Basin — has a high level of arsenic in groundwater, which many use as drinking water. Datong Basin is one of the most water-stressed regions (Xie et al., 2011c), in the world. In this water stressed region, surface water supply is usually very limited; therefore, alternative sources for portable, drinking water and house hold and irrigation water is mostly groundwater. The nexus of population growth and climate change in the region has exacerbated the use of groundwater resources with dramatic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

impact both on its quality and quantity (Wang et al., 2009). The current challenge is to understand the process and also focus on how to decontaminate the widely occurring geogenic arsenic in groundwater in this region of China so the water supply meets the required WHO standards (Xie et al., 2012).

Table 2. *Arsenic information in provinces of China*

S. No	Province	Areas	Max As (μg/L)	References
1	Anhui	Fuyang,Dangshan,Wuhe, Tianchang	1146	Wen et al., 2013; Guo, 2014; Sun et al., 2011; Jin et al., 2003; Li et al., 2006; Qin and Xu, 2010; Yu et al., 2007
2	Beijing	Shunyi	143	Guo, 2014; Wen et al., 2013; Jin et al., 2003; Pang et al., 2003
3	Guangdng	Fuoshan	>21	Guo, 2014; Huang et al., 2010, 2014; Wen et al., 2013; Fan, 2014
4	Gansu	Yumen, Qin'an, Datong, Menyuan	≥250	Guo, 2014; Wen et al., 2013; Jin et al., 2003; Yu et al., 2007
5	Henan	Qixian	≥500	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Li et al., 2010
6	Hunan	Shimen	≥50	Guo, 2014; Wen et al., 2013; Yu et al., 2007
7	Heilongjiang	Lindian, Zhaoyuan, Dorbod, Anda as parts of Songnen Plain	200	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Hao and Xing, 2010
8	InnerMongolia	Dengkou, Linhe, Hangjinhouqi,Wuyuan,Wulateqianqi, Wulatehouqi,Wulatezhongqi, Tuoketuo, Tumotezuoqi, Tumoteyouqi, Alashanzuoqi, as Hetao Plain; Keshenketengqi, Sunidyouqi, Sonidzuoqi, Naimanqi, Ewenkizuzizhiqi, Xinbaragzuoqi, Taibusqi, Horinger	1860	Guo, 2008, 2014; Wen et al., 2013; Bo and Luo, 2010; He et al., 2010; Luo et al., 2006; Luo, 1993; Yang et al., 2008
9	Jilin	Tongyu, Yaonan, Daan, Shuangliao as parts of Songnen Plain	≥500	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Bian et al., 2012; Tang et al. 2010
10	Jiangsu	Sihong, Nantong, Xuyi	333	Guo, 2014; Wen et al., 2013; Han et al., 2009; Zhang et al., 2010
11	Liaoning	Kangping	25	Guo, 2014; Wen et al., 2013; Liu et al., 2003
12	Ningxia	Pingluo, Helan, Huinun, Qingtongxia, Xixia as parts of Yinchuan Plain, Zhongwei, Zhongning	177	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Han et al., 2010
13	Qinghai	Guide, Datong, Menyuan	318	Guo, 2014; Wen et al., 2013; Jin et al., 2003; Shi et al., 2010
14	Sichuan	Jinchuan, Luding	287	Guo, 2014; Wen et al., 2013; Deng et al., 2004
15	Shandong	Dongchangfu, Yanggu, Yuncheng, Tengzhou, Jiaxiang, Guanxian, Liangshan	≥50	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Shen et al., 2005
16	Shanxi	Shanyin, Yingxian, Shuozhou as parts of Datong Basin; Fenyang, Xiaoyi, Pingyao, Wenshui, Jiexiu, Yuci,Qixian, Tianzhen,Xiaodianqu, Qingxu, Loufan, Dingrang, Yicheng, Yanhu, Yongji, Tengchong, Gengma, Eryuan	1932	Guo et al., 2005, 2013, 2014; Wen et al., 2013; Jin et al., 2003; Pei et al., 2005; Wang et al., 2010; Xie et al., 2008, 2011a, 2011b
17	Taiwan	Jiayi,Tainan	600	Guo, 2014; Wen et al., 2013; Li et al., 2006; Chen et al., 2003; Tseng et al., 1968
18	Xinjiang	Kuiteng,Wusu, Tacheng, Sulei, Bachu, Luntai, Awat, Shawan, Bohu	830	Guo, 2014; Wen et al., 2013; Yu et al., 2007; Huang et al., 1985; Wang et al., 1993; Zhu et al., 2009
19	Yunnan	Changning, Mengla	687	Guo, 2014; Yu et al., 2007; Chen et al., 2012; Liu et al., 2009; Yang et al., 2011
20	Zhejiang	Nanxun, Tongxiang	80	Guo, 2014; Jin et al., 2003; Jiang et al., 2010

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DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

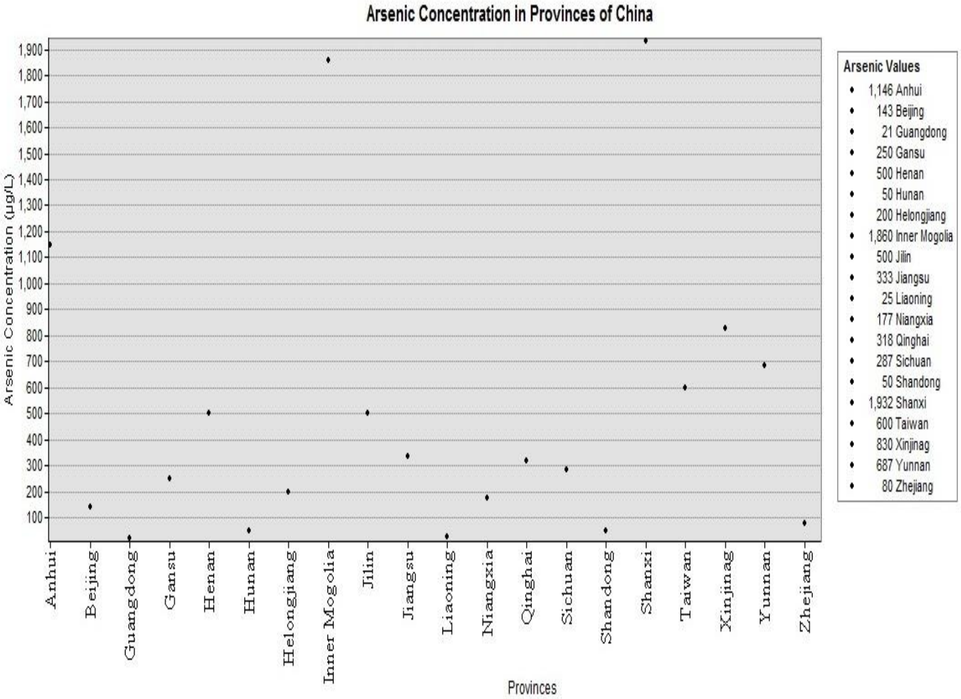

Figure 2. *Arsenic contamination level in different provinces of China*

Sources and Mobility of Arsenic

Natural occurrences of arsenic-contaminated groundwater have brought severe associated health risks. Arsenic may enter water bodies, air and land by transportation through water runoffs and wind-blown dust etc. Arsenic in the atmosphere comes from geological activities such as volcanoes, and organisms — especially microorganisms— and industrial activity in as the burning of fossil fuels as well as other activities. It has been previously accepted that the major sources of arsenic contamination in the groundwater of China are geological deposits, which is present in 200 minerals. Except geo and mining environments, other two main environmental conditions are known to be linked to natural arsenic in groundwater systems (Deng et al., 2009) (i) aerobic alkaline environments in closed basins in semiarid and in arid regions, where pH is high and leads to alkaline desorption of arsenic from mineral oxides, and (ii) aquifers are in under reducing conditions, where the release of arsenic is closely related to reductive dissolution of arsenic-bearing iron (hydro)oxides in sediments. In groundwater, there are two common types of forms of arsenic including Arsenite and Arsenate. Arsenite is always present under more reducing conditions whereas arsenate almost always present under oxidizing conditions. Several related studies in China have indicated the arsenic-enriched groundwater to be classified as reducing conditions (Smedley et al., 2003; Guo and Wang, 2005; Zhang and Guo, 2007; Guo et al., 2010; Hagiwara et al., 2011; Xie et al., 2013), particularly in the arid regions, where such conditions in alluvial and lacustrine aquifer sediments are often concomitant with high salinity and/or alkalinity. In some areas, mostly Taiwan, arsenic release has also been associated with anoxic aquifers (Lee et al., 2009).

As stated earlier, previous studies indicate that the sediment–water interactions cause geogenic enrichment of arsenic in groundwater. Furthermore, arsenic content ranging

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

from 1 to 10 mg/kg can cause enrichment of dissolved arsenic in groundwater may exceed 1000 μg/L (Smedley and Kinniburgh, 2002). It is critical to document the source of arsenic in sedimentary aquifers to improve our understanding of arsenic enrichment mechanism. Efforts have been made by different organizations to document the potential sources of arsenic in aquifers and it has been indicated that the sources of arsenic in sedimentary aquifers are related to chemical weathering of the minerals, which contain arsenic within it (Saunders et al., 2004; Zheng, 2007). Studies have reviewed and explained the link between natural arsenic contaminated aquifers and weathering. Several minerals related to arsenic and occurrences are documented in detail in *Table 3*.

Table 3. *Arsenic minerals occurring in nature*

S. No	Mineral	Composition	Occurrence
1	Native arsenic	As	Hydrothermal veins and deposits that contain other arsenic minerals
2	Niccolite	NiAs	A minor-component of Ni-Cu ores in high-temperaturehydrothermal veins
3	Safflorite	$(Co,Fe)As_2$	Hydrothermal veins
4	Realgar	AsS	Vein deposits often associated with orpiment clays, and lime stones, deposits from hot springs
5	Orpiment	As_2S_3	`Hydrothermal veins, hot springs, volcanic sublimation products
6	Cobaltite	CoAsS	Medium-temperature hydrothermal deposits, metamorphic rocks
7	Arsenopyrite	FeAsS	The most abundant arsenic mineral, dominantly in vein sofhydrothermal origin, found in pegmatites, high-temperature gold, quartz and tin veins, in contact metamorphic sulfide deposits, also in gneisses, schist sand other metamorphic rocks
8	Arsenianpyrite	$Fe(As,S)_2$	Hydrothermal veins, accessory mineral in igneous rocks, pegmatites and contact metamorphic deposits
9	Lollingite	$FeAs_2$	Mesothermal deposits associated with other sulfides and calcite gaunge
10	Tennantite	$(Cu,Fe)_{12}As_4S_{13}$	Hydrothermal veins and contact metamorphic deposits
11	Enargite	Cu_3AsS_4	Hydrothermal vein deposits formed at medium temperatures
12	Arsenolite	As_2O_3	Secondary mineral formed by oxidation of FeAsS, native arsenic and other arsenic minerals
13	Scorodite	$FeAsO_4 \cdot 2H_2O$	Secondary mineral formed by oxidation of arsenic-bearing sulfides
14	Annabergite	$(Ni,Co)_3(AsO_4)_2 \cdot 8H_2O$	Secondary mineral formed by the alteration of Co-Ni-bearing arsenides and sulfides, in the oxidized zone of hydrothermal mineral deposits
15	Hoernesite	$Mg_3(AsO_4)_2 \cdot H_2O$	Secondary mineral, in limestone blocks and volcanic tuff
16	Symplesite	$Fe^{2+}{}_3(AsO_4)_2.8H_2O$	Secondary mineral in the oxidized zone of some arsenic rich Hydrothermal mineral
17	Conichalcite	$CaCu(AsO_4)OH$	Secondary mineral in the oxidized zone of Cu deposits, and alteration product of enargite

Source: Modified from (Smedley and Kinniburgh, 2002; Anthony et al., 1990; Anthony et al., 2000)

Although large areas such as the Tarim basin (Xinjiang), the Ejina basin (Inner Mongolia), the Heihe basin (Gansu), the Qaidam basin (Qinghai), the Northeastern plain (Inner Mongolia, Jilin, and Liaoning), and the North China plain (Henan and Shandong) are known as being potentially affected, the worst situation of arsenic problems in China has been documented in Inner Mongolia and the Datong Basin, where several studies have been conducted. More recently, the controlling factors

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

(Saunders et al., 2004; Xie et al., 2008, 2009a, 2009b) have been documented to understand the arsenic mobilization in aquifers system at the Datong basin. Fe-hydroxides/oxides were the major causes for arsenic in aquifer systems at Datong (Xie et al., 2008, 2009a, 2009b). However, the source of the geogenic arsenic in some areas is a controversial issue and has yet to be determined. Although results of the preliminary geochemical survey have indicated that the bedrocks around the basin were the potential sources for arsenic in the aquifer system (Xie et al., 2013), more detailed work is still needed to answer this open question.

It is already generally accepted that the reduction of arsenic-bearing Fe oxides/hydroxides through organic carbon oxidation can be said to promote the mobilization of arsenic. However, the mechanisms involved are still not fully understood. Studies are being conducted to understand the processes controlling the mobilization of arsenic. Therefore, it is important to understand the transformation of Fe minerals within the aquifer system in order to elucidate the mechanisms involved in the release of arsenic into groundwater. Studies (Jiang, 2001; Xie et al., 2008, 2013) have shed a light on arsenic mobilization and the associated dissolution/precipitation and transformation of Fe minerals. Studies have presented Fe isotope compositions of Fe leached from aquifer sediments which were collected from a well-known arsenic-contaminated site in China at the Datong Basin; the results demonstrate that in various Fe pools, the Fe isotopic composition may be produced through microbial Fe(III) reduction and the secondary Fe(II) phases formation. The Fe isotope compositions and the distinct arsenic concentrations in HCl extracted poor-crystalline Fe phases and crystalline Fe(III) provide a framework to interpret arsenic mobilization in aquifers in the Datong Basin, China (Jiang, 2001, Xie et al., 2008, 2013; Zhu et al., 2009; Lee et al., 2009).

Generally, Arsenic pyrites/ferrous hydroxides are arsenic-rich minerals which are stable in reducing environment under water-table and are mostly concentrated in organic deposits. However, the different anthropogenic activities accelerate the oxidation process and arsenic release from the minerals. Absorption onto iron hydroxide is not a problem,while adsorption of arsenic onto iron hydroxide returns to the reduced-environment under the water-table and mixes with water and ultimately poisons the water. The hypothesis elaborates the recent phenomenon that the origin of arsenic-rich groundwater is human-made. Moreover, the different geological processes such as weathering, erosion, sedimentation, and use of irrigation and fertilizers accelerate the processes (Xie et al., 2008, 2009b, 2012; Hagiwara et al., 2011; Maity et al., 2012; Jiang, 2001).

Exposure pathways and toxic effects of arsenic to human health

Several studies have been conducted to document the toxicity of arsenic and its effects on human health in various arsenic-contaminated regions around the globe (Engel and Smith, 2004; Raven, 2009). Usually arsenic enters human-beings through two pathways; first, direct consumption of drinking water that contains arsenic and second, for populations not exposed to elevated arsenic in drinking water to have indirect contact through food intake and the cultivation of crops. Undetectable in its early stages, arsenic poisoning takes between 8 and 14 years to impact health, depending on the amount of arsenic ingested, nutritional status, and immune response of the individual (Raven, 2009; Fontcuberta et al., 2011). Toxicity depends on the amount of arsenic intake, which can be classified into acute, sub-acute and chronic toxicity

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

respectively. Arsenic contamination in drinking-water threatens health around the world and affects an estimated amount of more than 150 million people. While around 110 millions belong to highly affected ten countries in South and South-east Asia including Bangladesh, Vietnam, China, Taiwan, Laos, Cambodia, Myanmar, India, Pakistan, and Nepal (WHO, 1999; Hsueh et al., 1995; Brammer, 2008; Raven, 2009; Fontcuberta et al., 2011). Recent studies have also documented that human intakes of arsenic, especially inorganic in a range of 0.05 mgL^{-1} leads to arsenicosis. Arsenic is associated with a several non-neoplastic diseases, such as cerebrovascular disease, cardiac disease, leuco-melanosis and hyperker- atosis, diabetes mellitus, pulmonary disease and diseases of the capillaries, arteries and arterioles (Fontcuberta et al., 2011). Chronic arsenic ingestion from drinking water is known to cause skin cancer affected by arsenite and arsenate. Chronic exposure to inorganic As causes several disorders upon different biological systems such as the digestive system, respiratory system, cardiovascular system, hematopoietic system, endocrine system, renal system, neurological system, and reproductive system. These diseases ultimately increase the risk for bladder, kidney, liver, lung cancer, and diseases of the blood-vessels of the legs and feet, and possibly high blood pressure which is shown in *Figure 3* (Mandal et al., 1996; Maharjan et al., 2005; Saha, 2009; Shukla et al., 2010; Fendorf et al., 2010b; Chakraborti et al., 2010; WHO, 2011).

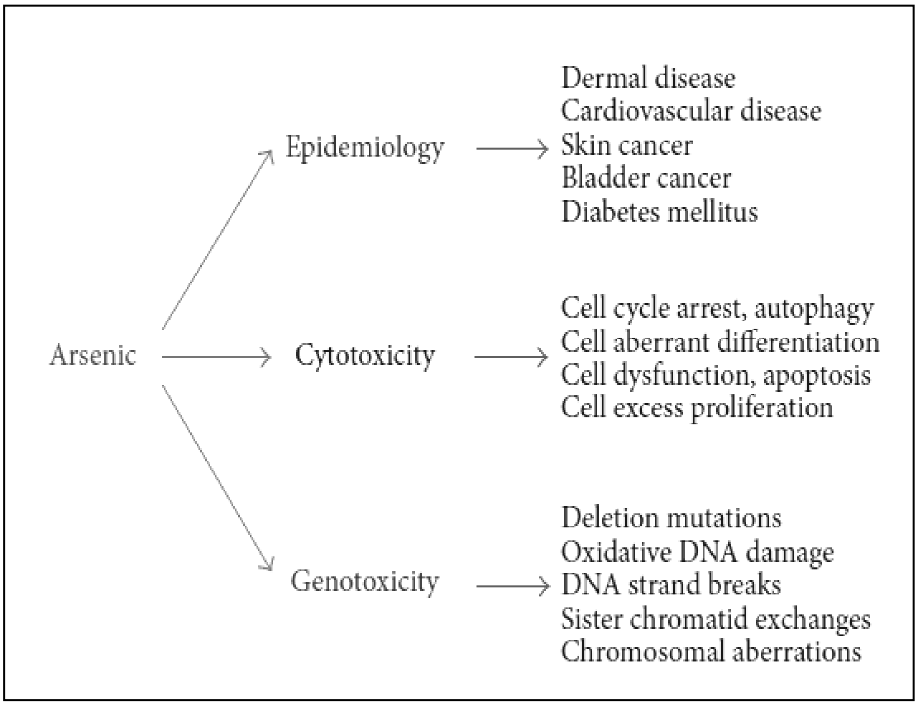

Figure 3. *Arsenic toxicity in human (Saha, 2009; Shukla et al., 2010)*

The major arsenicosis regions are presently documented in large deltas and along the largest rivers merging from the Himalayas (Fendorf et al., 2010a) such as in the Bengal delta (Chakraborti et al., 2010), other parts of India (Saha, 2009; Shukla et al., 2010), Nepal (Thakur et al., 2011), Pakistan (Malik et al., 2009), Myanmar, Vietnam, Cambodia (Berg et al., 2007; Polya et al., 2008) and China (He and Charlet, 2013). Therefore, arsenic free water is urgently needed to mitigate arsenic toxicity and protect

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the health and well being of people living in acute arsenic problem areas — especially in rural areas where the groundwater is the only source for drinking water. The populations exposed to to arsenic around the world are shown in the *Table 4*. It was first realized in the late 1970s and early 1908s that there were arsenic issues in China; firstly patients from Kuitun area of Xinjiang province were diagnosed to have arsenicosis. Later Inner Mongolia was documented as another areawith the worst arsenicosis in 1989, followed by the Shanxi province around 1994, and recently the Datong Basin has the worst arseni ccontamination levels, which has brought health risks communities belonging to rural areas (Sun, 2004; Kongkea et al., 2010, Xie et al., 2013, He and Charlet, 2013).

Table 4. *Estimated Number of people exposed to arsenic contamination in selected countries*

Countries	Population exposed to As	Reference
India	102585	WHO 1999, UNICEF 2013
China	31385	
Bangladesh	30969	
Pakistan	23202	
Vietnam	15603	
USA	6225	
Italy	5210	
Mexico	5041	
Japan	4910	
Taiwan	4133	
Argentina	2551	
Cambodia	2422	
Nepal	1465	
Hungary	1422	
Canada	1404	
Chile	1046	

In China, several provinces have arsenic contamination in its groundwater, which crosses the standard range of 10 ug/ml. However, epidemiological studies on the effects of arsenic exposure from drinking water on public health indicated a carcinogenic effect. As arsenic bring cancers of the skin, liver, lung, bladder and kidney, and probability, experts indicated that a concentration level of 50 µg As/L could lead to cancer in 1 in 100 individuals. In the case of chronic poisoning, this deadly element accumulates in skin, hair and nails, resulting in strong pigmentation of hand and foot, also known as keratosis, and problems in other body system such as respiratory, neurological, high blood pressure, endocrine, cardiovascular and metabolic dysfunctions/disorders (Yunus et al., 2011; McClintock et al., 2012; Ferlay et al., 2015).

Treatment options for remediation of arsenic contaminated water

Several methods are available for the determination of arsenic in water by WHO and several studies have been conducted for arsenic removal (Kartinen and Martin, 1995; Joshi and Chaudhury, 1996; Gates, 1998; Leupin et al., 2005; Mondal et al., 2013; Sharma et al., 2005a; Sharma, 2007a, 2007b). The most common of these methods include:

(i) Atomic absorption spectroscopic method – (a) hydride generation atomic absorption (AAS – HG) and (b) electrothermal atomic absorption (AAS – GF).

(ii) Silver diethyldithiocarbamate method (SDDC).

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DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

(iii) Inductively coupled plasma (ICP) method – (a) mass spectrometry (ICP-MS) and (b) atomic emission spectrometry (ICP-AES).
(iv) Anodic stripping voltammetry (ASV).

There are also several available options suited for arranging drinking water with low arsenic content such as:

(i) Explore alternative source of water with low arsenic content.
(ii) Treatment/removal of arsenic from the water source.

It is accepted that arsenic is found in shallow groundwater, while deep groundwater is either free of arsenic content or have low content. However, the depth to arsenic free aquifers differs between the locations so a detailed server is needed. The second option is to use surface water i.e lakes, rivers and ponds which are arsenic free or have a low arsenic content. The third option is to harvest rainwater, historically it is a way to utilize rainwater for domestic water supply and it has been used around the globe (BGS, 2004; Ahmed et al., 2005; WHO, 2011).

The aim of this section is to review and update the recent advances made in the technological development in arsenic removal technologies and to explore the potential of those advances so we can document the problem of arsenic contamination. There will be a special focus on the efficiency, applicability and social acceptability of the various technologies. In the 1990s, several methods were introduced for the removal of arsenic from water through large conventional treatment plants. The most commonly used technologies include adsorption onto sportive media, ion exchange resin and membrane processes, oxidation (biological and chemical), co-precipitation and adsorption onto coagulated flocs, lime treatment, and bacterial treatment, which can be applied for arsenic removal (Gates, 1998; Leupin et al., 2005; Sharma et al., 2005a; Sharma, 2007a, 2007b; Mondal et al., 2013) These techniques are still being used all around the world, several techniques are defined below.

Arsenic removal by Oxidation

Arsenic [As(III) and As(V)] is present in groundwater so oxidation is a pretreatment process which is conducted for converting arsenite to arsenate/AsIII, then to AsV and finally AsV precipitation is takes place. This process is important for anoxic/anaerobic groundwater because AsIII is the pre-dominant form of arsenic at aneutral pH (Sharma et al., 2007). Free chlorine, chlorine dioxide, hypochlorite, chloroamine, ferrate, ozone, oxygen, hydrogen peroxide, permanganate, and fulton's reagent are used for the oxidation of AsIII into AsV (Sharma et al., 2005b; Sharma and Sohn, 2009), but in developing countries, hypochloride, atmospheric oxygen and permanganate are being used. During the process of oxidation — which can take up to a couple of weeks hydrogen peroxide and chloroamine are slower than permanganate, chlorine and ozone (Islam, 2005, 2007). It is accepted that free Chlorine or hypochlorite is very effective for the oxidation of AsII, while theoxidation of AsIII is well achieved by ozone. However, chlorine dioxide is prohibited to use for surface water, American environmental agencies paymuch attention to this factor (Sarker, 2010). About the removalof arsenic, Utilizing FeO42- forpurifying water is recommended HFO (hydrous ferric oxide) appears to be the most important phase responsible for removing the arsenic from ground water around the world. It is well documented that High arsenic came into contact with iron oxides in the shallow aquifer, which could possibly lead to arsenic removal (Young, 1996; BGS, 2004; Ahmed et al., 2005; Lena et al., 2014; Shankar et al., 2014; Wegelin et al., 2000). Thus, for the removal of arsenic from water,

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DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

oxidation/precipitation technology is very effective (Yoon and Lee, 2005; Sharma et al., 2005b).

Arsenic removal by In-situ Oxidation

DPHE Danida Arsenic Mitigation Pilot Project is applied for In-situ oxidation of iron and arsenic in the aquifer. Stored aerated tube-well water is released back into the aquifer. The dissolved oxygen in water oxidizes arsenite to arsenate (less mobile) and the ferrous iron in the aquifer is oxidized to ferric iron which leads to a reduction in arsenic content in tube-well water. Results of the experiment in-situ oxidation show that arsenic in tube-well water is reduced to about half due to underground-precipitation and adsorption on ferric iron. In-situ oxidation has also been achieved by pumping the oxygenated water into the groundwater aquifer to reduce the arsenic content in the pumped groundwater. While its potential for the removal of arsenic is only little, the results indicate that arsenic concentrations can be reduced in the groundwater zone before water extraction (Dutta et al., 2004; Sharma et al., 2007).

Arsenic removal by Photochemical, Solar photo-catalytic Oxidation

Photochemical and photo-catalytic oxidation of AsIII have been used in many studies for arsenic removal. The most widely tested chemical oxidant in presence of naturally occurring iron in the field is UV-light assisted oxidation of AsIII (Miller et al., 2011) because FeIII - hydr-oxide and chloride species can absorb photons to provide highly oxidizing hydr-oxyl and di-chloro radicals which converts AsIII to AsV (Ryu et al., 2013), as SORAS is a simple technique of solar oxidation for arsenic in transparent bottles to reduce arsenic content of drinking water (Emett and Khoe, 2001). It is also indicated that the efficient oxidation of AsIII to AsV can be achieved by photo-catalytic oxidation (PCO) (Bissen et al., 2001). Here ultraviolet radiations speedup the oxidation of AsIII in presence of other oxidants or oxygen. Ultraviolet radiation/solar light help to create/generate the hydroxyl-radicals via the the process of photolysis of Fe(III) i.e (FeOH2+) and both oxygen and hydroxyle radicals accelerate the rate of oxidation.

It has also been investigated that adsorption of arsenic on TiO2 takes place while the oxidation process of AsIII to AsV is through photo-catalytic oxidation and TiO2. This can provide safe water; we know this because from experiments formentioned, it shows that it reduced arsenic levels to less than the standard limit given by WHO for drinking-water (Anthony et al., 1990, 2000; McNeill and Edwards, 1995; Dutta et al., 2004, 2005; Choong et al., 2007; Ravenscroft, 2009). This experiment is still being used in Bangladesh and indicated that this process averagely can reduce one-third of arsenic content from water.

Arsenic removal by Biological oxidation

Study (Pallier et al., 2010) documented that some micro-organisms such as Gallionella ferruginea and Leptothrixochracea support and accelerate biotic-oxidation of iron. The study (Pallier et al., 2010) was conducted in the laboratory where iron-oxides and micro-organisms (Gallionella ferruginea and Leptothrixochracea) were deposited in a filter-medium, which has a favorable environment for the adsorption of arsenic, as AsIII cannot be efficiently absorbed onto iron-oxides. These micro-organisms are assigned to oxidize AsIII to AsV, which got adsorbed in FeIII. This leads to up-to 95% removal of arsenic. Kinetics of bacterial oxidation of AsIII and oever all removal of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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AsV by sorption onto bio-genic manganese-oxides during ground-water treatment was also studied (Sharma et al., 2007; Pallier et al., 2010; Hu et al., 2012). Biological oxidation is a new technique of the oxidation of iron and manganese as a treatment technique for arsenic removal. These biological treatment techniques are the natural biological processes, and it takes a couple of days for remediation of metals in soil and groundwater by certain plants and micro-organisms. During treatment, the following sequences of reactions have taken place in the treatment system:

a. FeII to FeIII and MnII to MnIV (oxidation).
b. AsIII to AsV (oxidation).
c. MnO_2 (Precipitation).
d. Abiotic-oxidation of AsIII by MnO_2.
e. AsV sorption via MnO_2.

Whereas steps (a) and (b) are biotic steps (c) to (e) are abiotic. This natural process for treatment has been found excellent as it can lead to up-to 95% of the removal of arsenic (Pallier et al., 2010).

Arsenic removal by Coagulation–flocculation

Coagulation-flocculation has got attention for the removal of arsenic from water, it is most commonly used for larger-capacity facilities and it requires the formation of a floc used to remove arsenic from groundwater. Coagulation-flocculation usually requires Fe and Al based coagulants among other various chemical coagulants i.e ferric chloride, or ferric sulfate or aluminium sulfate, need to be added and dissolved in water under efficient stirring for 1-10 minutes. Positive-charged cat-ionic coagulants have to decrease the negative charge of colloids. In this process, aggregation of particles forms larger particles. It is described that this technique obtained enough amount for removal, which roundedout to more than 90% of AsV and 77% of AsIII. While using 9.2 ppm of Fe3+ aluminium or when ferric hydroxide micro-flocs are formed, As trivalent arsenic occurs in non-ionized form. For efficient removal, oxidation of As(III) to As(V) is thus required as a pretreatment. This surely can be achieved with the addition of bleaching powder (chlorine) or potassium permanganate. It has been documented that this technique is good for removal of arsenic below standard drinking water and also Fe based coagulants have also been investigated by several authors (Cheng et al., 1994; Appelo et al., 1999; Song et al., 2006; Sharma et al., 2007; Andrianisa et al., 2008; Gupta et al., 2009; Baskan and Pala, 2010; Lakshmanan et al., 2010; Van Halem et al., 2010; Lacasa et al., 2011). Recently, Hu et al. (2012) has used it and found the same.

Some of chemical coagulants are being used, whereas Fe based coagulants have been documented to be most efficient in water treatment than others e.g Al based coagulants (D.P.H.E, 2001; Katsoyiannis et al., 2004). For efficient removal of arsenic from water, the arsenic needs to be adsorbed on amorphous metal hydr-oxides formed from coagulant. There is critical limitation in the process of coagulation/flocculation, it produces a large amount of sludge along with a big concentration of arsenic (Hering et al., 1996; Appelo et al., 1999; D.P.H.E, 2001; Song et al., 2006; Sharma et al., 2007; Andrianisa et al., 2008; Gupta et al., 2009; Baskan and Pala, 2010; Lakshmanan et al., 2010; Van Halem et al., 2010; Lacasa et al., 2011). Real management of the contaminated sludge is needed for safeguarding the environment from secondary pollution and thus reduces the applicability of this method in field conditions.

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Arsenic removal by Electro-coagulation

Coagulation/flocculation has an alternative way known to be electro-coagulation which has a different the process, instead of adding a chemical reagent as ferric-chloride; metallic cat-ions are directly generated in the effluent to be treated while applying current between iron electrodes for dissolving soluble anodes. Generally, Electrolytic-oxidation of a sacrificial iron anode creates FeIII oxy-hydroxides / precipitates in the arsenic contaminated water in electro-coagulation. While with FeIII precipitated arsenic produces bi-nuclear inner-sphere complexes and it further aggregates to form a floc. Then metallic cat-ions and hydroxides are formed and colloids neutralize negatively charged which allow them to coagulate and finally create helpful results (Ghurye et al., 2004; Katsoyiannis and Zouboulis, 2006a, 2006b).

Arsenic removal by Electro-Chemical

In this method, a small amount of electricity is needed to create rust in arsenic contaminated water. The rust binds to arsenic, settling and/or filtering. The rust can then remove from the water (Jain and Singh, 2012).

Arsenic removal by Adsorption

This is one of the processes of adsorption, in which, solid particles are used for removing substances from solutions such as gas or liquid. Removal of arsenic by this technique onto activated/coated surfaces has been famous and used the most in many countries. Some of its benefits are its low cost, high removal efficiency, easy operation and handling and also its sludge-free day-to-day operations. There is an extra advantage of this technology a number of the adsorbents can be reused and regenerated. Several studies have addressed the arsenic-removal by adsorption via many adsorbents (Wilkie and Hering, 1996; Mohan and Pittman, 2007), including commercial activated carbon, activated alumina, layered double hydroxide, natural/modified clays and zeolites. Mohan and Pittman (2007) and Wilkie and Hering (1996) have reviewed and addressed approximately 40 different types of adsorbents using published literature, with more than 500 references. Recently, gaps in exploration and improvement in new adsorbents are being found. A few years back adsorption based on zeolites (Raven et al., 1998), iron oxides (Grafe et al., 2001; Zhu et al., 2013), alumina (Kanematsu et al., 2013), clays (Swarnkar and Tomar, 2012), etc. to adsorb arsenic from water and the removal of arsenic by adsorption processes/techniques probably depended on pH and the speciation of arsenic with better AsV removals as compared to AsIII at a pH level lower than 7 (Giménez et al., 2010; Anjum et al., 2011; Sun et al., 2013; Han et al., 2013; Zhu et al., 2013). Several other studies such as Kanematsu et al. (2013) reviewed that the general rate of arsenic adsorption and capacity adsorbents further depend on the presence of other ions such as: silicate, phosphate, $HCO^{-}3$, and Ca2+. These ions compete for the adsorption sites; some other studies (Lin and Wu, 2001; Singh and Pant, 2004) also substantiated this fact. It is documented that the most widely tested aluminium-oxide is activated alumina (AA) (Giles et al., 2011; Genuchten et al., 2012; Kanematsu et al., 2013). There are some other adsorption methods used for removal of arsenic from water, which are discussed in the section below.

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Arsenic removal by Bucket Treatment Unit

The Techniques of Bucket Treatment Unit (B.T.U) is an extremely effective and affordable treatment technique for the removal of arsenic within the household. Locally available materials can be used to build the treatment unit and if operated properly and smoothly, it is effective for removing of arsenic from water. These technology units units are generally based on chemical doses of 2 mg/L of potassium permanganate and 200 mg/L aluminum sulfate in a crushed powder form. In both lab and field conditions, these units were documented to provide good performances in removing arsenic.

Bucket Treatment Unit (B.T.U) was proposed by the DPHE-Danida Project which is based on the principles of co- precipitation, coagulation and adsorption techniques/processes. In this technique, there are two buckets, each one has a 20 liter capacity, and are placed one above the other. The further the process is conducted; chemicals are put manually with arsenic contaminated water in the upper red bucket. Mixing of chemicals is done by vigorous stirring with a wooden stick for about 30 to 60 seconds, followed by the flocculation process by gentle stirring for about 90 second. It takes 1-2 hours to settle. Once the water is settles, the water from the top red bucket is further allowed to flow via plastic pipe into the lower the green bucket, where a filter is already installed. In the lower bucket, which should have sand filter and a valve sludge does not build up in the upper bucket. This is how practically a treated water container (EPA, 2000a, 2000b; Giles et al., 2011).

Arsenic removal by Membrane technology (MT)

Membrane technologies, i.e RO, have been documented as reliable and efficient for arsenic removal. RO has proven to be the best practiced technology which can completely purify water. It is generally accepted that RO is superior in both pilot-scale and lab experiments; results have shown more than 95% As(V) and 74% As(III) removal efficiencies (BAMWSP, 2001; Tripathy and Raichur, 2008). Membrane processes get rid of arsenic through electric repulsion, filtration, and adsorption of arsenic containing compounds. With the help of poly-sulfone thin film and nano-filtration membrane (BQ01), the distinction between the removal of As(III) and As(V) was explored (Sarkar et al., 2000). It has been observed that the elimination of As(V) was much higher than that of As(III). Furthermore, another study (Kohnhorst and Paul, 2000) was conducted on the nano-filtration operating conditions, it was concluded that there is no effect on arsenic removal by the trans-membrane pressure, temperature or cross-flow velocity (Kohnhorst and Paul, 2000). This technique is proven to be the best and results shows that up to 99% of the arsenic can be removed (Sheng et al., 2006; Xi et al., 2014) through the conducted studies.

Arsenic removal by nano-filtration Technology

Arsenic is being removed from groundwater by nano-filtration (NF) membrane configuration. It has played a key role in controlling operation performances and membrane fouling. The nano-filtration (NF) membrane surface puts negatively charged above its iso-electric point (approximately at pH 5.9–6.4). Generally, alkaline/neutral pH is adjusted for raw groundwater so it brings up the effect of electrical charge repulsion and ion removal efficiencies, which is improved by nano-filtration (NF). This nano-filtration (NF) system has an efficiency of up to 92˜94% for arsenic removal (Xie et al., 2015; Song et al., 2015).

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Arsenic removal by Activated alumina (AA)

Activated alumina (AA) successfully removed arsenal from water supply systems and it was the first adsorptive medium for water supplies (Xie et al., 2008). Activated alumina is defined as a physical/chemical process, which is used particularly for the removal of arsenic and other ions. In this process, ions in feed water are sorbed to the oxidized Activated alumina (AA) surface. It is the widely tested aluminum oxide. It is prepared at high temperature, generally by the thermal de-hydration of aluminium hydroxide Al(OH)3. It has diameter approximately 0.3–0.6 mm; in addition, it has a high surface area for impressive sorption properties as it is a porous, granular material. The AA is used in the contaminants such as fluoride, arsenic NOM, silica and selenium. (Holl, 2010). It has been reported that, alum-impregnated AA can be adsorbent for AsV rather than untreated AA at a pH range of 2.8–11.5. When employed in batch mode, AsV concentration could be decreased down from $10mgL^{-1}$ (10,000 ppb) to 40 ppb (Katsoyiannis and Zouboulis, 2006a, 2006b).

Arsenic removal by Stevens Institute Technology

In this technology, the process resembles the previously mentioned B.T.U as Bucket Treatment Unit (B.T.U) proposed by DPHE-Danida Project which is mostly based on the principles of co-precipitation, coagulation and adsorption techniques/processes. This technology is also involved in two buckets, the first bucket is to mix chemicals (such as calcium hypo-chloride and iron sulphate) then supply in packets. The other bucket is used to separate flocs, where two processes have taken place (a) sedimentation and (b) filtration. The second bucket has a second inner bucket with slits on the sides to help sedimentation and to keep the filter sand bed in place (EPA, 2000a). This technology has been mostly used in Bangladesh and has been effective. The results of tested samples show that 80 to 95% of the samples reduce arsenic concentration to less than 0.05 mg/L (EPA, 2000a, 2000b).

Arsenic removal by iron coating

An experimental study has been conducted (Seidel et al., 2001), where an iron coating method is used as a simple groundwater remediation technology, which has great potential. Moreover, it is affordable, especially in villages and rural areas where groundwater is used by communities for drinking purposes. Under different conditions, coated iron oxide/hydroxide was investigated for the arsenic removal mechanism using an electron probe microanalysis, scanning-electron microscopy(SEM)/X-ray absorption spectroscopy, and fourier transformation infrared spectroscopy. It has proven to be an effective method for arsenic removal. Generally, the 4-step develop aquifer iron coating method, alternating injection of oxidant, iron salt and oxygen-free water. In situ the removal of arsenic from ground water in an aquifer was achieved by simultaneous injections of As(V) and Fe(II) reagents. The technique of adsorption or co-precipitation brings arsenic fixation with fine particles of goethite by way of bi-dentate bi-nuclear complexes. Therefore, the study documents that the technology results in high arsenic removal efficiency by the situ aquifer iron coating technique. This is likely resulted by the expanded particular surface area of the small goethite particles, which enhanced arsenic sorption capability and/or from coprecipitation of arsenic on the surface of goethite particles.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Arsenic removal by hollow fiber nano-filtration membranes with a sulfo-nated poly ether ether ketone coating

In 2015, this technique got high attention when experimental studies were done (Saitua et al., 2005). The overall performance of this technique for arsenic removal from drinking water was tested. The membrane works well and does not allow above 95% of As(V) to contaminate the water. Furthermore, the membrane properties were studied, and rejects arsenic in the presence of interfering ions. Results indicated that di-electric exclusion might be able to rationally explain the rejection deviation from the Donnan exclusion effect. The valence of the ions played a major role, where there was a presence of interfering ions. Finally, cost estimation was conducted at 1000 m3 h^{-1}, which is about 0.15 US$ per 3 m when using the proposed SPEEK coated hollow fiber membrane for a plant. Results from different sources indicate that a SPEEK composite nano-filtration membrane is mostly suitable and efficient for removing arsenic from groundwater (Pinon-Miramontes et al., 2003; Saitua et al., 2005; Shih, 2005; Harisha et al., 2010).

Arsenic removal by Naturally Occurring Iron

This method has been found effective in Asian countries, where naturally occurring iron is used to precipitate the ground water and for removing arsenic by adsorption (Ahmed, 2001).

Concluding Remarks and future perspective

Arsenic is worldwide problem; arsenic has been documented in the Americas, Africa, Asia, Europe and Pacific countries. Arsenic in groundwater can found in several geographical regions in mainland China. Studies identify areas of high-risk arsenic contamination in the north of China, including Xinjiang, Shanxi, Inner Mongolia, Henan, Shandong and Jiangsu. The most affected areas to date, rounded about 3998 individuals with arsenicosis, including children, with most living in rural areas of the Shanxi province, in northern China.

Arsenic has created health problems around the world. The major arsenicosis regions are presently documented in large deltas and along biggest rivers merging from the Himalayas, such as in the Bengal delta, other parts of India, Nepal, Pakistan, Myanmar, Vietnam, Cambodia and China. Usually, arsenic enters human-beings through two pathways; first, direct consumption of drinking water contaminated by arsenic and second, for populations not exposed to elevated arsenic in drinking water, the indirect intake through foods and crops cultivated using arsenic-contaminated water represent the main sources of arsenic intake for humans.

Several available options suited for arranging drinking water with low arsenic content such as: (i) Explore alternative source of water with low arsenic content and (ii) Treatment/removal of arsenic from the water source. The most commonly used technologies include adsorption onto sorptive media, ion exchange resin and membrane processes, oxidation (biological and chemical), co-precipitation and adsorption onto coagulated flocs, lime treatment, and bacterial treatment, which can all be applied for arsenic removal.

Aeration/Fe precipitation/filtration is developed to remove arsenic; this technology has been accepted in China. The modification of commercial adsorbents (e.g., activated

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carbon and activated alumina) and modification of natural mineral materials (e.g., zeolites) seem to be an approach to lower the operating cost and improve arsenic removal efficiency. Recently, several technologies to remove arsenic from drinking water have been introduced. Many of them are described in this review; each have their own advantages and disadvantages. Most of these technologies for removal of arsenic involve the direct removal of AsV or converting AsIII to AsV followed by removal of AsV.

While talking about future perspective, the government should monitor and document industrial and agricultural activities as they brought the arsenic pollution issue to the bodies of water in the first place.

Mining or chemical plants should be documented to deal with sewage, sludge storage and waste treatment. Sub departments should be developed and should be supervised by well-known persons; there should also be sampling and analysis of the discharge from industrial plants, which aim to supply safe drinking water to people in rural areas, including arsenic control. The government should take additional should take steps and put restrictions, if there is a not safe water source. Investment should be employed on the great engineering system for water transportation and water quality should be well documented. Small treatment facilities installations should be recommended in rural areas.

Acknowledgements. This research was supported by the Fuling Shale Gas Environmental Exploration Technology of National Science and Technology Special Project (grant no.: 2016ZX05060), the Demonstration of Integrated Management of Rocky Desertification and Enhancement of Ecological Service Function in Karst Peak-cluster Depression (grant no.:2016YFC0502400).

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APPENDIX

Table S1. *Arsenic affected areas in China*

S.NO	PROVINCES	Max As (µg/L)	Coordinates in decimal	
			Lat	Long
1	Anhui	1146	31.833333	117
2	Beijing	143	39.916667	116.383333
3	Guangdong	21	23.4	113.5
4	Gansu	≥250	38	102
5	Henan	≥500	33.9	113.5
6	Hunan	≥50	27.4	111.8
7	Helongjiang	200	48	129
8	Inner Mogolia	1860	44	113
9	Jilin	≥500	43.7	126.2
10	Jiangsu	333	32.9	119.8
11	Liaoning	25	41.1	122.3
12	Niangxia	177	36.6	105.32
13	Qinghai	318	35	96
14	Sichuan	287	30.133333	102.933333
15	Shandong	≥50	36.4	118.4
16	Shanxi	1932	37.7	112.4
17	Taiwan	600	25.033333	121.633333
18	Xinjinag	830	41	85
19	Yunnan	687	25.05	101.866667
20	Zhejiang	80	29.2	120.5

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DOI: http://dx.doi.org/10.15666/aeer/1702_16551683

BIOFORTIFICATION: A SUSTAINABLE AGRONOMIC STRATEGY TO INCREASE SELENIUM CONTENT AND ANTIOXIDANT ACTIVITY IN GARLIC

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(Received 29th Aug 2018; accepted 5th Nov 2018)

Abstract. The process of breeding to enhance nutrients, such as vitamins and minerals, in food crops through biofortification is a sustainable, eco-friendly and powerful strategy to overcome micronutrient deficiency. Therefore, the aim of the present investigation was to increase selenium content in edible parts of garlic (*Allium sativum* L.) supplied with either 20g/ha or 50g/ha aqueous solution of anhydrous sodium selenate. This was done either through foliar spray or soil flood application under open-field conditions. Results indicated that the 50g/ha concentration of sodium selenate application in the form of foliar spray significantly enhanced the selenium content in garlic bulb (3.23±0.16mgSe/Kg) and vegetative part (15.46±0.71mgSe/Kg) that is, a 12.52 and 7.8 fold increase was observed respectively, as compared to control. A significant increase in total phenolic content (4.72±1.79GAE/100g), total flavonoid content (18.50±1.82 mgQE/100g) and total antioxidant capacity (IC_{50} of 0.81mg/ml determined through DPPH radical scavenging assay) was also observed in the bulbous part of garlic. The results suggested that the consumption of 16g of dried garlic bulb, biofortified with 50g Se/ha, could cover the daily recommended dose of selenium for human beings. Selenium biofortified garlic crop can hold a market value as selenium functional food and can be used as an alternative to synthetic selenium supplements to overcome selenium deficiency.
Keywords: *Allium sativum; antioxidant activity; flavonoid content; malnutrition; polyphenols*

Introduction

Many people, particularly those living in developing countries are facing a silent crisis of malnutrition or hidden hunger (Swaminathan, 2012, Muthayya et al., 2013). About half of the world's inhabitants suffer in macronutrients and micronutrients deficiency which is responsible for 20 million adult deaths and more than five million childhood mortalities annually (Zhao and McGrath, 2009, Bouis and Welch, 2010) . In developing countries many food systems cannot provide adequate micronutrients to meet the demands of their citizens, especially families having fewer income resources (Bouis and Welch, 2010, El-Ramady et al., 2015). It is reported that among the six billion people in the world more than 15% are suffering from selenium deficiency (Fordyce, 2013). A range of chronic diseases have been associated with selenium deficiency including Keshan (an endemic congestive cardiomyopathy in China, with high rate of fatality) (Yang et al., 2008), cardiovascular diseases (Kardinaal et al., 1997, Oropeza-Moe et al., 2015), cancer, viral diseases (Clark et al., 1996, Beck et al., 2003), inflammatory conditions, diabetes mellitus, hepatopathies and HIV infection (Holben and Smith, 1999, Burk et al., 2015). Under this scenario, boifortification offers a cost

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DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

effective and sustainable strategy in modern agriculture to allow the access of more nutritious and healthy food to large population (Bouis et al., 2011, Carvalho and Vasconcelos, 2013).

Biofortification is the process of increasing the bioavailable micronutrient density of staple crops through conventional plant breeding and modern biotechnology to achieve a measureable and positive impact on human health (Pfeiffer and McClafferty, 2007). Such an intervention can be used to enhance the uptake and accumulation of specific nutrients in edible part of plants (Rouached, 2013). It is attained through genetic engineering, conventional breeding and manipulation of agricultural practices such as rhizosphere fertilization, soil and crop management strategies (Zuo and Zhang, 2011) etc. Selenium is a vital micronutrient for human beings and animals (Hartikainen, 2005, Lobanov et al., 2008). Plant based foods are significant nutritional sources of selenium (Se) supply for both human beings and livestock to meet their daily requirement for selenium. For adults, daily consumption of 40 to 50µg selenium for women to 75 µg for men and 8.7-10 µg for infants is recommended and an intake exceeding 400µg/day is assumed to be toxic (Burk, 2002, Fordyce, 2013). Selenium poisoning, referred to as selenosis is related to dietary intake of approximately 5 mg of selenium per day. In 1960, an outbreak of endemic human selenosis was reported in China, associated with the consumption of food containing more than 300mg/Kg selenium (Fordyce, 2013). In almost all European countries, the selenium fortified foods and the use of dietary selenium supplements are quite popular to overcome the Se deficiency (Yadav et al., 2007).

To overcome the animal Se deficiency, different practices are commonly employed, e.g., the use of dietary supplements, injections, salt licks and drenches (Yadav et al., 2007). Alternatively, consumption of selenium enriched plants and their products is beneficial because selenium present in organic form is more bioavailable than in inorganic form (Terry et al., 2000, Li et al., 2017). The process of selenium accumulation in agricultural plants varies according to the plant species, soil properties and the chemical nature of selenium (Mikkelsen et al., 1989).Vegetable crops belonging to the Allium family (*Allium sativum, Allium cepa,* etc) are important part of the human daily diet. Biofortification of the vegetables, known as seleniferous plants, can contribute to the alleviation of selenium deficiency. Among these alliaceous species, garlic is one of the most popular vegetables around the globe (Ghasemi et al., 2015). In 2007 according to FAO (United Nations Food and Agriculture Organization), approximately 1.01million hectare of land was used to produce about 10 million metric tons of garlic annually in the world. China is the largest producer of garlic, accounting more than 75% of the global production (Chen et al., 2013). *Allium sativum* has the ability to uptake the inorganic Se from the soil through the roots and is able to convert it into organic forms that are accumulated in its edible parts (Yadav et al., 2007). The chemistry of selenium in seleniferous plants simply relates to sulfur chemistry because selenium share great likeness in its chemical properties with sulfur and exists in oxidation states as elemental selenium (Se^0), selenide (Se^{2-}), selenite (Se^{4+}) and selenate (Se^{6+}). Within biological systems, selenium is incorporated as a constituent of selenocysteine (SeCys) and selenomethionine (SeMet) amino acids during translation of primary structure that comprise selenoprotiens. They are stored in the form of selenium methylselenocysteine (SeMSC) in seleniferous plants including garlic, Indian mustard (*Brassica juncea* L.), onion *(Allium cepa*), broccoli (*Brassica oleraceae* L.), sugar beet (*Beta vulgaris* L.)etc (Zayed et al., 1998, Fordyce, 2013). The main available form of

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DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

selenium to plants is sodium selenate which is actively taken up by seleniferous plants through sulfate transporter and assimilated as organic form (SeMet and SeCys) with the help of enzymes, including ATP reductase, ATP sulfurylase, SeCys-lyase and O-acetyl serine transferase (*Figure 1A*) (Adhikari, 2012).

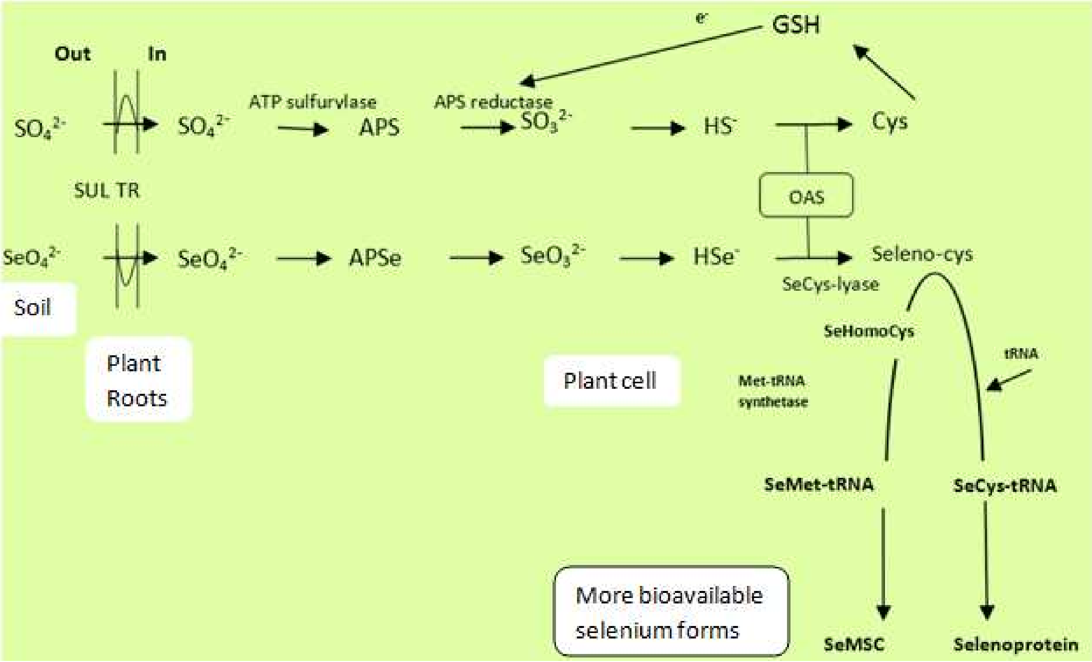

Figure 1A. *Flow diagram showing sulfur and selenate uptake and assimilation pathway in seleniferous plants. SeCys-lyase is the enzyme that is highly specific to selenium substrate. Sulfate transporter (SULTR), 5' adenylylsulfate (APS), 5' adenylylselenate (APS), O-acetylserine (Rehse et al., 2016)*

In the past few decades, interest of scientists in naturally occurring compounds that act as antioxidants and regarding particularly dietary selenoenzymes has been increasing. Selenoenzymes play a vital role in protecting the body from oxidative damage/harmful effects of reactive oxygen species (ROS) and contain one or more unpaired electrons (Birringer et al., 2002). ROS are produced either from external sources, such as chemicals/pollution, or from internal sources e.g. aerobic respiration. They react quickly with other compounds and a chain reaction starts as the other molecule loses electrons and becomes a free radical. The result is the oxidation of vital cellular parts like DNA and proteins, the disintegration of cell membrane that lead to diseases (Kaur and Kapoor, 2002). In human beings, important selenoproteins(also known as selenoenzymes) are catalase, glutathione peroxidase (GPx) and superoxide dismutase which act as antioxidant and protect cells from ROS (Steinbrenner and Sies, 2009). *Allium sativum* is a natural source of various bioactive phytomolecules, including selenoproteins, allyl thiosulfinates, flavonoids, organosulfur compounds, phenolic acids and vitamins (Choi et al., 2014). Previous studies have reported on the health-promoting benefits of garlic because of its biologically active phenolic compounds with interesting medicinal properties (González-Morales et al., 2017). The extract of garlic has a remarkable antioxidant capacity and provides protection from oxidative DNA damage (Park et al., 2009), decreases the risk of chronic diseases,

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mitigates atherosclerosis and cancer (Morihara et al., 2010) etc. The objective of this study is to enrich garlic with selenium through biofortification, to analyze its impact on garlic quality parameters including phytochemical content and biological potential as free radical scavenging property. Additionally, there is possibility to introduce the selected garlic species for selenium phytoextraction in to selenium laden soils of Punjab Pakistan.

Materials and Methods

Site description and Experimental design

A field experiment was conducted at botanical garden of PCSIR Labs Complex, Lahore, Pakistan. Local garlic variety was sown season in January 2016 and harvested in May 2016. The experimental site is situated between 31.52° North latitude, 74.33° East longitude at the altitude of 217 m above sea level. A randomized complete block (RCB) design with three replicates was used with two factors (conc. of selenium salt applied and way of applications). Garlic sets were planted in the field divided into five plots. Treatments were control (no selenium application), selenium foliar spray and selenium soil flooding and two selenium salt concentrations (20g/ha and 50g/ha). Standard agronomic practices were used. The size of each individual plot was 2 m length x 3.66m width= 29.28 m^2, with a density of 15 plants per square meter. Each plot was consisted of 11 rows, with 6 plants in each row and the distance between rows was 0.65 m. A basal dose of N-P-K in ratio of 11-5-18 kg/ha was applied prior to planting. Melathion was sprayed as a herbicide after 8 weeks of sowing.

Chemicals

All chemicals were analytical grade. sodium selenate, Folin Ciocaltaeu reagent, ascorbic acid, butylated hydroxyl Toluene (BHT), sodium carbonate, aluminium chloride potassium acetate, quercetin and gallic acid were purchased from sigma aldrich chemical Co (St.Louis, MO, USA). DPPH dye was purchased from Alfa Aesar, Germany. Ethanol, hydrochloric acid, dimethylsulphoxide (DMSO), methanol were obtained from Merck (Darmstadt, Germany).

Selenium treatments

Selenium was applied as sodium selenate (Na_2SeO_4) in the experimental field. Aqueous sodium selenate solution (1.0 g/Liter) was applied at the concentration of 20g/ha) and 50g/ha in two ways i.e. by foliar spray and through water flooding in selected plots. First application was carried out on day 9 and afterward 30 days interval from sowing, during whole growing season (*Figure 1B*). Garlic crop was harvested in May after120 ± 2 days of growth.

Preparation of sample

Finely ground sample 0.5 g was accurately weighed in a china crucible. The sample was kept in the muffle furnace at 500°C for 4-6 hours or until white ash is formed. The ash was dissolved in 5 ml of 6 N HCl by heating on a hot plate, filtered through Whatman no.1 filter paper and the final volume was made up to 100 ml with double distilled water (AOAC, 1990).

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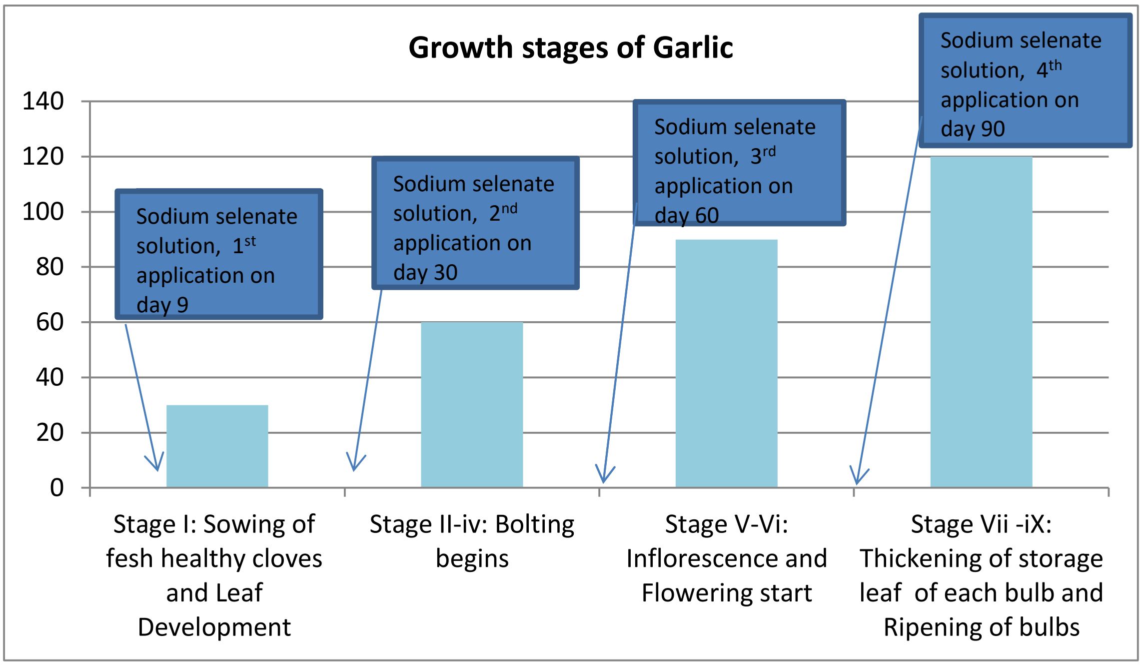

Figure 1B. *General symbolic representation of growth stages of garlic (I to ix) along with selenium application at various time periods. Information related to garlic growth stages was obtained from Meier et al. (2009)*

Determination of selenium by inductively coupled plasma-mass spectrometer using dynamic reaction cell

Shimadzu Sequential type plasma Emission Spectrometer model ICPS-1000 111 and JY 24 spectroanalyzer (ICP-MS) was used for the determination of Se. A glass Meinhard nebulizer and a glass cyclonic spray chamber were used to introduce the sample. Experimental Instrument Conditions were RF Power 1200 watts, plasma gas flow 15 L min^{-1},auxiliary gas flow 1.2 Lmin^{-1}, RPq 0.5, cell gas (O_2)flow rate(DRC) 0.4 Lmin^{-1}. A series of standards containing selenium (0.01 -0.5 mg/L) were prepared from standard stock solution of Se (1000 ± 2mg/L, Merck, Darmstadt, Germany), and used to calibrate instrument. Standard solutions of Selenium with 1.0 to 50 mg L^{-1} were used for quantification. Method validation was performed by analyzing three replicates of artificially spiked garlic powder with a final concentration of 5mg/kg of selenium. A standard deviation of 0.05 mg/kg and coefficient of variation of 2.71% was obtained with a recovery of 97%. The limit of detection (LOD=3SD) as calculated by the Eurachem Guide (Guide, 1998) was 0.15mg/kg. Measurement of uncertainty of the method (K=2) was 0.03.

Phytochemical analysis

An appropriate amount (20g) of garlic foliar mass and bulb powder were separately extracted in 80% ethanol by stirring at 25℃ for 24h in closed vessel system, according to the method described by Peschel et al. (2006) with minor modification. After solvent evaporation under vacuum, extracts were resuspended in DMSO and stored at 4 °C.

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Estimation of total phenolic content (TPC)

Total phenolic content of each treatment (garlic bulb & foliar mass) were determined by using Folin Ciocalteu reagent for color development along with sodium carbonate, by following the method with slight modifications reported by Singleton and Rossi (1965). Absorbance of developed blue coloured complex was taken at 755 nm with a spectrophotometer (Nicolet, Evlution-300, Germany). TPC of extracts was quantified through the standard curve of gallic acid (r^2 = 0.9972). The results are given in mg gallic acid equivalent (GAE)/ 100 g of dry wt.

Estimation of total flavonoid content (TFC)

Total flavonoid content was estimated by using aluminum chloride colorimetric method (Chang et al., 2002). Appropriate quantity (100µl) of each sample extract was taken and mixed with suitable amount of methanol, 10% aluminum chloride and 1 M potassium acetate for development of coloured complex. After 30 minutes of incubation period, absorbance of the developed colour was taken at 415 nm with a spectrophotometer. Quercetin standard curve (r^2 = 0.9985) was used for the quantification of TFC of experimental samples and expressed in mg quercetin equivalent (QE) /100g of dry wt.

In vitro 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

In current study the hydrogen atoms donating capacity of garlic leaves and bulb extracts were determined through DPPH free radical assay (Brand-Williams et al., 1995). Ethanolic solutions of each extract were prepared in the range of 0.02 mg/ml to 0.1 mg/ml, following the mixing of ethanolic dilutions of extract (100 µl each) with DPPH (0.1mM) solution. BHT (Butylated hydroxytoluene) and Ascorbic Acid were used as positive controls. After an incubation period of 30 minutes in the dark at ambient temperature, absorbance of reaction mixtures were taken at 517 nm through UV-spectrophotometer. Finally, duplicate measurements were taken and percentage DPPH radical scavenging ability was calculated by using *Equation 1*.

$$DPPH\ scavenging\ activity(\%) = \left\{(Abs._{(control)} - Abs._{(sample)} / Abs._{(control)}\right\} \times 100 \quad \text{(Eq. 1)}$$

where Abs. (control) was absorbance of DPPH radical + ethanol and Abs. (sample) was absorbance of DPPH radical + sample.

Statistical analysis

All data are presented as mean ± SD. The calculated mean values were based on the data obtained from at least three independent experiments. Two ways Analysis of Variance (Webb et al., 2012) was performed by Graph pad Prism 5 at a confidence interval of 95% to see the significant difference among results (GraphPad Software). Results showing probability value of < 0.05 were considered to be statistically significant.

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Results

Fresh weight yield m^{-2}, dry matter and climatic conditions

Observations were taken for the fresh weight of plantlets both in the case of control set as well as those treated after harvest, which showed that maximum fresh wt. yield of garlic plants was obtained in treatment III (1723 ± 32.12 g/m^2) and minimum in treatment II (1653 ± 25.86 g/m^2) in comparison to control plants (1650 ± 33.82 g/m^2). Present results were non significantly different (P > 0.05) in total fresh weight yield per square meter as shown in *Figure 2*. Dry matter of garlic plants enhanced as the concentration of selenium salt was increased. The highest amount of dry matter was obtained in treatment III (13.49 ± 0.71%) and treatment I (13.01 ± 2.46%) which were non significantly different (P > 0.05) from control (12.12 ± 1.57%) (*Figure 3* and *Table 1*).

Table 1. *ANOVA for fresh weight yield of garlic*

Source of Variation	% of total variation		P value	
Interaction	0.00		1.0000	
t	75.13		0.0045	
p	0.00		1.0000	
Source of Variation	**P value summary**		**Significant?**	
Interaction	ns		No	
t	**		Yes	
p	ns		No	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	0.0000	0.0000	0.0000
t	4	5203	1301	7.553
p	1	0.0000	0.0000	0.0000
Residual	10	1722	172.2	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1696	31.50	-13.74 to 76.74
fresh wt.yeild g/m2	1664	1696	31.50	-13.74 to 76.74
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	31.50	2.400	P > 0.05	ns
fresh wt.yeild g/m2	31.50	2.400	P > 0.05	ns
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1661	-3.000	-48.24 to 42.24
fresh wt.yeild g/m2	1664	1661	-3.000	-48.24 to 42.24
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	-3.000	0.2286	P > 0.05	ns
fresh wt.yeild g/m2	-3.000	0.2286	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1700	36.00	-9.243 to 81.24
fresh wt.yeild g/m2	1664	1700	36.00	-9.243 to 81.24
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	36.00	2.743	P < 0.05	*
fresh wt.yeild g/m2	36.00	2.743	P < 0.05	*
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
fresh wt.yeild g/	1664	1688	23.50	-21.74 to 68.74
fresh wt.yeild g/m2	1664	1688	23.50	-21.74 to 68.74
p	**Difference**	**t**	**P value**	**Summary**
fresh wt.yeild g/m2	23.50	1.791	P > 0.05	ns
fresh wt.yeild g/m2	23.50	1.791	P > 0.05	ns

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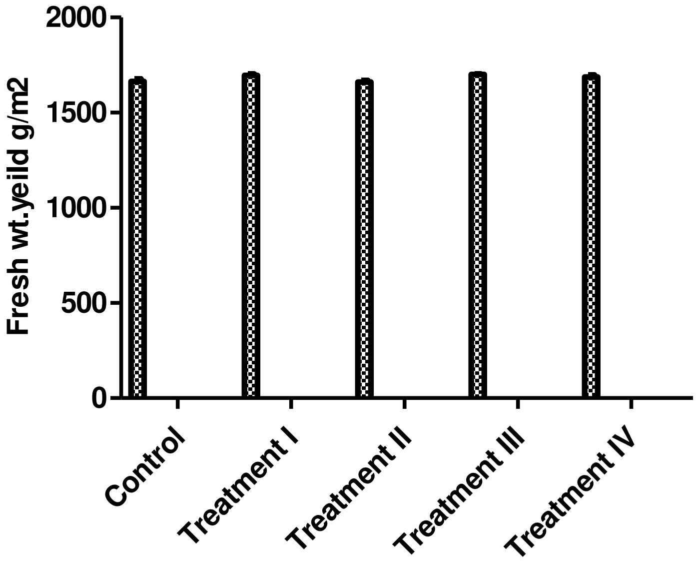

Figure 2. *Total fresh wt. yield of variously treated garlic plants. Error bars indicate standard error of the mean*

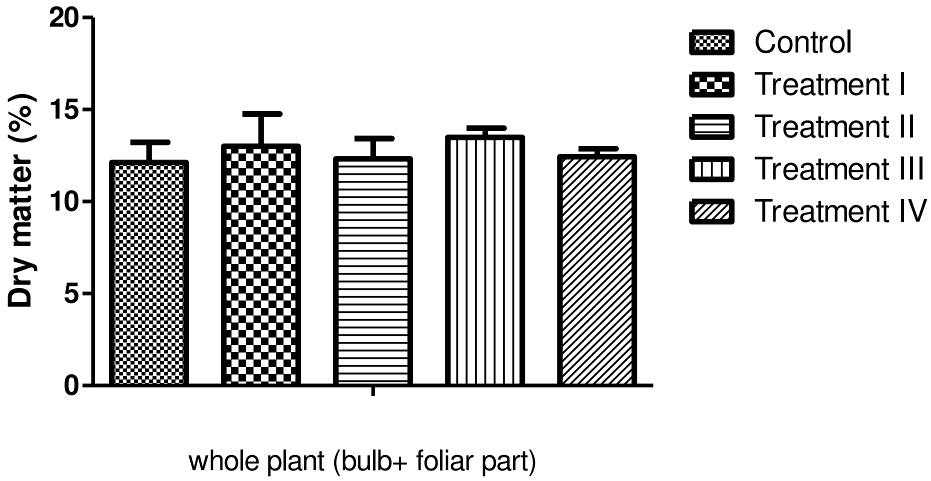

Figure 3. *Dry matter content in percentage for all four treatments and control garlic plant. Error bars indicate standard error of the mean*

Data related to monthly mean temperature (℃) and mean rainfall (mm) from the period of transplanting to harvest was collected from Pakistan Meteorological department (PMD), Lahore, Pakistan. The monthly average rainfall, maximum and minimum temperature for the garlic field location during the whole growing season were 0.62 mm, 29.05℃ and 16.13℃ respectively (*Figure 4*).

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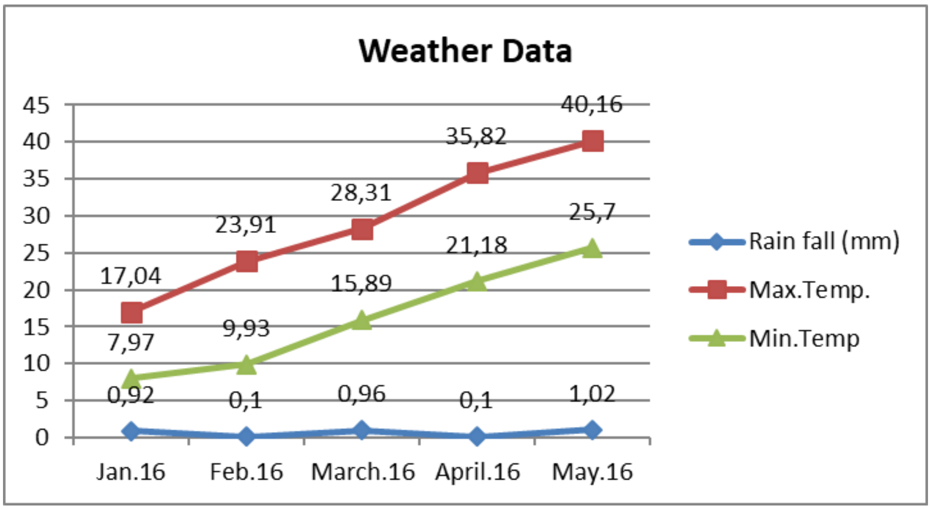

Figure 4. *Monthly average rainfall, maximum and minimum temperature recorded at local weather station during growing season*

Selenium content

Total selenium content of plant samples was determined by ICP-MS. Current results depicted that Se concentration was enhanced with increasing fertilization for all treatments. However, foliar application was found to be most effective in garlic selenium enrichment as compared to soil application (*Figure 5*). The highest average selenium concentration 3.23±0.16mgSeKg^{-1} in bulbs and 15.46±0.71 mgSeKg-^{1} in garlic vegetative part were observed in treatment III in comparison to control plant exhibiting 0.369±0.078 mgSeKg^{-1} in bulbous part and 4.96±0.49 mgSeKg^{-1} in vegetative part, respectively (*Table 2*).

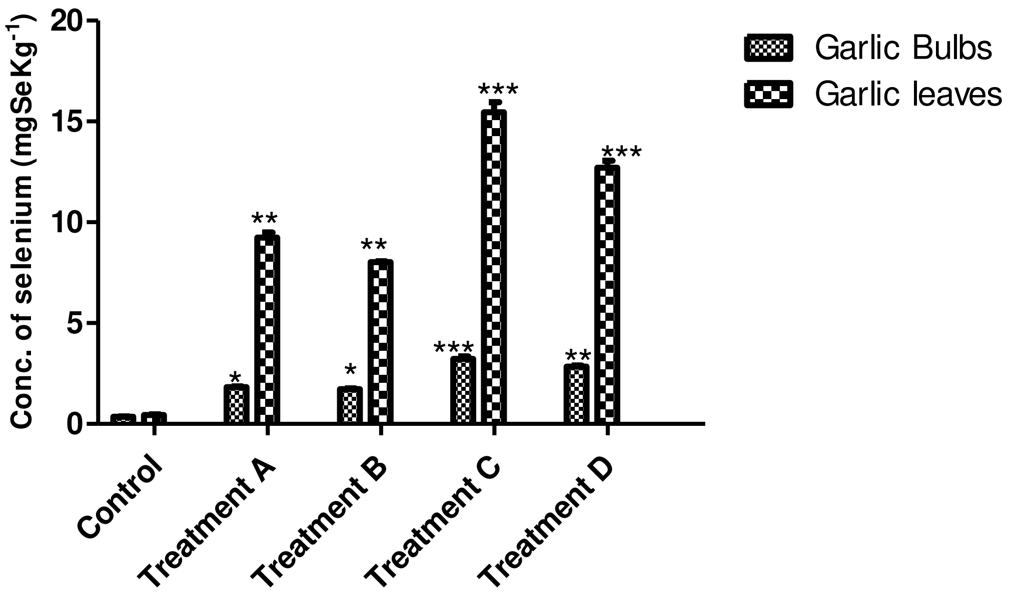

Figure 5. *Selenium concentration in foliar part and bulbs of Allium sativum subjected to four various Se treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Table 2. *ANOVA for selenium estimation in garlic*

Source of Variation	% of total variation		P value	
Interaction	15.89		< 0.0001	
t	48.81		< 0.0001	
p	35.12		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	83.83	20.96	229.1
t	1	257.5	257.5	2815
p	4	185.3	46.32	506.4
Residual	10	0.9146	0.09146	
Number of missing values	30			
Bonferroni posttests				
Garlic Bulbs vs Garlic leaves				
t	**Garlic Bulbs**	**Garlic leaves**	**Difference**	**95% CI of diff.**
Control	0.3550	0.4450	0.0900	-1.121 to 1.301
Treatment A	1.828	9.240	7.413	6.201 to 8.624
Treatment B	1.732	8.020	6.289	5.077 to 7.500
Treatment C	3.225	15.46	12.24	11.02 to 13.45
Treatment D	2.850	12.71	9.855	8.644 to 11.07
t	**Difference**	**t**	**P value**	**Summary**
Control	0.0900	0.2976	P > 0.05	ns
Treatment A	7.413	24.51	P<0.001	***
Treatment B	6.289	20.79	P<0.001	***
Treatment C	12.24	40.46	P<0.001	***
Treatment D	9.855	32.59	P<0.001	***

Total phenolic content (TPC)

Total phenolic content of all treated and control garlic samples exhibited significant results (*Figure 6*). The results showed that higher phenolic content are present in treatment III vegetative part (23.46±2.12 mg GAE/100g dry wt) followed by treatment IV (21.71±0.51mg GAE/100g dry wt), treatment I (19.24±0.35mgGAE/100g dry wt) and treatment II (18.82±1.17 mgGAE/100g dry wt) vegetative part, respectively. Treatment III was more effective for enhancing TPC of garlic bulbs i.e. 4.72±1.79 mgGAE/100g dry wt (1.82 fold increase) in comparison to control containing total phenolic content of 2.59±0.707mgGAE/100g dry wt which are significantly different from each other (*Table 3*).

Table 3. *ANOVA for total phenolic contents in garlic*

Source of Variation	% of total variation		P value	
Interaction	0.39		0.3750	
t	2.53		0.0044	
p	96.26		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	ns		No	
t	**		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	5.872	1.468	1.184
t	4	37.85	9.462	7.635
p	1	1442	1442	1164
Residual	10	12.39	1.239	
Number of missing values	0			

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Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	2.940	0.3500	-3.488 to 4.188
Garlic leaves	18.54	19.24	0.7050	-3.133 to 4.543
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	0.3500	0.3144	P > 0.05	ns
Garlic leaves	0.7050	0.6333	P > 0.05	ns
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	2.640	0.05000	-3.788 to 3.888
Garlic leaves	18.54	18.82	0.2850	-3.553 to 4.123
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	0.05000	0.04491	P > 0.05	ns
Garlic leaves	0.2850	0.2560	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	4.720	2.130	-1.708 to 5.968
Garlic leaves	18.54	23.46	4.925	1.087 to 8.763
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	2.130	1.913	P > 0.05	ns
Garlic leaves	4.925	4.424	P<0.01	**
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
Garlic bulb	2.590	3.950	1.360	-2.478 to 5.198
Garlic leaves	18.54	21.71	3.170	-0.6682 to 7.008
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	1.360	1.222	P > 0.05	ns
Garlic leaves	3.170	2.848	P < 0.05	*

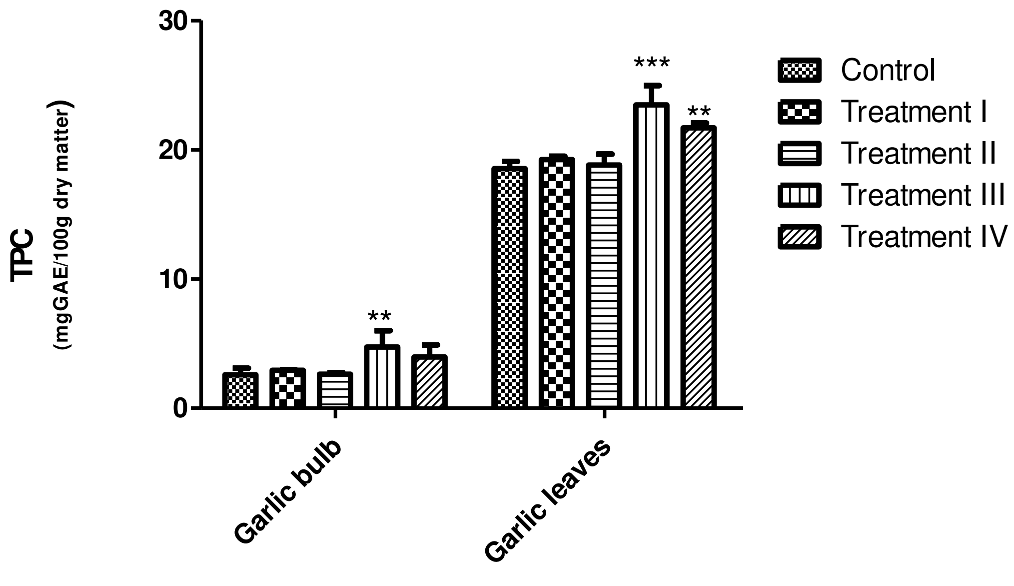

Figure 6. *Estimation of total phenolic content in foliar part and bulbs of Allium sativum subjected to four various Se treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Total flavonoid content (TFC)

Significantly (P > 0.05) elevated amount of total flavonoid content were observed in *Allium sativum* leaves and bulb (*Figure 7*). Current results depicted that total Flavonoid content of treated *A. sativum* bulbs and leaves were in the range of 18.19 ± 1.21 to 18.50 ± 1.82 mgQE/100g dry wt and 34.13 ± 1.36 to 34.99 ± 1.54 mgQE/100g dry wt, respectively in comparison to control bulb (11.32 ± 0.95 mgQE/100g dry) and leaves (13.53 ± 0.76 mgQE/100g dry wt. as in *Table 4*).

Table 4. *ANOVA for total flavonoid contents in garlic*

Source of Variation	% of total variation		P value	
Interaction	9.20		< 0.0001	
t	37.09		< 0.0001	
p	52.77		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	4	155.9	38.99	24.46
t	4	628.3	157.1	98.56
p	1	894.1	894.1	561.0
Residual	10	15.94	1.594	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.43	7.115	2.762 to 11.47
Garlic leaves	13.53	34.24	20.71	16.36 to 25.06
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	7.115	5.636	P<0.001	***
Garlic leaves	20.71	16.40	P<0.001	***
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.20	6.880	2.527 to 11.23
Garlic leaves	13.53	34.13	20.60	16.25 to 24.95
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	6.880	5.450	P<0.001	***
Garlic leaves	20.60	16.32	P<0.001	***
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.50	7.185	2.832 to 11.54
Garlic leaves	13.53	34.99	21.46	17.11 to 25.81
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	7.185	5.691	P<0.001	***
Garlic leaves	21.46	17.00	P<0.001	***
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
Garlic bulb	11.32	18.26	6.945	2.592 to 11.30
Garlic leaves	13.53	34.67	21.14	16.79 to 25.49
p	**Difference**	**t**	**P value**	**Summary**
Garlic bulb	6.945	5.501	P<0.001	***
Garlic leaves	21.14	16.75	P<0.001	***

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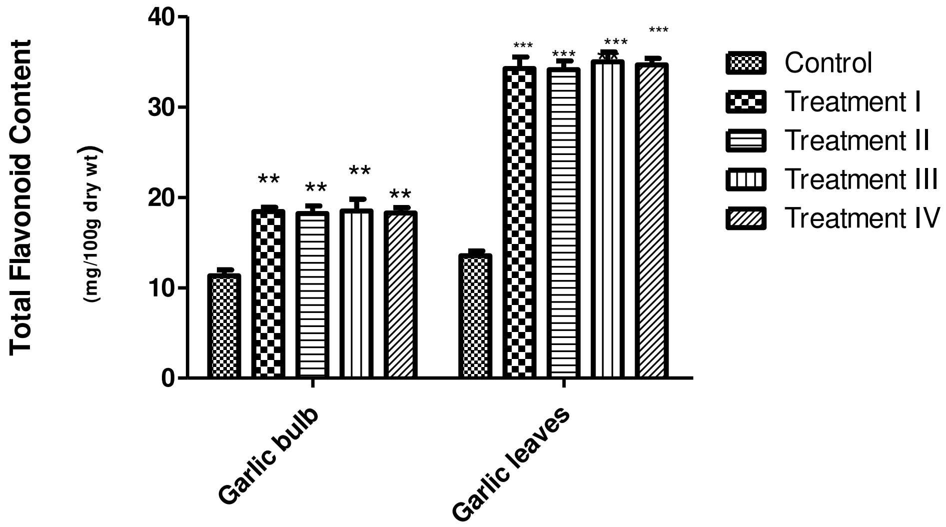

Figure 7. *Total flavonoid content in garlic bulbs and leaves subjected to four various treatments. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

In vitro antioxidant activity

The percentage DPPH radical scavenging capacity of garlic extracts with different treatments as well as controls is depicted in *Figure 8*. The IC_{50} values (sample concentration needed to scavenge 50% of DPPH dye) of all garlic extracts as well as both standards (Vitamin C and BHT) were calculated by linear regression of plots. The lowest IC_{50} indicates the higher antiradical activity of extract. Garlic extract of treatment III had an IC_{50} of 0.81 mg/ml, followed by treatment IV (0.82 mg/ml), treatment I (0.84 mg/ml) and treatment II (0.898 mg/ml). The IC_{50} value of non treated (control) garlic extract was 0.97 mg/ml. Of the two positive controls, BHT had the lowest IC_{50} (0.50 mg/ml) than vitamin C (0.51 mg/ml) but both showed significantly (P< 0.05) higher DPPH scavenging activity than treated garlic extracts (*Table 5*).

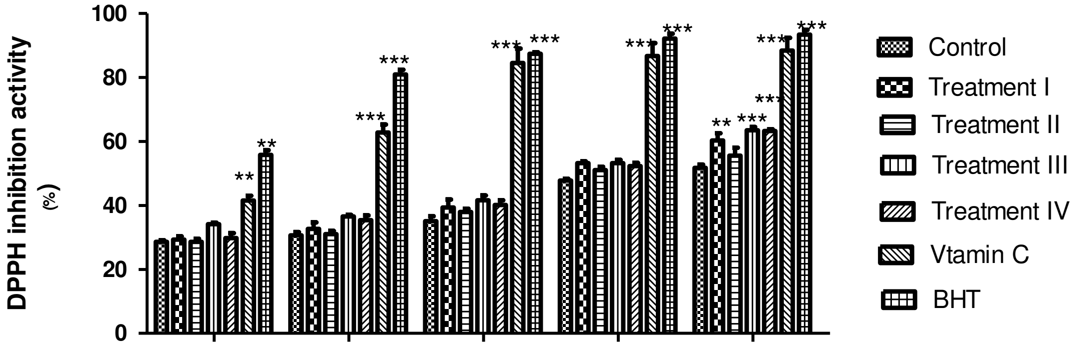

Figure 8. *DPPH radical scavenging activity of treated Allium Sativum bulbous part extracts, synthetic antioxidant BHT and Vitamin C in various concentrations. Error bars indicate standard error of the mean. (*) significantly different at P<0.05. (**) significantly different at P<0.01. (***) significantly different at P<0.001*

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Table 5. *ANOVA for antioxidant activity of garlic*

Source of Variation	% of total variation		P value	
Interaction	5.22		< 0.0001	
t	60.99		< 0.0001	
p	33.02		< 0.0001	
Source of Variation	**P value summary**		**Significant?**	
Interaction	***		Yes	
t	***		Yes	
p	***		Yes	
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	24	1547	64.46	9.849
t	6	18080	3013	460.3
p	4	9786	2447	373.8
Residual	35	229.1	6.545	
Number of missing values	0			
Bonferroni posttests				
Control vs Treatment I				
p	**Control**	**Treatment I**	**Difference**	**95% CI of diff.**
0.2mg	28.59	29.32	0.7300	-7.986 to 9.446
0.4mg	30.64	32.66	2.020	-6.696 to 10.74
0.6mg	35.11	39.39	4.280	-4.436 to 13.00
0.8mg	47.81	53.27	5.460	-3.256 to 14.18
1.0mg	51.74	60.30	8.560	-0.1557 to 17.28
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	0.7300	0.2853	P > 0.05	ns
0.4mg	2.020	0.7896	P > 0.05	ns
0.6mg	4.280	1.673	P > 0.05	ns
0.8mg	5.460	2.134	P > 0.05	ns
1.0mg	8.560	3.346	P<0.01	**
Control vs Treatment II				
p	**Control**	**Treatment II**	**Difference**	**95% CI of diff.**
0.2mg	28.59	28.59	0.0000	-8.716 to 8.716
0.4mg	30.64	31.05	0.4100	-8.306 to 9.126
0.6mg	35.11	37.99	2.880	-5.836 to 11.60
0.8mg	47.81	51.07	3.260	-5.456 to 11.98
1.0mg	51.74	55.58	3.840	-4.876 to 12.56
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	0.0000	0.0000	P > 0.05	ns
0.4mg	0.4100	0.1603	P > 0.05	ns
0.6mg	2.880	1.126	P > 0.05	ns
0.8mg	3.260	1.274	P > 0.05	ns
1.0mg	3.840	1.501	P > 0.05	ns
Control vs Treatment III				
p	**Control**	**Treatment III**	**Difference**	**95% CI of diff.**
0.2mg	28.59	34.14	5.550	-3.166 to 14.27
0.4mg	30.64	36.57	5.930	-2.786 to 14.65
0.6mg	35.11	41.63	6.520	-2.196 to 15.24
0.8mg	47.81	53.22	5.410	-3.306 to 14.13
1.0mg	51.74	63.55	11.81	3.094 to 20.53
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	5.550	2.169	P > 0.05	ns
0.4mg	5.930	2.318	P > 0.05	ns
0.6mg	6.520	2.549	P > 0.05	ns
0.8mg	5.410	2.115	P > 0.05	ns
1.0mg	11.81	4.616	P<0.001	***
Control vs Treatment IV				
p	**Control**	**Treatment IV**	**Difference**	**95% CI of diff.**
0.2mg	28.59	29.82	1.230	-7.486 to 9.946
0.4mg	30.64	35.41	4.770	-3.946 to 13.49
0.6mg	35.11	40.16	5.050	-3.666 to 13.77
0.8mg	47.81	52.32	4.510	-4.206 to 13.23
1.0mg	51.74	63.31	11.57	2.854 to 20.29

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DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

p	Difference	t	P value	Summary
0.2mg	1.230	0.4808	P > 0.05	ns
0.4mg	4.770	1.864	P > 0.05	ns
0.6mg	5.050	1.974	P > 0.05	ns
0.8mg	4.510	1.763	P > 0.05	ns
1.0mg	11.57	4.522	P<0.001	***
Control vs Vtamin C				
p	**Control**	**Vtamin C**	**Difference**	**95% CI of diff.**
0.2mg	28.59	41.52	12.93	4.214 to 21.65
0.4mg	30.64	62.84	32.20	23.48 to 40.92
0.6mg	35.11	84.55	49.44	40.72 to 58.16
0.8mg	47.81	86.76	38.95	30.23 to 47.67
1.0mg	51.74	88.43	36.69	27.97 to 45.41
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	12.93	5.054	P<0.001	***
0.4mg	32.20	12.59	P<0.001	***
0.6mg	49.44	19.32	P<0.001	***
0.8mg	38.95	15.22	P<0.001	***
1.0mg	36.69	14.34	P<0.001	***
Control vs BHT				
p	**Control**	**BHT**	**Difference**	**95% CI of diff.**
0.2mg	28.59	55.80	27.21	18.49 to 35.93
0.4mg	30.64	80.92	50.28	41.56 to 59.00
0.6mg	35.11	87.40	52.29	43.57 to 61.01
0.8mg	47.81	92.20	44.39	35.67 to 53.11
1.0mg	51.74	93.40	41.66	32.94 to 50.38
p	**Difference**	**t**	**P value**	**Summary**
0.2mg	27.21	10.64	P<0.001	***
0.4mg	50.28	19.65	P<0.001	***
0.6mg	52.29	20.44	P<0.001	***
0.8mg	44.39	17.35	P<0.001	***
1.0mg	41.66	16.28	P<0.001	***

Discussion

Micronutrients malnutrition is the insufficient availability of essential dietary microminerals to the population that will negatively impact the health of people and increase the risk of diseases (El-Ramady et al., 2015). Improvement of selected nutrients such as selenium in plants edible part through the process of biofortification will increase the nutritional value of food (Hirschi, 2008) which is proved through findings of current results that selenium concentration and polyphenolic content of selected garlic cultivar was enhanced through biofortification. Present results indicated that there is no considerable difference in fresh wt yield of treated and control sets of garlic plants. These results are in consistent with the findings of Põldma et al. (2013), who reported that effects of selenium treatment on yield of onion bulb (*Allium cepa* L.) was not significant and at Se50 (50µg/ml) there was no reduction in bulb size as compared to Se100 (100µg/ml). However, these observations are contradictory to Yadav et al. (2007) who reported that leaves and bulbs of *Allium cepa* were reduced in size at high concentration of 50µg/g Se spiked soil. High concentration of selenium (50g/ha) foliar application enhances the dry matter content of the whole plant. Current findings showed that on increasing the concentration of available selenium salt (Na_2SeO_4), accumulation of selenium content was increased in garlic plants. Foliar application of 20mgSem^{-2} and 50mgSem^{-2} to garlic plants resulted in 7.8 and 12.52 fold increase of selenium content in garlic bulbs as compared to control. Similarly, 3.52 fold increase of selenium content was observed in vegetative part of garlic plants on 50mgSem^{-2} foliar spray (*Figure 5*), which could be used as fodder for animals to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

improve their nutritional value regarding selenium content.Hegedűsová et al. (2017) reported that foliar application of selenium salt to Ambassdor pea variety at two concentrations i.e. 5mgSe/m^2 and 10mgSe/m^2 resulted in 25.4 and 49.1 fold enhancement of selenium content, depending on applied doses. Similar observations were reported by Yadav et al. (2007) that selenium accumulation in tissues of *Allium cepa* was improved from 278 to 1248.8 µg/g along with increasing Se concentration from 25µg/g to 50µg/g of soil, respectively. Whanger et al. (2000) was also reported that selenium uptake of *Allium tricoccum* was enhanced with increasing concentration of available Selenium, despite the nature of experimental media including peatmoss (I), vermiculite and hydroponics (III). Seleniferous plants has the potential to mobilize inorganic form of selenium from soil, and to accumulate it in the biomass in organic form making it more bioavailable to animals and human beings, which is proved by the study of Yan and Johnson (2011). Due to this inherited ability of the crop plants belonging to Allium family, they can be grown in those geographical areas that naturally enriched with selenium loaded soil, to do the work of phytoremediation. Hasanuzzaman et al. (2010) reported that selenium accumulators have the ability to accumulate 4000mg/kg selenium without exhibiting signs of toxicity in comparison to non seleniferous plants like rice, which showed 10% yield reduction on selenium threshold level of 2mg/kg in shoot tissues. Thus biofortification can be indirectly linked with phytoremediation (Yadav et al., 2007). These selenium biofortified garlic can be exported as food commodity in those specific areas of the world such as China (Tan et al., 2002) that naturally deficient for selenium. Daily intake portion (80g) of selenium biofortified rice for 20days can significantly increase the serum selenium level, which is confirmed by Giacosa et al. (2014). Based on the results shown in *Figure 5*, it can be assumed that daily intake of 16g of dried garlic bulb procured in treatment III can cover the daily recommended dose (40ug to 50ug for adults) of selenium (Burk et al., 2003).

Food enriched with polyphenolic compounds such as phenolics, flavonols and flavonoids have been reported to exhibit strong antioxidant activities which protects the cells from damaging effects of free radicals and reduces the risk of chronic diseases (Otunola and Afolayan, 2013).In this study, selenium accumulation in garlic biomass enhances the nutritional value and antioxidant capacity of garlic plant. Significant value of phenolic content that is 4.72 mgGAE/100 g dry wt of garlic was observed in the present study. Beato et al. (2011) had reported that the total phenolic content in four garlic cultivars varied from 3.4 mg GAE/100 g dry wt to 10.8 mg GAE/100 g dry wt with a mean value of 6.5 mg GAE/100 g dry wt grown at Andalusia, Spain. They reported ferulic acid and caffeic acid were the major polyphenols present in garlic with mean values of 2.6 and 2.9 mg/kg of dry matter, respectively. In the present study, considerable amount of total flavonoid content (TFC) were observed in garlic extracts of all treatments depending on the amount of available selenium in comparison to control plant. Higher value of TFC of garlic extract (18.50 ± 1.82 mgQE/100 g dry wt) in treatment III could be related to increased concentration of available selenium salt (50g/ha). Stable DPPH free radical scavenging assay is a commonly used method for the estimation of free radical scavenging ability of various compounds (Ghasemi et al., 2015). In the current study, results showed that there was a significant ($P< 0.05$) increase in the scavenging ability of DPPH-radical as dose of garlic extract increased (*Figure 8*). This trend is similar to results of Park et al. (2009) who accounted that garlic extracts exhibited remarkable scavenging properties by reducing stable radical DPPH to yellow colored diphenyl picrylhydrazine. This could be due to the hydrogen donating

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

ability of various vegetable extracts from their phenolic hydroxyl groups. Previously, Velioglu et al. (1998) reported a considerable association between phenolic content and antioxidant activity of various fruits, cereals and vegetable extracts. Kavalcová et al. (2014) reported statistically considerable value of antioxidant activity (4.05% to 5.07%) in association with polyphenolic content (260 t0 279 mg/Kg) in garlic samples collected from Pruzina, Strazov. Similarly, experimental garlic bulb obtained in treatment III exhibited a significant relation between higher value of polyphenolic content (2.59 to 4.72mg/100g) and total antioxidant capacity (93.75±1.54%).

Conclusion

The present study revealed that garlic selenium content was increased through biofortification process in field conditions. The process of selenium fertilization through foliar spray was more effective than soil irrigation and positive for all biochemical parameters analyzed. High polyphenolic content and antioxidant properties were observed in biofortified garlic in concordant with high selenium content which could be used as a powerful source of natural antioxidants along with selenium to combat hidden hunger of micronutrient. Further field experiments conducted in the present study will shade a new light to improve selenium content in other seleniferous crops which could be valuable considering agronomic and human health benefits.

Acknowledgements. The authors are grateful to the management of PCSIR labs complex Lahore for financial assistance.

Conflict of interests. Authors declare no conflict of interests.

Geographic information. Field experiments were conducted at botanical garden of PCSIR Labs Complex, Lahore, Pakistan. The experimental site is situated between 31.52° North latitude, 74.33° East longitude and altitude of 217 m above the sea level.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_16851704

SHORT TERM SOIL AND VEGETATION RECOVERY AFTER *ACACIA MEARNSII* REMOVAL IN VHEMBE BIOSPHERE RESERVE, SOUTH AFRICA

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(Received 12th Sep 2018; accepted 26th Nov 2018)

Abstract. Short term monitoring of soil and vegetation recovery following alien plant removal is required to reveal how ecological restoration is progressing. This study examined the recovery of soil physical properties and vegetation following *Acacia mearnsii* removal at Zvakanaka farm in Limpopo Province, South Africa. Soil and vegetation measurements were conducted in paired cleared, invaded and natural sites on 10 x 10 m plots. Results of the study show significantly ($P < 0.001$) higher soil moisture content in invaded and natural compared to cleared sites. Soil penetration was significantly ($P < 0.001$) higher in cleared than invaded and natural sites. Both infiltration rate and hydraulic conductivity showed no significant ($P > 0.05$) difference between the three sites. Strongly repellent soils were recorded in cleared sites only. Results showed a significant ($P < 0.05$) increase in measured diversity indices (species richness, Shannon-Wiener, Simpson's and evenness index) in cleared and natural than in invaded sites. However, most secondary woody invasive alien plants were recorded in cleared sites. The study concludes that *A. mearnsii* clearing triggers varying changes in soil physical properties. Although native plants are present in cleared sites, recovery may be hampered by the growth of secondary woody invasive alien plants.
Keywords: *post-clearing monitoring, invasive plants, ecosystem repair, revegetation, secondary invaders*

Introduction

Invasion by Australian *Acacia* species in South Africa has resulted in biodiversity loss (Werner et al., 2010; Gaertner et al., 2011), altered ecosystem functioning and service provisioning (Kull et al., 2008; Le Maitre et al., 2011). *Acacias* are known to stimulate changes in soil communities (Yelenik et al., 2004; Gaertner et al., 2011) which enables them to dominate native species, resulting in native species displacement (Le Maitre et al., 2011). Negative impacts of *Acacia* invasion on biodiversity and ecosystems ultimately affect human well-being (Le Maitre et al., 2011). For example, stands of *A. mearnsii* invasion in South Africa have been shown to utilize more water compared to fynbos biome native vegetation (Dye et al., 2001; Dye and Jarmain, 2004), this resulting in water reduction for agriculture, industry, recreation, conservation and domestic use (Görgens and van Wilgen, 2004). Also, the high biomass of *Acacia* species has been known to increase fire severity (van Wilgen and Richardson, 1985), thus not only affecting re-sprouting native plants but also housing properties.

Management interventions to address the impacts of *Acacia* invasions, and may other invasive plants, are underway in South Africa (Le Maitre et al., 2011). The Working for

Water (WfW) programme, a government initiative to manage and control invasives, has adopted a passive restoration approach which aims to remove the invader as well as limit and prevent their regeneration (Le Maitre et al., 2002; Esler et al., 2008; van Wilgen et al., 2012). However, the passive restoration approach by WfW has yielded mixed results when it comes to soil and vegetation recovery (Galatowitsch and Richardson, 2005; Blanchard and Holmes, 2008; Pretorius et al., 2008; Ruwanza et al., 2013a; Nsikani et al., 2017; Fill et al., 2018). As a result, there is a need to improve understanding of factors that hinder or facilitate soil and vegetation recovery after alien plant clearing.

For ecological restoration to be successful, periodic monitoring of cleared areas is required to reveal how restoration is progressing and were management interventions are required (Ruiz-Jaen and Aide, 2005; Fill et al., 2018). However, a complicating factor in monitoring ecological restoration projects is the defining of appropriate variables to be measured (Ruiz-Jaen and Aide, 2005). In cases were the restoration goal is to return natural vegetation structure, function and processes to reference condition, monitoring native vegetation diversity and soil processes e.g. physical (soil structure), chemical (soil nutrients) and biological (soil bacteria) becomes important (Ruiz-Jaen and Aide, 2005; Wortley et al., 2013). Unfortunately, both short and long-term monitoring in most WfW alien clearing projects is rarely done (Fill et al., 2018).

Methodological constraints associated with monitoring biological invasions have been reported in the past (Stricker et al., 2015). Although most observational studies compare differences between invaded, uninvaded and cleared areas (Fill et al., 2018), a more appropriate method is to observe changes before and after invasion (Stricker et al., 2015). The challenges associated with before and after observational experiments is time, given that invasion could have occurred several years ago, and observations prior-invasion might not have been done. Observing changes between invaded, uninvaded and cleared areas does not allow deduction on causation to be made, because observed differences may be driven by other ecosystem processes and not necessarily by invasive plants (MacDougall and Turkington, 2005; Guido and Pillar, 2015; Guido and Pillar, 2017). Despite these methodological limitations, observing invaded, natural and cleared areas can assist in generalizing recovery patterns that can be used to inform recovery trajectories. This study present findings on soil and vegetation recovery post *A. mearnsii* removal. The goal is to assess changes in both soil physical properties and native vegetation diversity after *A. mearnsii* clearing, to provide a picture of ecological recovery. Results will guide future ecological restoration initiatives following *A. mearnsii* removal.

Materials and methods

Study area and site identification

The study area was Zvakanaka farm (22°97'72.23"S and 29°95'30.90"E; *Fig. 1*), some 10 km outside Louis Trichardt in Limpopo Province, South Africa. The farm is used for tourism purposes, with a few guest houses and camp sites. Vegetation in the farm is classified as both Soutpansberg Summit Sourveld and Soutpansberg Mountain Bushveld by Mucina and Rutherford (2006). Soils in the study area are derived from shale and siltstones of the Soutpansberg group (Mucina and Rutherford, 2006). They are generally shallow and drain quickly leading to leached and acidic soils. Average annual rainfall is between 450 and 900 mm, with most rain falling in summer between October

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and March. Temperature ranges from approximately 5°C in winter to 35°C in summer (Mucina and Rutherford, 2006).

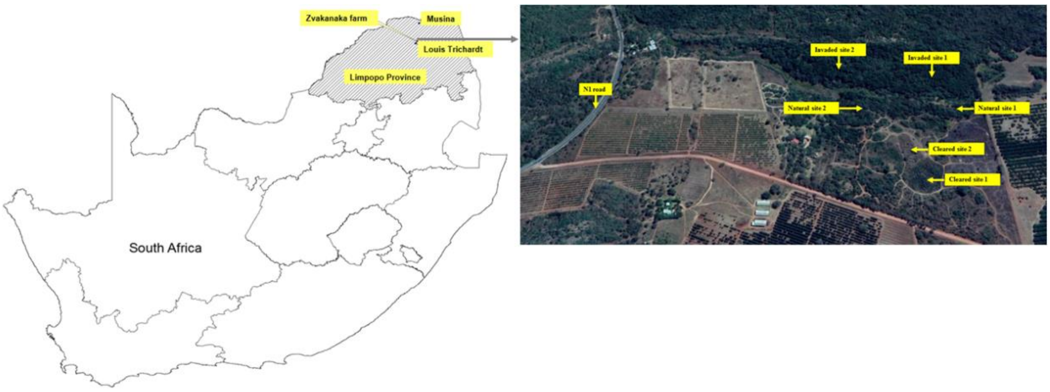

Figure 1. *The location of the study area in Zvakanaka farm located outside Louis Trichardt in the Limpopo province of South Africa*

Within Zvakanaka farm three invasion conditions (approximately 2 km apart), namely cleared, natural and invaded were identified (*Table 1*). Two sites were setup at each of the above-mentioned invasion condition (paired sites were approximately 50 m apart). Due to the small size of the cleared area, only two sites were possible in the cleared area, therefore the experimental design was limited to site pairing per each of the above-mentioned invasion condition.

Table 1. *Characteristics of the study area showing the three invasion conditions namely cleared, invaded, and natural sites. Each site's Universal Transverse Mercator coordinate location is shown*

Invasion condition	Site name	Coordinates	Site characteristics
Cleared site	CS 1	22°58'47.02"S, 29°57'25.77"E	- Cleared of *A. mearnsii* and other woody invasive in early 2016 - Follow-up clearing treatment in progress
	CS 2	22°58'46.86"S, 29°57'21.37"E	
Natural site	NS 1	22°58'38.59"S, 29°57'21.70"E	- Dominated by stands of native species - Canopy cover > 60%
	NS 2	22°58'38.32"S, 29°57'17.36"E	
Invaded site	IS 1	22°58'32.64"S, 29°57'26.08"E	- Invaded by huge stands of *A. mearnsii* with little underground vegetation - Canopy cover > 60%
	IS 2	22°58'32.43"S, 29°57'20.54"E	

Cleared sites had *A. mearnsii* (and any other existing woody invasives) removed by WfW in early 2016 (Maytham, 2017, personal communication). Clearing by WfW involved the felling of alien trees and herbicide application on cut stumps to prevent re-growth. Cleared plant material were stack burnt on site. The cleared area had received only one follow-up treatment (meant to remove all alien plant saplings) since the initial clearing was conducted. No soil or vegetation surveys were conducted before WfW clearing, therefore, the condition of the cleared areas prior both invasion and clearing is

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unknown. Close to the cleared site is a mature stand of *A. mearnsii* that is yet to be cleared. The stand which is dominated by *A. mearnsii* represented the invaded site. In-between the cleared and invaded sites, dense stands of native species exist, and these acted as the natural reference sites. Therefore, the three invasion conditions comprised two cleared sites where *A. mearnsii* was removed in 2016, two invaded sites where *A. mearnsii* dominate (canopy cover above 60%), and two natural sites where stands of native species dominate (canopy cover above 60%).

Survey design and field sampling

In winter 2017, five randomly distributed replicated plots, each measures 10 m x 10 m, were set-up on each of the above-mentioned site. A total of 30 plots were setup (5 plots x 2 sites x 3 invasion conditions). Within each plot soil and vegetation surveys were conducted. Soil cores (30 in total) measuring 10 cm in diameter and 10 cm depth were collected from the center of each plot for gravimetric soil moisture and soil water repellency measurements which were conducted under laboratory conditions at the University of Venda. Before the above-mentioned laboratory measurements were conducted, soils were first sieved using a 2-mm sieve to remove debris. Gravimetric soil moisture (expressed as a percentage) was assessed by weighing wet soils, dry them in an oven at 105°C for 72 hours, then re-weighing them to obtain the water content (Black, 1965). The Water Droplet Penetration Time (WDPT) method was used to measure soil water repellency (Doerr and Thomas, 2000). Sieved soils were air dried for seven days under laboratory conditions (temperature 18±2°C which is the average Thohoyandou winter temperatures) before being set into petri dishes and levelled. The WDPT test was conducted by placing five water droplets on the soil surface and record the time taken for the water droplet to penetrate the soil (Doerr and Thomas, 2000). The penetration time was averaged to represent the WDPT for each soil sample. Soils were classified based on repellecy classes suggested by Bisdom et al. (1993) (see *Table 2*).

Table 2. *Classification of soil water repellency based on the The Water Droplet Penetration Time (WDPT) method*

Classification	The Water Droplet Penetration Time (in seconds)
Non-repellent	< 5
Slightly water repellent	6 - 60
Strongly water repellent	61 - 600
Severely water repellent	601 - 3600
Extremely water repellent	> 3 601

Soil penetration resistance levels and infiltration rates were measured under field conditions 30 cm away from soil collection points at the center of each plot. Soil penetration resistance levels were measured using a pocket penetrometer (SOILTEST, Inc., Evanston, Illinois, USA). To take measurements, the penetrometer is pushed into the soil and a metal ring is pushed up to mark the resistance value in kg cm^{-2} (Leung and Meyer, 2003). Infiltration rate and hydraulic conductivity in the soil were measured with a mini disk infiltrometer (Decagon Devices, Pullman, WA, USA). The infiltrometer is an acrylic tube with a semipermeable plastic disk, a suction tube inside, and a rubber stopper (Latorre et al., 2013). Suction rate was set at 2.0 cm in this study. Both the upper and lower chambers were filled with water before taking measurements

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on flat soil surfaces following the hand removal of litter. The water infiltration rate was measured from the drop of water level in the lower chamber in mL after every 30-s interval for 5 minutes. The cumulative infiltration rate over time was determined using the method suggested by Zhang (1997). Similarly, hydraulic conductivity was calculated from the infiltration data using the van Genuchten-Zhang method (Zhang, 1997, 1998). For more information see the mini disk infiltrometer manual which can be found at Decagon Devices (2014).

A detailed vegetation survey was conducted in all plots. Richness of trees and shrubs was determined from counts of the total individual plant species in the plot. Richness of herbs and graminoids was determined from counts of all the individuals in a 1 m^2 quadrat placed at the center of the plot. All recognized plant species in the plot were collected for identification at University of Venda herbarium in the Department of Botany. Plant species were assigned to four broad growth form classes based on morphology and height. The four-growth form used in this study are trees, shrubs, forbs and grasses, as described by Goldblatt and Manning (2000).

Data analysis

To avoid pseudo-replication, soil and vegetation results from the ten plots per invasion condition (two sites x five plot replicates) were averaged. Gravimetric soil moisture, soil penetration resistance levels, infiltration rates**,** and hydraulic conductivity for the different sites, were analysed using one-way ANOVA in Statistica version 13.1 (Statsoft Inc, 2016). For each site, species richness, Shannon-Wiener diversity index (H'), Simpson's index of diversity and Evenness index (J) using Pielou's 'J' (Zar, 1996) were calculated per plot and used to examine the effects of alien plant clearing on species diversity. The effects of the above-mentioned diversity indices on invesion conditions were compared using one-way ANOVA in Statistica. Proof of normality and homogeneity of varience were tested using Kolmogorov-Smirnov tests and Levene test respectively. Data was normally distributed and where ANOVAs were significant, Tukey's HSD unequal n test was used to determine differences between sites. Soil water repellency classes were analyzed using the Chi-squared test. Plant species occupancy frequencies, which is the number of species occupying different plots per site independent of their abundance, were calculated as a percentage for all the identified species at each site (presented as *Appendix*).

Results

Comparisons between the three sites showed significant ($P < 0.001$) differences in gravimetric soil moisture levels (*Fig. 2A*). Soil moisture levels were higher in invaded and natural sites than in cleared sites. Contrary, soil penetration resistance levels were significantly ($P < 0.001$) higher in cleared sites than in the invaded and natural sites (*Fig. 2B*). The average infiltration rate in the cleared and natural sites was 3.2 ± 0.58 cm and 3.2 ± 1.11 cm respectively after 5 minutes, compared to 2.0 ± 1.26 cm in the invaded sites. However, the above-mentioned results on infiltration rates showed no significant ($P > 0.05$) differences between the three sites (*Fig. 2C*). Similarly, soil hydraulic conductivity showed no significant differences between the three sites (*Fig. 2D*).

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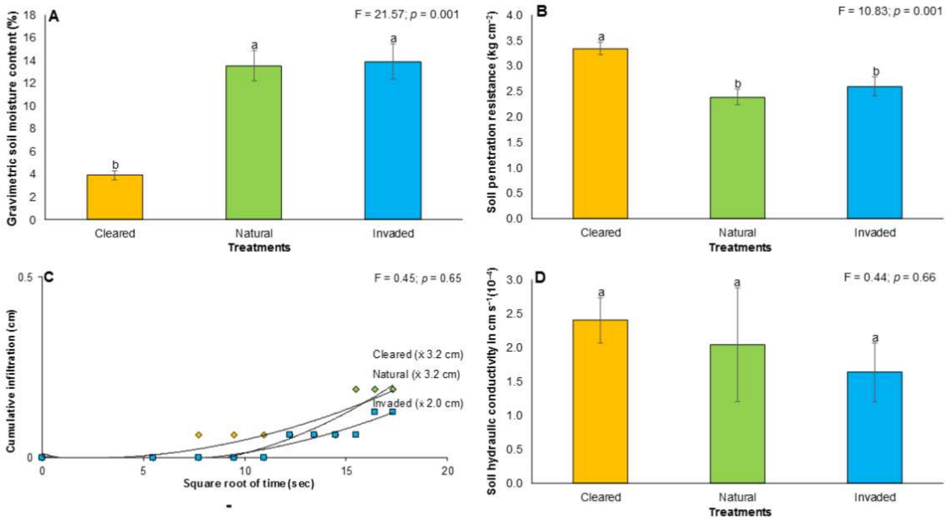

Figure 2. *Results show (A) gravimetric soil moisture content (%), (B) soil penetration resistance levels (C) cumulative infiltration and (D) hydraulic conductivity in soil samples taken from cleared, natural and invaded sites. Bars represent mean ± se and ANOVA results are shown. Bars with different superscripts are significantly different at p < 0.05*

The chi-squared analysis of WDPT classes showed no significant (*P > 0.05*) differences between the three sites (*Fig. 3*). The above-mentioned result is because a greater percentage (80%) of soils in all the three sites were slightly repellent. The remaining 20% in cleared sites were strongly repellent, whereas in the natural and invaded sites the remaining 20% of collected soils were wettable (*Fig. 3*).

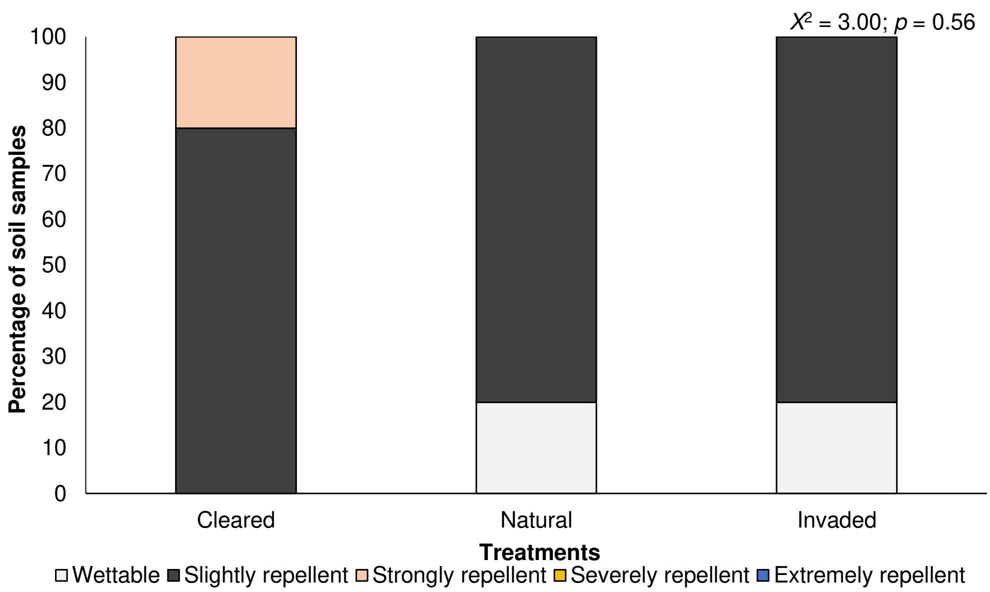

Figure 3. *Distribution of water repellency classes (based on the Water Droplet Penetration Time (WDPT) method) in soil samples taken from cleared, natural and invaded sites. Chi-squared analysis results are shown*

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A total of 42 plant species were identified of which 27 were trees and shrubs, six were herbs and nine were grasses (*Appendix*). Cleared sites recorded a higher occurrences of woody alien invasive plants compared to natural and invaded sites. The most commonly occurring woody invasive alien plants in the cleared sites were *A. mearnsii*, *Lantana camara*, *Psidium guajava*, *Solanum mauritianum*, *Eucalyptus spp.* and *Rubus rigidus*. Species richness showed significant ($P < 0.001$) differences between all the three sites (*Table 3*). Higher species richness was recorded in cleared sites compared to natural and invaded sites. Comparisons on species richness per growth form showed significant ($P < 0.05$) differences between all three sites for all the growth forms (*Table 3*). Trees, shrubs and herbs were higher in cleared and natural sites compared to invaded sites, whereas grasses were higher in cleared sites than in natural and invaded sites. Both Shannon-Wiener and Simpson's index of diversity were significantly ($P < 0.01$) higher in cleared and natural sites compared to invaded sites (*Table 3*). Evenness index showed no significant ($P > 0.05$) differences between all the three sites.

Table 3. *Comparison of indices of diversity between cleared, natural and invaded sites. Data are means ± se and One-ANOVA results are shown*

	Cleared	**Natural**	**Invaded**	**One-way ANOVA**	
				F - values	***P* - values**
Species Richness	23.20 ± 1.83^a	16.20 ± 1.83^b	10.20 ± 1.02^b	16.45	0.001
Shannon-Wiener	2.40 ± 0.07^a	2.29 ± 0.12^a	1.71 ± 0.17^b	12.14	0.001
Simpson's index of diversity	0.87 ± 0.01^a	0.87 ± 0.02^a	0.75 ± 0.03^b	10.37	0.002
Evenness index	0.77 ± 0.02^a	0.83 ± 0.02^a	0.74 ± 0.03^a	3.73	0.06
Species richness per growth form					
Richness of trees and shrubs	12.20 ± 1.16^a	9.20 ± 0.80^a	6.40 ± 0.75^b	9.94	0.003
Richness of herbs	5.00 ± 0.89^a	4.20 ± 0.37^a	2.40 ± 0.25^b	5.32	0.02
Richness of grasses	6.00 ± 1.05^a	2.80 ± 0.74^b	1.40 ± 0.25^b	9.82	0.003

Discussion

The removal of *A. mearnsii* has triggered varied changes in soil physical properties, decreased soil moisture content, increased soil compaction, and intensifying soil water repellency. Soils underneath both *A. mearnsii* and natural areas exhibited higher soil moisture content compared to soils were *A. mearnsii* was removed. The above findings agree with previous findings by Ruwanza et al. (2013b) who showed that soils in cleared areas have lower moisture content than soils in natural and invaded areas, though the above study was conducted in *Eucalyptus* cleared sites. The reasons for higher soil moisture content underneath natural and *A. mearnsii* invaded areas could be linked to higher stand densities compared to the cleared areas. Generally, soils underneath vegetated areas have higher water holding capacity than soils in areas where vegetation has been removed (Wang et al., 2013; Schoonover and Crim, 2015). High water holding capacity in vegetated areas could be a result of hydraulic redistribution, thus the transportation of water via plant roots from wet to drier parts of the soil profile (Leffler et al., 2005). Orwa et al. (2009) indicated that *A. mearnsii* develop a superficial lateral root system whose taproot development is largely depends upon the depth of the soil, thus allowing the plant access to water from the water table. Besides hydraulic redistribution as a factor contributing to the observed soil moisture content differences

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between sites, increased litter content cover underneath both natural and invaded areas could explain the higher soil moisture under these areas compared to cleared areas. Litter is known to provide soil cover, which facilitates the capture of rainwater and avoid evaporation (Dormaar and Carefoot, 1996). Besides, the canopy of both natural and *Acacia* species has the potential to provide shelter for soil moisture thus making it high upon being captured by litter (Mugunga and Mugumo, 2013).

The removal of *A. mearnsii* is expected to cause soils to become less repellent given that some studies have shown that invasion by *A. mearnsii* causes soils to be repellent (Ruwanza, 2017). Contrary, results of this study showed that soils in cleared areas were strongly repellent with the bulk being slightly repellent. The reason why some soils in the cleared areas were strongly repellent compared to the natural and invaded areas could be linked to reduced soil moisture content and soil compaction that was reported in the cleared areas. Soil with low moisture content, which are generally compact, are known to be repellent (Diehl, 2013). Soil compaction on cleared areas could be linked to the clearing method used to remove the invasive trees, especially were mechanical harvesting is used. Mechanical harvesting of plants has been found to trigger soil compaction and repellency, which can persist for years after clearing (Titshall et al., 2013).

The removal of alien plants by WfW assumes that natural vegetation will recover unassisted (Esler et al., 2008; Fill et al., 2018). Although results of this study indicate that the clearing of *A. mearnsii* facilitates an increase in native vegetation diversity, the presents of woody invasive alien plants may hinder this recovery process. Previous studies have reported that removal of *Acacias* facilitates native species recovery (Pretorius et al., 2008; Ndou and Ruwanza, 2016; Fill et al., 2018). However, rapid secondary invasion by invasion alien grasses and herbs was observed in all the above-mentioned studies. However, this study observed the dominance of woody invasive alien plants in cleared areas. The reason for the dominance of these recruiting woody invasives could be that the removal of *Acacias* facilitated soil stored seed bank of other invasives to germinate. Also, the clearing of *Acacias* is known to facilitate the recruitment of woody invasive alien plants due to the increased availability of soil nutrient resources (Pretorius et al., 2008). The recruitment of woody invasive alien plants can negatively affect native species recovery and slow down the native vegetation recovery process. This is because the fast-growing recruiting woody invasive alien plants have the potential to outcompete recruiting native species for resources (e.g. soil nutrients) thus negatively affecting the recovery process.

Conclusions and recommendations

Although native vegetation recovery is improving in cleared sites, it can be slowed down by the recruitment of secondary woody invasive alien plants. The above results point to the need for effective follow-up and monitoring of cleared areas so that recruiting secondary invaders are removed. Effective monitoring may include increasing the follow-up interval to facilitate continues removal of any recruiting invasive plants. Besides, removing recruiting invasive alien plants during follow-up, effective monitoring should include developing appropriate interventions to improve both soil and vegetation recovery. Such interventions during follow-up can include, soil nutrient manipulation and native plant species introduction in cleared areas. However, the efficacy of these interventions will need to be tested.

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Our comparison of cleared, invaded and natural sites has revealed some changes in measured soil and vegetation variables. However, the study results cannot infer that the observed changes were a result of clearing alone, since knowldge on pre-invasion and pre-clearing condition is limited. Given that previous studies have showed that *A. mearnsii* invasion trigger changes in soil properties and vegetation diversity (see Le Maitre et al., 2011; van der Waal et al., 2012), the reported changes in soil and vegetation diversity in this study's cleared sites can be a consequence of *A. mearnsii* removal. The above explanation is further supported by the fact that plant composition in invaded and natural sites were significantly different, this pointing to the notion that invasion cause changes to plant composition, so does removal of the invader in this case *A. mearnsii* (Kumschick et al., 2015; Guido and Pillar, 2017).

Acknowledgements. We are grateful to the owners of Zvakanaka farm for permission to conduct this research on their farm. We also thank Prof. Tshisikhawe M.P for plant identification.

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APPENDIX

Appendix 1. *Forty-two frequently occurring species in relation to invasion condition and sites. Values indicate calculated species occupancy frequencies (as a %)*

	Cleared sites		Natural sites		Invaded sites	
Plant name	**Site one**	**Site two**	**Site one**	**Site two**	**Site one**	**Site two**
Trees and shrubs						
Brachylaena discolor	40	40	40	20	10	10
Conostomium natalense	35	5	30	10	0	0
Rhus pentheri	20	0	15	45	0	0

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17051716

	Cleared sites		Natural sites		Invaded sites	
Plant name	**Site one**	**Site two**	**Site one**	**Site two**	**Site one**	**Site two**
Nuxia floribunda	20	60	0	20	0	0
Vernonia spp.	0	80	5	15	0	0
Carissa edulis	15	45	50	10	0	0
Lantana camara	45	65	0	0	40	40
Caesalpinia decapetala	60	0	0	80	35	25
Acacia mearnsii	5	80	0	0	40	60
Eucalyptus spp.	55	25	0	0	0	0
Athrixia phylicoides	70	10	10	10	0	0
Asparagus spp.	15	5	5	15	0	0
Vachellia karroo	20	40	50	10	0	0
Psidium guajava	20	20	0	0	0	0
Landolphia kirkii	15	5	0	0	0	0
Euclea natalensis	0	40	10	50	10	30
Lippia javanica	30	30	20	40	0	0
Combretum kraussii	5	15	30	30	0	0
Ziziphus mucronata	30	10	0	0	0	0
Zanthoxylum capense	0	0	30	50	20	20
Nerium oleander	0	0	10	30	0	0
Asparagus falcatus	5	35	20	60	5	15
Diospyros lycioides	0	0	30	10	0	0
Jacaranda spp.	15	25	0	0	10	30
Dombeya rotundifolia	10	10	40	0	15	5
Solanum mauritianum	5	55	0	0	20	60
Rubus rigidus	30	30	0	0	10	10
Herbs						
Bidens pilosa	45	15	0	0	0	0
Felicia sp.	10	30	20	40	5	15
Dicoma anomala	15	5	5	15	20	0
Tylophora sp.	30	10	0	40	0	0
Vernonia natalensis	60	0	5	15	0	0
Ipomoea sp.	20	0	0	0	5	15
Grasses						
Panicum maximum	35	45	20	20	20	60
Cyperus spp.	0	40	0	0	0	0
Setaria sphacelata	20	40	10	10	0	0
Urochloa spp.	5	15	0	0	0	0
Cyperus rotundus	30	10	20	40	25	15
Cynadon dactylon	60	40	5	15	0	0
Themeda triandra	40	0	20	0	0	0
Eragrostis spp.	0	0	0	20	0	0
Aristida transvaalensis	0	20	0	0	0	0

ALLELOPATHIC POTENTIAL OF *PINUS ROXBURGHII* NEEDLES AGAINST SELECTED WEEDS OF WHEAT CROP

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(Received 13th Sep 2018; accepted 5th Dec 2018)

Abstract. This study was aimed at evaluating allelopathic activity of various solvent extracts of *Pinus roxburghii* (Pine tree) against some important weeds viz. *Phalaris minor* (bunchgrass), *Avena fatua* (wild oat), *Chenopodium album* (pigweed), *Euphorbia helioscopia* (sun spurge) and *Rumex dentatus* (toothed dock) of bread wheat (*Triticum aestivum*) by employing sandwich method (powdered needles) on filter paper, soil and agar. The data attained from statistical analysis revealed that methanolic needle extract possessed the highest germination percentage inhibition for *T. aestivum*, followed by *C. album* and *A. fatua* applied in soil. Similarly, maximum radical length suppression was observed for *R. dentatus*, followed by *C. album*. The plumule length retardation was noted best in *R. dentatus*, followed by *A. fatua.* The application of methanolic *P. roxburghii* extract was responsible for reduction in seed germination, radical and plumule length of wheat. Based on results, it can be concluded that methanolic *Pinus* needles extract possesses potential inhibitory effects that required for further detailed analysis to establish allelopathic potential and onward application to be used as phytoherbicide.
Keywords: *Allelopathic potential, Bread wheat, Pinus needles, Growth retardation, Methanol extract, Weed management*

Introduction

Allelopathy is the negative effects of one plant on the germination, growth, and/or development of other plants. The chemicals which execute allelopathy are commonly known as allelochemicals. Plant release chemical into the environment and these chemicals affect physiological functions of other plants in immediate vicinity, such as seed germination, photosynthesis, respiration, transpiration, stomatal behavior and ion uptake (Anwar et al., 2017a). Allelochemicals come from the class of secondary metabolites that are produced as by-products in the primary metabolic pathways of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

plants. Like many other natural compounds, these chemicals have the capacity to cause a wide array of biological effects and can be quite useful for agriculture systems as well as weed control practices (Anwar et al., 2013). Allelochemicals include alkaloids, flavonoids phenoloids, and glucosionates. These are produced by the plants during their growth and developmental processes (Ahmed et al., 2014). Allelochemicals have been found to act as agent in the formation and disintegration of plant hormones, for instance they play role in the activation of Abscisic acid (ABA) synthesis via the action of ferulic acid (Zafar et al., 2010; Zhou et al., 2004).

Pinus roxburghii Sarg. (Family: Pinaceae) commonly known as “chir pine” is native to Himalaya, the region extends from northern Pakistan, across northern India and Nepal to Bhutan. Dried needles of Pine trees forms a dense carpet on the forest floor, which are gathered by the locals in large bundles to serve as bedding for their cattle, for the year round. Still a large quantity of these needles is left on the forest floor and with the rain water these needles are weathered and the leachates from them are mixed with the surrounding soil environment. Needles and bark of *P. roxburghii* is reported to possess secondary metabolites such as alkaloids, glycosides, flavonoids, saponins, tannins and triterpines which may have potential aliphatic actions (Baroniya and Baroniya, 2014). It is concept that Wheat in the high mountain areas, do not reach to maturity in most cases and as such the crop is harvested premature and used as fodder. However, past temperature trends in the high mountain areas (Chitral district) have led to shortening of the growing season length which certainly has helped in increasing wheat yield as well as crop area in high mountain areas (Hussain et al., 2005). In this context, a study was carried out to evaluate allelopathic activity of *Pinus roxburghii* (Pine tree) against some important weeds viz. *Phalaris minor* (bunchgrass), *Avena fatua* (wild oat), *Chenopodium album* (pigweed), *Euphorbia helioscopia* (sun spurge) and *Rumex dentatus* (toothed dock) of wheat crop.

Materials and methods

Collection and mechanical processing of P. roxburghii needles

Mature needles (500 g) of *P. roxburghii* were collected from district Rawalpindi (73° 02’ E longitude and 33° 36’ N latitude, 508 m above sea level) Punjab, Pakistan during November, 2017 and washed for several times under running tap water. The needles were dried for 4 weeks in shade at 30°C. The dried needles were crushed using heavy duty blender to make fine powder by passing through mesh size 2mm and kept in air tight plastic zip lock bags at 4 °C (Anwar et al., 2013).

Procurement and surface sterilization of test species seeds

Seeds of major weeds of *T. aestivum* viz. *P. minor, A. fatua, C. album, E.helioscopia* and *R. dentatus* were procured from Barani Agricultural Research Institute (BARI), Chakwal, District Rawalpindi, Punjab, Pakistan. Seeds (15g) of each test species were surface sterilized with 2% (w/v) solution of Sodium hypochlorite (NaOCl) for 1-2 min. After disinfection seeds were washed with distilled water (Anwar et al., 2016; Biljana and Kragujevac, 2015).

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Filter paper and soil bioassay with P. roxburghii needles solvent extracts

Dried needles powder was extracted in distilled water, hexane and methanol separately at 30°C for 24h on an orbital shaker (160rpm). The extract was filtered through Whatman filter paper No.1. The stock solution was diluted to prepare different concentration i.e. T_1 (100%), T_2 (75%), T_3 (50%) and T_4 (control) (Sahu and Devkota, 2013; Anwar et al., 2017a).

An aliquot of 15 ml extract was added on 25g soil and 5 ml extract was added on filter paper per Petri dish. Distilled water, blank hexane and methanol was used as control in respective solvent extract bioassay. Ten seeds of selected test species were used per Petri dish. Each treatment was replicated for three times. The Petri dishes were wrapped with aluminium foil and incubated in growth chamber (NTS Model MI-25S) at 28°C for 15 days. The germination percentages, lengths of radical and plumule were calculated for each test species by comparing with respective control (Khan et al., 2008).

Filter paper and soil bioassay with P. roxburghii needles dried powder

Dried powder (10mg) of *P. roxburghii* needles was added on filter paper along with 5ml distilled water per Petri dish. Similarly, 50mg powder was added on 25g soil along with 15ml distilled water per Petri dish (Raana et al., 2012). Ten surface sterilized seeds of each test species were placed in sterilized Petri dishes. The Petri dishes were wrapped with aluminium foil and incubated in growth chamber (NTS Model MI-25S) at 25°C for 15 days. The germination percentages, lengths of radical and plumule were calculated for each test species (Anwar et al., 2017b).

Sandwich method

Five ml of 0.75% (w/v) agar (Nalge Nunc Intl., Roskilde, Denmark) was poured in each of the six-well ($10cm^2$ area/well) into multi-dish plastic plate. The agar solution was left for solidification. The powder of *P. roxburghii* needles was placed @ 10 and 50 mg in wells of plate and roofed by a thin layer of 0.75% (w/v) agar. After solidification, 10 seeds of each test species were placed on agar gel in each well of the plate (Fujii et al., 2003, 2004). The multi-well plastic plates were incubated in growth chamber (NTS Model MI-25S) at 25°C for 15 days. Each treatment was replicated three times. The germination percentages, lengths of radical and plumule for each test species were calculated.

A completely randomized design (CRD) was used for experiment analysis. The statistical analysis was done using STATISTIX 9. Means were separated by Fisher's protected LSD test (Nekonam et al., 2014).

Results

Allelopathic potential of P. roxburghii aqueous extract

Germination percentage

The results revealed that aqueous extract inhibited 54%, 48% and 43% germination percentage of *T. aestivum, C. album* and *A. fatua*, respectively on filter paper (*Table 1*), whereas, no significant inhibitory effect on the germination of *R. dentatus*, *P. minor* and *E. helioscopia* was observed supposed to be resistance against *P. roxburghii* extract.

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Similarly, *P. roxburghii* aqueous extract on soil significantly inhibited 59%, 50% and 44% seed germination of *T. aestivum*, *C. album* and *A. fatua*, respectively compared to the control (*Table 2*). It has been observed that maximum (98%) germination was observed for *R. dentatus*, *P. minor* and *E. helioscopia*. During the experimentation, minimum germination was noted for *T. aestivum* (i.e. 46% and 41%) on filter paper and soil, respectively. The results revealed that germination percentage reduction of the *T. aestivum, C. album* and *A. fatua* were concentration dependent, with the increase of concentration, the suppression potential was gradually enhanced (*Fig. 1a*).

Radical length

The aqueous extract of *P. roxburghii* exhibited radical length inhibition of *C. album* (40%) followed by *R. dentatus* (39%) on filter paper (*Table 3*), whereas, no significant effect was noted for *T. aestivum*, *P. minor, E. helioscopia* and *A. fatua* showing resistance against extract. Similarly, the applications of extract into soil significantly suppressed radical length of *C. album* and *R. dentatus* with 46% and 41% respectively as compared to control (*Table 4*). The maximum (98%) radical length was observed for *T. aestivum*, *P. minor, E. helioscopia* and *A. fatua.* The final data concluded that minimum radical length was noted for *C. album* i.e. 60% and 64% on filter paper and soil, respectively (*Fig. 1b*).

***Table 1.** Allelopathic effect of P. roxburghii aqueous extract (AE) on germination percentage of test species on filter paper*

Treatments	**Test species**					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	42[d]	51[d]	80[a]	82[a]	84[a]	47[d]
$T_{2\ AE}$	66[c]	65[c]	82[a]	84[a]	86[a]	68[c]
$T_{3\ AE}$	79[b]	78[b]	83[a]	85[a]	87[a]	79[b]
$T_{4\ AE}$	91[a]	89[a]	84[a]	86[a]	88[a]	91[a]
[1]LSD	12.554	18.510	14.844	13.08	15.580	15.541
[2]F-value	14.63*	21.36*	23.04*	39.81*	52.38*	44.98*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

***Table 2.** Allelopathic effect of P. roxburghii aqueous extract (AE) on germination percentage of test species on soil*

Treatments	**Test species**					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	38[d]	50[c]	81[a]	83[a]	85[a]	46[d]
$T_{2\ AE}$	55[c]	53[c]	83[a]	85[a]	87[a]	56[c]
$T_{3\ AE}$	70[b]	72[b]	84[a]	86[a]	88[a]	72[b]
$T_{4\ AE}$	92[a]	90[a]	85[a]	87[a]	89[a]	92[a]
[1]LSD	19.808	18.990	16.60	16.435	18.67	22.33
[2]F-value	10.99**	18.19*	34.19*	22.97*	31.71*	18.61*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

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Table 3. *Allelopathic effect of P. roxburghii aqueous extract (AE) on radical length (cm) of test species on filter paper*

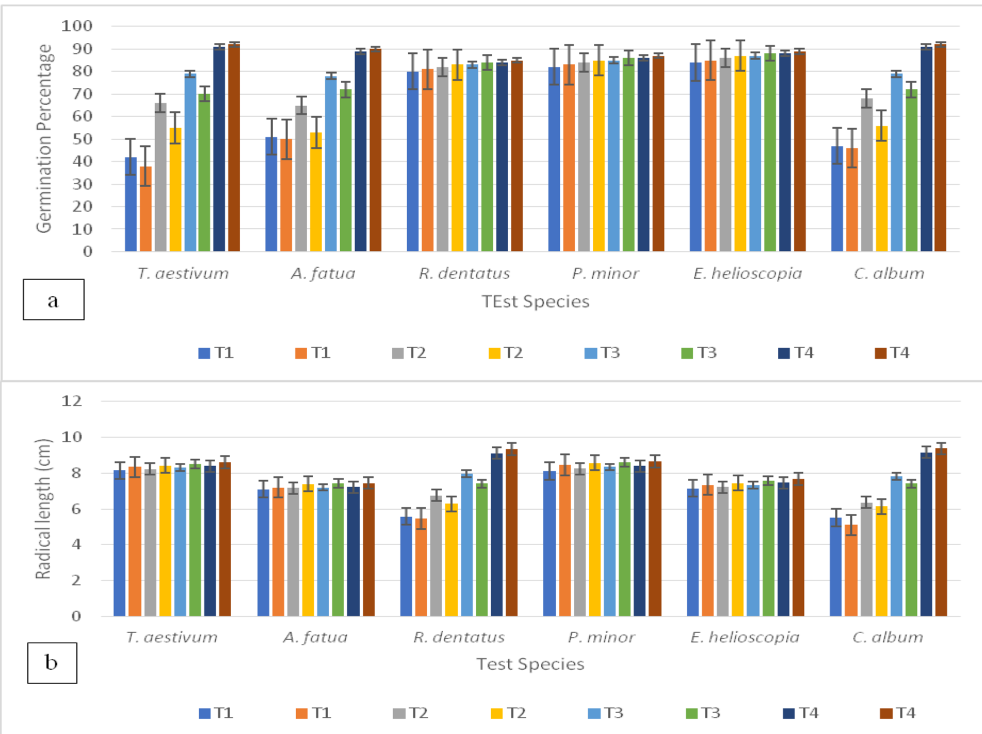
Treatments	**Test species**					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	8.15^{a}	7.11^{a}	5.59^{d}	8.12^{a}	7.16^{a}	5.52^{c}
$T_{2\ AE}$	8.24^{a}	7.18^{a}	6.76^{c}	8.25^{a}	7.23^{a}	6.37^{c}
$T_{3\ AE}$	8.31^{a}	7.21^{a}	7.99^{b}	8.34^{a}	7.34^{a}	7.81^{b}
$T_{4\ AE}$	8.39^{a}	7.22^{a}	9.12^{a}	8.39^{a}	7.46^{a}	9.17^{a}
[1]LSD	1.640	0.899	1.2125	1.1662	1.420	0.8677
[2]F-value	46.47*	65.33*	112.84*	95.02*	55.72*	134.19*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 4. *Allelopathic effect of P. roxburghii aqueous extract (AE) on radical length (cm) of test species on soil*

Treatments	**Test species**					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	8.34^{a}	7.21^{a}	5.46^{c}	8.46^{a}	7.35^{a}	5.11^{d}
$T_{2\ AE}$	8.42^{a}	7.39^{a}	6.28^{c}	8.57^{a}	7.45^{a}	6.15^{c}
$T_{3\ AE}$	8.51^{a}	7.43^{a}	7.41^{b}	8.62^{a}	7.57^{a}	7.41^{b}
$T_{4\ AE}$	8.61^{a}	7.45^{a}	9.34^{a}	8.65^{a}	7.68^{a}	9.38^{a}
[1]LSD	1.1027	2.7087	1.2058	0.9098	0.8741	0.7677
[2]F-value	93.48*	46.38*	98.75*	217.04*	162.39*	135.19*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$



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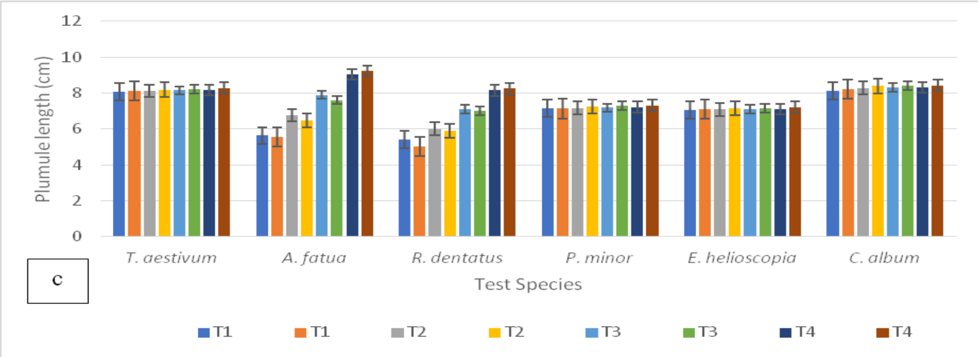

Figure 1. *Allelopathic potential of P. roxburghii aqueous extract against test species on filter paper (FP) and soil (S) on: (a) germination percentage (b) radical length (c) plumule length (T_1 = 100%, T_2 = 75%, T_3 = 50% and T_4 = control)*

Plumule length

The aqueous extract of *P. roxburghii* significantly inhibited the plumule length of *A. fatua* (38%) and *R. dentatus* (34%) as compared control on filter paper (*Table 5*). Remarkably, there was no momentous effect on plumule elongation of *T. aestivum, P. minor, E. helioscopia* and *C. album*. Likewise, *P. roxburghii* aqueous extract significantly inhibited plumule length of *A. fatua* (40%) and *R. dentatus* (39%) as compared to control in soil (*Table 6*). The statistical figures also proposed that maximum plumule length (98%) was noted for *T. aestivum, P. minor, E. helioscopia* and *C. album*. The statistical data concluded that minimum plumule length was noted for *A. fatua* showing 62% and 60% on filter paper and soil, respectively (*Fig. 1c*).

Table 5. *Allelopathic effect of P. roxburghii aqueous extract (AE) on plumule length (cm) of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	8.08^a	5.63^d	5.41^c	7.14^a	7.06^a	8.12^a
$T_{2\ AE}$	8.11^a	6.78^c	6.01^c	7.17^a	7.08^a	8.29^a
$T_{3\ AE}$	8.15^a	7.89^b	7.11^b	7.19^a	7.10^a	8.31^a
$T_{4\ AE}$	8.18^a	9.04^a	8.15^a	7.22^a	7.11^a	8.33^a
[1]LSD	2.3332	1.4291	0.8679	1.2428	0.7319	0.8297
[2]F-value	27.95*	23.83*	81.03*	29.85*	219.87*	222.41*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Table 6. *Allelopathic effect of P. roxburghii aqueous extract (AE) on plumule length (cm) of test species on soil*

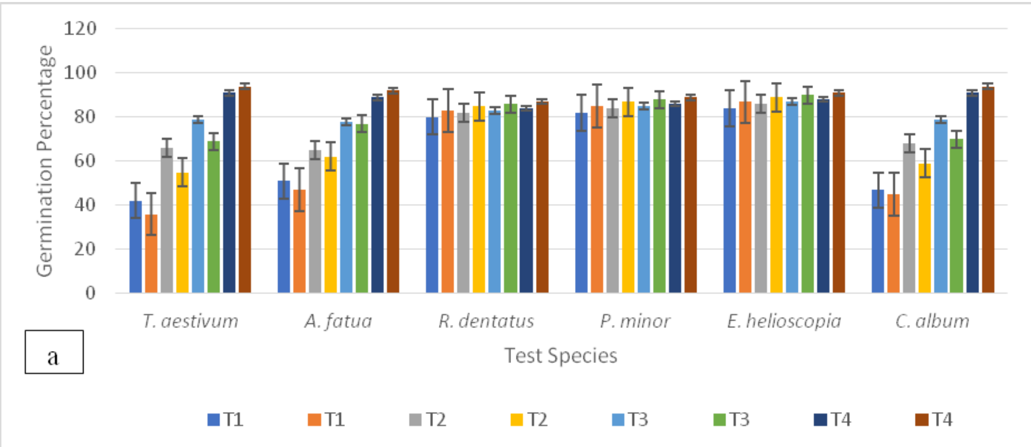
Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ AE}$	8.13^{a}	5.54^{c}	5.01^{c}	7.13^{a}	7.12^{a}	8.22^{a}
$T_{2\ AE}$	8.19^{a}	6.48^{c}	5.89^{c}	7.26^{a}	7.14^{a}	8.39^{a}
$T_{3\ AE}$	8.23^{a}	7.61^{b}	6.99^{b}	7.31^{a}	7.17^{a}	8.41^{a}
$T_{4\ AE}$	8.28^{a}	9.24^{a}	8.25^{a}	7.32^{a}	7.21^{a}	8.43^{a}
[1]LSD	2.4502	0.9978	0.7720	1.1434	0.8468	1.1019
[2]F-value	9.54**	72.85*	102.44*	72.9*	36.04*	86.41*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Allelopathic potential of P. roxburghii hexane extract

Germination percentage

It has been observed from the results that there was significant reduction of germination of *T. aestivum, C. album* and *A. fatua* showing 54%, 48% and 43% respectively as compared to their respective control on filter paper (*Table 7*), whereas, no significant effect on the germination of *R. dentatus*, *P. minor* and *E. helioscopia,* showing resistance to the allelopathic *P. roxburghii* hexane extract. Similarly, *P. roxburghii* hexane extract applied into soil showed the highest degree of inhibition of seed germination of *T. aestivum, C. album* and *A. fatua* with 62%, 52% and 49% respectively as compared to their respective control (*Table 8*). The maximum (98%) germination was noted for *R. dentatus*, *P. minor* and *E. helioscopia*. In the present study it was noted that minimum germination was noted for *T. aestivum* i.e. 46% and 38% on filter paper and soil. The statistics also recommended that allelopathic inhibitory effect was concentration dependent for *T. aestivum, C. album* and *A. fatua* with concentration increase, suppression potential was gradually enhanced (*Fig. 2a*).



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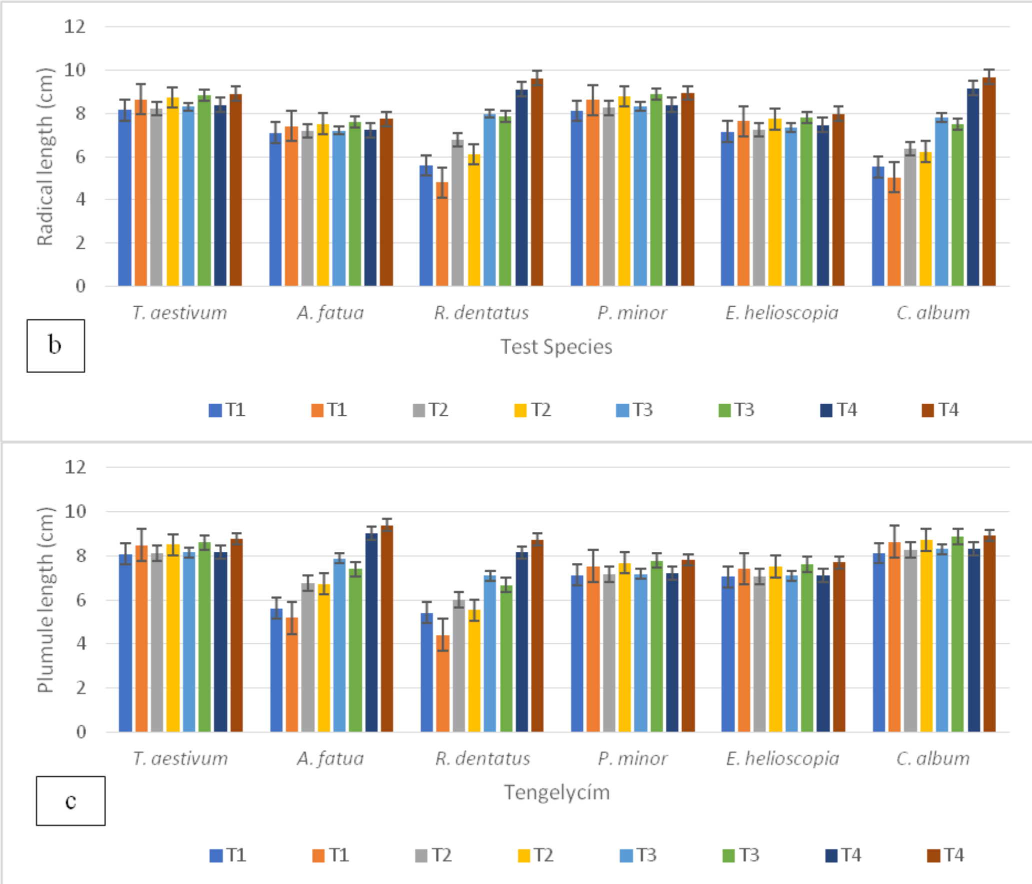

Figure 2. *Allelopathic potential of P. roxburghii hexane extract against test species on filter paper (FP) and soil (S) on: (a) germination percentage (b) radical length (c) plumule length (T_1 = 100%, T_2 = 75%, T_3 = 50% and T_4 = control)*

Table 7. *Allelopathic effect of P. roxburghii hexane extract (HE) on germination percentage of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	42^d	51^d	80^a	82^a	84^a	47^d
$T_{2\ HE}$	66^c	65^c	82^a	84^a	86^a	68^c
$T_{3\ HE}$	79^b	78^b	83^a	85^a	87^a	79^b
$T_{4\ HE}$	91^a	89^a	84^a	86^a	88^a	91^a
[1]LSD	12.554	18.510	14.844	13.08	15.580	15.541
[2]F-value	14.63*	21.36*	23.04*	39.81*	52.38*	44.98*

Means followed by different letters within one column differ significantly at *P* < 5%
*Significant at *P* < 1%

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Table 8. *Allelopathic effect of P. roxburghii hexane extract (HE) on germination percentage of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	36^{d}	47^{d}	83^{a}	85^{a}	87^{a}	45^{d}
$T_{2\ HE}$	55^{c}	62^{c}	85^{a}	87^{a}	89^{a}	59^{c}
$T_{3\ HE}$	69^{b}	77^{b}	86^{a}	88^{a}	90^{a}	70^{b}
$T_{4\ HE}$	94^{a}	92^{a}	87^{a}	89^{a}	91^{a}	94^{a}
[1]LSD	13.722	18.26	18.774	13.74	14.529	17.77
[2]F-value	25.64*	22.09*	15.39*	16.90*	20.73*	32.81*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Radical length

It is also clear from the result that *C. album* (40%) followed by *R. dentatus* (39%) exhibited radical length inhibition in *P. roxburghii* hexane extract on filter paper (*Table 9*), whereas, no noteworthy effect on radical length of *T. aestivum*, *P. minor, E. helioscopia* and *A. fatua* showing resistance to extract. Likewise, *P. roxburghii* hexane extract on soil cause significant radical length reduction of *R. dentatus* (50%) and *C. album* (48%) as compared to control (*Table 10*).

Table 9. *Allelopathic effect of P. roxburghii hexane extract (HE) on radical length (cm) of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	8.15^{a}	7.11^{a}	5.59^{d}	8.12^{a}	7.16^{a}	5.52^{c}
$T_{2\ HE}$	8.24^{a}	7.18^{a}	6.76^{c}	8.25^{a}	7.23^{a}	6.37^{c}
$T_{3\ HE}$	8.31^{a}	7.21^{a}	7.99^{b}	8.34^{a}	7.34^{a}	7.81^{b}
$T_{4\ HE}$	8.39^{a}	7.22^{a}	9.12^{a}	8.39^{a}	7.46^{a}	9.17^{a}
[1]LSD	1.640	0.899	1.2125	1.1662	1.420	0.8677
[2]F-value	46.47*	65.33*	112.84*	95.02*	55.72*	134.19*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 10. *Allelopathic effect of P. roxburghii hexane extract (HE) on radical length (cm) of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	8.66^{a}	7.42^{a}	4.79^{d}	8.63^{a}	7.65^{a}	5.04^{d}
$T_{2\ HE}$	8.73^{a}	7.52^{a}	6.11^{c}	8.78^{a}	7.74^{a}	6.23^{c}
$T_{3\ HE}$	8.85^{a}	7.61^{a}	7.85^{b}	8.88^{a}	7.83^{a}	7.49^{b}
$T_{4\ HE}$	8.91^{a}	7.75^{a}	9.64^{a}	8.95^{a}	7.98^{a}	9.68^{a}
[1]LSD	3.8879	2.0971	0.6773	1.2360	1.2027	1.2205
[2]F-value	12.78*	116.95*	319.12*	118.48*	94.48*	66.72*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

The maximum (98%) radical length was observed for *T. aestivum*, *P. minor, E. helioscopia* and *A. fatua*. The final data concluded that minimum radical length was noted for *C. album* (60%) and *R. dentatus* (50%) on filter paper and soil, respectively (*Fig. 2b*).

Plumule length

P. roxburghii hexane extract significantly retarded the plumule length of *A. fatua* (38%) followed by *R. dentatus* (34%) as compared to control on filter paper (*Table 11*), whereas, no momentous effect on plumule length of *T. aestivum, P. minor, E. helioscopia* and *C. album*. Likewise, the plumule length of *R. dentatus* (49%) and *A. fatua* (45%) was suppressed significantly as compared to control in extract applied into soil (*Table 12*).

***Table 11.** Allelopathic effect of P. roxburghii hexane extract (HE) on plumule length (cm) of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	8.08^a	5.63^d	5.41^c	7.14^a	7.06^a	8.12^a
$T_{2\ HE}$	8.11^a	6.78^c	6.01^c	7.17^a	7.08^a	8.29^a
$T_{3\ HE}$	8.15^a	7.89^b	7.11^b	7.19^a	7.10^a	8.31^a
$T_{4\ HE}$	8.18^a	9.04^a	8.15^a	7.22^a	7.11^a	8.33^a
[1]LSD	2.3332	1.4291	0.8679	1.2428	0.7319	0.8297
[2]F-value	27.95*	23.83*	81.03*	29.85*	219.87*	222.41*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

***Table 12.** Allelopathic effect of P. roxburghii hexane extract (HE) on plumule length (cm) of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ HE}$	8.49^a	5.18^c	4.42^d	7.54^a	7.43^a	8.64^a
$T_{2\ HE}$	8.51^a	6.72^b	5.53^c	7.69^a	7.52^a	8.73^a
$T_{3\ HE}$	8.62^a	7.41^b	6.67^b	7.79^a	7.62^a	8.88^a
$T_{4\ HE}$	8.78^a	9.39^a	8.75^a	7.82^a	7.71^a	8.93^a
[1]LSD	2.3501	0.8878	0.7630	1.6434	1.3019	0.5319
[2]F-value	9.65**	75.85*	101.54*	73.1*	84.41*	239.87*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

The maximum plumule length (98%) was noted for *T. aestivum, P. minor, E. helioscopia* and *C. album*. The statistical data concluded that minimum plumule length was noted for *A. fatua* (62%) and *R. dentatus* (61%) on filter paper and soil, respectively (*Fig. 2c*).

Allelopathic potential of P. roxburghii methanolic extract

Germination percentage

P. roxburghii methanolic extract on filter paper showed significant inhibitory activity on seed germination of *T. aestivum* (57%), *C. album* (49%), and *A. fatua* (46%), respectively as compared to control (*Table 13*). Likewise, *P. roxburghii* methanolic extract on soil exhibited the highest degree of inhibition germination for *T. aestivum*

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(62%), *C. album* (52%), and *A. fatua* (49%) respectively as compared to control (*Table 14*). The statistical data also suggested that the there was no significant effect on germination %age of *P. minor*, *R. dentatus* and *E. helioscopia*. The maximum (98%) germination was observed for *P. minor*, *R. dentatus* and *E. helioscopia*. The statistical results recommended that minimum germination was noted for *T. aestivum* showing 43% and 38% on filter paper and soil, respectively. The statistics also recommended that allelopathic inhibitory effect was concentration dependent for *T. aestivum, C. album* and *A. fatua* (*Fig. 3a*).

Table 13. *Allelopathic effect of P. roxburghii methanolic extract (ME) on germination percentage of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	40^d	49^d	82^a	84^a	86^a	47^d
$T_{2\ ME}$	64^c	69^c	84^a	86^a	88^a	62^c
$T_{3\ ME}$	83^b	80^b	85^a	87^a	89^a	77^b
$T_{4\ ME}$	93^a	91^a	86^a	88^a	90^a	93^a
[1]LSD	18.606	17.890	17.535	18.50	19.67	21.68
[2]F-value	10.29**	18.19*	23.85 *	33.69*	33.71*	14.19*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 14. *Allelopathic effect of P. roxburghii methanolic extract (ME) on germination percentage of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	36^d	47^d	83^a	85^a	87^a	45^d
$T_{2\ ME}$	55^c	62^c	85^a	87^a	89^a	59^c
$T_{3\ ME}$	69^b	77^b	86^a	88^a	90^a	70^b
$T_{4\ ME}$	94^a	92^a	87^a	89^a	91^a	94^a
[1]LSD	13.722	18.26	18.774	13.74	14.529	17.77
[2]F-value	25.64*	22.09*	15.39*	16.90*	20.73*	32.81*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Radical length

The data revealed that the highest radical length inhibition activity exhibited by *R. dentatus* (47%) followed by *C. album* (43%), measuring 47% and 43% in *P. roxburghii* methanolic extract on filter paper (*Table 15*). Likewise, methanolic extract on soil caused significant radical length reduction of *R. dentatus* and *C. album* measuring 50% and 48%, respectively as compared to control, while *T. aestivum P. minor, E. helioscopia* and *A. fatua* remained unaffected (*Table 16*). The maximum radical length was noted for *T. aestivum, P. minor, E. helioscopia* and *A. fatua* (98%). The results also

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illustrated that minimum radical length was noted for *R. dentatus* i.e. 53% and 50% on filter paper and soil, respectively. The results revealed that allelopathic inhibitory effect was concentration dependent for *R. dentatus* and *C. album* (*Fig. 3b*).

Table 15. *Allelopathic effect of P. roxburghii methanolic extract (ME) on radical length (cm) of test species on filter paper*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	8.31^a	7.31^a	4.97^d	8.45^a	7.46^a	5.39^d
$T_{2\ ME}$	8.45^a	7.42^a	7.13^c	8.59^a	7.55^a	6.92^c
$T_{3\ ME}$	8.63^a	7.51^a	8.81^b	8.67^a	7.69^a	8.17^b
$T_{4\ ME}$	8.71^a	7.55^a	9.44^a	8.75^a	7.78^a	9.48^a
[1]LSD	3.7087	1.2858	0.7098	0.7741	0.9677	0.998
[2]F-value	45.38*	99.75*	216.14*	151.39*	124.19*	64.33*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 16. *Allelopathic effect of P. roxburghii methanolic extract (ME) on radical length (cm) of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	8.66^a	7.42^a	4.79^d	8.63^a	7.65^a	5.04^d
$T_{2\ ME}$	8.73^a	7.52^a	6.11^c	8.78^a	7.74^a	6.23^c
$T_{3\ ME}$	8.85^a	7.61^a	7.85^b	8.88^a	7.83^a	7.49^b
$T_{4\ ME}$	8.91^a	7.75^a	9.64^a	8.95^a	7.98^a	9.68^a
[1]LSD	3.8879	2.0971	0.6773	1.2360	1.2027	1.2205
[2]F-value	12.78*	116.95*	319.12*	118.48*	94.48*	66.72*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Plumule length

P. roxburghii methanolic extract on filter paper significantly inhibited plumule length of *R. dentatus* (46%) and *A. fatua* (42%) as compared to the control (*Table 17*). Likewise, the highest degree of inhibition in plumule length was measured for *R. dentatus* (49%) and *A. fatua* (45%) in methanolic extract applied into soil (*Table 18*). The data further suggested that there was no significant effect on germination of *T. aestivum, P. minor, E. helioscopia* and *C. album*. The statistical results recommended that highest plumule length (98%) was exhibited by *T. aestivum, P. minor, E. helioscopia* and *C. album*. The results further indicated that minimum plumule length noticed for *R. dentatus* i.e. 68% and 61% on filter paper and soil, respectively (*Fig. 3c*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Table 17. *Allelopathic effect of P. roxburghii methanolic extract (ME) on plumule length (cm) of test species on filter paper*

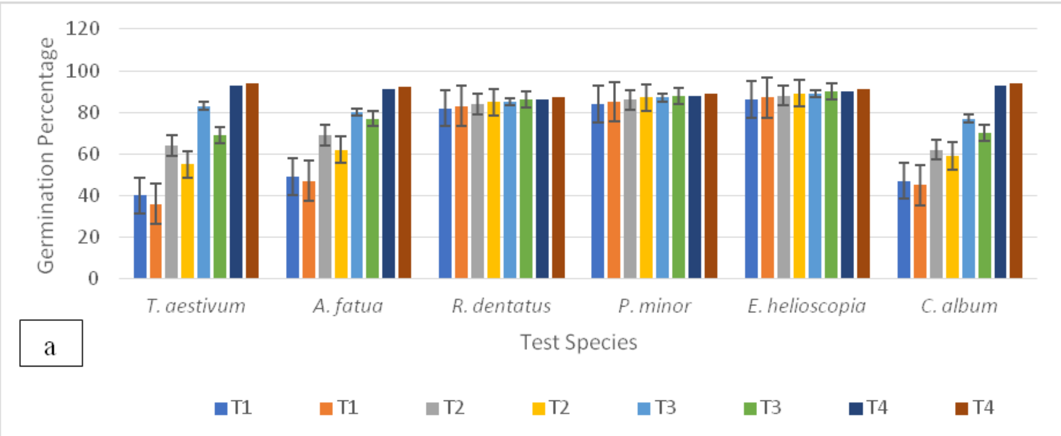
Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	8.18[a]	5.31[d]	4.53[d]	7.39[a]	7.14[a]	8.34[a]
$T_{2\ ME}$	8.29[a]	7.18[c]	6.12[c]	7.43[a]	7.29[a]	8.43[a]
$T_{3\ ME}$	8.35[a]	8.51[b]	7.23[b]	7.51[a]	7.35[a]	8.55[a]
$T_{4\ ME}$	8.48[a]	9.14[a]	8.45[a]	7.52[a]	7.41[a]	8.63[a]
[1]LSD	2.1332	1.5092	0.6679	1.5428	0.7497	0.6297
[2]F-value	28.55*	24.81*	84.03*	27.95*	220.41*	221.91*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 18. *Allelopathic effect of P. roxburghii methanolic extract (ME) on plumule length (cm) of test species on soil*

Treatments	Test species					
	T. aestivum	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
$T_{1\ ME}$	8.49[a]	5.18[c]	4.42[d]	7.54[a]	7.43[a]	8.64[a]
$T_{2\ ME}$	8.51[a]	6.72[b]	5.53[c]	7.69[a]	7.52[a]	8.73[a]
$T_{3\ ME}$	8.62[a]	7.41[b]	6.67[b]	7.79[a]	7.62[a]	8.88[a]
$T_{4\ ME}$	8.78[a]	9.39[a]	8.75[a]	7.82[a]	7.71[a]	8.93[a]
[1]LSD	2.3501	0.8878	0.7630	1.6434	1.3019	0.5319
[2]F-value	9.65**	75.85*	101.54*	73.1*	84.41*	239.87*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$



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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

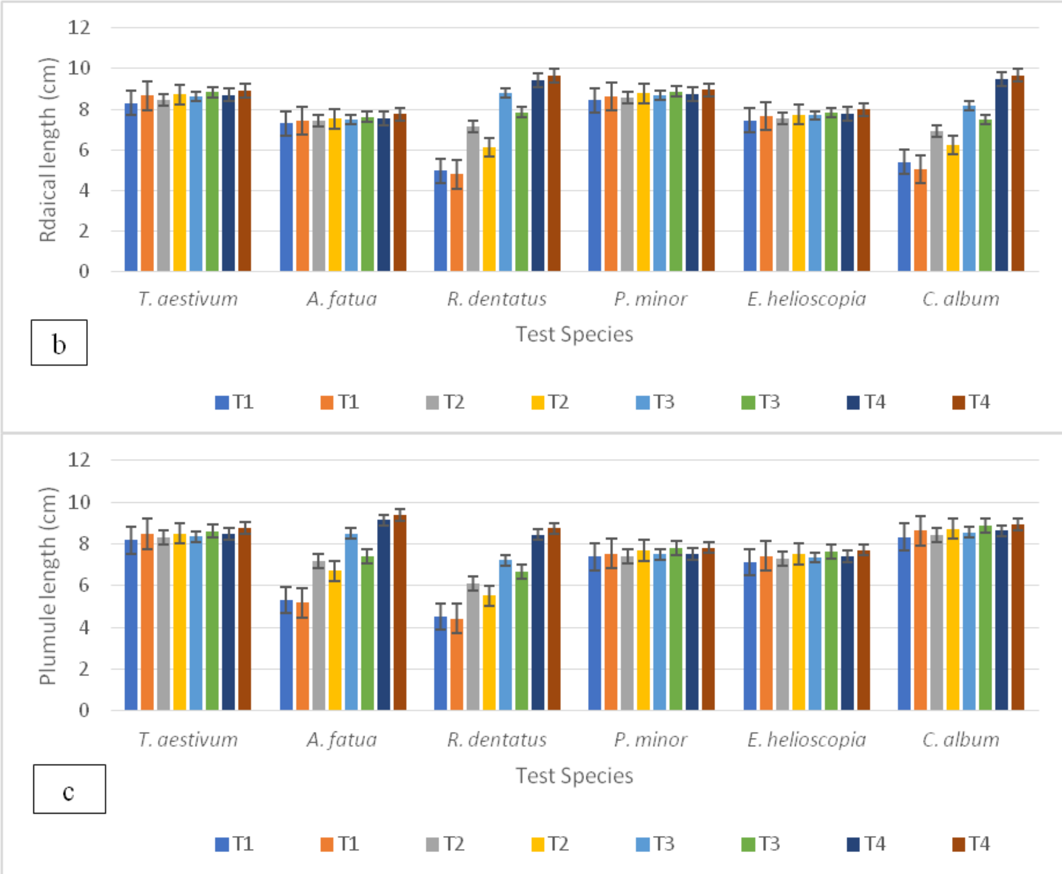

Figure 3. *Allelopathic potential of P. roxburghii methanolic extract against test species on filter paper (FP) and soil (S) on: (a) germination percentage (b) radical length (c) plumule length (T_1 = 100%, T_2 = 75%, T_3 = 50% and T_4 = control)*

Allelopathic potential of P. roxburghii in sandwich method

Germination percentage

The data revealed that *T. aestivum, C. album* and *A. fatua* showing 52%, 47% and 44% germination inhibition respectively as compared to control in *P. roxburghii* needles powder on filter paper, whereas, no significant effect on germination of *R. dentatus, P. minor* and *E. helioscopia* showing resistance to dry powder. The results also declared that maximum (97%) germination was noted for *R. dentatus, P. minor* and *E. helioscopia.* In the present study, it was demonstrated that minimum germination was noted for *T. aestivum* i.e. 48% and 45% on filter paper and soil, respectively. The experimental results of the current study indicated on agar the highest germination reduction was noted for *T. aestivum* (51%), followed by *C. album* (36%) and *A. fatua* (35%) at 10 mg conc. Similarly, the highest germination reduction was noted for *T. aestivum* (56%), followed by *C. album* (48%) and *A. fatua* (43%) at 50 mg conc (*Table 19*). The statistical data concluded that minimum germination was noted for *T. aestivum* measuring 49% and 44% at 10 mg and at 50 mg conc., respectively. The statistics also recommended that with the increase of concentration, the inhibitory effect was progressively increased for *T. aestivum, C. album* and *A. fatua*. The statistical

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

results recommended that the germination of *P. minor, E. helioscopia* and *R. dentatus* were completely resistant to dry powder (*Fig. 4a*).

Table 19. *Allelopathic effect of P. roxburghii leaf powder on germination percentage of test species*

Treatments		Test species					
Media		*T. aestivum*	*A. fatua*	*R. dentatus*	*P. minor*	*E. helioscopia*	*C. album*
Filter paper	10 mg	43^{b}	44^{b}	86^{a}	74^{a}	83^{a}	48^{b}
	Control	90^{a}	78^{a}	88^{a}	79^{a}	84^{a}	91^{a}
Soil	50 mg	41^{b}	43^{b}	87^{a}	80^{a}	85^{a}	45^{b}
	Control	91^{a}	80^{a}	90^{a}	81^{a}	86^{a}	93^{a}
Agar	10 mg	46^{b}	54^{b}	93^{a}	82^{a}	88^{a}	60^{b}
	50 mg	41^{b}	47^{b}	91^{a}	80^{a}	86^{a}	49^{c}
	Control	93^{a}	83^{a}	93^{a}	83^{a}	89^{a}	95^{a}
[21]LSD		13.36	18.44	19.53	18.957	23.14	15.64
[2]F-value		16.69*	14.95*	31.64*	32.00*	19.62*	16.2*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Radical length

The data revealed that *C. album* and *R. dentatus* showing 35% and 32% radical length inhibition respectively as compared to control in *P. roxburghii* needles powder on filter paper, whereas, no significant effect on radical length of *T. aestivum*, *A. fatua, P. minor* and *E. helioscopia* showing resistance to dry powder. It is also clear from the result that *C. album* and *R. dentatus* showed 45% and 42% radical length inhibition respectively as compared to control in powder applied into soil. The results also declared that maximum (96%) radical length was noted for *T. aestivum*, *A. fatua, P. minor* and *E. helioscopia.* In the present study, it was demonstrated that minimum radical length was noted for *C. album* i.e. 65% and 55% on filter paper and soil, respectively (*Table 20*). The experimental results of the current study indicated on agar the highest radical length reduction was noted for *R. dentatus* (35%), followed by *C. album* (34%) at 10 mg conc. Similarly, the highest radical length reduction was noted for *R. dentatus* (41%), followed by *C. album* (36%) at 50 mg conc. The statistical data concluded that minimum radical length was noted for *R. dentatus* i.e. 65% and 69% at 10 mg and at 50 mg conc., respectively (*Fig. 4b*).

Plumule length

The data revealed that *A. fatua* (39%) and *R. dentatus* (37%) showing plumule length inhibition respectively as compared to control in *P. roxburghii* needles powder on filter paper, whereas, no significant effect on plumule length of *T. aestivum*, *C. album*, *E. helioscopia* and *P. minor* showing resistance to dry powder. It is also clear from the result that *R. dentatus* (49%) and *A. fatua* (46%) showed and plumule length inhibition respectively as compared to control in powder applied into soil. The results also declared that maximum (95%) plumule length was noted for *T. aestivum*, *C. album*, *E.*

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helioscopia and *P. minor*. In the present study, it was demonstrated that minimum plumule length was noted for *A. fatua* (61%) and *R. dentatus* (51%) on filter paper and soil, respectively (*Table 21*). The results of the current study indicated on agar the highest plumule length reduction was noted for *R. dentatus* (42%), followed by *A. fatua* (40%) at 10 mg conc. Similarly, the highest plumule length reduction was noted for *R. dentatus* (49%), followed by *A. fatua* (44%) at 50 mg conc. The statistical data concluded that minimum plumule length was noted for *R. dentatus* measuring 68% and 61% at 10 mg and at 50 mg conc., respectively (*Fig. 4c*).

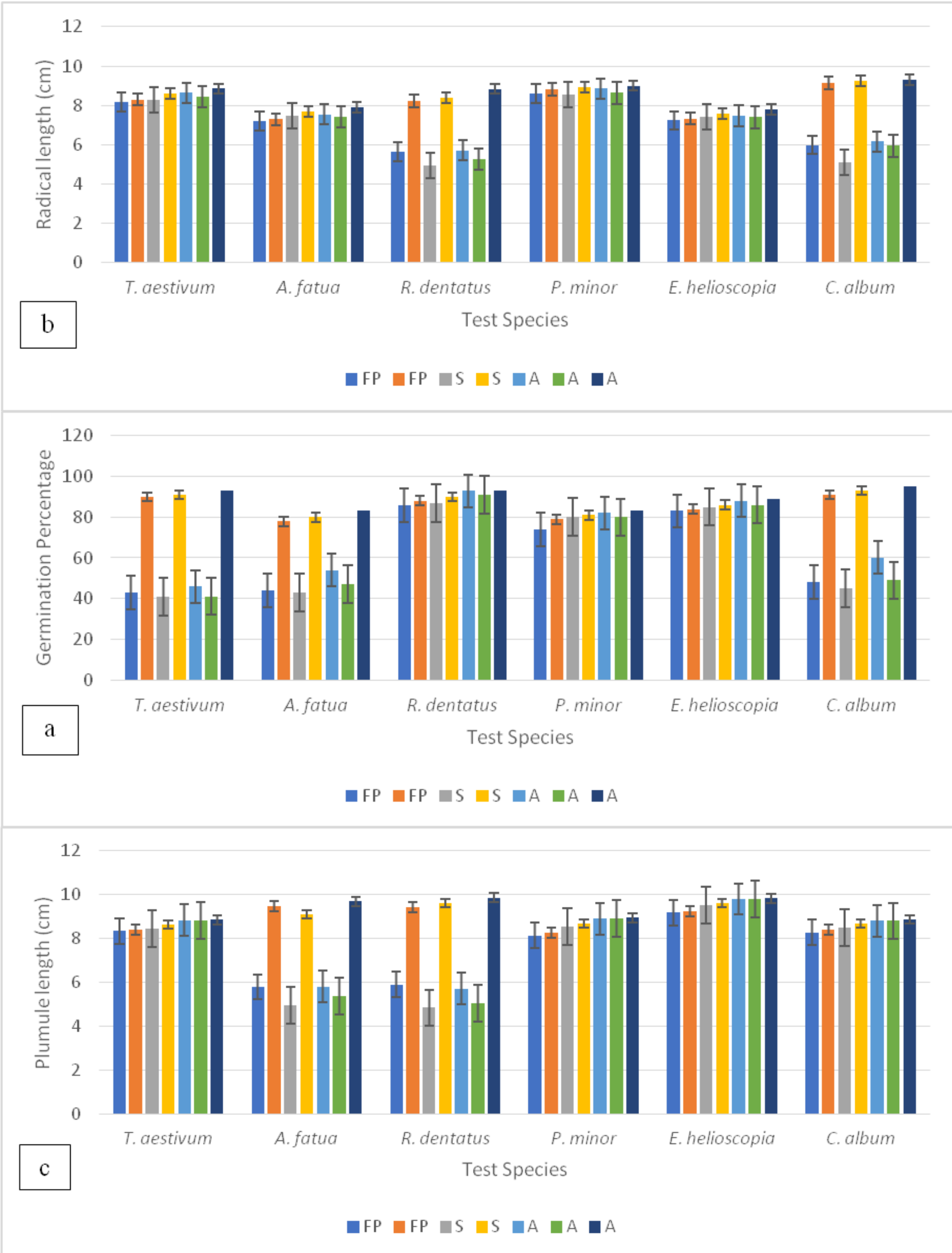

Figure 4. *Allelopathic potential of P. roxburghii needles powder against test species on filter paper (FP) and soil (S) on: (a) germination percentage (b) radical length (c) plumule length (T_1 = 100%, T_2 = 75%, T_3 = 50% and T_4 = control)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

Table 20. *Allelopathic effect of P. roxburghii leaf powder on radical length (cm) of test species*

Treatments		Test species					
Media		***T. aestivum***	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
Filter paper	10 mg	8.16^{a}	7.21^{a}	5.63^{b}	8.60^{a}	7.23^{a}	5.98^{b}
	Control	8.29^{a}	7.29^{a}	8.23^{a}	8.82^{a}	7.33^{a}	9.15^{a}
Soil	50 mg	8.27^{a}	7.46^{a}	4.91^{b}	8.54^{a}	7.41^{a}	5.09^{b}
	Control	8.61^{a}	7.68^{a}	8.40^{a}	8.93^{a}	7.56^{a}	9.23^{a}
Agar	10 mg	8.64^{a}	7.54^{a}	5.71^{b}	8.85^{a}	7.46^{a}	6.15^{b}
	50 mg	8.42^{a}	7.41^{a}	5.25^{b}	8.63^{a}	7.39^{a}	5.93^{b}
	Control	8.85^{a}	7.89^{a}	8.83^{a}	8.99^{a}	7.78^{a}	9.31^{a}
[21]LSD		3.1879	1.9971	0.6373	1.6360	1.7027	1.0523
[2]F-value		14.78*	132.95*	301.12*	126.48*	95.48*	48.12*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Table 21. *Allelopathic effect of P. roxburghii leaf powder on plumule length (cm) of test species*

Treatments		Test species					
Media		***T. aestivum***	***A. fatua***	***R. dentatus***	***P. minor***	***E. helioscopia***	***C. album***
Filter paper	10 mg	8.35^{a}	5.80^{b}	5.91^{b}	8.15^{a}	9.19^{a}	8.28^{a}
	Control	8.41^{a}	9.48^{a}	9.44^{a}	8.27^{a}	9.25^{a}	8.40^{a}
Soil	50 mg	8.46^{a}	4.95^{b}	4.85^{b}	8.56^{a}	9.52^{a}	8.49^{a}
	Control	8.66^{a}	9.09^{a}	9.60^{a}	8.68^{a}	9.62^{a}	8.68^{a}
Agar	10 mg	8.84^{a}	5.82^{b}	5.72^{b}	8.90^{a}	9.80^{a}	8.82^{a}
	50 mg	8.82^{a}	5.38^{b}	5.05^{b}	8.92^{a}	9.82^{a}	8.81^{a}
	Control	8.86^{a}	9.69^{a}	9.86^{a}	8.95^{a}	9.84^{a}	8.87^{a}
[21]LSD		4.6846	1.5971	0.9340	0.8018	0.3182	1.5127
[2]F-value		7.14**	43.74*	55.31*	55.01*	14.14*	75.45*

Means followed by different letters within one column differ significantly at $P < 5\%$
*Significant at $P < 1\%$

Discussion

Aqueous extracts of *Pinus albicaulis, P. contorta, P sabiniana* and *P. ponderosa* needles inhibited radical length of *Hordeum vulgare* and *Bromus mollis* (Heisey and Delwiche, 1983). *P. densiflora* needles water extract inhibited seed germination and seedling growth in *Clematis apiifolia, Ledebouriella seseloides, Melandrium firmum, Bidens pinnata,* and *Platanus orientalis* (Kil and Yim, 1983). Aqueous extracts of *Pinus divaricata* and *P. resinosa* fresh needles and litter reduced seed germination, root elongation and growth of *Poa pratense, Epilobium angustifolium, Agropyron repens* and *Phleum pratense* (Jobidon, 1986). *P. koraiensis* water extracts from leaves affected the seed germination and seedling elongation of selected weeds of wheat crops (Kil et

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al., 1991). *P. sylvestris* water soluble root secretions exerted negative biochemical effect on *Picea excela* (Kolesnichenko and Andryushchenko, 1978) and inhibited germination and early seedling growth of *Betula pendula. Betula pubescens* and *Pinus sylvestris* (Hytonen, 1992). Aqueous extracts of *P. thunbergii* needles inhibited seed germination and seedling length of *Lactuca sativa*, Carpesium *abrotanoides* and *Oenothera odorata* (Kil, 1989). The allelopathic potential is due to the presence of 9α, 13β-epidioxyabeit-8(14)en-18-oic acid reported from the aqueous methanolic extract of red pine needles that inhibited the growth of *Lepidium sativum*, *Lactuca sativa*, *Medicago sativa*, *Lolium multiflorum* and *Digitaria sanguinalis* (Kato-Noguchi et al., 2009). Another compound abscisic acid-β-D-glucopyranosyl ester (ABA-GE) was also isolated and found to have allelopathic activity (Kato-Noguchi et al., 2011). The needle extract from *P. nigra* inhibited seed germination of rye grass (Terzi, 2013). Similarly, aqueous extract of *P. roxburghii* needles suppressed the growth of mustard and wheat seedlings (Baroniya and Baroniya, 2014), while that of *P. brutia* needles suppressed the growth of *Lolium multiflorum* and *Poa pratensis* seedlings (Aliloo et al., 2012).

The current study was in accordance with Singh et al. (2001), who determined that seedling growth and seed germination of *Capsicum annuum, Pisum sativum* and *Oryza sativa* was significantly retarded by *Pinus* needles and the inhibitory effect was concentration dependent. Likewise, *Amaranthus paniculatus* and *Trifolium pratense* seeds treated with leaf extract of *P. roxburghii* and *Rhododendron arboreum* that resulted momentous consequence on germination of tested species (Madgil and Kapil, 1990). Poisonous compounds produced by *P. densiflora* checked growth and seed germination of adjacent species (Kil and Yim, 1983). The phenolic composites of *P. rigida* exhibited both retardation and development effect on *Cassia mimosoides* revealing concentration dependent. Fresh, senesced and decaying needles from *P. halepensis* exhibited potent inhibitory potential on *Festuca arundinacea*, *Cyanodon dactylon*, *Avena sativa* and *Lemna minor* (Nektarios et al., 2005).

Different species of *Pinus* had exhibited allelopathic potential against other plant species (Kato-Noguchi et al., 2009). *P. roxburghii* extract possessed significant inhibitory potential on different plants (Melkania, 1984). Allelopathic effects of *P. halepensis* possessed herbicidal activities (Hamrouni et al., 2015; Anwar et al., 2019). A significant herbicidal activity of *P. halepensis* against common weeds of cereal crops (Amri et al., 2013). The inhibitory effect of the *Pinus* needles, being more pronounced in the fresh, moderate in the senesced, and low in the decaying conditions (Monnier et al., 2011). Allelopathic potential of *P. halepensis* had been broadly calculated from various plant parts, which can be autotoxic and thus prevent the germination of seeds in a forest plants as was observed for inhibition of *Stipa tenacissima* grasslands (Fernandez et al., 2013; Navarro-Cano et al., 2009). Allelochemicals are reported to be present in stems, roots, leaves and fruit of *P. halepensis* (Baroniya and Baroniya, 2014), which exhibited allelopathic effects (Hamrouni et al., 2015) and strong herbicidal activity of essential oil was reported against common weeds of cereal crops (Fujii et al., 2004). *P. halepensis* extracts inhibited germination and growth of *Lactuca sativa* and *Linum strictum*. The strong allelophatic potential of *P. halepensis* could be attributed to the presence of numerous phenolic compounds such as benzeneacetic, 4-hydroxybenzoic, vanillic, veratric, syringic and p-coumaric acids, and non-phenolic acids such as lactic, succinic, palmitic acids in *P. halepensis* (Fernandez et al., 2006). Green needles are found to have higher amounts of phenolic compounds and condensed tannins (Refifa et al., 2016). Reduced germination and suppressed growth in different

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plants could be a result of damage in the membrane integrity. It has been observed that seeds supplemented with Pinus needle extract showed enhance electrolyte leakage which reveals higher damage to membranes (Baroniya and Baroniya, 2014).

The black pine showed to inhibit the growth of *Phalaris canariensis*, *Trifolium campestre* and *Sinapis arvensis* seeds (Amri et al., 2013; Anwar et al., 2018c). Different pinene isomers exhibited allelopathic potential against *Zea mays* seed germination (Areco et al., 2014). The leaf methanolic extract from *P. nigra suppressed* the seed germination of perennial ryegrass and tall fescue (Robert, 1986; Terzi et al., 2013). Valera-Burgos et al. (2012) noticed inhibitory potential of *Pinus pinea* needles extract on seedling growth and seed germination 3 Mediterranean shrub species. Allelopatic substance was isolated from the exudates of Japanese red pine trees the substance was identified as phenylacetic acid which inhibited shoot and radical elongation of *Cryptantha crassipes* I. M. Johnst (Khan et al., 2008; Anwar et al., 2018d). *P. densiflora* needles contain toxic substances that inhibit seed germination and growth of plants (Fernandez et al., 2013), these inhibitory effects can be attributed to direct molecular alteration (Hamrouni et al., 2015). The main inhibitory substance from methanol extract of the pine needles was identified as 9alpha, 13beta-epidioxyabeit-8(14) en-18-oic acid, this substance is responsible for the inhibition of root and shoot growth of *Echinochloa crus-galli* (Baroniya and Baroniya, 2014).

Powdered *P. roxburghii* needles reduce germination and, root and shoot growth of *Achyranthes aspera* L. (Khosla et al., 1981; Anwar et al., 2018a). Plants growing at higher altitudes are observed to accumulate phenolic compounds in higher concentrations consequently inhibit plant growth (Baroniya and Baroniya, 2014). Gymnosperm trees showed strong allelopathic effect on the germination, growth, and development of other plant species in the forest community (Silva et al., 2015) due to presence of allelochemicals, mostly phenolic compounds and terpenoids (Rice, 1984). *P. halepensis* had strong inhibitory effect on seedling establishment of various species suggesting allelopathic effects of litter or root exudates (Maestre, et al., 2003). Needle essential oil identified components are (*E*)-caryophyllene, terpinen-4-ol, α-humulene, and α-terpineol (Satyal et al., 2013; Anwar et al., 2018b), it was reported that (*E*)-caryophyllene inhibited the germination and seedling growth of *Brassica campestris* and *Raphanus sativus* (Wang et al., 2009). Application of *α*-pinene inhibited the growth of *Cassia occidentalis, Amaranthus viridis, Triticum aestivum, Pisum sativum and Cicer arietinum* seedlings due to the oxidative damage in root tissues (Singh et al., 2006). Allelochemicals in chir pine needles suppressed the growth of radical and plumule length of canary grass (Refifa et al., 2016). Blum (1998) observed that *P. divaricata* and *P. resinosa* needles leachates suggestively checked seedling growth and germination of *Epilobium angustifolium, Agropyron repens and Phleum pretense. The* seedling growth of *Lepidium virginicum* was significantly checked by *P. roxburghii* needles (Williams and Hoagland, 1982). The mechanism of retardation on the seedling growth produced by phytochemicals checked cell elongation and division (Node et al., 2003). Similarly, *P. densiflora* cones have high biological activity against select plant species (Lee and Monsi, 1963).

Conclusions

Present results indicated that pine needles extract and dried powder at higher concentrations reduced the seed germination, radical and plumule length of weeds

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DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

associated with the wheat crop. *P. roxburghii* is located in the mountain region of Pakistan and in every season, the fallen needles form a bed on the forest floor. During the rainy season, pine needles get dissolved with water and mixed into the soil and resultantly caused in crop reduction. Further work is needed to appraise the potential inhibitory effects of allelochemicals from the pine needles.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17171739

A COMPARATIVE STUDY OF ARTIFICIAL NEURAL NETWORKS AND MULTIPLE REGRESSION ANALYSIS FOR MODELING SKIDDING TIME

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(Received 23rd Oct 2018; accepted 20th Dec 2018)

Abstract. One of the most important functions of forests is providing raw material for wood. Timber extraction is among the most technically demanding, expensive and time consuming activities for wood raw material production. The analysis of timber extraction activities is complex and it is quite difficult to model them. Therefore, Artificial Neural Networks (ANN) frequently used as a modelling tool in the analysis of complex problems have been used to solve this issue. The aim of this study is to investigate the feasibility of ANN's including Multilayer Perceptron (MLP), Cascade Forward Back Propagation (CFBP) and compare the predictions for total time during log skidding operations stations in Eastern Black sea region (Giresun Forest District Directorates) of Turkey with those of the Multiple Regression Analysis (MRA). Moreover, standard times are calculated, and affective factors are determined, after which the effectiveness levels are evaluated at each working stage by way of timing determinations when skidder is used for timber extraction. The comparison of models were carried out by using the correlation coefficient, mean squared error, root mean square errors and mean absolute error. The comparison results indicate that MLP and CFBP models are better at predicting the total time during log skidding operations in comparison with the MRA model. These results have put forth that artificial neural networks have a greater prediction in comparison with multiple regression analysis for predicting the skidding time in timber extraction operations and that less erroneous results are obtained. It is observed that artificial neural networks can be preferred in cases for which the multiple regression analysis predictions have not been met and the analysis cannot be performed.
Keywords: *timber extraction, log skidding, multilayer perceptron, cascade forward back propagation, Turkey*

Introduction

Turkey has a total of 22.7 million hectares of forest area, which makes up 27% of its total land area. Almost 46% of the total area is on steep land with slopes greater than 40%. Thus, harvesting in mountainous regions has always played a significant role (GDF, 2017). Forestry operations in Turkey are carried out under different conditions on a forest area of approximately 22.7 million hectares, located at different parts of the country (Çalışkan et al., 2017).

Timber harvesting, as a succession of interrelated and interdependent operations in timber production, felling, processing and timber transport. Timber transport consists of two sub phases: timber extraction and further transport that are mutually dependent (Bayoğlu, 1962; Seçkin, 1978; FAO, 1982; Erdaş, 1986; Haarlaa and Jurvelius, 1987; Acar, 1994; Dykstra and Heinrich, 1996; Heinimann, 1999; Karaman, 2001; Heinimann and Stampfer, 2003; Pentek, 2008; Çalışkan et al., 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17411756

Different tools and methods are used according to technical, economic and environmental factors for timber extraction operations which is one of the stages of wood raw material production. Skidding the wood raw material on skid trails via forest tractors or agricultural tractors to place on temporary stacking locations (ramp) is one of these methods. Land topography is among the most distinctive factors in skidding work. Timber extraction methods are generally determined according to ground slope classes. The slope groups taken as basis for determining production type are as follows: using agricultural tractor and animals for skidding 0-33% ground slope, using skidder (MB Trac 900) for cable drawing 34-50% ground slope and cable timber extraction 50%< ground slope (Erdas et al., 1986; Heinimann, 1999). Timber extraction is the most difficult and expensive stages of producing wood raw material and also has the highest environmental damage. A planned approach is required for shortening this process, making the work easier, improving efficiency and thus attaining an economical process (Marchi et al., 2014).

When wood raw material skidding activities are taken into consideration with a system approach, there is a necessity for determining the standard times for different conditions that can be used while planning, applying and inspecting skidding activities which will also form a basis to ensure that skidding workers receive equal pay under equal conditions. Effectiveness of operating conditions while calculating standard time is quite high especially for mountainous forests. Determining the wages according to these factors is of vital importance for ensuring wage justice among employees and for the cost effectiveness of forest management for employers. There are various studies on timber extraction operations in which time studies have been carried out for cable drawing via agricultural tractors and forest tractors (Acar, 1995, 1997; Karaman, 1997; Öztürk, 2005; Tunay et al., 2002; Öztürk, 2010; Çağlar, 2016).

ANN is used as a popular method in different engineering applications by many researchers. ANNs are software designed for simulating the operation of simple biological neural systems (Yurtoğlu, 2006). The main reason why a human being may develop solutions for issues that require thinking and observation skills is his/her ability to learn through experience (Sağıroğlu et al., 2003). Therefore, ANN may be a beneficial tool in engineering applications (Topçu and Sarıdemir, 2008). ANN is a strong tool especially for data models with low regression coefficients (Esteban et al., 2009). ANN's are used for modelling complex operations in many engineering fields ranging from aeronautics, electronic, production, robotics, communication, construction, forest etc.

ANN's was successfully implemented in the field of forest modelling. Particularly, ANN approach has been used for many objectives modeling individual tree survival probabilities (Guan and Gertner, 1991), forest age using TM images (Jensen et al., 1999), tree mortality (Hasenauer et al., 2001; Castro et al., 2013), forecasting of industrial wood demand (Güngör et al., 2004), pine bark volume (Diamantopoulou, 2005), tree volume (Diamantopoulou, 2005a; Diamantopoulou and Milios, 2010; Özçelik et al., 2008, 2010), tree stem diameters (Diamantopoulou, 2005b; Leite et al., 2011), tree felling times (Karaman and Çalışkan, 2009), tree heights (Diamantopoulou and Özçelik, 2012; Özçelik et al., 2013), diameter distributions (Cai et al., 2012) predict of skidding time (Naghdi and Ghajar, 2012), trunk volume estimates (Bayati and Najafi, 2013), prediction of cable drawing time (Bayati and Najafi, 2015), tree diameter increments (Ercanlı et al., 2016), describing diameter distribution (Bolat et al., 2016), bark volume estimation (Çatal et al., 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The use of artificial neural networks in timber extraction is at a starting stage in Turkey. Hence, there are a few studies in forestry literature which compare the performance of artificial neural networks with other models. For this reason, the aim of this study was to investigate the feasibility of two different reputed types of ANN's and compare the results with those of MRA for predicting the total time during log skidding operations stations. Thus, the network architecture, training algorithm and transfer function that yielded the best result were determined. The data set was separated into three sets as training, test and validation. The training data set used for the training of ANN which makes up the majority of the input data set is used to maximize the ability of the network to predict the correct results and the minimize the error. Test data set is not considered during learning and is used to test the success rate for the prediction of the network after the learning stage. Control data set used during the training of the network. If the performance of the trained data set is very low, but the performance of the control set is high; this raises a suspicion that the network has memorized. In such cases, the network has to be trained again (Çelik, 2004).

Material and Methods

Study Area

This study was carried out at the Anbardağ forest planning unit covering an area of approximately 5975.0 ha in the Giresun province in the north-eastern Black Sea region of Turkey. The area is located between 40° 42' 47" – 40° 30' 13" North, and 38° 01' 49" – 38° 13' 16" East. The average terrain gradient is 30%, and altitudes range from 1.100 m to 1.500 m above sea level. Dominant tree species used for production purposes are natural oriental spruce (*Picea orientalis* Link.) and oriental beech (*Fagus orientalis* Lipsky). Felling and delimbing operations were carried out via chainsaws. Agricultural and skidder (MB Trac 900) are mostly represented as off-road machines and have been widely used (Çalışkan et al., 2017.

Field Data Collection

Timber extraction was done by skidder with cable drawing and on-road skidding system. Measured data for the timber operations have been recorded in study forms. Time values for each stage have been measured as 1/100 minute (PM) using a chronometer, the amount of work done has been determined in units and as m^3, factors that have an impact on the work done (tree type, diameter, height, ground slope, cable drawing distance, number of logs, log volume, skidding distance) have also been recorded in the work form. No intervention was made on the workers regarding issues such as starting and stopping of work, breaks, pauses, dealing with other operations.

Variables that were considered to have an impact on the work time for skidder timber extraction operations have been evaluated as $X_{ii}\left(X_{11}-X_{17}\right)$ and expressed in numerical values. These variables have been briefly explained in *Table 1*.

Continuous time measurement method was used for time measurements and the work phases, total turn time and waiting times were determined using a digital chronometer. The measured time values were for a two person working group with units of 1/100 minute. Work phases and related time values have been expressed as $y_{ii}\left(y_{11}-y_{21}\right)$ for tractor timber extraction operations and related time values have been briefly explained in *Table 1*.

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Measurements and observations have been carried out related with the timber extraction operations for spruce trees using skidder (preparation time on skidder, pulling time to empty cable, hooking time, time to take the load, wait time to obstacle, waiting time to cable dissolution, waiting time, skidding time, unhooking time, stacking time).

Table 1. *Work phases and their descriptive statistics*

Work phases	*Aver.*	*Std. Dev.*	*min.*	*max.*	Work phases	*Aver.*	*Std. Dev.*	*min.*	*max.*
y_{11} : Preparation time on Tractor	72.00	49.74	0.00	205.0	y_{fa} : Total activity(skidding) time	936.0	462.3	250.0	2800
y_{12} : Pulling time to empty cable	143.0	88.28	10.00	420.0	y_{tr} : The tractor is actively operating time	327.0	157.1	60.00	730
y_{13} : Hooking time	160.0	201.8	10.00	980.0	y_{ge} : For cable drawing, actual skidding time	609.0	357.9	130.0	2080
y_{14} : Time to take the load	146.0	88.55	20.00	470.0	X_{11} : Cable haulage distance	26.92	15.45	2.00	90.00
y_{15} : Wait time to obstacle	60.00	12.88	0.00	550.0	X_{12} : Land slope	57.08	12.48	25.00	85.00
y_{16} : Waiting time to cable dissolution	9.00	36.65	0.00	210.0	X_{13} : Diameter of logs	51.48	19.70	17.00	100.00
y_{17} : Load take-off time	34.00	64.83	0.00	300.0	X_{14} : Length of logs	6.61	3.90	1.00	24.00
y_{18} : Waiting time	192.0	731.8	0.00	6340	X_{15} : Number of log	3.15	3.09	1.00	15.00
y_{19} : Skidding time	40.00	46.90	0.00	210.0	X_{16} : Log volume	0.96	0.72	0.09	3.74
y_{20} : Unhooking time	92.00	50.35	0.00	230.0	X_{17} : Skidding distance on the road	9.67	11.13	0.00	50.00
y_{21} : Stacking time	36.00	68.21	0.00	250.0					

In addition, times for work groups have been determined as such by combining certain work phases:
Active operating time for the tractor(skidder):

$$y_{tr} = y_{11} + y_{14} + y_{17} + y_{19} + y_{21} \quad \text{(Eq.1)}$$

Real skidding time required for cable drawing:

$$y_{ge} = y_{12} + y_{13} + y_{14} + y_{15} + y_{16} + y_{20} \quad \text{(Eq.2)}$$

Total activity time:

$$y_{fa} = y_{tr} + y_{ge} \quad \text{(Eq.3)}$$

The definitions of all the y parameters mentioned in the equations are shown in *Table 1*. The ANN models were generated using Matlab software. Statistical analyses were carried out via “SPSS 21.0” software.

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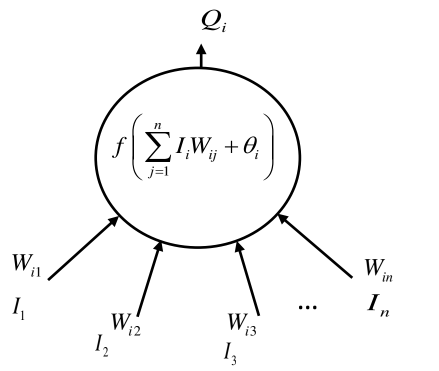
Artificial Neural Network (ANN) modelling approach

General background

ANN is a methodology developed based on the biological operating principle of the human brain which is applied on complex problems. An artificial neural network has three main elements: neurons, connections, and training rules (*Figure 1*) (Dağlı, 1994; Fausett, 1994; Haykin, 1994). In addition, an ANN is comprised of three layers with interconnected neurons which are input layer, output layer and hidden layer. The hidden layer just receive signals from the input layer and send signals to the output layer and their number is determined by way of trials (Benli, 2002).



Here;
I_j : input signal from the jth neuron
W_{ij} : connection weight for the connection from the jth neuron to the ith neuron
θ_i : initial value of the ith neuron
O_i : output signal of the ith neuron
f (..) : activation function

Figure 1. *Topology of a Neuron*

One of the most important aspects of an ANN is the connections that enable the neurons to transfer data to each other with weight values (w_{ji}). The weight values have an impact on each input of every operating element (Yıldız, 2001). Training of an ANN is defined as making changes in the weights matrix. It can be classified in two groups as supervised and unsupervised learning (Dağlı, 1994; Karaman and Çalışkan, 2009): MLP and CFBP methods have been used in this study for the prediction of the total time during log skidding operations.

Multiple Layer Perceptron (MLP)

Back propagation (BP) algorithm is used for training MLP networks in this study since it is easy to understand and prove mathematically. The BP artificial neural network models have already been described and are used widely (Rumelhart et al., 1986; Fausett, 1994; Haykin, 1994; Özçelik et al., 2010).

BP algorithm uses two parameters that control the speed at which training takes place. The learning coefficient determines the amount of change in the weights. It is observed that generally values between 0.2 and 0.4 are used and that the value of 0.6 yields the most successful results (Öztemel, 2003). The momentum coefficient plays a role on training performance. It is observed that selecting a value ranging between 0.6 and 0.8 would be best (Öztemel, 2003). Levenberg-Marquardt is highly recommended for neural networks since it is one of the most efficient algorithms (Yu et al., 2011). Levenberg–Marquardt (LM) algorithm has been developed by Kenneth Levenberg and Donald Marquardt (Yu et al., 2011). Considering the features of the problem in this

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study, LM (trainlm) was chosen as the training function. Network architecture, training rate and momentum factor have been determined in our study after examining different combinations. The general structure of MLP is shown in *Figure 2*.

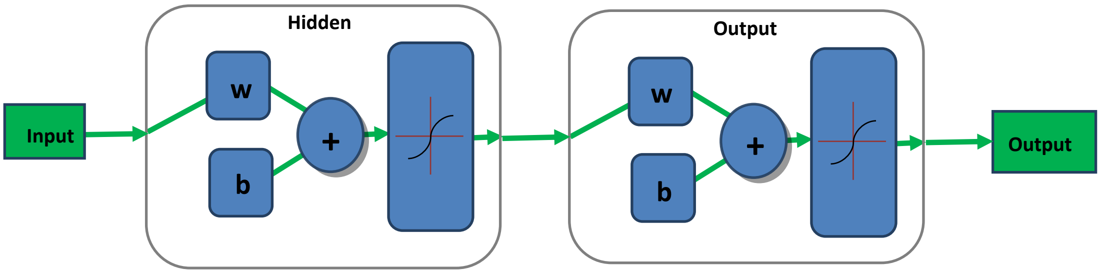

Figure 2. *Multilayer Perceptron (MLP) network architecture*

Cascade Forward Back Propagation (CFBP)

Different than MLP, the input values in CFBP are connected with all layers. CFBP has a learning property just like MLP (*Figure 3*) (Demuth et al., 2009). Considering the features of the problem in this study, LM (trainlm) was chosen as the training function. Network architecture, learning rate and momentum factor were determined in the study after examining different combinations. The general structure of CFBP is shown in *Figure 3*.

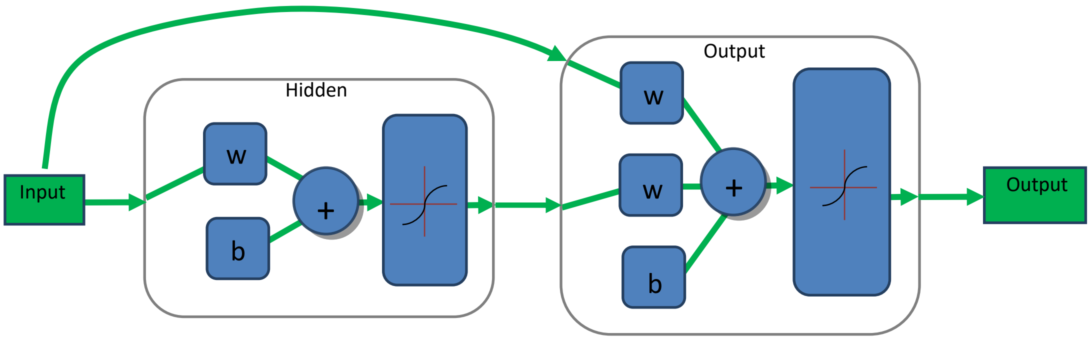

Figure 3. *Cascade Forward Back Propagation (CFBP) network architecture*

Multiple Regression Analysis (MRA)

Regression is one of the methods used for testing whether there is a relationship between two or more variables and to express the relationship between the variables by way of linear or curvilinear equations (Öztürkcan, 2009). Regression analysis using more than one independent variable is called multiple regression analysis.

The general structure of the equation in cases when there is more than one independent variable (such as $x_1, x_2, x_3, \ldots$);

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n \pm \varepsilon \qquad \text{(Eq.4)}$$

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where Y: Dependent variable, X_i: Independent variable ($i=1,2,3\ldots n$) β_i: Regression parameters ($i=1,2,3\ldots n$), ε: Random error and n: number of unknown parameters.

Statistical calculations on variables that are independent from (x_{ii}) and dependent on (y_{ii}) measurement results have been carried out as;

- Calculation of the average and deviations.
- Examination of the variables that are effective on the actual time spent for each work phase or the unit time value.
- Examination of the relations between variables.
- And determination of the impact of independent variables on the total time spent for work phases (multiple regression analysis).

Model evaluation criteria

In this study, the corrected determination coefficient (R), Mean Squared Error (MSE), Root Mean Square Error (RMSE) and mean absolute error (MAE) were used as criteria for comparing artificial neural networks (MLP, CFBP) and multiple regression analysis (MRA). Accordingly, high R and low MSE, RMSE and MAE values indicate the best model. MAE and MSE values were close to 0 and the R value was close to 1, thereby indicating that the predicted value strongly converges to the right (Hocking, 1986; Law, 1999; Cho, 2003; Arıkan Kargı, 2014).

a) The Correlation coefficient (R)

$$R = \frac{\sum\left(X_i - \bar{X}\right)\left(Y_i - \bar{Y}\right)}{\sqrt{\sum\left(X_i - \bar{X}\right)^2\left(Y_i - \bar{Y}\right)^2}} \qquad \text{(Eq.5)}$$

b) Mean Squared Error (MSE)

$$MSE = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)^2 \qquad \text{(Eq.6)}$$

c) Root Mean Square Error (RMSE)

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - y_i\right)^2}{n}} \qquad \text{(Eq.7)}$$

d) Mean absolute error (MAE)

$$MAE = \frac{\sum_{i=1}^{n}\left(x_i - y_i\right)}{n} \qquad \text{(Eq.8)}$$

where X_i and Y_i are the observed and predicted data, respectively; $\bar{X}$ and $\bar{Y}$ are the mean of the observed and predicted and n the number of observations in the dataset.

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Results and Discussion

The arithmetic average, standard deviation, max and min values for the actual time values measured with a unit of 1/100 minutes as the variables of observed values regarding the work phases of cable drawing by forest tractor have been calculated and presented in *Table 1*.

Single input variance analysis was used to examine whether the impact of the correlation matrix indicating the relationship between the variables and X_{ii} groups on the values of y_{ii} was statistically significant or not.

The developed ANN models (both MLP and CFBP models) used Cable haulage distance (X_{11}), Land slope (X_{12}), Diameter of logs (X_{13}), Length of logs (X_{14}), Number of logs (X_{15}), Log volume (X_{16}) and Skidding distance on the road (X_{17}), as input variables and the Total activity time (Skidder, y_{fa}) as the output variable.

In this study, all data were first normalized (0-1) and training, validation and testing data sets that are randomly partitioned into training (65% of all data). The validation (10%) and test data sets (the remaining 25%) were used for conquering general patterns between input and output variable while building the ANN model. The training set adjusts the connection weights and the parameters of the model; the validation set checks the performance of the model through the training process and stops the training to avoid overfitting; while the testing set evaluates the trained ANN performance and generalization power (Ghajar et al., 2012a,b; Ghorbani et al., 2016).

A typical MLP model with a BP algorithm is constructed for predicting the total activity time for skidder. The most important characteristic of multi-layered artificial neural networks is that they can be designed to contain more than one hidden layer. However, it has been determined that networks designed with one or two hidden layers display a good performance, whereas networks with more than one or two hidden layers do not have any advantage (Yeşilnaçar et al., 2005; Rumelhart, 1986). The number of neurons in the hidden layer is also an effective element in network performance. In some cases, networks with two hidden layers with a smaller number of neurons may have a better performance in comparison with networks that contain many neurons in one hidden layer (Yılmaz, 2009).

In this study, the number of neurons in the hidden layer was determined by trial and error. In this context, one hidden layer with 30 neurons was included in the model. Neurons ranging between 1 to 40 were given to each layer for determining the number of neurons included in the hidden layer. Each model was tested 15 times to determine the best model for our study. Therefore, the most suitable model was identified as the model with a 7-30-1 network structure. This study, the hyperbolic tansig transfer function was used between the input and hidden layers, and a pureline transfer function was used between the hidden and output layers. The Levenberg-Marquardt (LM) method was used for the optimization of the algorithms.

In this model, each combination of learning rates and momentum factors were tested for different numbers of hidden neurons. The network was trained in 930 epochs using the LM learning algorithm with a learning rate of 0.001 and a momentum coefficient of 0.3. This was the best combination that conducts to the smaller values of R, MSE, RMSE and MAE in *Table 2*. Regression values for the data used in the training, validation and testing of the MLP have been given in *Figure 4*.

Figure 4 shows the R values graph for the training, test and validation stages of the studied model. The values determined were R= 0.9027 for the learning stage, R= 0.7767

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for the test stage, R= 0.7427 for the validity testing of the model and R=0.8536 in total. *Figures 5 and 6* show the graphs comparing the model predictions and observed values for the MLP model. It can be observed from *Figures 5-6* that the values of the total time during log skidding operations were usually predicted near the observed value.

Table 2. *Regression Equalities for calculating the total activity time of timber extraction via skidder*

Nu	Total Time	*b*	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	R-sq
1	y_{fa}	55.39	15.09	2.34	-3.05	6.73	26.14	385.4	-1.16	.777
2	y_{fa}	71.14	14.95	2.05	-3.07	6.21	26.70	380.3		.776
3	y_{fa}	137.0	14.88	1.97	-3.82		26.07	404.0		.775
4	y_{fa}	261.9	14.80		-3.90		25.59	399.8		.773

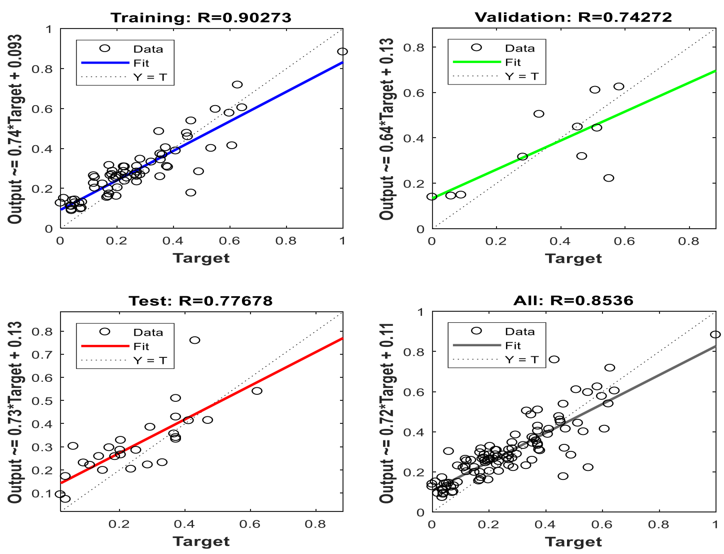

Figure 4. *Training, test, validation distribution graphs for the MLP prediction model*

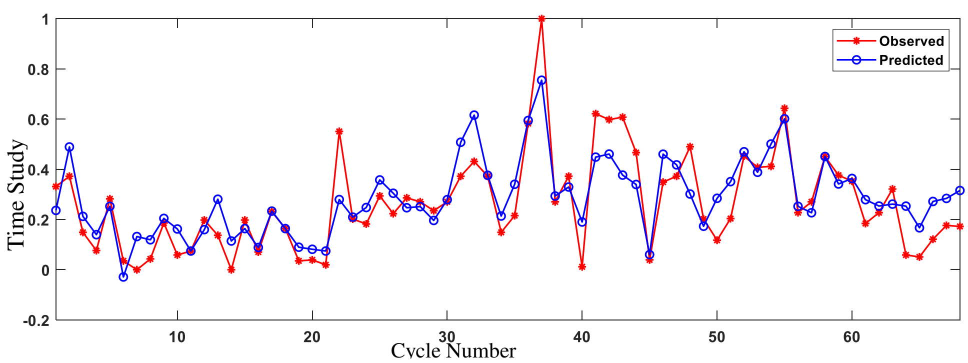

Figure 5. *Comparison of predicted(blue line) and observed(red line) values for training sets using MLP*

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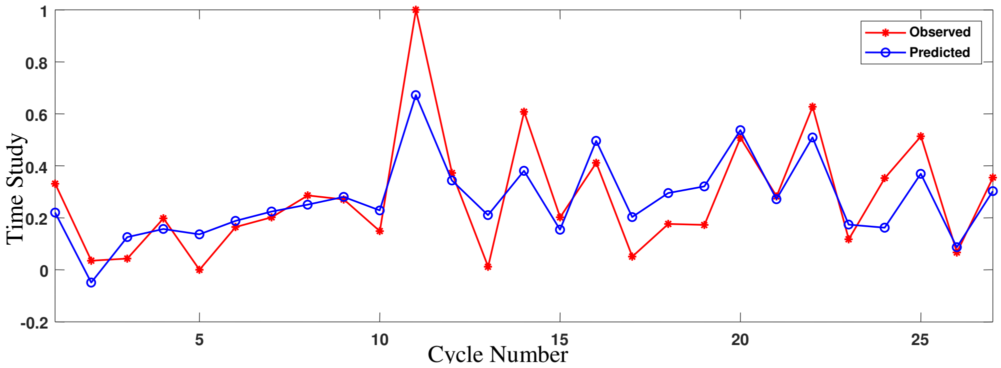

Figure 6. *Comparison of predicted (blue line) and observed (red line) values for test sets using MLP*

The hyperbolic tansig transfer function was used between the input and hidden layers, and a pureline transfer function was used between the hidden and output layers in CFBP model which gave the best results. The LM method was used for the optimization of the algorithms. CFBP model had a single hidden layer with 16 neurons. According to results, the most suitable model for CFBP is 7-16-1 network structure. In this model, the network was trained in 900 epochs using the LM learning algorithm with a learning rate of 0.001 and a momentum coefficient of 0.3. This was the best combination that conducts to the smaller values of R, MSE, RMSE and MAE in *Table 2*. Regression values for the data used for the training, validity and testing of the CFBP model have been given in *Figure 7*.

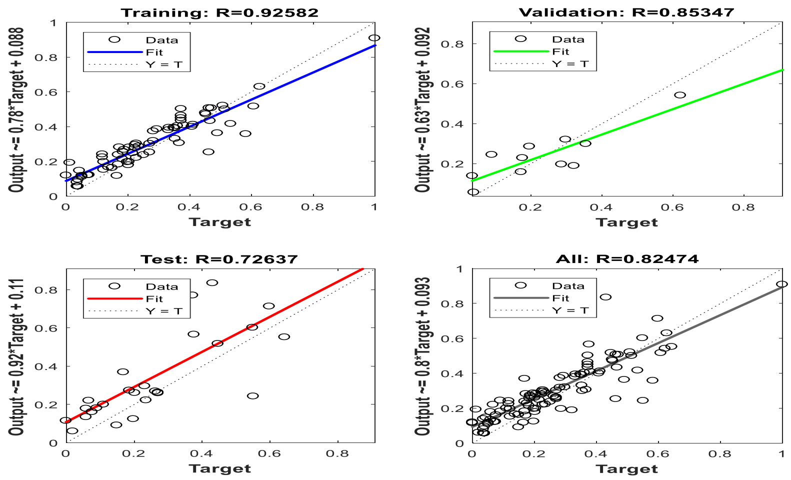

Figure 7. *Training, test, validation distribution graphs for the CFBP prediction model*

Figure 7 shows the R value graphs for the training, test and validation t stages for the studied model. The values determined were R= 0.9258 for the learning stage, R= 0.7263 for the test stage, R= 0.8534 for the validity testing of the model and R= 0.8247 in total. *Figures 8 and 9* show the graphs comparing the model predictions and observed values for the CFBP model.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17411756

It can be observed from *Figure 8-9* that the values of the total time during log skidding operations are usually predicted near the observed value.

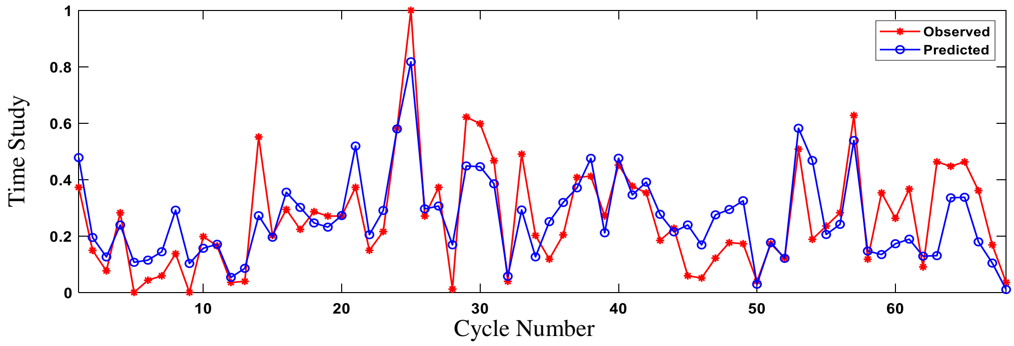

Figure 8. *Comparison of predicted and observed values for training sets using CFBP*

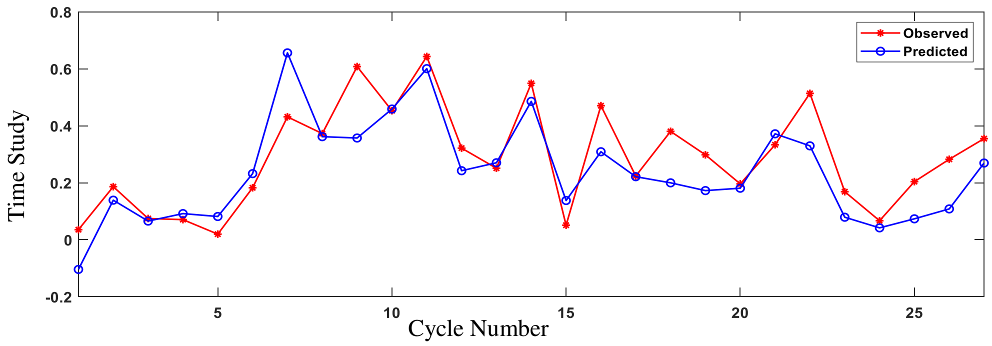

Figure 9. *Comparison of predicted and observed values for test sets using CFBP*

Alternative equalities have been generated via multiple regression analysis. Operations carried out for the timber extraction total activity time with forest tractor (y_{fa}) have been given in detail.

Regression equalities based on the $y_{fa} = f\left(X_{11}, X_{12}, X_{13}, X_{14}, X_{15}, X_{16}, X_{17}\right)$ relationship have been given in *Table 3*. Consistency of the equation was tested using the coefficients obtained from the regression equation and test data. Graphs that compare the model predictions obtained from the MRA model and the observed values have been given in *Figure 10*.

Table 3. *A Comparison of the MLP, CFBF and MRA Models*

Model	*Topology*	*R*	*MSE*	*RMSE*	*MAE*
MLP	7-30-1	0.85	0.0098	0.0991	0.0760
CFBP	7-16-1	0.82	0.0125	0.1120	0.0784
MRA	7-1	0.77	0.0127	0.1126	0.0877

It can be observed from *Figure 10* that the values of the total time during log skidding operations usually predicted near the observed value.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17411756

The performances for predicting total activity time for skidder are compared using three techniques of MLP, CFBP and MRA. The values of performance measures are given in *Table 3*.

Values of R, MSE, RMSE and MAE were compared at the end of the study for determining the algorithm with the best performance. MLP had the best performance in the study followed by CFBP and finally MRA with the lowest performance.

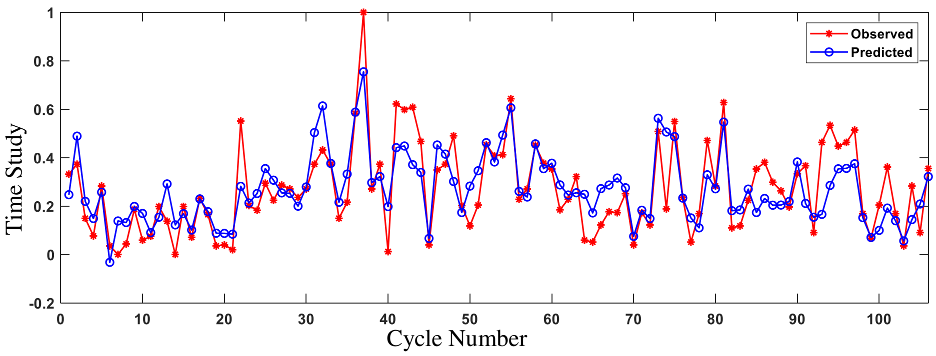

Figure 10. *Comparison of predicted and observed values using MRA*

Conclusion

The aim of this study was to investigate the feasibility of two different reputed types of ANNs including MLP, CFBP and compare them with the MRA model with regard to predicting the total time during log skidding operations stations in Eastern Blacksea region (Giresun Forest District Directorates) of Turkey.

Determination coefficient (R) and the expressions that indicate the error variance (MSE, RMSE and MAE) have been taken into consideration for determining the model with the best results. Accordingly, the model with high R and low MSE, RMSE and MAE values was taken into consideration as the best model. The R values obtained in the study were determined to vary between 0.85 and 0.82 for MLP and CFBP and as 0.77 when multiple regression was used.

Thus, it can be observed that artificial neural networks have higher prediction accuracy in comparison with multiple regression analysis and yield results with lower error values. In this case, it can be predicted that artificial neural networks may be preferred in cases when regression analysis predictions are not met and the analysis cannot be carried out.

MLP yielded better results in comparison with CFBP when ANN methods are compared among themselves for the prediction of total time during log skidding operations stations. The results of this study can be used for preparing and inspecting machine operating programs and for calculating production cost. MLP models can be used reliably for calculating the operating time of different land and operating conditions.

The results of this study can be used for determining the time dependent machinery demand to be used in timber extraction operations by private companies, for cost calculation and for putting forth the alternatives to carry out the work at minimum cost.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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TECHNICAL AND ECONOMICAL EVALUATIONS OF CALABRIAN PINE (*PINUS BRUTIA* TEN.) SEMI-ARID PLANTATIONS IN THE ŞANLIURFA-HARRAN PLAIN OF TURKEY

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(Received 28th Oct 2018; accepted 11th Jan 2019)

Abstract. Determinations of success of semi-arid plantations are very important. This paper was handled to evaluate the success of Calabrian pine plantations established in the semi-arid Harran Plain of Turkey between 1996 and 1999 regarding technical and economic aspects. The data were obtained from both 12 sample plots at different aspects in 2015 and from the Şanlıurfa Forest Enterprise Directorate's records. Also data of the yield tables of plantation areas and natural stands, and the product range table of Calabrian pine were used. Variance analysis was administered to control the growth performance of Calabrian pine plantations. The differences of diameter and height growths and the number of trees per hectare were determined. The criteria of net present value, internal rate of return and benefit cost ratio were used for economic evaluation. In conclusion, diameter and height growth and success rate were found higher in eastern, northern and western aspects, the mean survival percentage ranged from 18.60-57.13%, and the high percentage of survival was found in the north aspect. Economic return of Calabrian pine plantations in semi-arid areas in terms of wood production was found to be lower than that of the natural stands and of the plantations in other areas. However, it was concluded that the plantations in semi-arid regions are crucial because of the benefits such as the biological diversity, the protection of natural balance, prevention of erosion and carbon sequestration, and incorporation of the wastelands to the production. It was suggested that the plantations in semi-arid area should be improved and maintained for sustainable environmental management.
Keywords: *benefit cost ratio, internal rate of return, net present value, Pinus brutia, plantation growth performance, semi-arid region, sustainable environmental management*

Introduction

As one of the countermeasures against global warming, afforestation in arid and semi-arid lands had been proposed and has been tested from 1999. In this areas, harsh environmental condition constrains plant growth and survival, and then biomass productivity is usually quite low (Suganuma et al., 2014). As plantations become increasingly important sources of wood and fiber in arid and semi-arid places, they have also become increasingly criticized for their hydrological impacts (Sadeghi et al., 2016). In arid and semi-arid regions, where rainfall is not sufficient to sustain a good seedling/tree growth, water harvesting for afforestation is applied (Prinz, 2001). On the other hand, in combating desertification and erosion, the most significant issue is to conduct successful plantation activities. With this aim, it needs well-designed plantation projects, a consideration of all details and accurate implementation of the projects

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

(Niskanen, 1997; Ürgenç, 1998; Turna et al., 2007; Tunçtaner, 2007; Yılmaz and Tonguç, 2010). Hence, the destruction of ecological balance brings the environmental problems that threaten the community life such as drought, erosion and flood, and such catastrophes led many people to say that forest sources are of significant functions in protecting community health care (Koçer et al., 2009; Oğuz et al., 2009). Other biological and mechanical measures should be taken into consideration alternatively in case of the lack of convenient conditions for plantations (Anonymous, 1999).

Afforestation area has grown rapidly over the last few decades in the semi-arid grasslands in the world in an effort to restore mountain vegetation. The reasons for this land-use change included an increasing demand for timber production (Chen et al., 2016). Hence, analyses of the rationale of forest plantations in many developing countries have often been focused on efficient wood production and financial return on invested capital (Niskanen, 1997). Afforestation is a key technique for the control of desertification and environmental deterioration in arid and semiarid regions. Therefore, it is important to quantify the influence of the succession that results from afforestation on biodiversity conservation and ecological environment (Liu et al., 2015). The most common afforestation efforts in the arid and semi-arid regions of Asia include planting plantations of *Pinus eldarica, Cupressus arizonica, Robinia pseudoacacia*, and *Fraxinus rotundifolia* (Harrington et al., 1989; Michelozzi et al., 2008; Motahari et al., 2013; Sadeghi et al., 2016). These species are frequently preferred because they are drought resistant and grow fast. For this aim, *Pinus halepensis, Pinus brutia* and *Eucalyptus camaldulensis* are also used with success in some countries.

Although the slumping hills or the ones prone to slump in erosion areas were temporarily stabilized through technical facilities with alluvial cones, avalanche corridors, sand and gravel deposits, the most effective and permanent measures are taken through plantations in the basin (Ürgenç, 1998). In order for a successful plantation in the areas with shallow and poor soil, little and irregular rainfall, high evaporation, insufficient organic materials and stony surface, it is important to provide funds for plantation activities without industrial concerns as well as recruiting knowledgeable and experienced technical staff. In arid and semi-arid planting lands, it is essential for a successful plantation to use local species and local races among these species which are compatible with the existent ecological conditions. Also, deep-rooted tree species should be used in these areas (Turna et al., 2006). Besides, it will also achieve success in terms of combating erosion by using advanced nursery techniques and certain methods reducing crop water stress (mulching, soil improver materials, seedling shelter etc.).

In Turkey, combating desertification and erosion began in 1955 and the activities continued in a more planned way after the foundation of General Directorate of Afforestation and Erosion Control. According to the data of the General Directorate of Forestry, 2.2 million ha of Turkey's forest area, which is 22.3 million ha, are subject to afforestation from ecological, technical and social aspects. The areas to be afforested in Turkey will reach to a total of 4.8 million ha when a million hectare public lands to be afforested and the areas, which are in 0.11-0.40 canopy and afforested with difficulty in terms of technical aspect since it has lost its natural regeneration conditions. And, this has added to 2.2 million ha (Ürgenç et al., 1993; Anonymous, 2001).

There is a report that 40% of Turkey's total area is suffering from drought and desertification is indispensable unless satisfactory measures are taken (Ürgenç, 1998). Furthermore, the most important issues as a result of global warming causing serious

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

changes in climate systems are drought, desertification and erosion (Koçer et al., 2009). In this respect, it was stated that drought and desertification directly threatened more than 4 billion ha of area and a population of 1.2 billion people in 110 countries in "the United Nations Convention to Combat Desertification" which was accepted by the Intergovernmental Negotiating Committee in the United Nations Conference on Environment and Development on 17 June 1994 and was adopted by Turkey since 16 May 1998 (Anonymous, 2013). That is, desertification will result in erosion and, therefore, lead to yield loss, hunger and poverty in arable lands, pastures and woodlands (Turna et al., 2007). Only successful regeneration and plantation activities will make it possible to prevent this situation (Saatçioğlu, 1976; Atay, 1987; Ata, 1995).

Arid and semi-arid regions are fragile ecosystems. Sustainable management of arid and semi-arid areas requires good planning and careful implementation, as they have ecological, biological, technical and socio-economic constraints. Decisions related to the species and provenance selection, site preparation, spacing and other techniques and details should be made by paying attention to the specific conditions of these areas (Boydak and Çalışkan, 2015). In semi-arid area afforestation, it is the front plan to offer various benefits such as biodiversity service, protection of natural balance, prevention of erosion, etc., which often do not have a price in the market, rather than wood production. By estimating indirectly the economic values of such benefits and services, it is possible to add them in plantation incomes. In this case, it is obvious that the benefits will be very high. Although wood production is not for the purpose of production, there is also time to time wood production in these fields. Therefore, it is necessary to make economic evaluations in terms of wood production and compare with the natural stands in the same conditions.

This study was carried out in the plantation fields in the semi-arid and arid Şanlıurfa-Harran Plain in Turkey. These plantations established in the region between 1996 and 1999 years are arid/semi-arid plantations in the scope of combating erosion. Despite some studies related to technical and economical evaluations of plantations in general (Türker, 1986; Niskanen, 1997; Ahtikoski and Pulkkinen, 2003; Daşdemir and Şahin, 2006; Turna et al., 2007; Tuçtaner et al., 2012; Suganuma et al., 2014; Kulik et al., 2015; Wu et al., 2015), there are not many regarding particularly the economic evaluation of plantation activities in semi-arid areas. In this study, technical and economical evaluations were carried out in the plantation area in the Şanlıurfa-Harran Plain by making measurements in the field in 2015 after 16-18 years of field installation. So, this research was conducted in order to investigate the success rate of plantations with Calabrian pine in semi-arid areas, determine the diameter and height growth as a result of half of the rotation age, and make economic evaluations based on wood production.

Materials and methods

Study area

The study was carried out in the Calabrian pine (*Pinus brutia* Ten.) plantation area established as Tofaş Memorial Forest in the period 1996-1999 in the Eyyübiye district of Şanlıurfa (Harran Plain) of Turkey (*Fig. 1*). Calabrian pine is also known as Turkish pine, East Mediterranean pine and brutia pine. According to data of the Şanlıurfa Meteorological Station (TSMS, 2015), the average annual temperature in the region was 18.4°C. While the maximum temperature was 46.8 °C in July and the minimum was -

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

11.4 °C in February. The mean annual precipitation amount was 382 mm and the precipitation was 223 mm in the vegetation period. Since the places which receive a rainfall of 300 mm and below are considered to be arid and those which receive a rainfall between 300-600 mm are semi-arid, Şanlıurfa is understood to be semi-arid. The general acreage of the plantation area is 77.33 ha and 64.40 ha of the total area were planted. Through this plantation was aimed to prevent moderate and heavy sheet erosion and gully erosion (Anonymous, 1997). The average height of the field is 423 m and soil structure is clay loam. In the plantations, 0 + 1 aged, potted and Hatay originated Turkish pine seedlings were used. The planting distance was 3 × 3 m. The tilth was made with tractor + ripper plough in the plantation area and planting was made with labor force.

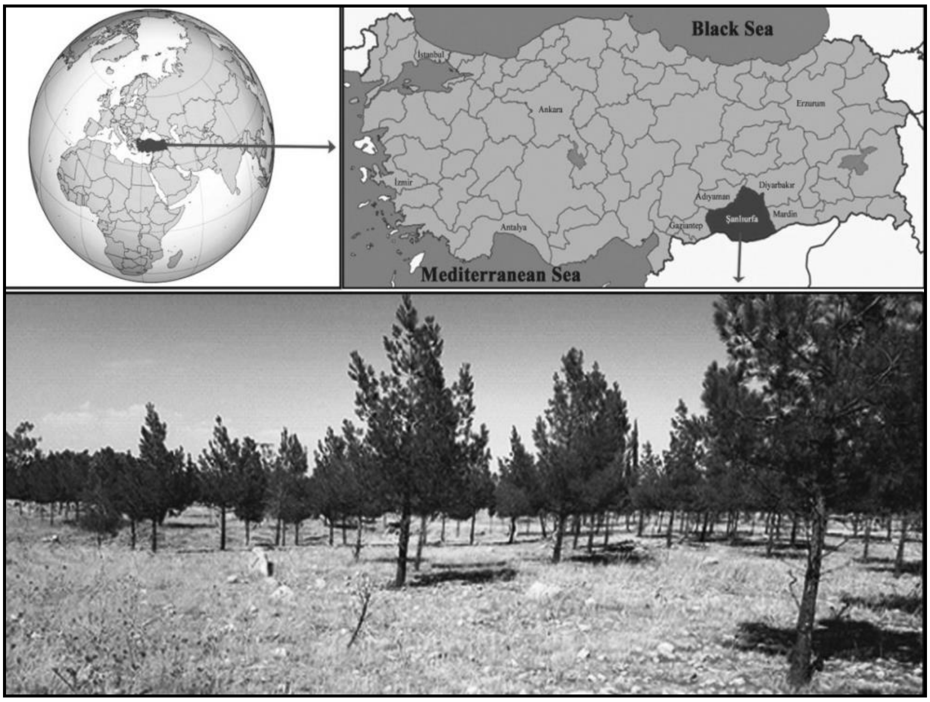

Figure 1. *The location and a general view of the study area (up left: URL-1 2016, up right: URL-2 2016)*

Data and evaluation

The materials for the study are as follows: the data obtained from the diameter, height measurements and the number of trees and their ages in the sample plots from different aspects in the plantation areas in 2015 was used for technical evaluations, the data from the Şanlıurfa Forest Enterprise Directorate (plantation expenses, wood production costs, sale prices of wood, etc.). The yield table for Turkish pine plantation areas (Usta, 1991), the yield table for Turkish pine natural stands (Erkan, 1996) and barked volume ratios in the product range table of Turkish pine (Sun et al., 1977) was used for economical evaluations.

A total of 12 sample plots at 4 different aspects and heights, each measuring 20 × 20 m square sized with 3 repetitions, were taken in 2015 (*Table 1*). The number of trees, the heights and their 1.30 m diameters, and ages of five predominant (the tallest) trees were determined in each sample plot.

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Table 1. *The features of sample plots*

Research area	Species	Sample plots			
		Aspect	Altitude (m)	Size (m^2)	Number (piece)
Harran Plain-Tofaş Land	Calabrian pine	North	400	400	3
		South	425	400	3
		East	431	400	3
		West	457	400	3

The planting distance at the beginning (3 × 3 m) was taken as a reference in order to find out the success rate in the plantation areas after 16-18 years. Thus, the success rate was measured by converting the current number of trees in the sample plots into hectare and proportioning them to the required seedling number (1,111 pcs/ha) in the plantations with the distance at the beginning. Moreover, the mean diameter and height was determined at the sample plot level by calculating the arithmetic mean of diameters and heights. The mean success rate, diameter and height values were obtained from the sample plots according to different aspects. In this study one way variance analysis (ANOVA) were administered and the differences between the mean rates were determined by Duncan Range Test. For this purpose, SPSS 22.0 package program was used.

The economic evaluations of the plantation areas were made in relation to income and expense accounts of wood production (ŞFED, 2015). Firstly, the site index of the plantation area was determined. For this, the heights and ages of five predominant trees were measured. By taking the top height and age means of five predominant trees as a reference, the top mean height and age of sample plots at each aspect were measured. The site class of the sample plots, rotation age (u), and the highest mean increment in that site class were determined based on the top mean height, age and 3 × 3 m potential growth area by using the yield table for Turkish pine plantation areas (Usta, 1991). The intermediate yields (D_1, D_2, …) during the rotation age benefiting from the yield table and final yield amounts (A_u) were determined. As the plantation areas were bare at the beginning, the yield from the area was taken as null before the application. Likewise, the site index and yield table made by Erkan (1996) for Turkish pine natural stands, the rotation age for the related site index, intermediate and final wood production volumes were determined. The intermediate and final yield sizes were classified into product ranges using barked volume ratios in the product range table for Turkish pine by Sun et al. (1977).

The production costs (stumpage price + loading + transportation + stacking-sorting + storage) and auction sale prices according to product range were taken from the Production Cost Sheet and General Sale Sheet in the 2015 balance sheet of the Şanlıurfa Forest Enterprise Directorate (ŞFED, 2015). The net selling prices were calculated by extracting production costs from selling prices of the products. The net incomes were found by multiplying the net selling prices by the intermediate and final yields in terms of product ranges for both plantation areas and natural stands. Also, Calabrian pine plantation costs (establishment, maintenance, sapling cost and other expenses) were taken from the records of the Directorate for the machine + labor force production technology with 2015 prices (US$/ha) and were added into the economic analyses.

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DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

Taking into consideration of the determined incomes and expenses, economical analyses were made by the use of the following criteria as Net Present Value (NPV) (*Eq. 1*), Internal Rate of Return (IRR) (*Eq. 2*) and Benefit Cost Ratio (BCR) (*Eq. 3*) (Geray, 1986; Türker, 1986; Erkan, 2002; Daşdemir and Şahin, 2006; Tunçtaner et al., 2012; Daşdemir, 2012, 2015; Bijarpas et al., 2015):

$$NPV = \sum_{t=0}^{n} \frac{B_t}{(1+i)^t} - \sum_{t=0}^{n} \frac{C_t}{(1+i)^t} \quad \text{(Eq.1)}$$

$$IRR\ (i_r) \rightarrow NPV = 0 = \sum_{t=0}^{n} \frac{B_t - C_t}{(1+i_r)^t} \quad \text{(Eq.2)}$$

$$BCR = \frac{\sum_{t=0}^{n} \frac{B_t}{(1+i)^t}}{\sum_{t=0}^{n} \frac{C_t}{(1+i)^t}} \quad \text{(Eq.3)}$$

In the formulas, B_t shows revenues in the t year, C_t shows costs in the t year, n is rotation age, i is interest rate and i_r is internal rate of return (IRR). In the research, P = 3% forestry little interest rate was used in NPV and BCR calculations. IRR is calculated by iteration method using different interest rates in the Excel software. The current prices of 2017 have been used in the calculations.

In this study, the economic analyses were made only in terms of wood production without considering the advantages such as biodiversity service of semi-arid regional plantations, the protection of natural balance, prevention of erosion, etc., and they were compared with the economic returns of natural brutia pine stands under the same conditions.

Results

Results related to the technical analysis of plantations

According to the results of the variance analysis, the survival percentage, height and breast height diameter ($d_{1.30}$) of the young saplings were found to vary according to the aspects (*Table 2*).

When examined the results of the variance analysis related to the variables such as diameter, height and survival percent in *Table 2,* it was found that there is a significant difference among the aspects of three variables at a confidence level of $P < 0.01$. Therefore, a Duncan test was conducted in order to determine homogenous groups in terms of the examined variables (diameter, height, survival percentage). The Duncan test results related to the variables of diameter, height and survival percent, which are important in terms of growth and adaptation in the evaluation of plantation activities, were shown in *Table 3*.

From the mean values examined in *Table 3,* it is understood that breast height diameter growth ranges from 13.49 to 15.20 cm. According to the Duncan test, three different groups were emerged in terms of breast diameter growth at a confidence level of $P < 0.01$. When taking into consideration age difference in the Calabrian pine

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plantations at different aspects, it was ascertained that on the northern aspects growth was better than other aspects in terms of mean diameter growth, and it decreased especially among those towards the south aspects despite the age difference. The height was found to range 3.66-6.25 m according to the aspects. In the result of the Duncan test at a confidence level of $P < 0.01$, three different homogenous groups were determined among the aspects in terms of the variable of height (*Table 3*). One of the significant variables in determining the adaptability of plantations is the mean survival percentage. It was found out that the mean survival percentage ranges from 18.60 to 57.13%. According to Duncan test results; the survival percentage at a confidence level of $P < 0.01$ forms three different homogenous groups in terms of the aspects (*Table 3*). These results show that the high percentage of survival is seen in the north aspect while the lowest one is in the south aspect. Despite the age difference, the mean survival percentage is seen to be higher in north and eastern (shady) aspects rather than southern and western (sunny) aspects.

Table 2. *The results of one way variance analysis related to Calabrian pine plantations*

Variation source		Sum of squares	Degree of freedom	Mean square	F value	Significance level
Diameter	Between groups	5.693	3	1.898	25.484	0.000
	Within groups	0.596	8	0.074		
	Total	6.289	11			
Height	Between groups	13.531	3	4.510	1061.276	0.000
	Within groups	0.034	8	0.004		
	Total	13.565	11			
Survival percentage	Between groups	2615.469	3	871.823	42.789	0.000
	Within groups	163.000	8	20.375		
	Total	2778.469	11			

Table 3. *Duncan range test results related to Calabrian pine plantations*

Research area	Aspect	Diameter (cm)	Height (m)	Survival percentage (%)
Harran Plain	South	13.49^{a}	3.66^{a}	18.60^{a}
	West	14.49^{b}	5.90^{b}	23.97^{ab}
	East	15.13^{c}	6.14^{c}	32.33^{b}
	North	15.20^{c}	6.25^{c}	57.13^{c}
	Mean	14.58	5.49	33.01

a: fist group, b: second group, c: third group

Results related to the economic analysis of plantations

Site index, rotation age, thinning age and intensity, volume results

Based on the top age and height values from the sample plots, the top height and age averages were measured at the aspects. From the yield table produced for Turkish pine plantation areas by Usta (1991), it was found out that all the sample plots were in the third (bad) site class based on the top height, age and 3 × 3 m (9 m^2; 1,111 trees in per

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hectare) potential growth area (*Table 4*). According to this table, the top height in partially shady (northern, eastern and western) aspects is higher than that in the south. This shows that water-humidity is the minimum factor limiting the growth in semi-arid areas.

Table 4. *The top height, age and site index at the aspects of Calabrian pine plantation area*

Sample plot		**Top height (m)**	**Age (year)**	**Site index (Third class = III = bad site)**
Aspect	**Number (piece)**			
North	3	6.26	18	III
South	3	3.80	16	III
East	3	6.53	18	III
West	3	6.06	17	III
Mean		5.66	17	III

By using the yield table prepared by Usta (1991), the 33rd age with the highest average volume increment was identified as rotation age for the 3 × 3 m (9 m^2) potential growth area in the third site index. Likewise, the only thinning age was taken as the 25^{th} age determined by Usta (1991), and it was assumed that the thinning treatments was applied at 35% intensity. Also, 35% (21.35 $m^{3)}$ of 61 m^3 volume given by Usta (1991) for the 25^{th} age was taken as the intermediate yield (D_{25}). Assuming that a thinning treatment of 35% intensity applied at the 25^{th} age increased the gap-distance by 35%, the volume of the final yield (A_{33}) at the 35^{th} age was taken as 95.5 m^3 corresponding to 12 m^2 potential growth area.

For natural Turkish pine stands the yield table and site index prepared by Erkan (1996) were used. The site class which is close to the top height (5.66 m) and age (17 years old) values in the sample areas in the triple site classification table of Erkan (1996) belong to third class. The yield table of 13.3 site index which is close to 12.5 as the average of this class was used. The rotation age in this table was 45 years, and it was supposed that first thinning was at the age of 25 (D_{25} = 15.68 m^3), second thinning was at the age of 35 (D_{35} = 20.67 m^3) and the final yield was at the age of 45 (A_{45} = 138.23 + 19.25 = 157.48 m^3).

Income and cost findings

The intermediate and final yield volumes determined for both natural Calabrian pine stands and plantation areas were distinguished into product ranges based on the medium diameter values that corresponded to these volumes in the related yield table and using the barked volume ratios in the product range table prepared by Sun et al. (1977). The net selling prices were calculated by extracting production costs from the auction sale prices in terms of production range of the Şanlıurfa Forest Enterprise Directorate (*Table 5*). The net incomes were calculated by multiplying the number of production range by net selling prices.

On the other hand, the cost of 1 ha plantation area (establishment, maintenance, sapling cost and other expenses) was determined as 2,467.17 US$/ha at 2015 prices based on unit prices of the General Directorate of Forestry for the option of machine + labor production technology.

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Table 5. *Production costs and sale prices according to product types of Calabrian pine (as of 2015)*

Tree species	Product types	Production cost (US$)	Sale price (US$)	Net sale price (US$)
Calabrian pine	Log (m^3)	21.87	78.57	56.69
	Mine pole (m^3)	21.87	65.06	43.19
	Industrial wood (m^3)	15.31	61.89	46.58
	Fuel wood (m^3)	17.19	28.10	10.91

Findings of net present value, internal rate of return and benefit cost ratio

The rotation age, thinning ages and third site class were determined in the way described above for brutia pine plantations and natural stands. The economic analyses were made using NPV, IRR and BCR criteria based on net selling prices, plantation costs, and the intermediate and final yield volumes determined by the help of the related yield tables and product range table. The calculations were made based on the 2015 prices (*Table 6*).

In order to eliminate the effect of rotation age, the annual mean NPVs were calculated by dividing NPVs (US$/ha) calculated for plantation areas and natural stands to rotation age. According to this, the annual NPVs are calculated as - 29.52 US$/ha/year for the option of *plantation area + bad site + 33 year rotation age* and - 15.64 US$/ha/year for the option of *natural stand + bad site + 45 year rotation age*. Therefore, it was found that natural brutia pine stands in the same site index had a higher NPV than that of brutia pine plantations in semi-arid regions in terms of wood production according to NPVs calculated for both the whole rotation age and annually.

In the above calculations, NPV, IRR and BCR values were calculated according to the assumption that success of plantation areas was 100% (optimal). However, since the success was determined to be 33.01% in the plantation areas because of several reasons (*Table 3*), the incomes from the fields were multiplied by these success rates and thus actual economic results were achieved and the results were shared in *Table 7*. Because the yield tables for the natural stands reflected the real situation of the areas, the optimal and actual situations of them were the same.

According to *Tables 6* and *7,* NPVs of both natural stands and plantation areas with 3% interest rate in the third site index areas were found negative. However, the NPV of plantation areas is smaller. Considering the actual success of plantation areas, the NPV is even smaller. Similarly, IRR and BCR values of plantation areas are smaller in natural stands. This most probably stems from the fact that the plantation area is in semi-arid regions and the success rate is low.

Discussion and conclusion

In this study, technical and economical evaluations were carried out by making measurements in the plantation area in the Şanlıurfa-Harran Plain of Turkey as a result of half of the rotation age in 2015 after 16-18 years from establishment of the area. In this research, it was determined that the top mean height value was 6.25 m for Calabrian pine in the plantation area of the Harran Plain, the top mean breast diameter value was 15.20 cm and the top mean survival percentage was 57.13% in northern and eastern

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(shady) aspects (*Table 3*). According to the variance analysis, significant differences were estimated at a confidence level of $P < 0.01$ in spite of the age differences in the aspects (*Table 2*). The mean values of such variables were determined to decrease from the shady aspects towards sunny (southern and western) aspects by Duncan test (*Table 3*).

Table 6. *Net present value, internal rate of return and benefit cost ratio calculations for the Calabrian pine plantation area and the natural stand*

Site	Site index	Rotation period	Return years	Product types	Amount of product (m^3/ha)	Net sale price (US$/$m^3$)	Reduced net incomes (US$/ha) P = 0.03	Reduced net incomes (US$/ha) P = 0.01
Afforestation Site of Calabrian pine	III (Bad)	33	D_{25}	Fuel wood	6.6	10.91	34.40	56.17
				Industrial wood	6.73	46.58	149.73	244.46
				Mine pole	8.03	43.19	165.63	270.41
			A_{33}	Fuel wood	36.67	10.91	150.89	288.19
				Industrial wood	26.84	46.58	471.39	900.33
				Mine pole	31.99	43.19	520.87	994.84
				Log	0	56.69	0.00	0.00
			Total of incomes (US$/ha)				1,492.91	2,754.40
			Total of outcomes (US$/ha)				2,467.17	2,467.17
			NPV (US$/ha)				- 974.14	287.24
			IRR (%)				1.46	
			BCR				0.61	
							P = 0.03	P = 0.02
Natural forest stand of Calabrian pine	III (Bad)	45	D_{25}	Fuel wood	4.85	10.91	25.28	32.26
				Industrial wood	4.94	46.58	109.91	140.27
				Mine pole	5.9	43.19	121.69	155.31
			D_{35}	Fuel wood	6.95	10.91	26.96	37.93
				Industrial wood	6.26	46.58	103.63	145.81
				Mine pole	7.46	43.19	114.49	161.09
			A_{45}	Fuel wood	67.24	10.91	194.06	301.02
				Industrial wood	41.1	46.58	506.28	785.34
				Mine pole	49.13	43.19	561.07	870.33
				Log	0	56.69	0.00	0.00
			Total of incomes (US$/ha)				1,763.37	2,629.36
			Total of outcomes (US$/ha)				2,467.19	2,467.19
			NPV (US$/ha)				- 703.79	162.07
			IRR (%)				2.19	
			BCR				0.71	

NPV: net present value, IRR: internal rate of return, BCR: benefit cost ratio

The mean height, mean diameter and mean survival percentage of the research area are generally at a low rate. But, when considered that the general ecological structure of the research area falls under the domain of a semi-arid climate zone, the stated diameter and height growth and survival percentage in this plantation area can be said to be at a

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satisfactory level and value in terms of prevention of erosion in the region and soil conservation. In a research study that was conducted in the black pine plantation areas of the Konya-Karapınar region which had similar ecological conditions to those of this research (Özel, 2010); its growth and survival percentage were found to be low. The function of black pine in order to protect especially agricultural areas against endemic wind erosion and the consequential soil erosion was of crucial importance. Similarly *Eucalyptus camaldulensis* was considered as the most suitable tree species for arid land afforestation of Western Australia, by comparing biomass growth and survival ratio of planted trees (Suganuma et al., 2014). Along with this, the variable of survival percentage is a significant one in order to determine adaptability of the species used in the plantation activities (Ürgenç, 1998; Tunçtaner, 2007). That this variable reaches up to 57.13% at the northern aspects in a poor plantation circumstances under semi-arid climate conditions like the study area will be useful in terms of the implementation and sustainability of protective forest functions such as erosion control, carbon sequestration and recreation. A study made in the semiarid desert steppe of China showed that afforestation greatly affected the community and habitat characteristics, and promoted the formation of the crust on the soil surface with the succession of vegetation (Liu et al., 2015). In this regard, it is important to establish new forest areas in different plantation conditions and to manage them functionally against catastrophic disasters such as erosion, flood and avalanche as a result of the destructions of Turkey's forest resources, irregular urbanization and industrialization.

Table 7. *Net present value, internal rate of return and benefit cost ratio values for Calabrian pine according to optimal and actual situation*

Stand type	Site index	Rotation period (year)	According to optimal situation			According to actual situation		
			NPV (US$/ha)	IRR (%)	BCR	NPV (US$/ha)	IRR (%)	BCR
Afforestation	Bad	33	- 974	1.46	0.61	- 1,974	1.25	0.20
Natural forest stand	Bad	45	- 704	2.19	0.71	- 704	2.19	0.71

NPV: net present value, IRR: internal rate of return, BCR: benefit cost ratio

In Turkey, a total of 57.17 million ha are exposed to moderate and severe erosion. The amount of soil loss due to erosion eliminates the primary sources for agricultural production, cultivated for nutrition in semi-arid and arid regions especially of the countries whose forests have been destroyed. While a total of 585.6 ton/km^2 topsoil is lost because of floods in Turkey, this rate is 31.3 ton/km^2 in Europe (Çepel, 1992; Dağdaş, 2007). The places which experience erosion are mostly arid and semi-arid areas in Turkey and the world (Fidan, 2006). In the plantation of such areas where there is poor and shallow soil and of erosion risk, some applications should be used such as the selection of appropriate species and origins, the use of potted seedlings which are of quality root-shoot balance and mycorrhizal vaccines as well as some cultural rehabilitation activities (Ürgenç, 1998; Boydak and Çalıkoğlu, 2006). Especially the use of quality seedlings has a special importance in terms of the plantation success. The quality seedling should be of a balanced root-shoot rate and sufficient capillary root density (Turna et al., 2007). What is more, by protecting the existent vegetation where there is a risk of drought, desertification and severe erosion, it will make the plantations successful to make use of natural species and local races which have adaptability. In this regard, the successful samples of erosion control, dune and arid area plantations that need special techniques can be found in Turkey (Boydak and Çalıkoğlu, 2006; Ayan et al., 2007).

Arid and semi-arid afforestations have many environmental and protector impacts. Afforestation decreases percent cover and aboveground biomass, and increase plant diversity of herbaceous community. Afforestation also decreases soil bulk density and pH, and increased soil water content (Chen et al., 2016). Moreover, afforestation increases soil organic carbon and nitrogen storage in semi-arid grass lands, which has important implications for C sequestration. In the arid and semi-arid regions, water supply is the main factor limiting sustainable development of planted forests (Wang et al., 2011; Chen et al., 2016).

On the other hand, a thorough plantation in semi-arid areas reduces seedling survival percentage significantly. It is thought to be an important factor on the low survival percentage of brutia pine seedlings in the study area. In this regard, it is necessary first to protect the area and provide soil stability by placing endemic and existent annual and perennial plant species in order to reduce earth movement. Since greening which is also called as temporary and pre-stabilization activity enables to improve physical soil properties and increase organic ingredients in soil, it boosts significantly the achievement of the main plantation activity or the adaptation success of permanent stabilization activity. In this issue, similar outcomes are achieved in the research studies aimed at controlling erosion in the Eastern Anatolia Region and it is recommended that the natural vegetation should be protected and prohibited for pasturage before plantations (Daşdemir et al., 1996; Daşdemir, 2006). Besides, it is observed that the research areas are anthropogenic steppe areas where the black pine and oak forests are cleared in order to grow crops and consequently they are faced with a moderate and severe risk as a result of denudation. Hence, it is more convenient that the plantation is maintained with the drought-tolerant origins of a local species, black pine, in such plantation areas. Also, leaved species should be partly utilized where local climatic factors are suitable. Therefore, it will enable plantation activities to be carried out swiftly and genetic diversity to be protected.

Economic analyses were made by taking third class (IIIth =bad) site, 33 and 45 year rotation age and machine + labor force production technology into consideration in terms of only wood production for the natural Calabrian pine stands and the Calabrian pine plantations in the Şanlıurfa-Harran Plain (*Table 6*). The NPV, IRR and BCR values of each option were calculated according to optimal and actual situation. According to the both situations, the NPV, IRR and BCR values of natural stands are greater than those of plantation areas (*Table 7*). According to this, NPV = - 974 US$/ha, IRR = 1.46% and BCR = 0.61 for the option of *plantation area + bad site + 33 years rotation age*; NPV = - 704 US$/ha, IRR = 2.19% and BCR = 0.71 for the option of *natural stand + bad site + 45 years rotation age*.

In this issue, in a research study carried out on the Turkish pine plantations of the Bartın region (Daşdemir and Şahin, 2005), the lowest NPV and IRR values were found to be as follows: NPV = 217.45 US$/ha and IRR = 4.28% for the option of *bad site + 33 years rotation age + labor force*. On the other hand, in another study carried out in the Antalya region by Erkan et al. (2002), the lowest NPV = - 23.845 US$/ha and IRR = 4.48% in 40 years rotation age for brutia pine without the exception of production techniques. Economical profitability with 10% discount rate was found as a value

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DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

chancing between 311 and 3,863 US$/ha according to different afforestation options in afforestation with fast-growing eucalyptus species in Northeast Thailand (Niskanen, 1997). Hence, NPV and IRR values of the Calabrian pine plantations in the Şanlıurfa-Harran Plain are lower than those of both natural and planted Calabrian pine stands in other regions in the same site index. It mainly stems from that the plantation areas are in semi-arid regions, the growth-increment is limited, the success rate is low and plantation expenses are high.

Even if Calabrian pine plantations in semi-arid areas are of low income in terms of wood production, they play an important role because they bear significant benefits and services such as the services of biological diversity, the production of natural balance, prevention of erosion, carbon sequestration, recreation, employment in addition to wood production. Among the studied environmental impacts of afforestation, carbon sequestration and transpiration are most important in economic terms (Niskanen, 1997). As a result of taking into consideration these benefits and services, which are priceless but is estimated indirectly, it will be seen that semi-arid area plantations are of high benefit. In this regard, in a study of erosion control by Daşdemir et al. (1996) in the Erzincan region, the price of such benefits and services was estimated as 4.50 US$/ha with 1994 prices and BCR = 1.37. Similarly, in a research study in the Bartın region (Daşdemir and Seğmen, 2009), such benefits and services were estimated to be 33.58 US$/ha at 2004 prices. Provided that these benefits and services are taken into consideration, it is obvious that the plantations in semi-arid areas will be of high income. Therefore, the potential semi-arid plantations should be accelerated as well as industrial plantations for wood production in good site areas. In addition, economic efficiency and cost minimization principles should be obeyed as much as required in semi-arid plantations and the highest success should be targeted with a minimum cost.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17571772

BIOSORPTION OF Cr^{3+} AND Pb^{2+} FROM TANNERY WASTEWATER USING COMBINED FRUIT WASTE

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(Received 29th Oct 2018; accepted 5th Dec 2018)

Abstract. In recent years, tanning industries have been proved to be among the major contributors of heavy metals. Different convectional and biological method has been used to remove these heavy metals from tannery wastewater but most of these technologies are costly and not easily accessible. This research is aimed at determining the efficiency of combining the waste rind of *Citrulus lanatus* and waste peel of *Citrus sinensis* as a low cost biosorbent for the removal of Cr^{3+} and Pb^{2+} pollutants present in tannery wastewater. The selected biosorbents where surface characterized and point of zero (pH_{PZC}) was determine with pH adjusted using NaOH solution to be between 2-12. The adsorption process of Cr^{3+} and Pb^{2+} by the selected biosorbent was determined under factors such as contact time, pollutants dosage, absorbent dosage, and particle size. The Lagmuir equation and Freundlich isothermal were used to determined adsorption equilibrium while pseudo first order and pseudo second order were used to determine the adsorption kinetics. The result obtained shows that *Citrulus lanatus* has better adsorption of Pb^{3+} (86%) while the combined fruit better adsorption of Cr^{3+} (85%) under the all factors. The Lagmuir equation and Freundlich shows suitability of indicating equilibrium sorption for Cr^{3+} and Pb^{2+}. The adsorption kinetic study shows that pseudo-second order obeys kinetics model signifying that absorption occurs by chemisorptions. The combination of the waste rind of *Citrulus lanatus* and waste peel of *Citrus sinensis* is an effective low cost biosorbent for the removal of Cr^{+3} and Pb^{+2} pollutants present in tannery wastewater.
Keywords: *adsorption isotherm, heavy metal pollution, adsorption kinetics, Citrus sinensis, Citrulus lanatus*

Introduction

Heavy metal pollution resulting from anthropogenic activities is a serious problem to man and his environment due to the persistence nature and ability of heavy metals to accumulate in the food chain (Dudgeon et al., 2006; Lintern et al., 2016; Liu et al., 2018). In developing countries, industries channel wastewater into nearby water bodies either untreated or not properly treated due to their proximity to these water bodies (Desrosiers et al., 2019).The wastewater produce by most of these industries are rich in heavy metals which pose serious toxicity at low level exposure (Hughes et al., 2015). In recent years, tanning industries have been proved to be among the major contributors of heavy metals (Cr, Pb, Zn, Cu, Cd, As and Se) in to water bodies (Aravindhan et al., 2004; Di Iaconi et al., 2003; Panizza and Cerisola, 2004). These heavy metals release into water bodies have been shown by many researchers to be associated with chronic and acute effects to man and detrimental to other organisms such as algae, plants, other animals, microorganisms etc (Costa-Boeddeker et al., 2018; Wang et al., 2018; Xun et al., 2018). It is thereby paramount to treat wastewater before discharge (Tong and Elimelech, 2016; Zhang and Anadon, 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17731787

A lot of technology is available for the treatment of wastewater before discharging into water bodies, many of which have contributed in minimizing pollution resulting from chemical industries but biosorption is gaining acceptance owing to the fact that most researchers have reveal the potentiality of some bio sorbents to effectively remediate wastewater with high heavy metal pollutants removal if match up to other biological and conventional technologies (Nahar et al., 2018). Biosorption is a remediation technology that depends on the mechanism of heavy metal accumulation by agricultural or biological adsorbents from an aqueous solution as a result of binding site present on this bio sorbents (He and Chen, 2014; Ileri et al., 2014).

Several research have been conducted showing the efficiency of different biosorbent removing Cr^{3+} from wastewater among which include egg-shell, sawdust, rice husk and lemon peel (Nahar et al., 2018), phosphate treated sawdust (Ajmal et al., 1996), cotton hull and soybean (Marshall and Champagne, 1995), sawdust carbon (Selvi et al., 2001), egg-shell (Park et al., 2007; Mashangwa et al., 2017), Litchi peel (Manikandan et al., 2016), modified groundnut hull (Owalude and Tella, 2016), Powdered potatoes peel (Mutongo et al., 2014), *Citrus limetta* (Saha et al., 2013) etc.

Nevertheless, Pb^{2+} has also attracted researchers for use in the biosorption of wastewater. This curiosity have led to the success of the used of different agricultural waste as low-cost biosorbents, among which include banana peel (Anwar et al., 2010), different cortex fruits (Al-Qahtani, 2016), Agare bagasses (Velazquez-Jimenez et al., 2013), Garden grass (Hossain et al., 2012), modified orange peel (Guo et al., 2011), modified lentic husk (Basu et al., 2015), olive stone raw (Fiol et al., 2006), ponkan peel (Pavan et al., 2008) etc.

A lot of success has been recorded for the biosorption of different pollutants by *Citrus sinensis* (Feng et al., 2011; Guiza, 2017; Pérez-Marín et al., 2008) and *Citrulus lanatus* (Reddy et al., 2014; Liu et al., 2012; Jawad et al., 2018), however more research is needed to investigate how those agricultural wastes can be improved for effective pollutants removal from waste water. The current research is aimed at determining the efficiency of combining the waste rind of *Citrulus lanatus* and waste peel of *Citrus sinensis* as a low cost biosorbent for removal of Cr^{3+} and Pb^{2+} pollutants present in tannery wastewater.

Material and Method

Sample Collection and Preparation

The waste peel of C*itrus sinensis* peel and *Citrulus lanatus* rind were collected from road side fruit sellers at kasuwan mata, Kaduna South Local Government, Kaduna State, Nigeria. Both bio-wastes were cut into small pieces, washed several times with borehole water then distilled water. *Citrus sinensis* peel was dried at 40°C in the oven as described by Kelly-Vargas et al. (2012). *Citrulus lanatus* rind was first dried in sunlight for 48 hours then oven dried at 80°C. The fruit wastes were grounded using a mechanical blender (Greenis, FGR-8840) and then sieved through standard sieve to obtain various size of C*itrus sinensis* peel and *Citrulus lanatus* rind. The fruit waste powders were stored separately in an airtight container before use. To obtain a combined fruit waste of the two peels, an equal amount of dried, grounded peels were weighed and mixed together. Tannery effluent samples were collected from the main discharge point/network pipeline of Unique Leather Industry located at Sharada industrial area, Kano. Standard sample bottles were used in collecting the wastewater

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DOI: http://dx.doi.org/10.15666/aeer/1702_17731787

sample. Using distilled water, the bottles were washed thoroughly. The samples were placed in an storage box containing ice packs in order to preserve and maintain their composition thus preventing degradation by microbes. In this regard, each sample bottle is added with 5 ml 2.0 M nitric acid per litre (Lugo-Lugo et al., 2012). Digestion of samples was carried out according to the Standard method by Ahuja (2012). Samples were filtered using Whatman filter paper of pore size 0.45µm and analysed using AAS for heavy metal content as described by Lugo-Lugo et al. (2012). pH was measured using Toledo pH meter of model FE20/EL 20.

Characterization of Biosorbents

Citrus sinensis peel, *Citrulus lanatus* rind and Combine fruit waste were pre-treated using H_2O_2 according to Shen et al. (2011). Surface characterization was done using the method of Boehm (1966) in which 0.4g of *Citrus sinensis* peel, *Citrulus lanatus* rind and Combine fruit waste was were neutralize separately using 50ml of Cr^{+3} and Pb^{+2} solution by using acid based titration techniques for 5 days. Point of zero (pH_{PZC}) of *Citrus sinensis* peel, *Citrulus lanatus* rind and Combine fruit wastewater determined by adjusting the pH value using 50ml 0.01 NaCl solution to between 2-12. The samples were agitated and stored for 24 hours at 12rpm at room temperature. The P_{PZC} was then calculated graphically according to Banerjee et al. (2017). Scanning electron microscope (JEOL JSM-6100) was used to observe how the surface of *Citrus sinensis* peel, *Citrulus lanatus* rind and Combine fruit waste is affected by the adsorption process.

Effect of Particle Size and Contact Time on Cr and Pb Adsorption

The effect of contact time and particle size was studied together by placing 5g of *Citrus sinensis* peel, *Citrulus lanatus* rind and Combine fruit waste of two particle sizes (40mm (coarse) and 60 mm (fine)) in separate conical flasks containing 100 ml effluent sample and agitated at 150 rpm in a shaker. Each set of flasks were agitated for 20, 40, 60, 80, 100, and 120 minutes, respectively. The samples were filtered using Whatman No 41 filter paper after each interval. Residual heavy metal concentration of the sample was measured, using AAS.

Biosorption was calculated using the mass balance formula below:

$$q = \frac{v(C_l - C_e)}{m} \quad \text{(Eq.1)}$$

where, q (mg/g) is the adsorption capacity, C_l and C_e are the initial and final concentrations (mg/l) of sample, V(l) is the volume of effluent sample and m is the weight (mass) of adsorbent (g).

Percentage removal: it was calculated using the formula:

$$q\%\ uptake = \frac{(C_o - C_e)}{Co} \times 100 \quad \text{(Eq.2)}$$

where C_0 and C_e are the initial and final concentrations (mg/l) of metals in sample before and after shaking.

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DOI: http://dx.doi.org/10.15666/aeer/1702_17731787

Effect of Cr^{3+} and Pb^{2+} dosage on Adsorption Process

To the 250 m/l of solution containing 15 mg/l, 20 mg/l, 25 mg/l, 30 mg/l and 35 mg/l Cr and 10 mg/l, 15 mg/l, 20 mg/l, 25 mg/l and 30 mg/l of Pb 5g of dried *Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combined fruit waste was added separately and shaked at room temperature at 150 rpm for 12 hours. Cr and Pb percentage reduction was thus calculated using the method employed by Kumar et al. (2018).

Percentage removal: it was calculated using the *Equation 2*.

Effect of pH on Cr and Pb adsorption Process

The absorption ability of the solvent was compared at a pH of 2, 4, 6, 7, 8 by placing 1g of *Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combined fruit waste in 100 ml each of the solution of Cr and Pb at 15 mg/l and 10 mg/l, respectively. The samples were filtered using Whatman No 41 filter paper after each interval. Residual heavy metal concentration of the sample was measured, using AAS. The pH of the solution was adjusted using diluted sodium hydroxide and Nitric acid as adopted from Salim et al. (2016).

Effect of Particle Size and Dosage

The effect of absorbent dosage was study by dissolving a fixed mass of 15mg/l of Cr^{3+} and a fixed mass of Pb^{2+} 10 mg/l in 100 ml of solution. 1 g, 2 g, 3 g, 4 g and 5 g each of *Citrus sinensis* peel, *Citrulus lanatus* rind, and combined fruit waste where placed in each solution. The samples were filtered using Whatman No. 41 filter paper after each interval. Residual heavy metal concentration of the sample was measured by using AAS as described above.

Adsorption Equilibrium Study

Adsorption equilibruim study for Cr and Pb using *Citrus sinensis* peel, *Citrulus lanatus* rind and hybrid fruit waste powder was performed by suspending 1g, 2g, 3g, 4g and 5g of *Citrus sinensis* peel, *Citrulus lanatus* rind and combined fruit waste powder in 15mg/l, 20mg/l, 25mg/l, 30mg/l and 35mg/l Cr^{+2} and 10mg/l, 15mg/l, 20mg/l, 25mg/l and 30mg/l of Pb^{+2} and shaked at room temperature at 150 rpm for 12 hours. Langmuir and Freudlich isotherm models were thus tested according to the method adopted by Manikandan et al. (2016).

Langmuir equation is represented below:

$$\frac{1}{q_x} = \frac{1}{b_i q_f} T_e + \frac{1}{q_f} \qquad \text{(Eq.3)}$$

The equation was adopted from Kumar et al. (2018), where T_e = Equilibrium Cr^{3+} and Pb^{2+} concentration in solution (mg/l), q_f is maximum Cr^{3+} and Pb^{2+} absorbed per unit weight of *Citrus sinensis* peel, *Citrulus lanatus* rind and combined fruit waste powder (mg/g), b_i is affinity adsorbate – biosorbent (l/mg), = is the amount of Cr^{3+} and Pb^{2+} adsorbed by the adsorbent at equilibrium (mg/g) the value of q_f and b_i is determined from the slope and intercept of. Z is the separation factor and is calculated using the formula below:

$$Z = \frac{1}{1 + b_I T_a} \qquad \text{(Eq.4)}$$

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where T_g = initial concentration of Cr^{+3} and Pb^{+2} in the solution.

Freundlich Isotherm is represented below:

$$Log\, qx = Log\, Kf + \frac{1}{r \log T_e} \quad \text{(Eq.5)}$$

The equation was adopted from Wang et al. (2010), where K_f is Freudlich Constant *r* is Freudlich Coefficient, and K_f and *r* are determine by plotting a graph of q_x against T.

Adsorption Kinetics

Adsorption kinetics is fundamental in describing the characteristic of an absorbent. To ascertain the mechanism involve in the adsorption of Cr^{3+} and Pb^{2+} by *Citrus sinensis* peel, *Citrulus lanatus* rind and combined fruit. Pseudo first order and pseudo second order reaction was used according to the procedure of Ho and McKay (1998).

The pseudo first order is represented below:

$$Log\ (q_i - q_t) = log\ (q_i) - \frac{K_i T}{2{,}303} \quad \text{(Eq.6)}$$

The equation was adopted from Poonam et al. (2018), where q_i and q_t = Amount of Cr^{3+} and Pb^{2+} (mg/g^{-1}) absorbed at equilibrium and time *T*, respectively, *K* is the pseudo-first order rate constant (min^{-1}).

The pseudo second order is represented below:

$$\frac{T}{q_t} = \frac{1}{K_2 q_2} + \frac{T}{q_2} \quad \text{(Eq.7)}$$

$$\frac{1}{q_t} = \frac{1}{K_2 q_2 T} + \frac{1}{q_2} \quad \text{(Eq.8)}$$

The equation was adopted from Poonam et al. (2018), where q_i and q_t = Amount of Cr^{3+} and Pb^{2+} (mg/g^{-1}) absorbed at equilibrium and time *T*, respectively, K_2 is the pseudo second order rate constant ($g\ mg^{-1}\ min^{-1}$).

Desorption Study

Desorption of Cr^{3+} and Pb^{2+} was from *Citrus sinensis* peel, *Citrulus lanatus* rind and combined fruit waste powder was studied for 0.1 N HCl, 0.1 N HNO_3, 0.1 N H_2SO_4 and 0.1 N NaOH. The dried *Citrus sinensis* peel, *Citrulus lanatus* rind and combined fruit waste powder with absorbed Cr^{3+} and Pb^{2+} in the solvents and shake for 150 rpm at room temperature for 12 hours adopted from Rosales et al. (2016). In order to determine the mechanism involve in adsorption of Cr^{3+} and Pb^{2+} by *Citrus sinensis* peel, *Citrulus lanatus* rind and hybrid fruit waste.

Statistical Analysis

Experimental was conducted in triplicate and data were analyzed statistically using standard deviation to find significance difference at 5% level. The Residual Sum of Squares (RSS) was determined for both adsorption kinetic and isotherm models to check error in model fittings. All analysis was done using BM SPSS statistics version 23.

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Result and Discussion

Effect of Contact Time on Cr^{+3} and Pb^{+2} Adsorption

Figure 1 shows the uptake of the two metal ions, Cr^{3+} and Pb^{2+} the waste peels (C*itrus sinensis* waste peel, *Citrulus lanatus* waste rind and combine fruits waste) was effective and continued progressively at the various time intervals set for the experiment. However, at 80 min the uptake of both metals stopped.

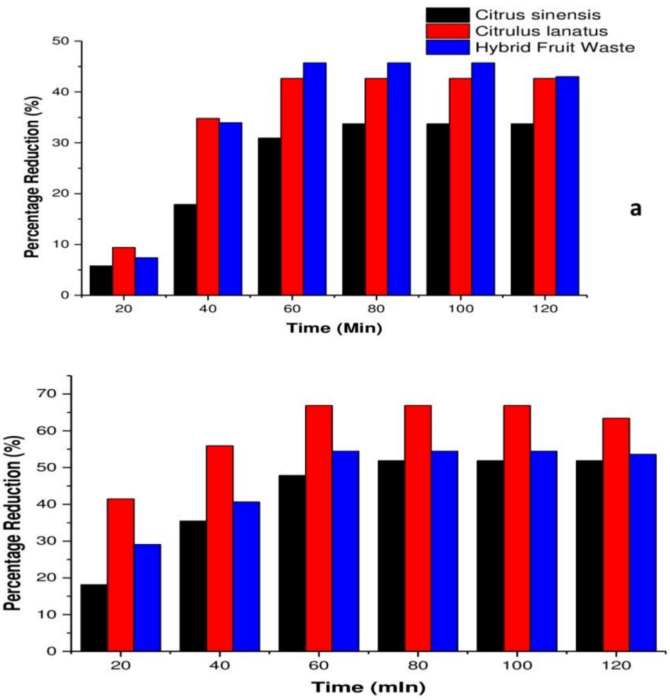

Figure 1. *Effect of Contact Time on Adsorption Cr^{+3} (a) and Pb^{+2} (b)*

The situation remained same from 100 min to 120 min. In other words, there was no net uptake of the metals after 80 min. The reason why the uptake of both Cr^{3+} and Pb^{2+} stopped from 80 min could be attributed to the fact that the particles of C*itrulus lanatus* waste rind, *Citrus sinensis* and the combined fruit waste peel posses selective potential for retaining of some heavy metals over a period of time (Ahad et al., 2017). With respect to contact time it is ascertain that Combined fruit waste was best in Cr^{3+} removal and *Citrulus lanatus* best in Pb^{2+} removal at all time interval used for the studies. This could be attributed to their surface areas. *Citrulus lanatus* waste rind and the combine waste fruit particles have a high surface area compare to *Citrus sinensis* waste peel thus providing more binding sites for metal ions. In contrast, *Citrus sinensis* has lesser surface area as such fewer sites for attachment by metal ions are available. Although, there was loss of metals at 120 min from the combine waste materials, it is likely that there was a synergistic effect by *Citrulus lanatus* and combine fruit waste (Gupta and Garg, 2015). The uptake of Cr^{3+} and Pb^{2+} also varied with size of the absorbent as higher uptake was noticed by fine absorbent (40mm) compare to coarse absorbent (60mm). The result obtained attribute high Pb^{2+} adsorption to fine particle of *Citrulus*

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lanatus if compare to coarse particle of *Citrulus lanatus*. The adsorption of Cr^{3+} by fine particles of combine fruit waste peel also follow the same trend as *Citrulus lanatus* if compare to coarse particle of combine fruit waste peel. The reason for the differences of pattern of adsorption of Pb^{2+} and Cr^{2+} by the absorbents based on size could be attributed to the fact that as the particle size increase so do the binding site increase resulting in the enhancement of metal absorption by the biosorbents (Al-Ghouti et al., 2003). Although, Senthil Kumar et al. (2012) shows that decrease of absorbent to a diameter of 240µm can cause reduction in adsorption ability of the adsorptive sites due to agglomeration of the particles that is not the case here because the lowest size used was 40mm.

Characterization of Biosorbents

Active site and surface area increase was recorded for *Citrus sinensis, Citrulus lanatus* and combined waste fruit waste peel although no significant differences (P = 0.05) exist between the increase of active sites and surface area of *Citrulus lanatus* rind compare to combine fruit waste but significant difference exist (0.05) between the increase of binding site and surface aarea of *Citrulus lanatus* rind and combined fruit waste compare to *Citrus sinesis* with both *Citrulus lanatus* and Combine fruit waste having more binding site and larger surface area. The pH (pH_{ZPC}) *Citrus sinensis, Citrulus lanatus* and combined waste fruit waste peel were 3, 4 and 7, respectively for Cr^{3+} but 2, 5, 4 for Pb^{3+}. The increase in active site and surface area is attributed to the collapsing of the adsorbent wall resulting to the modification of the adsorbent structure into porous structure. Result of scanning electron microscopic shows smooth surfaces for all the absorbent before adsorption study but rough surfaces after adsorption. The rough surface after adsorption signifies enhancement in adsorption capacity of the adsorbents and also the reason for the increase surface area.

Effect of Adsorbent Dosage on Cr^{+3} and Pb^{+2} Adsorption

Variation in dosage of *Citrus sinensis, Citrulus lanatus* and combined waste fruit waste peel affected the percentage uptake of Cr and Pb. Increase in dose of the test material from 1 – 5 g, was signified by an increase in percentage uptake of both metal ions (*Figure 2*). This could have resulted from the availability of more binding sites with each addition of waste materials. The difference in percentage uptake of Cr^{3+} by *Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combine waste rind was significant ($P < 0.05$) with combine waste fruit waste performing better than *Citrus sinensis* and *Citrulus lanatus.* The difference was also significant between *Citrus sinensis* waste peel, Citrulus lanatus and combines fruit waste for Pb^{2+} uptakes with *Citrulus lanatus* performing better than combined fruit and *Citus sinensis*. The reason could be similar to the same reason why both test material display the pattern of metal uptake under different duration.

Effect of pH on Adsorption Process

The adsorption process for Cu^{3+} and Pb^{2+} follow the decreasing pattern from pH of 2-12 with combine fruit waste material having higher adsorption for Cr^{2+} at the pH of 2 and *Citrulus lanatus* having higher adsorption for Pb^{2+} at pH of 2, pH was determined for these studies owing to the fact that Wang and Chen (2009) reported that the reason why biosorption occur is due to exchange of ion of which pH is the determinant factor

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for which proton compete with cation for binding sites (*Figure 3*). The higher adsorption recorded at the pH of 2 by all the materials used could be attributed to the fact that at lower pH (acidic medium), the removal of H^+ lead to availability of free binding sites for Cr^{3+} and Pb^{2+} (Poonam et al., 2018). Elhafez et al. (2017) had earlier reported that the removal of heavy metal by biosorption increases with increasing pH and decreases after pH of 4. This increasing pH tend to facilitate the precipitation of heavy metals thereby contributing to it removal.

Effect of Cr^{3+} and Pb^{2+} Dosages on Adsorption Efficiency

The test for the effect of Cr^{3+} and Pb^{2+} on adsorption efficiency was carried out taking into consideration the concentration of both heavy metals in the wastewater for effective treatment. The result obtain shows decreasing trend of adsorption efficiency with increase in mass of Cr^{3+} and Pb^{2+} ion concentration (*Figure 4*). This trend could be attributed to the fact that the increases in the concentration of Cr^{3+} and Pb^{2+} lead to decrease in the availability of free binding site on the solvent until the binding sites are completely saturated (Poonam et al., 2018; Zhang et al., 2018; Zhao et al., 2018). The difference in adsorption capacity of Cr^{3+} by *Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combine waste rind was significant at the different Cr^{3+} and Pb^{2+} concentration ($P < 0.05$) with combine waste fruit waste performing better than Citrus sinensis and Citrulus lanatus in Cr^{3+} adsorption and Citrulus lanatus performing better in Pb^{2+} adsorption.

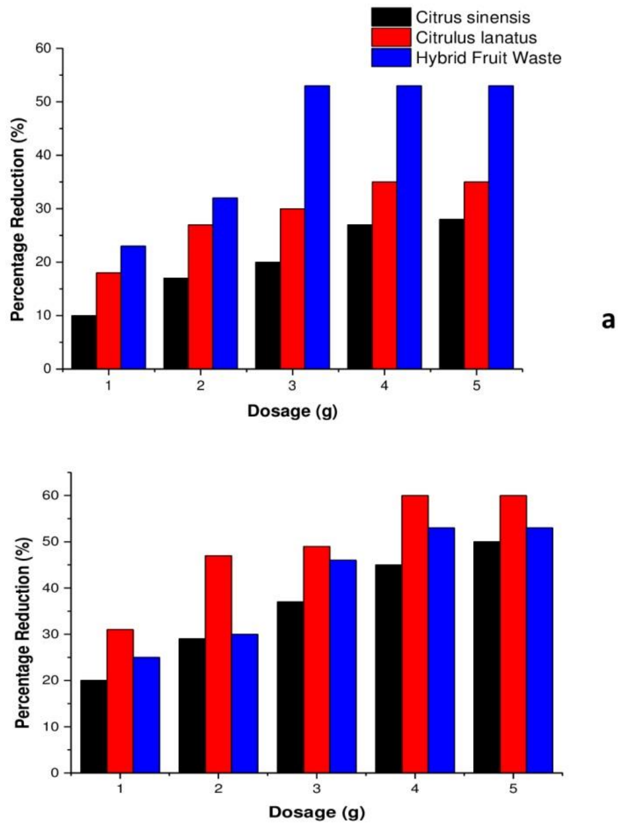

Figure 2. *Effect of Adsorbent Dosage on Adsorption (a) Cr^{3+} (b) Pb^{2+}*

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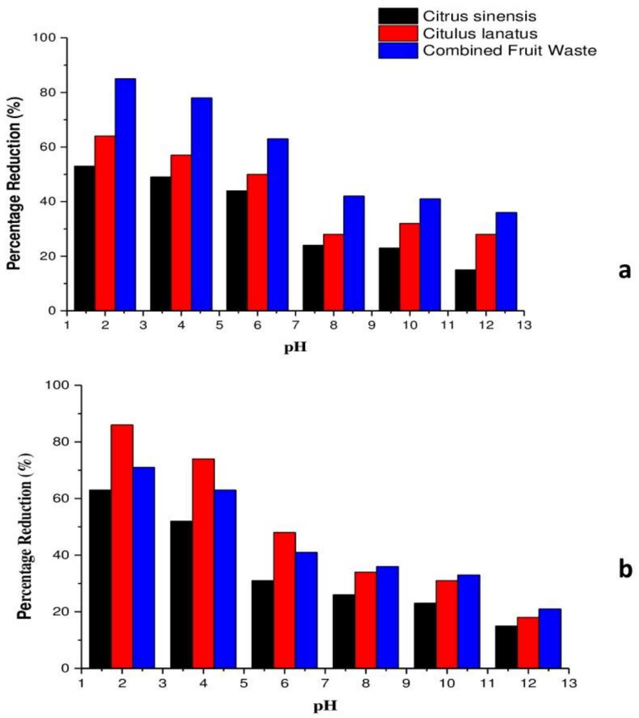

Figure 3. *Effect of pH on Adsorption Process (a) Cr^{3+} (b) Pb^{2+}*

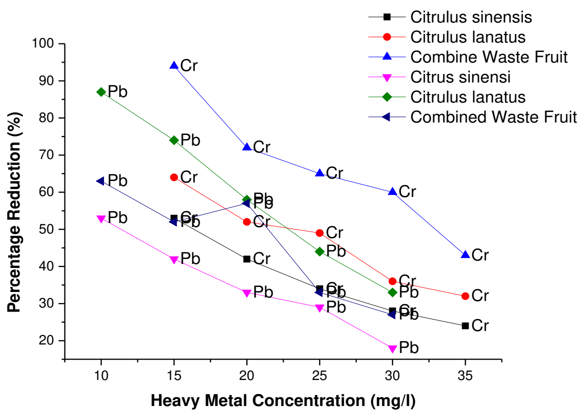

Figure 4. *Effect of Cr^{3+} and Pb^{2+} on Absorption efficiency*

Desorption Study

All the solution used for the desorption process recorded a high recovery of Cr^{3+} and Pb^{2+} from the biosorbent and are also non-polluting and non-damaging. This research is in agreement with the research available in literature (Zhang et al., 2018; Yang and Cui, 2013). This high efficiency recovery supports the fact that exchange ion depends on desorption. The recovered absorbents (*Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combine waste rind) still show high Cr^{3+} and Pb^{2+} removal even after triplicating the process indicating its high reuses capacity for commercial application (*Figure 5*).

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):1773-1787.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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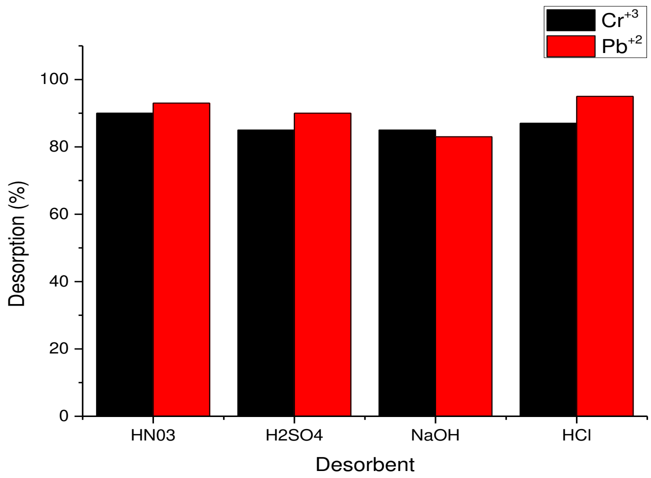

Figure 5. *Desorption Effeciency of Different Pollutants for the Desorption of Pb^{2+} and Cr^{3+}*

Comparison of the Removal Efficiency of Combined Fruit Waste and Other Biosorbents

Comparative study of the removal efficiency of the combination of *Citrus sinensis* peel and *Citrulus lanatus* rind with and other Biosorbents shows high removal efficiency by the combination of *Citrus sinensis* peel and *Citrulus lanatus* rind (*Table 1*). The difference in uptake by the Biosorbents could be attributed to difference in surface area, binding sites and experimental conditions.

Table 1. *Comparative Biosorption of Cr^{3+} and Pb^{2+} by Different Agricultural Waste*

SN	Biosorbent	Heavy Metal	Percentage Reduction (%)	References
1	*Citrus sinensis*	Cr^{+3} and Pb^{+2}	53, 63 (pH 2)	Present Study
2	*Citrulus lanatus*	Cr^{+3} and Pb^{+2}	64, 86 (pH 2)	Present Study
3	Combination of *Citrus sinensis and Citrulus lanatus*	Cr^{+3} and Pb^{+2}	85, 71 (pH 2)	Present Study
4	Eggshell	Cr^{+3}	99 (pH 7.2)	Park et al., 2007
5	*Citrus tangerine, Actinidia deliciosa, Musa acuminate* (waste)	Cr^{3+}	88, 91, 42	Al-Qahtani, 2016
6	*Brassica napus*	Pb^{+2}	94	Morosanu et al., 2017
7	*Saccharum officinarum* bagasse	Cr^{3+}	94	Rico et al., 2018
8	*Glycine max*	Pb^{+2}	79 (pH 4)	Gaur et al., 2018
9	*Saphora japonica* pod	Pb^{+2}	59 (pH 6)	Amer et al., 2015

Adsorption Isotherms

Langmuirs and *Freundlich* isotherms for Cr^{3+} and Pb^{2+} is approximately 1 signify the suitability of *Langmuir* and *Freundlich* isotherms in indicating equilibrium sorption (*Table 2*). The separation factor (Z) for *Langmuir* isotherms was found to be between the ranges of 0-2 signifying that the adsorption of Cr^{3+} and Pb^{2+} by the surfaces of the peels were favourable (Mallampati et al., 2015).

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***Table 2.** Isotherm Parameters for Adsorption of (a) Cr^{3+} (b) Pb^{2+}*

a,	Langmuir				Freundlich		
SN	**Fruit Waste**	**q_f (mg/g)**	**b_i (l/mg)**	**R^2**	**K_f**	**n**	**R^2**
1	*Citrus sinensis*	4.35	0.15	0.9557	0.5677	3.8878	0.9349
2	*Citrulus lanatus*	5.06	0.21	0.9756	0.8455	3.4588	0.9436
3	*Combine Fruit Peel*	5.35	0.29	0.9945	1.8873	5.5633	0.9971
b,	**Langmuir**				**Freundlich**		
SN	**Fruit Waste**	**q_f (mg/g)**	**b_i (l/mg)**	**R^2**	**K_f**	**n**	**R^2**
1	*Citrus sinensis*	3.17	0.15	0.9548	1.3878	3.4536	0.9677
2	*Citrulus lanatus*	4.04	0.24	0.9968	3.456	4.4565	0.9998
3	*Combine Fruit Peel*	3.27	0.17	0.9564	1.5733	3.4567	0.9456

Adsorption Kinetics

The correlation coefficient value (r) obtained follow pseudo-second order kinetics model and pseudo-first order kinetic model indicating that absorption occurs due to chemical and physical reaction between the surface of *Citrus sinensis* waste peel, *Citrulus lanatus* waste rind and combines wastes and Cr^{3+} and Pb^{2+} (Elhafez et al., 2017; Salmani et al., 2017; Xu et al., 2013). Since the adsorption of Cr^{3+} and Pb^{2+} by the waste fruit favour second order model, it signifies that the valence electron of the heavy metal binded with the negative charged surface site of the waste fruit in order to attain equilibrium (Elhafez et al., 2017) (*Table 3*).

***Table 3.** Kinetics Parameters for Adsorption of (a) Cr^{3+} (b) Pb^{2+}*

a,	Pseudo-Second Order Parameter				Pseudo-First Order Parameter		
SN	**Fruit Waste**	**K_2 (g mg^{-1} min^{-1})**	**q_i (mg/g)**	**R^2**	**K_1 (min^{-1})**	**q_i (mg/g)**	**R^2**
1	*Citrus sinensis*	0.1678	5.74	0.9923	0.1878	5.74	0.8701
2	*Citrulus lanatus*	0.2558	7.26	0.9776	0.2434	7.26	0.9433
3	*Combine Fruit Peel*	0.3455	7.78	0.9996	0.3558	7.78	0.9572
b,	**Pseudo-Second Order Parameter**				**Pseudo-First Order Parameter**		
SN	**Fruit Waste**	**K_2 (g mg^{-1} min^{-1})**	**q_i (mg/g)**	**R^2**	**K_1 (min^{-1})**	**q_i (mg/g)**	**R^2**
1	*Citrus sinensis*	0.216	3.60	0.8751	0.2763	3.60	0.8350
2	*Citrulus lanatus*	0.278	4.64	0.9973	0.3747	4.64	0.9387
3	*Combine Fruit Peel*	0.235	3.74	0.9278	0.3543	3.74	0.8756

Conclusion

Based on the findings of the current work, it could be concluded that *Citrus sinensis* peel and *Citrulus lanatus* rind as well as combination of the two fruit wastes can be used to reduce or control heavy metals from industrial effluent. Although, *Citrulus lanatus* is best for Pb^{2+} removal while combination of the two fruit wastes is best in Cr^{3+} removal. Further investigations involving the use of the agricultural wastes could lead to the discovery of more effective materials or compound materials for control of heavy metal pollutants in waste waters. Further research is needed on how to strengthen the uptake efficiency by such natural materials (the combination of *Citrus sinensis* peel and *Citrulus lanatus* rind) so as to enhance their effectiveness.

Acknowledgements. The first author appreciate Nigeria Petroleum Development Fund (PTDF) Nigeria for providing the fund and enabling environment to carry out the research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17731787

EFFECTS OF NANO-Fe_3O_4 AND HYDROGEN SULFIDE ON ALLEVIATING HEAVY METAL TOXICITY IN CUCUMBER SEEDLING

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(Received 15th Sep 2018; accepted 12th Nov 2018)

Abstract. The possible role of reducing effects of nano-Fe_3O_4 and hydrogen sulfide on alleviating heavy metal toxicity in germinating cucumber seedling was investigated. The toxic effects of Fe_3O_4 nanoparticles (NPs) (6, 50, 100 nm), and bulk were evaluated in cucumber plants. Among the synthesized nano-Fe_3O_4, only the small size (6 nm) had significant inhibit effect on fresh and dry biomass of cucumber seedlings. cucumber seedlings grown under lowest concentrations of 50 mg/L nano-Fe_3O_4 (6 nm) were affected by a decrease in biomass and enzyme activities compared to control. However, at higher concentration of nano-Fe_3O_4 dosage (2000 mg/L), there was significant increase in biomass and enzymatic activities of superoxide dismutase (SOD) and peroxidase (POD), respectively. The toxicity effects of the different sizes of nano-Fe_3O_4 particles and bulk-Fe_3O_4 might depend on the chemical composition, the structure properties/ particle sizes, the concentration in the tested medium, the time of incubation, as well as the plant species.
Keywords: *heavy metal toxicity, toxic mechanism, magneto dipole interaction, nano-Fe_3O_4, cucumber seedling*

Introduction

Due to the anticipated high-volume production and widespread use in coming years, engineered nano-materials will inevitably be released into the environment and interact with plants during manufacture, use, and disposal. Magnetic (Fe_3O_4) nanoparticles have been widely investigated for their scientific interests but also their technological application in many fields (ferrofluids, biomedicines, magnetic records, catalysts and electronic technique) due to their biological compatibility and magnetic properties (Shen et al., 2013). Iron oxide nanoparticles are among the primary nano-materials used in biomedical fields due to their magnetic property and high chemical stability (Xiao et al., 2011). Co-precipitation method and high-temperature decomposition are the two methods commonly used to prepare iron oxide nanoparticles. However, reactions of co-precipitation do not furnish excellent control of size distribution and crystallinity of the resulting particles; they are also thermodynamically driven (Laurent et al., 2008; Vinod et al., 2012; Jain et al., 2008). On the other hand, the use of FeOx NPs produced with high-temperature decomposition reactions of metal-organic compounds is limited because they are soluble only in organic solvents. The preparation of water-dispersible iron oxide nanoparticles with suitable surface coatings provides a major challenge in nanotechnology. In the present study, we synthesized water-soluble small iron oxide nanoparticles by the chemical reduction method using vitamin C as the reducing agent by oxidizing its C=C double bond. It has been reported that the magnetic property of magnetic (Fe_3O_4) nanoparticles could depend on their particle sizes (Roca et al., 2007). The polyol-mediated method is a versatile chemical system to prepare excellent-quality

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water-stable Fe_3O_4 nanoparticles (Shen et al., 2013). In this synthesis approach, polyols (ethylene glycol (EG), diethylene glycol (DEG), triethylene glycol or poly-ethylene glycol) are usually used as solvent and reductant and play a stabilizer role to control the growth of particles and prevent inter-particle aggregation (Liu et al., 2009; Miguel-Sancho et al., 2011). Magnetic (Fe_3O_4) nanoparticles synthesized with this method can exhibit some water dispersibility, compared to the thermal decomposition method. This might be dependent on the hydrophilic coating (Liu et al., 2009). In the present work, the polyol-mediated method was used as the second method to prepare magnetic (Fe_3O_4) nanoparticles: 50 nm and 100 nm, using ethylene glycol (EG) as the solvent and reductant. The TEM images, XRD, vibrating sample magnetometer (VSM), Fourier transform infrared (FTIR) spectroscopy and thereto gravimetric analyses (TGA) were used in this study to characterize the synthesized nanoparticles and bulk. The aim of this paper is to research on the effects of nano-Fe_3O_4 and hydrogen sulfide on alleviating heavy metal toxicity in germinating cucumber seedling.

Materials and Methods

Photosynthesis of cucumber can be best accumulated at 25~32 °C. During the morning, the temperature should be maintained at around 25 °C, and the temperature will be slightly reduced in the afternoon. Carbon dioxide fertilized in the morning. In order to promote nutrient transport and reduce plant consumption, the temperature should be controlled in the first half of the night at 16-18 °C and in the second half of the night at 10-14 °C. The optimum temperature for root growth is 20~23 °C, the lowest temperature is 8~12 °C, and the highest temperature is 32~38 °C. The temperature of root growth is no less than 15 °C.

Chemicals

Different chemicals were used to synthesize different sizes of magnetic (Fe_3O_4) nanoparticles. In general, ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium hydrogen carbonate ($NaHCO_3$), ascorbic acid or vitamin C ($C_6H_8O_6$), ethylene glycol $C_2H_6O_2$, 1,6-Hexanediamine ($C_6H_{16}N_2$), and sodium acetate (CH_3COONa), also abbreviated NaOAc were used as shown in the *Table 1* to synthesize nanoparticles of 6, 50 and 100 nm sizes.

Table 1. *Chemicals used to synthesis nano-Fe_3O_4 (6 nm, 50 nm, and 100 nm)*

N	Chemical composition				Autoclave		
					Temperature (°C)	Time (min)	Size (nm)
I	$FeCl_3.6H_2O$(0.54g)	$NaHCO_3$(1.52g)	VC(0.06g)	H_2O(35ml)	150	240	6
II		NaOAc(2g)	1,6-Hexa(6.7ml)		198		50
	$FeCl_3.6H_2O$(1g)			EG(30ml)		360	
III		NaOAc(1g)	1,6-Hexa(7ml)		205		100

The mini-Q quality water was used for all reactions and preparation procedures. All glassware used in the synthesis processes of magnetic (Fe_3O_4) nanoparticles were

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thoroughly cleaned several times in aqua regia (HCl: 3 parts and HNO_3: 1 part); then rinsed with water mini-Q and dried at 60 °C before being used (Burman et al., 2004; Garg et al., 2006).

The Magnetic (Fe_3O_4) Nanoparticles

Magnetic (Fe_3O_4) nanoparticles (6 nm) were synthesized following the procedure from Xiao et al. (2011), with some modification (*Figure 1*). In a typical Fe_3O_4 nanoparticles, a 15 mL of 0.54 g ($FeCl_3$. $6H_2O$) aqueous solution was first vigorously mixed with 20 mL of 1.52 g $NaHCO_3$ aqueous solution, then the mixture solution was stirred for 30 min to form a yellow solution. About 0.06 g of vitamin C was added into the first solution and the mixture stirred for 10 min before being transferred into an autoclave that was kept at 150 °C for 4h. After being allowed to cool at a room temperature, the black products were collected by magnetic separation and washed three times with water (Millipore, resistivity>18.2 MΩ.cm^{-1}) and ethanol. The residue obtained was dried at 60 °C before being analyzed and used.

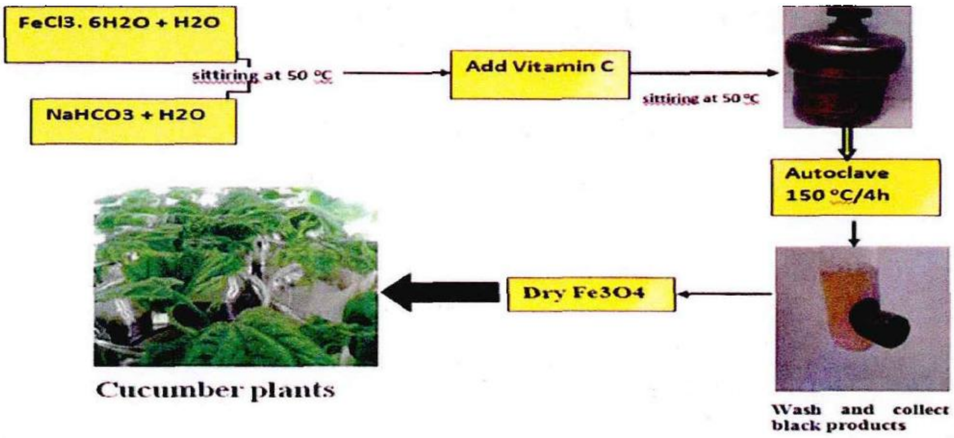

Figure 1. *Synthesis procedures of magnetic (Fe_3O_4) nanoparticles (6 nm)*

Magnetic (Fe_3O_4) nanoparticles (50 nm and 100 nm) were synthesized following some modification of the procedures from Yin et al. (2013). For Fe_3O_4 (50 nm), 1 g ($FeCl_3$. $6H_2O$) and 2 g (NOAc) were first vigorously mixed with 30 mL of EG (Ethylene glycol). Then 6.5 mL of 1,6-hexanediamine was added into the first solution and the mixture stirred for another 10 min before being transferred into an autoclave that was kept at 198 °C for 6 h. The synthesis procedure of 50 nm and 100 nm were similar. The only difference was in the addition of 1 g NOAc), 7 mL of 1,6-hexanediamine and boiled at 205 °C for 6 h in the synthesis of nano-Fe_3O_4 (100 nm). All procedures after autoclaving were similar to those of Fe_3O_4 (6 nm).

The morphology, particle shape and structure of Fe_3O_4 NPs were determined by Scanning Electron Microscopy (SEM, S-4800, HITACHI, Japan) and Transmission Electron Microscope TEM (JEM 200CX, Japan), respectively. For TEM analysis, the Fe_3O_4 NPs or bulk-Fe_3O_4 were dispersed in deionized water and sonicated for 40 min. Then, some suspension liquid was dropped on a copper grip for Transmission Electron Microscopy (TEM) observation (Zhang et al., 2011). The TEM pictures were obtained from Tecnai G2 20 S-Twin transmission electron microscope 119 (FEI company, Japan)

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operating at 200 kV. The mean size, size distribution, and Zeta potential of 50 mg/L magnetic (Fe_3O_4) nanoparticles or bulk-Fej04 were measured by Dynamic Light Scattering (DLS) equipment (ZETA SIZER 90, Nano series, Malvern, UK) in deionized water and nutrient solution (pH 5.5). FT-IR spectra were obtained using Fourier transform infrared spectroscopy (FTIR Tensor 27, Broker, Germany) analysis. The magnetic property was determined using the vibrating sample magnetometer (VSM, 7410, Lake Shore, USA). The crystal structure of the synthesized nanoparticles or bulk was obtained by X-ray diffraction, XRD (XRD, X'pert PRO MPD, Holland) measurement. To collect 20 data from about 10 °C to 90 °C, we used a continuous scanning mode. Thermo gravimetric analysis (TGA) was carried out for powder samples using a NETZSCH STA 449C thermo gravimetric analyzer.

To combat against adverse environmental heavy metal toxicity, plants have developed potential mechanisms such as enzymatic activities (SOD and POD) and the production of low molecular weight thiols. Naturally, plants show differences in their capacity of heavy metals tolerance. Some plants can grow in metal-contaminated soils with high level, while other groups could not grow even at low concentration. The fresh roots or shoots (0.2 g) from each treatment were homogenized with 1.8 mL of 0.05M sodium phosphate buffer (PH 7.8) under ice bath to make 10% sample compound liquid. The homogenate was centrifuged at 10.000 mg and 4 °C for 15 min. Then, the supernatant was used for superoxide dismutase (SOD) and MDA contents. The SOD activity was analyzed by determining the ability to inhibit the photochemical reduction of nitroblue tetrazolium at 550 nm.

Results

Figure 2 shows the XRD patterns of the synthesized magnetic (Fe_3O_4) nanoparticles with different sizes and bulk-Fe_3O_4.

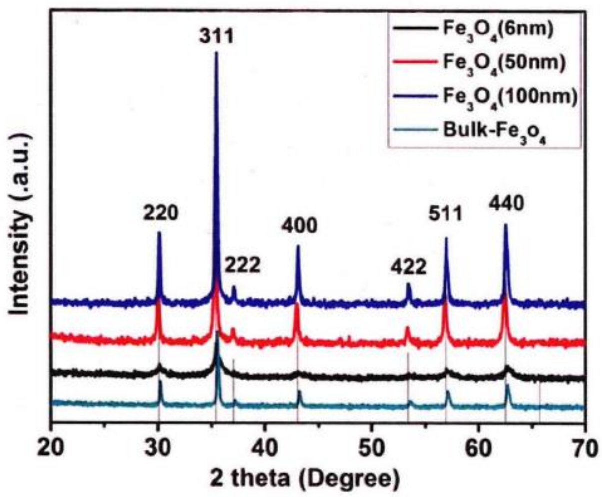

Figure 2. *XRD patterns of Fe_3O_4 nanoparticles (6 nm, 50 nm, and l00 nm), and Bulk-Fe_3O_4*

In this study, the characteristic peaks observed in the XRD pattern at 2θ of 30.09, 35.42, 37.05, 43.05, 53.39, 56.94, 62.51 were corresponding to the diffractions of 220, 311, 222.400, 422, 511 and 440 lattice planes of inverse cubic spinet structure of Fe_3O_4

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DOI: http://dx.doi.org/10.15666/aeer/1702_17891797

NPs and Fe_3O_4-Bulk. As shown in *Figure 2*, all XRD patterns had a diffraction peak at 2θ=35.42 °C corresponding to the spinet phase of the synthesized (Fe_3O_4) nanoparticles or bulk. The decrease in the intensity after the diffraction peaks at 2θ=35.42 °C could be attributed to the formulation of more crystalline phase particles in all annealed magnetic (Fe_3O_4) nanoparticles and the bulk-Fe_3O_4. The results show that the spinet structures are in good agreement with the XRD standard for the magnetic nanoparticles and no peaks of impurities were observed in the XRD pattern, indicating that the synthesized particles powders are magnetic (Fe_3O_4) nano-particles.

The properties of nanomaterial strongly depend upon the dimension of nanoparticles. Hence, the control of particle size is very important in nanotechnology study or nanoparticles application. In order to analyze the morphology and particles size of the synthesized magnetic (Fe_3O_4) nanoparticles and bulk-Fe_3O_4, SEM images of particles were taken. The results are presented in the *Figure 3*.

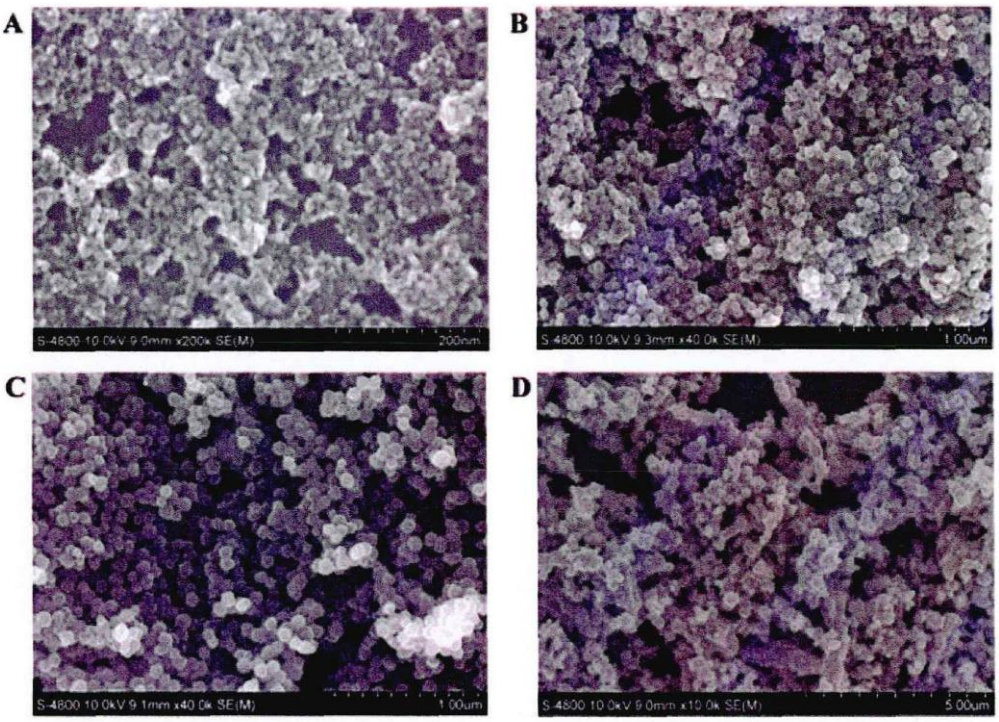

Figure 3. *SEM images of Fe_3O_4 nanoparticles 6 nm (A), 50 nm (B), 100 nm (C) and bulk-Fe_3O_4 (D)*

The SEM images showed that spherical magnetic (Fe_3O_4) nanoparticles (*Figure 3A,B and C*) were obtained from all methods of synthesis used in this study. The SEM images of the synthesized Fe_3O_4 nanoparticles (*Figure 3A*) showed that the materials obtained were made of small particles. As showed in *Figure 3*, Fe_3O_4 nanoparticles (small size) are found to be interconnected with least agglomeration which might be due to their high surface charge and the magneto dipole interaction.

Discussion

The *Figure 4* shows representative TEM images of the synthesized magnetic (Fe_3O_4) nanoparticles with diameters of 6.82±1.38 nm; 52.56±8.01 nm; 100.66±15.62 nm,

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respectively. We observed that the sizes of all synthesized nanoparticles were smaller compared to bulk-Fe_3O_4 (144.27±28.95 nm). The size histograms of the synthesized nanoparticles and bulk based on statistical analysis of over 90 particles are shown in the *Figure 4a-d*. The measurement of the particle diameters of all synthesized magnetic (Fe_3O_4) nanoparticles showed a normal distribution curve after ultra-sonication dispersal as compared to bulk (*Figure 4a-d*). Similar observation has been reported by Shen in 2013 (Shen et al., 2013), who synthesized and characterized water-soluble ultra-small Fe_3O_4, nanoparticles for potential bio-application.

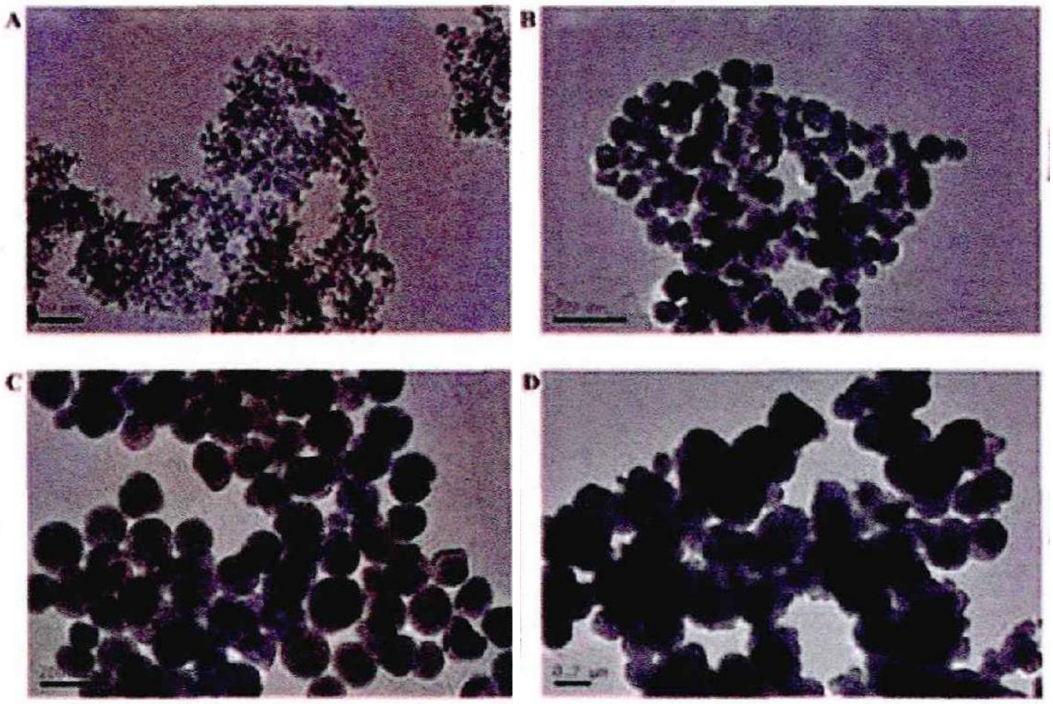

Figure 4. *TEM images and size distribution histogram of Fe_3O_4 NPs: 6 nm (A-a), 50 nm (B-b), 100 nm (C-c) and Bulk-Fe_3O_4, (D-d)*

When suspended in water or nutrient solution, the hydrodynamic diameters of the 50 mg/L of all sizes of the synthesized nanoparticles and bulk-Fe_3O_4 were larger than the results measured by their corresponding TEM images. This is due to their agglomeration. The *Table 2* shows that nano-Fe_3O_4 particles were largely aggregated.

Table 2. *Size distribution and zeta potentials of nano- and bulk- Fe_3O_4*

Nanoparticles or bulk	Water		Nutrient solution	
	<DH>nm	ξ-potential (mV)	<DH>nm	ξ-potential (mV)
Fe_3O_4 (6nm)	255±9.1	-12.66±0.4	433.3±42.2	-15.9±0.2
Fe_3O_4 (10nm)	242.2±0.6	27.9±0.4	741.1±39.6	-16.6±1.3
Fe_3O_4 (100nm)	184.7±4.1	29±0.1	911.5±35.3	-13.5±1.1
bulk- Fe_3O_4	1041±150.8	9.8±0.6	1402±118.9	-18.8±0.1

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In water, the aggregation level appeared in the order of 6 nm>50 nm>100 nm with the particles averaging 255±9.1 nm, 242.2±6.6 nm and 184:7±4.1 nm, respectively. The practical averages in nutrient solution were 433±42.2 nm, 741±39.6 nm, 911±35.3 nm, and 1402±118.9 nm respectively for 6 nm, 50 nm, and 100 nm. The change in the order of the aggregation level (100 nm>50 nm>6 nm) in the nutrient solution could be due to the presence of additional chemicals in the nutrient solution. We observed that the hydrodynamic diameters of all nanoparticles were smaller in water and nutrient solution compared to bulk-Fe_3O_4, (1041±150.8 nm in water and 1402±118.9 nm in nutrient solution).

The modification of the synthesized magnetic (Fe_3O_4) nanoparticles with different sizes (6 nm, 50 nm, and 100 nm) and bulk-Fe_3O_4 surfaces for their stabilization were analyzed and confirmed by Fourier Transform Infrared Spectroscopy (FTIR) as shown in the *Figure 5*.

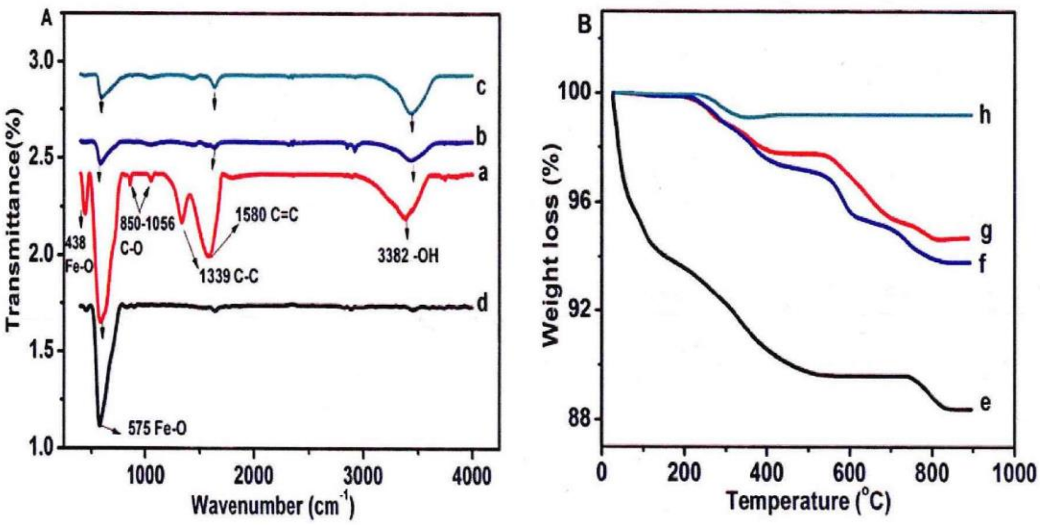

Figure 5. *FTIR Analysis of Fe_3O_4, nanoparticles (Aa-c) and Bulk- Fe_3O_4, (Ad), TGA curves of Fe_3O_4, nanoparticles (Be-f) of bulk- Fe_3O_4 (Bh)*

In general, the stretching absorption peaks of Fe-O at 575 cm^{-1} appeared in the FTIR spectra of all the synthesized nanoparticles and bulk (*Figure 2-SAa-d*). In addition, the peaks in the region numbers 438-575 cm^{-1} are also attributed to the Fe-O vibration (*Figure 5*). In the FTIR spectra of the nano-Fe_3O_4 (6 nm), the peaks around 850-1056 cm^{-1}, 1342 cm^{-1} and 1580 cm^{-1} were related to the group C-O, C-C and C=C respectively (*Figure 5*). The double bonds of C=C were due to the additional compounds, mainly the Vitamin C in using chemicals for the synthesis of nano-Fe_3O_4 (6 nm). The peak observed in the region number 3382 cm^{-1} was related to the vibration of -OH. This peak (around 3382 cm^{-1}) was found in the FTIR spectra of all synthesized nanoparticles (6 nm, 50 nm, and 100 nm). However, it was not observed in the bulk spectra (*Figure 5*). The FTIR spectra analysis suggests that the characteristic stretching absorption peaks at 575 cm^{-1} corresponding to the Fe-O was related to the magnetite phase. *Figure 5 (Be-h)* shows the weight loss curves of magnetic (Fe_3O_4) nanoparticles (6 nm, 50 nm, and 100 nm) and bulk-Fe_3O_4 at different stages of surface modification. It appeared that below 200 °C, the weight loss was insignificant for nano-Fe_3O_4 (50 nm,

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100 nm) and bulk, which probably; presents a relative good thermal stability. The weight loss of 1.93%, 2.29% and 0.84% for 50 nm, 100 nm, and bulk, respectively (*Figure 5 B-f, g, and h*), in the region of 200-400 °C, may be attributed to the release of crystal water. On the other hand, the TGA curve of the small size (6 nm) was very complex as shown in the *Figure 5*. The weight loss in the temperature range of 26-100 °C is about 4.85% corresponding to the release of physically absorbed water and the residual solvent in the sample. In the temperature range 100-450 °C there still exists a gradual weight loss of 5% which is due to the organic surface modification. There was another stage of weight loss of 1.2% observed in the temperature range of 730-830 °C, which may be due to the deoxidation of FeO. The total weight loss from the graph of synthesized nanoparticles (*Figure 5*) was 11.33%, 5.31% and 6.2%, respectively for 6 nm, 50 nm, and 100 nm indicating the transition phase from Fe_3O_4 to FeO. The experiment result shows the toxicity effects of the different sizes of nano-Fe_3O_4 particles and bulk-Fe_3O_4 might depend on the chemical composition, the structure properties/ particle sizes, the concentration in the tested medium, the time of incubation, as well as the plant species.

The phytoxicity effects of Fe_3O_4 NPs (6 nm, 50 nm, 100 nm), and bulk were evaluated in cucumber (Cucumis sativus L) plants grown in hydroponic conditions in terms of growth parameters, biomass production, TEM observation, antioxidant enzyme activities and MDA content. The results show that among the synthesized nano-Fe_3O_4 (6 nm, 50 nm and 100 nm), only the small size (6 nm) was observed to have significant inhibition on fresh and dry biomass of cucumber plants. Nano-and bulk-$Fe_3O_4$4 treatments (21days) caused more oxidative stress in cucumber plants. Cucumber plants grown under lowest concentrations of 50 mg/L nano-Fe_3O_4 (6 nm) were affected by a decrease in biomass and enzyme activities compared to the control. However, at higher concentration of nano-Fe_3O_4 dosage (2000 mg/L), there was significant increase in biomass and enzymatic activities (SOD and POD) respectively. The phytotoxicity effects of the different sizes of nano-Fe_3O_4 particles and bulk-Fe_3O_4 might depend on the chemical composition, the structure properties/particle sizes, the concentration in the tested medium, the time of incubation, as well as the plant species.

Conclusion

The results showed that,nano-Fe_3O_4 (2000 mg/L) did not change the toxicity of the tested heavy metals at high concentration (10 mM) in the seedlings of cucumber. Addition of magnetic (Fe_3O_4) nanoparticles (2000 mg/L) in each metal solution (1 mM), significantly decreased the growth inhibition and activated protective mechanisms to alleviate oxidative stress induced by heavy metals in the cucumber seedlings. The reducing effects of nano-Fe_3O_4 against heavy metals stress could be dependent on the increase in the enzyme activity (SOD and POD), but also their adsorption capacity of heavy metals.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No. 31271627) and Henan Provincial Research Foundation for Science and Technological Breakthroughs, China (Grant No. 152102110106).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17891797

SPATIAL CHARACTERISTICS OF URBAN GREEN SPACE: A CASE STUDY OF SHANGHAI, CHINA

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(Received 28th Sep 2018; accepted 28th Nov 2018)

Abstract. In China, rapid urbanization has profoundly transformed the spatial pattern of urban land use, including urban green spaces. The government plans to optimize green spaces to integrate them with urban development; this requires an understanding of the process of green space change. Quantifying landscape pattern and its change is essential for monitoring and assessing ecological consequences of urbanization. In this study, the moving window method and gradient analysis were used to analyze high-resolution satellite images and interpret Shanghai urban green space distribution data. A window of 5 km was found to be a rational size that can reflect urban green space patterns via changes in landscape metrics. Green space patterns can be quantified through gradient analysis and landscape metrics. Different types of green space exhibit obvious "spatial characteristics" along the landscape pattern gradient. Urban green space distribution patterns can be used to verify the urban space development mode. The 'concentric circle' development theory was applicable to the urban development of Shanghai. The research provides significant guidance for further study on the interrelations of urban green space pattern processes, for examination and assessment of urbanization processes and for urban planners and decision makers.
Keywords: *landscape pattern, spatial extents, gradient analysis, landscape index, urban green space*

Introduction

With the accelerated progress of globalization and urbanization, urban issues (such as urban shape and evolution) have become very topical issues across the world (Breuste et al., 1998; Pickett et al., 2001; Whitford et al., 2001). In 1940, only 8% of the global population lived in downtown areas. By the year 1980, more than one third of the population was living in urban areas (World Commission on Environment and Development, 1987). Experts predict that by the year 2025, 60% of the world population will be living in cities (Schell and Ulijaszek, 1999). With the rapid growth of population, downtown areas within cities develop quickly and the urban areas increase tremendously as well; this growth results in various modes of city development. The morphology and evolution of cities have been extensively studied by geographers, economists, and social scientists for centuries (von Thünen, 1825; Christaller, 1933; Hoyt, 1939; Harris and Ullman, 1945; Lösch, 1954; Wilson, 1976; Allen and Sanglier, 1979; Tobler, 1979; Wilson, 1981; Couclelis, 1985; Batty et al., 1989; Wong and Fotheringham, 1990; White and Engelen, 1993; Batty, 1997; Schweitzer, 1997; Portugali, 2000). The changes in urban morphology will have a profound and far-reaching influence in the urban environment, especially the transformation of urban green landscapes (Gordon, 1990). Furthermore, green land plays a critical role in urban

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

development. Thus, the rationality and scientific validity of urban green land distribution is a criterion for judging a city's sustainable development (Chiesura, 2004). At the same time, governments are beginning to recognize the importance of healing the rift between humans and nature. Planners, designers and researchers are in need of valid tools for making quantitative assessments and analyses of reasonable development models with the aim of giving a stronger scientific basis for decisions about urban development.

In previous research on urban shape and evolution, landscape pattern analysis has increasingly been applied to examine urban issues (Kong and Nobukazu, 2006). The window movement method and the gradient analysis method, both based on FRAGSTATS software (http://www.umass.edu/landeco/research/fragstats/fragstats.html) are especially widely used. A landscape index has been widely applied to analyze the urban landscape pattern quantitatively (Pickett et al., 1997, 2001; Whitford et al., 2001; Kong and Nobukazu, 2006). Gradient analysis is now repeatedly adopted in research aimed to investigate the influence of urbanization on urban morphology development (Whittaker, 1975; Kowarik, 1990; Pouyat and McDonnell, 1991; Pouyat et al., 1995; Frohn, 1998; Sukopp, 1998; Zhu and Carreiro, 1999; Wu, 2000; McGarigal and Cushman, 2008). For instance, Luck and Wu (2002) found that the spatial pattern of urbanization can be studied quantitatively by combining gradient analysis and a landscape index. Kong and Nobukazu (2006) utilized these methods of gradient analysis and landscape index to show that the urbanization had changed greatly Jinan's land use types. Li et al. (2004) utilized the landscape pattern index and gradient analysis method to research the landscape structure of Shanghai and the effects of its spatial characteristics on urbanization gradient; results showed that the farmland was the dominant landscape, accounting for about 60% of the landscape. Zhang et al. (2008) also combined the two methods to analyze changes in the landscape index under different extents in Huizhou city of Guangdong Province. However, these studies examined the range of a whole city region and different land types (Luck and Wu, 2002; Zhu et al., 2006). Research using the gradient analysis method and landscape index to study urban green space patterns is limited. Therefore, the use of these two methods to study the pattern of urban green space has a very important significance.

Shanghai, as a megalopolis in China, is undergoing rapid urbanization. A series of problems (such as short-term high-density investment, population explosion, industrial concentration, rapid urbanization of suburbs, and high-strength development features of increased building density and plot ratio) arising from urbanization are being developed at the sacrifice of urban green space and agricultural land and could endanger the sustainable development of Shanghai. One of the urgent issues to be solved by Shanghai is how to optimize the land utilization pattern to meet the land demand for residential, industrial and urban greening purposes. This must be accomplished while also addressing the impact of unlimited expansion of urban space on the urban ecological system and its ecological result during the process of urbanization. The landscape pattern index is an important tool for quantitative analysis of landscape space dynamics and has played an important role in urban ecology and urban landscape research in recent years (Luck and Wu, 2002; Zhang et al., 2004).

In the current study, high resolution remote sensing images were used to interpret urban green space vector diagrams. With the help of a geographic information system (GIS), a gradient analysis was conducted of the landscape belt transect across

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

downtown Shanghai, China. The objectives were to illustrate the space pattern characteristics of Shanghai's urban green space landscape, examine the extent of change in various green space landscapes using gradient analyses, and study the changes of landscape metrics in landscape stratification along the belt transect under different extents. Three main questions were studied. (1) What is the rational extent for the gradient analysis of Shanghai urban green space landscape pattern? (2) Do different urban green spaces have unique spatial characteristics? (3) What is the development pattern of Shanghai urban space? The research was intended to provide a scientific basis for further optimization of the landscape pattern and construction of an urban green space landscape system with a rational structure, scientific layout and ecological benefit.

Materials and Methods

The Shanghai metropolitan region (30°23′–31°37′ N, 120°50′–121°45′ E) is located on the eastern coast of China, with the Yangtze River estuary to the north, the East Sea to the east and Hangzhou Bay to the south (*Fig. 1a*). The region has an area of 6,340 km^2 and a population of 24.1527 million. Shanghai has a northern subtropical monsoon climate, with an average annual temperature of 16°C; however, summer temperatures average 28°C, while the winters are cold with an average temperature of 4°C. Average annual precipitation is approximately 1,200 mm, with 60% of the annual rainfall occurring during May–September.

The research area was mainly located within the outer ring road of Shanghai and had an area of 660 km^2. Included in the study area were Yangpu District, Hongkou District, Zhabei District, Jing'an District, Huangpu District and Luwan District, and partial areas of Putuo District, Changning District, Minhang District, Xuhui District, Baoshan District and Pudong New Area (*Fig. 1b*).

Shanghai has proposed a strategy of building a forested city, with the focus on constructing urbanized areas and public green space at various levels to form a greening network described as "surrounded by forest, internal greening network". The strategy is intended to realize the purpose of urban area greening through a large cycle of "ring, wedge, corridor, garden and forest". The network will situate the city in a forest to surround people with green area so as to lay a foundation for "Shanghai in forest" and "green Shanghai". Therefore, research on Shanghai's urban green space pattern is of great importance for urban greening construction in the city.

Primary data sources used in the study included: (1) 2015 Landsat-8 images (resolution 30 m; bands 6, 5 and 4); (2) Shanghai city image atlas in 2015 and 1:28,000 greater Shanghai map in 2015; and (3) urban planning data obtained from the Shanghai Planning Bureau and the Statistics Bureau (auxiliary information).

Green land information for Shanghai in 2015 was obtained using ArcInfo software (Version 8.2, ESRI, Redlands, CA, USA) as well as visual interpretation to classify green land. In addition, a field survey and data adjustment helped to define the urban green type vector diagram with attribute data and the corresponding spatial data. At various spatial scales (3 km, 4 km, 5 km, 6 km and 7 km), 160 belt transects were established in the directions from north to south and east to west; 320 sampling points were identified as well (*Fig. 1c*). The classification of urban green space types was determined using the Standard for Classification of Urban Green Spaces (CJJ/T85-2002) of the Ministry of Housing and Urban-Rural Development. The specific urban

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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green land function and characteristics were determined, as well as the types of land use, ownership, and physical factors (colors, tones, location, size and texture) (*Fig. 1c*). The accuracy of grading different urban green land samples was 97.5%, which was considered acceptable. ArcMap (Version 8.2, ESRI) was used for spatial analysis and for transforming the vector data of green land types into the raster format in 2 × 2 m pixel areas.

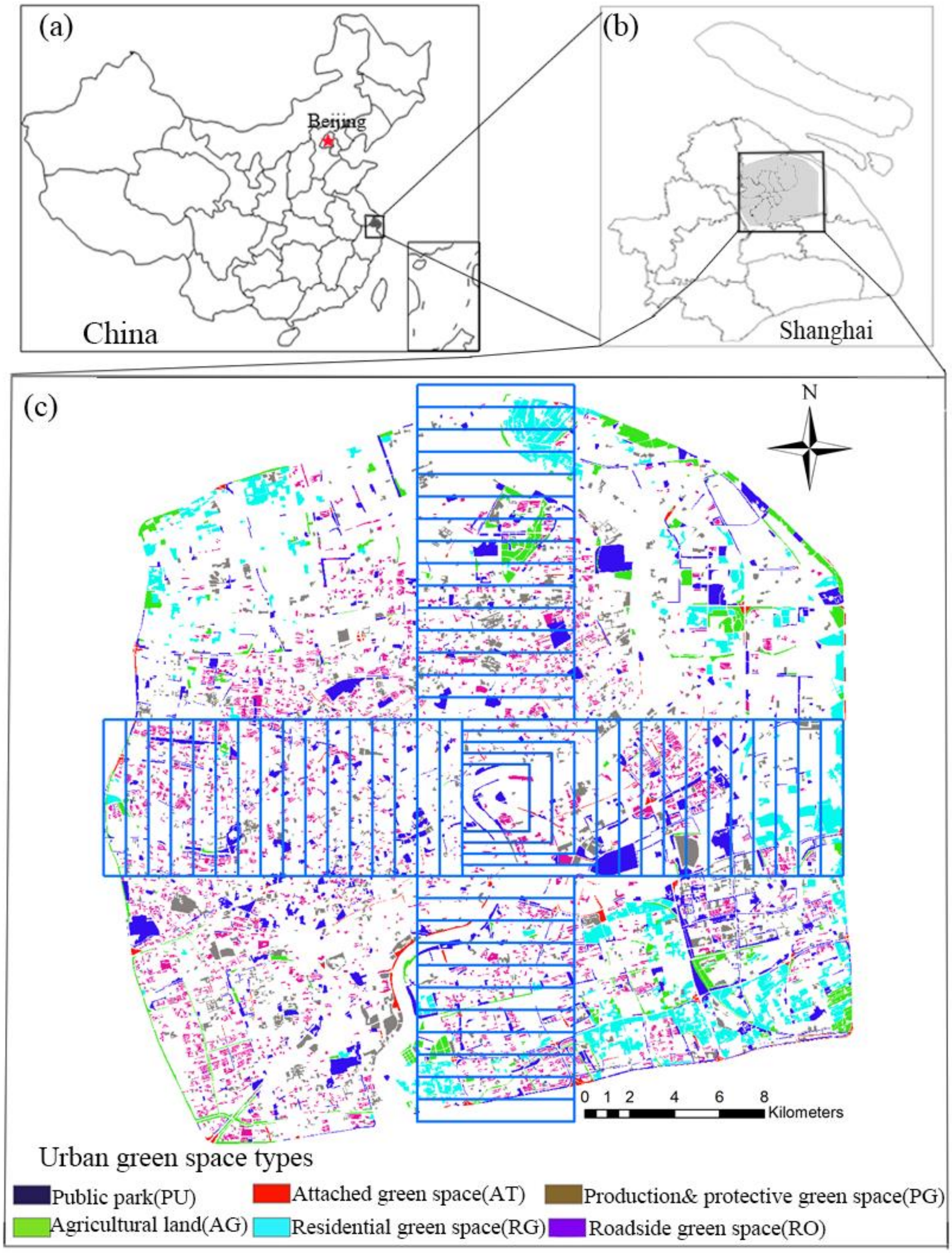

***Figure 1.** The study area showing: (a) location of Shanghai city in China; (b) regional location of Shanghai City; (c) urban green space map of Shanghai City and transects of windows used to sample green space*

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Based on the Standard for Classification of Urban Green Spaces (CJJ/T85-2002) of the Ministry of Housing and Urban-Rural Development, and in combination with Shanghai's urban green space characteristics, Shanghai's urban green spaces were divided into six categories, as follows:

- Public park (PU) - this type of green space included comprehensive parks, citywide parks, regional parks, community parks, parks in residential areas, gardens and parks in residential quarters, theme parks, children's parks, zoos, botanic gardens, historical gardens and parks, scenic spots, amusement parks, other theme parks, and belt-shaped parks.
- Residential green space (RG) - this type of green space included residential quarters and communities.
- Attached green space (AT) - this type of green space included public facilities, industrial green space, green space for warehousing, green space for external traffic, green space for municipal facilities, and special green space.
- Roadside green space (RO) - this type of green space included roadside verges and similar green fields.
- Production and protective green space (PG) - this type of green space included green space for production and protection.
- Agricultural land (AG) - this type of green space included cultivated land, garden plots, forest land, grassplots, agricultural land for facilities, field ridges, and country roads.

Identification of each green space type was determined (in combination with the comprehensive analysis of the specific situation of Shanghai) based on the features of the spaces that were visible on the remote sensing image including color, tone, shape, position, size and texture.

The combined method of gradient analysis and landscape metrics was used in the study. "Windows" of various sizes (3 km, 4 km, 5 km, 6 km and 7 km) were used to move along a selected belt transect and analyzes the landscape pattern changes so as to identify the urban green space landscape pattern and its gradient change. The Lujiazui central green space was the central window. The spatial step of each window along a transect was 1 km and windows moved from west to east and from south to north, obtaining a series of land pattern samples of various sizes. The central window location was the origin (0 km). Movements toward the east and north were designated positive values and toward the south and west were negative values. Land pattern characteristics were calculated using a 1-km sliding step to correct for noise caused by small scale and local variations, a technique similar to the analysis of time series data (*Fig. 1c*). ArcMap software (ESRI) was used to digitally cut the sample belts from the Shanghai green space vector diagram and convert them into 2-m × 2-m grid (raster) data for visual presentation.

The FRAGSTATS 4.2 software (McGarigal, 2002) was used to determine the correlation pattern characteristic index of green space landscape (McGarigal and Marks, 1995). The redundancy and overlap among landscape metrics have been investigated previously (e.g., O'Neill et al., 1999). Not all the available landscape metrics were needed to capture the changes in the composition and configuration of the landscape. A small set of metrics (13 in total) were chosen to be both sensitive to changes and numerically reliable (i.e., showing consistent trends) for depicting landscape patterns (*Table 1*). As in most cases in the existing literature, the term "landscape pattern" in this study included both the non-spatial composition (e.g., the number and relative

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abundance of patch types, patch size, and other related non-spatial measures) and spatial configuration (e.g., patch shape, juxtaposition, contrast, and boundary characteristics). Li and Reynolds (1994) defined spatial heterogeneity in a similar way.

Specifically, 10 indices were used as compositional measures: percentage of landscape (PLAND), patch density (PD), edge density (ED), patch richness (Hobbs, 1988) (PR), Shannon diversity index (SHDI), largest patch index (LPI), mean patch size (MPS), aggregation index (AI), patch size standard deviation (PSSD) and patch size coefficient of variation (PSCV). The remaining three indices were used as configurational measures: landscape shape index (LSI), area-weighted mean shape index (AWMSI) and area-weighted mean fractal dimension (AWMFD) (O'Neill, 1988). This dichotomy of compositional versus configurational indices is apparently an oversimplification, and many of these indices reflect both aspects of landscape pattern to varying degrees (McGarigal and Marks, 1995). However, this simple classification scheme was adopted so as to facilitate the organization of results and their interpretation.

Table 1. *Landscape metrics used to quantify the spatial pattern of urbanization in metropolitan Shanghai, China (based on McGarigal and Marks, 1995)*

Landscape metrics	Abbreviation	Description
Compositional measures		
Percentage of landscape (%)	PLAND	The proportion of total area occupied by a particular patch type; a measure of dominance of patch types
Patch density	PD	The number of patches of per 100 ha
Edge density (m ha^{-1})	ED	The total length of all edge segments per hectare for the class or landscape of consideration
Patch richness	PR	The number of patch types in the landscape; a measure of diversity of patch types
Shannon diversity index	SHDI	A measure of patch diversity in a landscape, which is determined by both the number of different patch types and the proportional distribution of area among patch types
Largest patch index (%)	LPI	The ratio of the area of the largest patch to the total area of the landscape
Mean patch size (ha)	MPS	The average area of all patches in the landscape
Aggregation Index	AI	The aggregation index reflects the patches in the landscape and the index of the decentralized state
Patch size standard deviation (ha)	PSSD	The standard deviation of patch size in the entire landscape
Patch size coefficient of variation (%)	PSCV	The standard deviation of patch size divided by mean patch size for the entire landscape
Configurational measures		
Landscape shape index	LSI	The total length of patch edges within the landscape divided by the total area, adjusted by a constant for a square standard
Area-weighted mean shape index	AWMSI	Mean patch shape index weighted by relative patch size
Area-weighted mean fractal dimension	AWMFD	The patch fractal dimension weighted by relative patch area

Results

Synoptic characteristics of the Shanghai area landscape

Data in *Table 2* show the six categories used to distinguish green space in Shanghai. PU had the largest PLAND (up to 30.32%), and its PSCV value (278.15%) was also relevantly large, indicating that PU was the dominant green space type in the belt

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DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

transects. AT had the second largest PLAND (26.46%) and the proportions of RG, AG, PG and RO decreased (in that order) such that RO occupied the smallest proportion (2.46%) of the sampled area. PD (the number of patches per unit area) reflects the degree of landscape breakage; i.e., the larger is PD, the smaller are the patches and the higher is the degree of fragmentation. The PD of RG was the largest and far higher than that of other green space types; AT had the most scattered distribution and highest degree of fragmentation among the belt transects. AG had the largest LPI (1.25%) and the largest AI (87.25%), indicating that AG green space was concentrated with a low degree of fragmentation.

The PD and LSI of the entire landscape were relevantly large, indicating that the overall degree of breakage of Shanghai urban green space was relevantly large and Shanghai was comprised of complex green space patch shapes. The AI of RO was the smallest of all green space types, and RO consisted of small areas and scattered distribution. The Shannon diversity index of the entire belt transect was 1.65, indicating a low level of diversity.

Table 2. *Class and landscape-level metrics of the land use transects*

Landscape metrics	Types						Landscape level
	PU	RO	RG	AT	PG	AG	
Percentage of landscape (%)	30.32	2.46	18.08	26.46	6.94	15.74	—
Patch density (No/100hm^2)	8.34	3.20	13.55	6.87	1.63	0.93	37.77
Largest patch index (%)	2.54	0.18	1.40	0.36	0.6235	1.25	4.34
Edge density (m/ha)	3.23	0.77	1.60	2.27	1.12	0.50	4.75
Landscape shape index	36.53	22.37	31.59	59.13	17.84	15.54	77.43
Patch size coefficient of variation (%)	278.15	176.615	236.06	146.54	231.16	269.14	365.74
Area-weighted mean shape index	2.017	1.99	1.937	2.18	2.52	4.15	2.41
Aggregation Index (%)	77.53	51.85	74.92	74.18	60.63	87.25	74.30
Shannon diversity index	—	—	—	—	—	—	1.65

Gradient analysis with landscape-level metrics at different spatial extents

Although there is a large amount of literature about the use of landscape gradients, the study area does not conform entirely to previously used methodology. Therefore, a rational spatial extent had to be chosen for analyzing the land use pattern in Shanghai. Based on the literature, five spatial extents were selected (3 × 3 km, 4 × 4 km, 5 × 5 km, 6 × 6 km and 7 × 7 km) to study the gradient distribution characteristics of Shanghai urban green space landscape pattern in an attempt to find the window size that was most appropriate.

Based on the landscape metrics at various spatial extents, PD, MPS, LSI and AWMSI exhibited obvious gradient change trends (*Figs. 2b, 2c, 2e and 2f*). Among these indices, PD gradually decreased from west to east and the highest value occurred at −8 km to −2 km from the origin. This area was the old city of Shanghai with small and scattered landscape green spaces indicating a high degree of breakage in the landscape (*Fig. 2b*). MPS gradually increased from west to east along the belt transect and displayed a J-

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shaped distribution; this distribution indicated that the areal extent of a single patch from west to east of the city gradually increased and was highly concentrated at the urban suburbs. Furthermore, there was a concentration of either of two types of green space based mainly on large patch green space (such as AG) with low breakage degree (*Fig. 2c*).

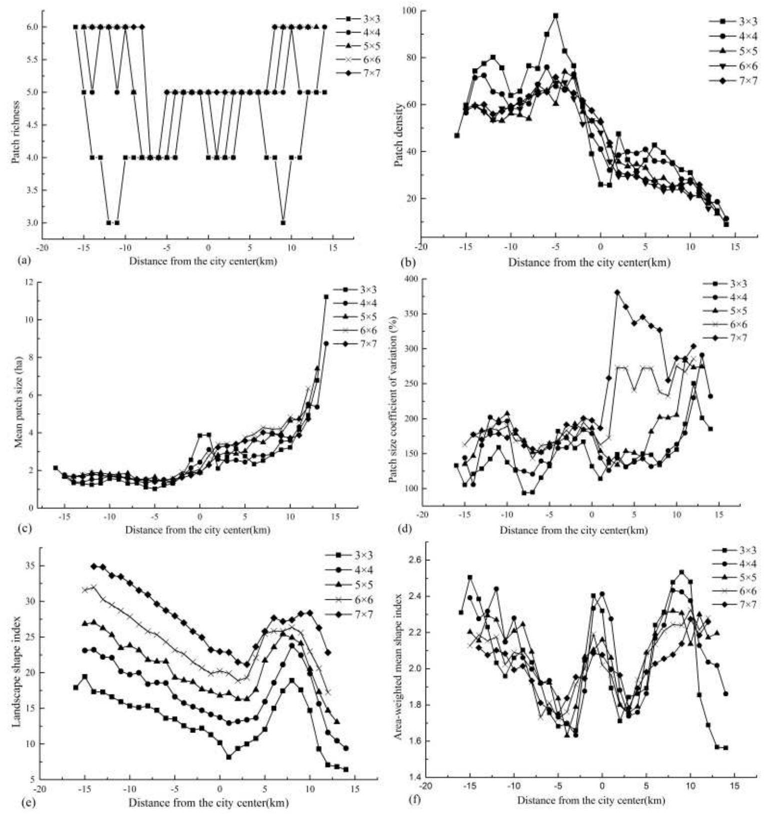

Figure 2. *Variation of landscape metrics along the east-west direction land use transect at different spatial extents: (a) patch richness, (b) patch density, (c) mean patch size, (d) patch size coefficient of variation, (e) landscape shape index, and (f) area-weighted mean shape index. To examine the effect of changing scale of analysis, five window sizes were used: 3 × 3 km, 4 × 4 km, 5 ×5 km, 6 × 6 km and 7 × 7 km blocks. See text for details*

LSI gradually decreased from west to east along the belt transect with the valley at −2 to +4 km from the center and peaked at +9 km; this occurred because at a distance of −16 to −4 km, there was a gradual transition from urban suburbs to the old city. The proportion of RG in the living area of the urban suburbs was large and, the larger the LSI value, the more dispersed were the green space patches. The peak occurred at +9 km this was the location of the Pudong New Area of Shanghai, which had a large RG area and therefore large LSI value (*Fig. 2e*).

The large values of AWMSI mainly occurred at −16 to −10 km, −3 to +2 km and +8 to +12 km because urbanization and government planning policy had resulted in patch shapes having greater complexity (*Fig. 2f*). Although PR and PSCV did not exhibit obvious gradient characteristics along the east-west belt transect gradient (*Figs. 2a and 2d*), they both showed increased fluctuation (and a zigzag trend) as the spatial extent of the analytical windows decreased (*Fig. 2b*). LSI progressively decreased, then

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progressive increased and finally progressively decreased again from west to east along the belt transect (*Fig. 2e*). The highest value of LSI occurred at −16 km from the city center and another peak occurred at +8 km because these were the locations of residential estates with large greening area and relevantly large dispersion.

Based on the change of spatial extent of the analytical windows and comprehensive analysis, PR, PD, MPS, PSCV, LSI and AWMSI fluctuated irregularly (with both increases and decreases of spatial extent) along the belt transect. Generally, when the window was large (≥ 5 km), the changes in landscape metrics were gradual along the belt transect and exhibited smooth trend lines. When the window was small (≤ 4 km), the landscape metrics fluctuated dramatically. Therefore, a spatial extent of 5 km was deemed to be an appropriate scale for the gradient analysis of Shanghai green space landscape pattern. This size of analytical window not only avoided large fluctuations in the landscape metrics, but also comprehensively reflected the pattern of change of the urban green space landscape gradient.

Variation of urban green space types percent coverage along the land use transect at different spatial extents

There was almost no distribution of AG along the belt transect gradient in the city and the percentage of AG in the landscape increased sharply at +10 km east of the origin, i.e. near the junction between the second ring road and third ring road (*Fig. 3a*).

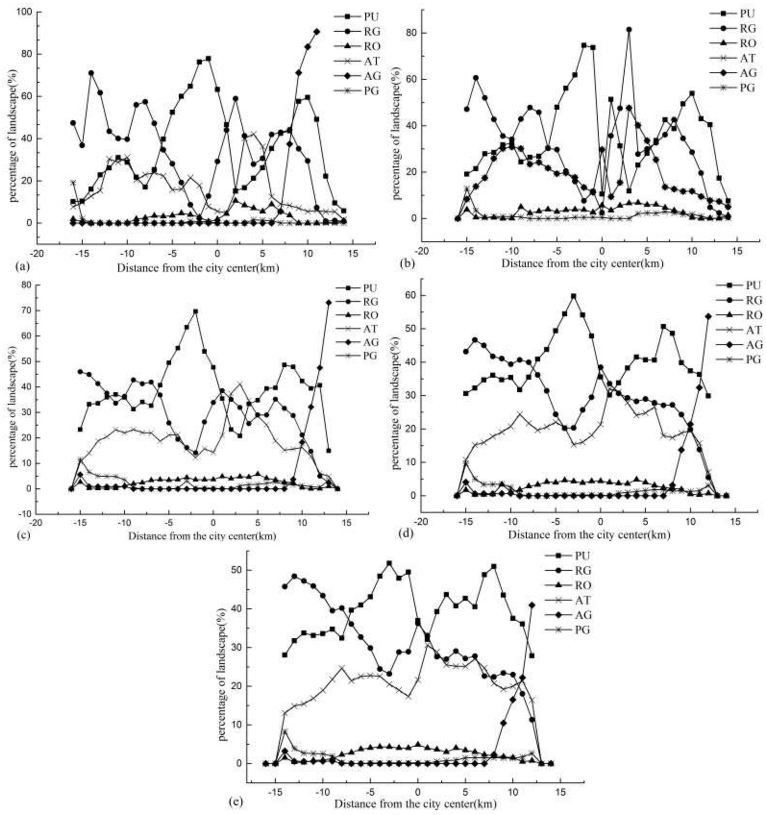

Figure 3. *Variation in the proportions of Shanghai urban green space types along the east-west direction transect at different spatial extents: (a) 3 km extent, (b) 4 km extent, (c) 5 km extent, (d) 6 km extent, (e) 7 km extent. RO: roadside green space; PU: public park; RG: residential green space; AT: attached green space; AG: agricultural land; PG: production and protective green space*

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The distribution of RG progressively decreased from west to east of the city with a low value within the range −4 km to −1 km. PU progressively increased from the suburbs to the central urban area with the highest value at the central urban area; this type of green space exhibited an M-shaped distribution with peaks at −4 km and +11 km, and a valley at +1 km to +3 km (*Figs. 3a, 3b, 3c, 3d and 3e*). PG reached its highest peak between −13 km and −14 km from the center, and its proportion in the landscape was small between −10 km to +14 km from the center with sporadic distribution and a value close to 0. AT generally exhibited an M-shaped distribution (*Figs. 3a, 3b, 3c, 3d and 3e*) and reached peak values at −10 km to −13 km and +1 km to +5 km, with a sharp decrease to a valley at −1 km to +5 km. RO changed in an irregular pattern, with a small proportion at both sides of the city and a large amount at −10 km to +8 km.

Changes in the proportion of the landscape occupied by various green space types were closely related to the land utilization space pattern of Shanghai. The downtown area within the second ring is the political and business center of Shanghai; due to its early construction it mostly consisted of AT and PU with a small percentage of RO, and the green space types were not fragmented (*Figs. 3a, 3b, 3c, 3d and 3e*). When the spatial extent of the windows along the transect was 6 km and 7 km, changes in the proportions of green space in the landscape along the belt transect exhibited a smooth trend line. However, when the extent was reduced to 3 km and 4 km, the changes exhibited erratic fluctuations, indicating that the landscape metrics used in this research were sensitive to pattern changes when the spatial extent of windows used to identify them was small. Therefore, we concluded that the window size of 5 km was a proper extent for studying Shanghai's urban green space landscape pattern.

Transect analysis using landscape-level metrics

Figures 4 and 5 portray the variations in landscape metrics in the study area. The cross-section analysis from east to west and from south to north using a 5-km window showed that variations in the six major types of green space exhibited the same pattern. As a proportion of the landscape (PLAND), PU and AT exhibited an M-shaped distribution and the largest proportions of PU occurred at the city center and at +9 km from the city center. The largest proportions of AT occurred at +4 km and +8 km from the city center, and PU mainly occurred between these two locations. The trends in the proportions of other categories of green space at the same distance from the city center were the same (*Figs. 4a and 4b*). The patch density (PD) of the six major categories of urban green space was basically the same from east to west and from south to north (*Figs. 4c and 4d*), and there was only some difference between PU and RO at −5 km to -16 km. However, at the landscape level, the overall PD trend of the six major categories of urban green space was the same (*Fig. 5a*).

The overall trend of the largest patch index (LPI) was also the same in the six major categories of urban green space, although there was some difference for PU at −7 km to −16 km, and for AT at +6 km to +16km. However, at the landscape level, the distribution trend of LPI from east to west and from south to north was the same (*Figs. 4e, 4f and 5b*). The overall trend for LSI was the same in all green space types, but there was some small difference between PU and the other types at −5 km to −16 km. At the landscape level, the distribution trend of LSI for the six major categories of urban green space was the same (*Figs. 4f, 4g and 5c*).

At the landscape level, the distribution trends of MPS and PSSD for the six major categories of urban green space from east to west and from south to north were the

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DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

same, exhibiting exponential growth (*Figs. 5d and 5e*). Furthermore, the distribution trends of AWMSI, AWMFD and SHDI were also the same (*Figs. 5f, 5g and 5h*). To sum up, the distribution trends of the six major categories of urban green space in Shanghai were basically the same for both cross-sections and at the landscape level, which is characteristic of a "concentric circle" development pattern. Thus, these results provide further evidence that Shanghai's urban development can be described by the "concentric circle" urban development theory.

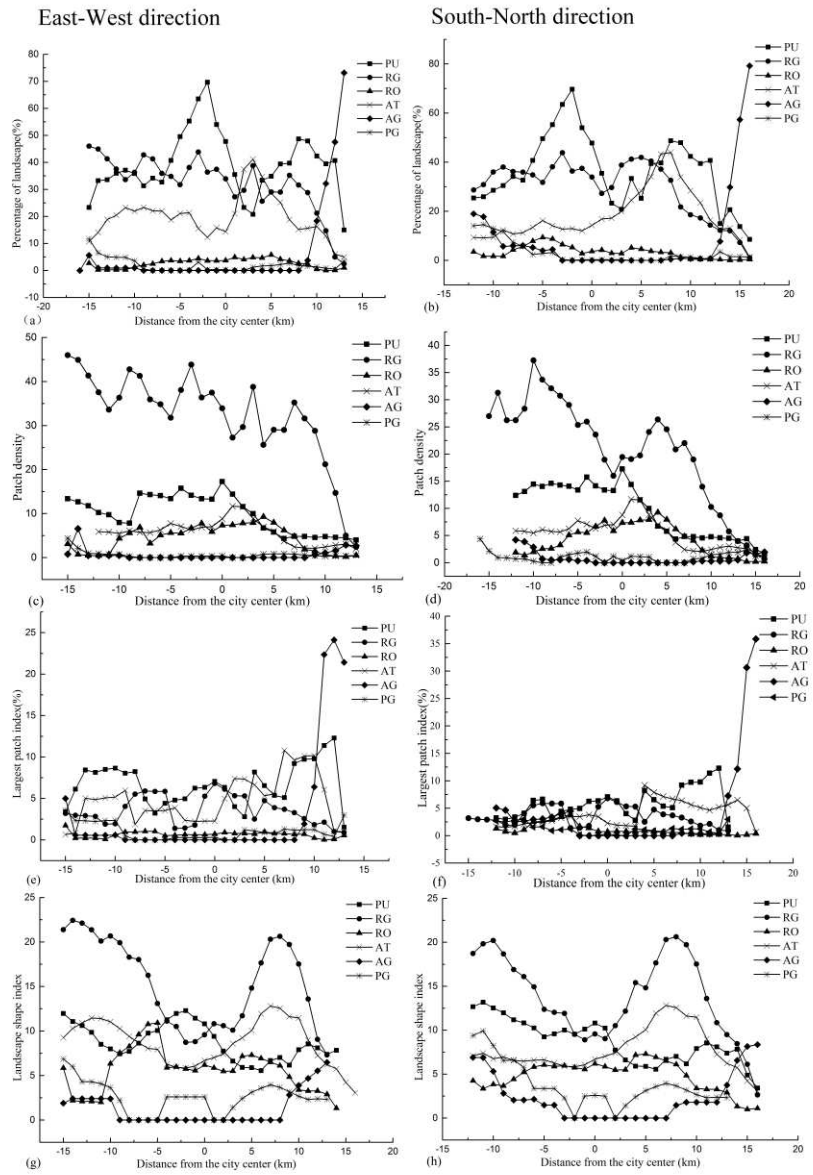

Figure 4. *Gradient changes of landscape indices in different types of urban green space along east-west and south-north transects in Shanghai: (a and b) Percentage of landscape (%); (c and d) Patch Density (No. per 100 ha); (e and f) Largest patch index (% total area); (g and h) Landscape shape index. RO: roadside green space; PU: public park; RG: residential green space; AT: attached green space; AG: agricultural land; PG: production and protective green space*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

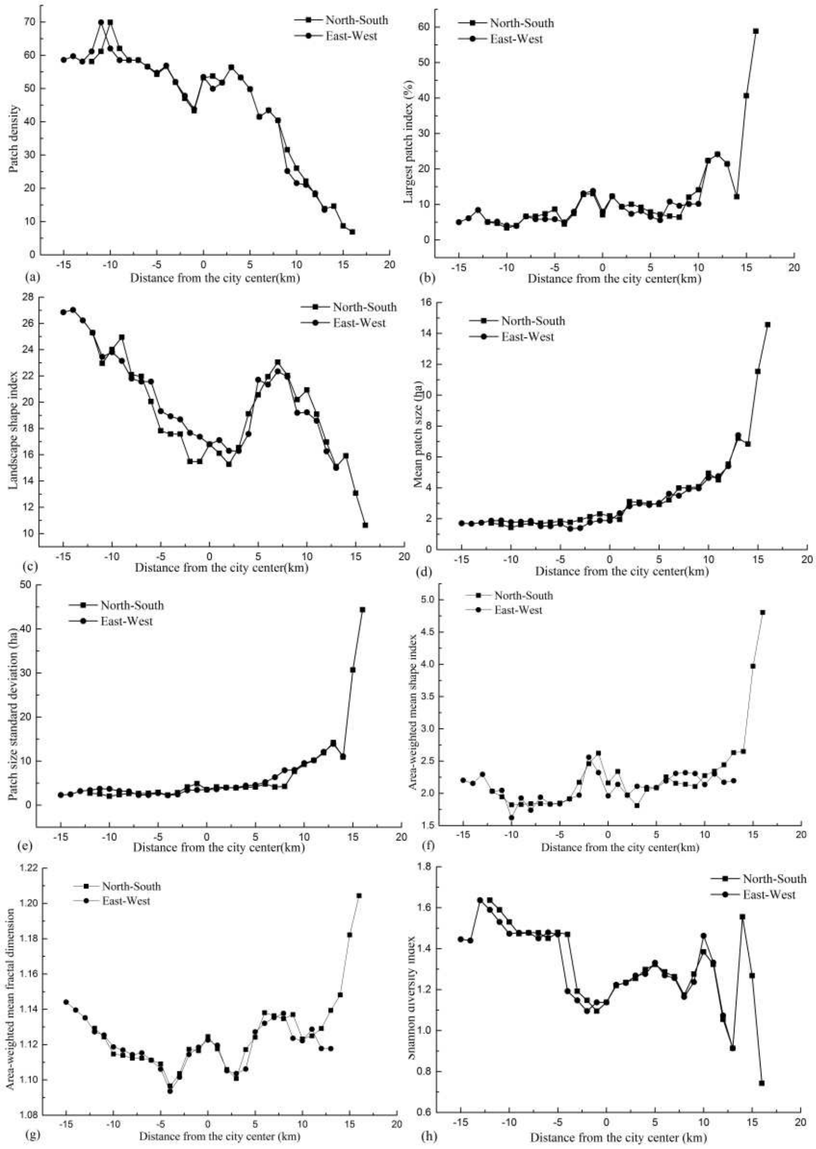

***Figure 5.** Gradient changes of urban green space at the landscape level: (a) Patch Density (No. per 100 ha); (b) Largest patch index (% total area), (c) Landscape shape index; (d) Mean patch size (ha); (e) Patch size standard deviation (ha); (f) Area-weighted mean shape index; (g) Area-weighted mean fractal dimension; (h) Shannon diversity index*

Discussion

The reasonable extent for landscape pattern analysis

The spatial resolution of remote sensing images and data processing accuracy have an effect on the identification of green space types and on research about landscape pattern changes (Turner, 1989; O'Neill et al., 1996; Baldwin et al., 2004; Wu, 2004; Buyantuyev and Wu, 2007; Weng, 2007; Castilla et al., 2009). Therefore, the selection of high spatial resolution images and reasonable study amplitudes is vital to quantitative research of urban green land distribution patterns (Buyantuyev et al., 2010). In this study, we examined a range of reasonable spatial extents (3, 4, 5, 6 and 7 km) by which

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

to analyze Shanghai's urban green land landscape pattern. The results showed that 5 km was the most suitable size of window, which corroborates the research findings of Shenyang (Tang, 2008).

Moreover, the spatial resolution of remote sensing images that suits the research on urban green land landscape pattern amplitude is determined by characteristics of the research area, by researchers' caution and by the analytical methods applied. According to our research, the spatial resolution of Landsat images (30 meters) was sufficient to determine the key information about urban green land needed in the research. For instance, LPS, AWMFD and SHDI (*Figs. 5c, 5g and 5h*) indicated that the green land area from the suburban district inward to the downtown area of Shanghai was increasing while the patch density was decreasing. This pattern reflects the fact that the government has put emphasis on the provision of urban green land and has made a reasonable plan for the city. The method that combines the use of FRAGSTATS, GIS analysis and landscape indices provides significant guidance for further research about urban landscape patterns and town planning.

However, some uncertainty and a use of subjective factors is involved when considering the impact of the "background" land uses and edge effects during execution of the moving window procedure, sampling methods, and the selection of landscape metrics at different periods. Yet, little thought is given to the combination of the moving window procedure, landscape metrics and transfer matrix method of quantification. Therefore, classification training, revision of parameters and mathematical modeling should be conducted according to the characteristics of different research areas so as to increase the analysis accuracy and the degree to which research methods are integrated.

The theories of urban development

Dietzel et al. (2005) proposed a "concentric" model to describe urban development. Under this model, urban growth starts with the expansion of urban core areas and diffuses from these core areas outward. Urbanization in the Shanghai metropolitan region has resulted in dramatic increases in patch density, edge density, and patch and landscape shape complexity, as well as sharp decreases in both the largest and mean patch sizes, agricultural area, and landscape connectivity (*Figs. 4 and 5*). The general pattern of urbanization revealed in this study was that the increasingly urbanized landscape became compositionally more diverse, geometrically more complex, and ecologically more fragmented. This pattern is consistent with those in Phoenix and Las Vegas, USA as reported by Wu et al. (2011). First, both 32-km long and 7-km wide west–east and south–north transects in this study provided an excellent opportunity for characterizing broad-scale landscape pattern change along a rural–urban–rural environmental gradient; this has not been reported previously in any Chinese city. The urbanization gradient revealed by our transect analysis seemed to suggest that the different types of urban green space that are located different distances away from the downtown center can reflect different types of land-use. Therefore, land-use regularly ranges from the suburban agricultural land to public land to residential land, and then again to public land and residential land (*Figs. 4a and 4b*). The classic concentric zone urban development theory seems to account adequately for the land use pattern of the Shanghai metropolitan region up to the year 2015, and this is partly because classic theories of urban morphology such as the "concentric circle" theory were developed based primarily on studies of old and well-established cities (i.e., Chicago, San Francisco and Boston in the USA).

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DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

Shanghai was growing rapidly in the beginning of the 21st century, which may be indicative of a fast-growing city, or urban development among Chinese coastal cities. Perhaps a new urban development theory will be proposed in the near future that is more suitable for understanding the metropolitan area of Shanghai urbanization development.

In particular, combining gradient analysis with landscape metrics, as illustrated in this study, can help to quantitatively identify and characterize the gradients and complex spatial pattern of urbanization, which can subsequently be related to ecological and socioeconomic processes (McDonnell et al., 1997). The expansion of this study to understand the mechanism of urban landscape pattern formation requires a more comprehensive study. (e.g., Luck et al., 2001).

Conclusions

The method that combines the use of FRAGSTATS software, GIS analysis and landscape indices provides a completely new way of thinking and methodology for the analyzing the gradient of urban green landscape from an urban area to a suburban area. Meanwhile, this technique also promotes the analysis and quantitative research of landscape ecology (Herold et al., 2003; Sawaya et al., 2003; Garrigues et al., 2006).

“Windows” of different spatial extents were applied in this study to determine the one most reasonable for analyzing Shanghai’s urban green space. Results confirm that the range of 5 km is the most suitable for research on Shanghai green landscape gradient analysis. Analysis at this scale not only avoids erratic changes in landscape indices, but also accurately identifies how urban green land types vary as the gradient changes. At the same time, a window of 5 km shows the significant “spatial characteristics” of urban green land changes with the different gradients.

Urban green landscape pattern can corroborate spatial development patterns, confirming that Shanghai urban development represents a mode of concentric circles. This conclusion relates closely to the leading urban development policies, and the coherence of urban land space and urban sustainable development.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_17991815

EFFECT OF NEPHTHYL ACETIC ACID FOLIAR SPRAY ON AMELIORATION OF SALT STRESS TOLERANCE IN MAIZE (*ZEA MAYS* L.)

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(Received 30th Sep 2018; accepted 28th Nov 2018)

Abstract. The present study was aimed to evaluate the effect of naphthyl acetic acid (NAA) on chlorophyll, protein, proline, sugar and carotenoid contents along with certain enzymatic activities, namely peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APOX) in two selected varieties i.e. Iqbal and Pahari of maize crop induced under salt stress of different concentrations (100mM, 80mM, 60mM, 40mm and 20mM). The experiment was conducted at the Department of Botany, Bacha Khan University Charsadda, Pakistan during maize growing season, 10-09- 2015. The result of the study revealed that different concentrations of NaCl affect the physiological and biochemical parameters. The anti-oxidative enzymes, such as peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APOX), and sugar, proline, and proteins were found maximum in treatment T2, T4, T10 and T12 in the Pahari variety which on physiological basis was found more tolerant to the saline condition and regarding the response to exogenous application of naphthyl acetic acid (NAA) the variety Iqbal was more sensitive to salt stresses.
Keywords: *nephthyl acetic acid, selective growth, antioxidant enzymes, action, salt stress tolerance*

Introduction

Maize (*Zea mays* L.) is one of the most important crops which are used as breakfast and the oil of maize for diabetic patient all over the world. Maize is also used as food

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

for animals (Hussain et al., 2010). It is important measure salt stress tolerance of plants because up- or down-regulation of salt changes physiological mechanisms in the plants (Tas and Basar, 2009). The salt was effect resolute at seed earlier stage of wheat from a decrease in germination percentage, fresh and dry weight of shoots and roots due to the translocation of several nutrient ions (Afzal et al., 2005). The presence of salt in the soil of marshlands or when salt is already a part of the soil is called primary or main salinization, but some plants are growing and adapted to saline soil. Minor or secondary salinization occurs in the soil where salt concentration is low and sometimes soil becomes saltier because of poor and low irrigation (Zhu, 2007). Soil salinization is one of the major factors of soil squalor or soil salinity. Soil salinization reached about 19.5% of the irrigation land and 2.1% of the dryland agriculture current in the world (FAO, 2000). In Pakistan, after wheat, cotton, and rice, maize is the fourth largest grown crop. In Pakistan one million hectares of maize production produce about 3.5 million metric tons. Punjab contributes 39% of the total area under maize and 30% of total production, Khyber Pakhtunkhwa contributes 56% of the total area production, Sindh contributes 63% of the production and Baluchistan 5% of the total area (PARC, 2010*).* Salt stress creates both ionic as well as osmotic stress on plants (Parvaiz and Satyawati, 2008). Salt stress is a major abiotic stress that can affect plant morphology and physiology, in this way the fresh and dry parts of shoots and roots are decreased perhaps due to the high concentration of salt ions or water-related qualities (Hajer et al., 2006). The leaf chlorophyll imbalance due to salt stress (abiotic stress) reduces photosynthesis (Turan et al., 2007). Salt stress is caused by high concentration of Na^+ and Cl^- in the environment that reduce the fertility of the soil so plant roots can't grow in the saline soil (Rasool et al., 2013). When maize, wheat, and rice etc. seeds are sown in saline soils, salt (Na^+ and Cl) cause many adverse symptoms on seed germination, as result less number of seeds germinate, plant growth is stunted, height of the plant becomes short, leaves are smaller and thicker than in normal size of plants and the color is dark green and bluish. When plants grow in saline soils, the various plant parts e.g. leaves, fruits, roots, stems etc. are very small and so they affect quality and quantity of fruits, vegetables, and agricultural products e.g. high concentration of Na^+ also decrease sugar production in sugar crops (Storey et al., 1977). Salinity causes two major effects osmotic stress and ionic toxicity in plants, these two major effects osmotic stress, and ionic toxicity affect the various processes in the plant (Yadav et al., 2011).

To use growth-stimulating chemicals (Naphthalene acetic acid) for the increase of plant production and also stimulate plant physiologists the world (Ahmad et al., 2010). To study the upward transport of nutrients due to growth regulator chemical Naphthalene acetic acid (NAA), these growth regulator chemical Naphthalene acetic acid (NAA), ameliorate growth of the physiological and proteins, carbohydrate, sugar etc. the of plants (Iqbal et al., 2009; Tůmová et al., 2018). Growth regulators or hormones stimulate and promote growth in plants. Naphthalene acetic acid (NAA) is a growth controller and also increases the production and yield of plants. Naphthaleneacetic acids (NAA) are synthetic growth regulator hormones which ameliorate the potential of plants with suitable concentration. Naphthaleneacetic acid (NAA) also affects the growth, yield, and production of tomatoes plants. (Jahan and Fattah, 1991). Keeping in view all the above information, the present study will be conducted to screen maize (Pahari and Iqbal) accessions for salt stress tolerance. In addition, physiological and biochemical variations are also assessed for some physiological traits that could be used as selection criteria for future breeding programs.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

Aims of the study

The present study was aimed at assessing the physiological mechanism of adaptation to salt tolerance in maize at the vegetative stage with variable levels of salt stress (20, 40, 60, 80, and 100mM of salt, accompanied by the application of naphthyl acetic acid (NAA) foliar spray). The reactions of maize to salt stress were assessed on the basis of selectivity of growth responses, osmoregulation, and antioxidant enzyme actions.

Materials and methods

Experimental design

The experiment was conducted during the 10- 09- 2015 corn growing season in the greenhouse of the Botanical Department of Bacha Khan University Charsadda (latitude 34.1509', east longitude 71.735'E, 908 feet above sea level). Seeds of two seed selections, Iqbal and Pahari, are from the Crop Research Institute Pirsabaq Nowshehra (CCRI). Seeds were sterilized with 5% oxychloride and 95% ethanol before planting and then rinsed three times with distilled water. After soaking, the seeds were sown in plastic pots (14 cm below the inner diameter, inner diameter 18.5 cm, height 15.6 cm, thickness 0.5 cm), filled with 3 kg of air-dried soil and sand (3:1), and placed in triplicate. The plants were exposed to 20, 40, 60, 80 and 100 mM salts for about 15 (26- 09- 2015) days after germination in an incubator with an average day and night temperature of 25°C (10 hours) and 16°C (14 hours), respectively. In the group experiments, all treatments were sprayed with NAA for 1 week; control plants were usually irrigated with distilled water. After 15 days, leaf samples were collected for analysis.

Chemical analysis of rhizospheric soil

Soil pH and electrical conductivity (EC)

The pH of the rhizospheric soil was measured by preparing a 1:1 (soil: water) suspension (McKeague, 1978; McLean, 1982). Air-dried soil samples (10 g) were mixed in 10 ml of distilled water and stirred for 1 hour in a magnetic stirrer for uniform mixing, and then the suspension was filtered through Whatman No. 42 filter paper. The pH of the filtrate was measured with an Electrical pH meter while the EC was recorded using an electrical conductivity (EC) instrument. The readings were measured in micro-siemens per centimeter (µS/cm). Soil Moisture (10 g) was taken from a uniform depth, 6 inches from the surface of the pot. The dry weight was determined by drying the soil in an oven at 70°C for 72 hours to constant weight.

Soil moisture content

The soil (10 g) was removed from the same depth, i.e. 6 inches from the surface of the pot. The dry weight was determined by drying the soil in an oven at 70°C for 72 hours to constant weight. The soil moisture content is calculated by the following formula:

$$\text{Parentage moisture content} = \frac{(\text{Fresh weight of soil - the Dry weight of soil}) \times 100}{\text{Fresh weight of soil}} \qquad \text{(Eq.1)}$$

Field capacity of the rhizospheric soil

The field capacity of rhizospheric soil was determined by the following method:

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

$$\text{Parentage Field Capacity} = \frac{(\text{Weight of wet soil (g) - Weight of dry soil (g)}) \times 100}{\text{Weight of dry soil}} \quad \text{(Eq.2)}$$

The agronomic character of maize under drought stress

Detailed agronomic characterization of maize under drought stress was performed, including:

- Root length
- Root fresh weight
- Root dry weight
- Root moisture content
- Shoot length
- Shoot fresh weight
- Shoot dry weight
- Shoot moisture content
- Number of leaves
- Leaf fresh weight
- Leaf dry weight
- Leaf moisture content
- Germination
- Leaf area
- Root/shoot ratio
- Vigorous index

Physiological and biochemical analysis

- The protein content of leaves was determined following the method of Lowry et al. (1951) using BSA as standard.
- Sugar estimation of fresh leaves was done following the method of Dubois et al. (1956).
- Chlorophyll content of leaves was determined by the method of Arnon (1949).
- The proline content of leaves was measured by the method of Bates et al. (1973).
- Peroxidase (POD) activity was determined by the method of Vetter et al. (1958) as modified by Gorin and Heidema (1976).
- Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction of nitro-blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971).
- Ascorbate peroxidase (APX) activity was determined according to Asada and Takahashi (1987).
- Catalase (CAT) was measured according to Asada and Takahashi (1987), with modification.

Statistical analysis

The data were analyzed statistically by the Analysis of Variance technique (Steel and Torrie, 1980) and comparison among treatment means was made by Duncan's Multiple Range Test (DMRT) (Duncan, 1955).

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

Results

Physicochemical characteristics of rhizospheric soil

The effect in *Table 1*, *Equations 1 and 2* shows that the extreme pH of the treatment for T6 (60 mM) was recorded in Iqbal and Pahari compared to the control (untreated). A decrease in the pH and EC of the soil collected from Iqbal and Pahari was noted in T10 (100 mM + NAA), reflecting the positive role of NAA in maintaining EC and pH under induced salt stress. In Iqbal and Pahari, the maximum moisture content percentage and field capacity percentage for T8 (80 mM + NAA) treatment were reported; T6 reported the minimum for both varieties.

Table 1. *Effect of salinity on field water holding capacity, moisture content, soil pH and soil conductivity of maize (Zea mays L.)*

Treatments	Field Capacity (%)		Moisture content (%)		Soil pH		Electric Conductivity (S/m)	
	VI	V2	VI	V2	VI	V2	VI	V2
Tl	18.20824±0.01	13.91949±0.02	15.05717±0.012	11.13425±0.013	7.3±0.012	7.3±0.013	700±0.071	302.5±0.072
T2	12.27679±0.04	11.19778±0.05	10.93439±0.06	10.070 15±0.07	7.5±0.06	7.4±0.07	995±0.02	700±0.03
T3	13.971 17±0.04	30.04483±0.05	12.23155±0.06	23.09645±0.07	7.05±0.06	7.1±0.07	1000±0.08	995±0.09
T4	18.69354±0.04	11.5757±0.05	15.74942±0.01	10.37475±0.02	7.6±0.01	7.1±0.02	1263±0.015	1000±0.016
T5	20.62099±0.027	18.5961±0.028	16.41353±0.08	15. 44271 ±0.09	7.25±0.08	7.25±0.09	1246±0.02	1263±0.03
T6	14.26551±0.08	0.813335±0.09	12.48453±0.015	0.806773±0.016	7.4±0.015	7.2±0.016	761±0.05	1246±0.05
T7	18.25423±0.08	18.52983±0.07	15.28907±0.02	15.36283±0.03	7.2±0.02	7.3±0.03	1510±0.033	761±0.034
T8	23.75444±1.01	1.993725±1.02	19.19482±0.06	1.954753±0.07	7.4±0.06	7.2±0.07	750±0.045	1510±0.046
T9	14.82779±0.06	22.93297±0.07	11.84361±0.06	18.6276±0.07	7.15±0.06	7.15±0.07	720±0.075	750±0.08
T10	11.34148±0.06	1.588586±0.07	10.18621±0.011	1.56374±0.012	7.4±0.011	7.1±0.012	1080±0.027	720±.028
T1l	19.4188±1.03	15.44447±1.02	15.98667±0.034	12.66257±0.035	6.9±0.034	7.05±0.035	670±0.035	1080±0.036
T12	15.73781±0.01	11.52747±0.02	13.59781±0.015	10.33599±0.016	7.1±0.015	7.10±0.016	1846±0.071	670±0.072

V1= (Iqbal), V2= (Pahari), Tl concentration) T6= (60mM + NAA). (Control + NAA). (100mM NaCl concentration), T2= (l00mM + NAA), T3= (80mM NaCl concentration), T4= (80mM + NAA), T5= (60mM NaCl T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mMNaCl concentration) T10 (20mM + NAA), T11= (Control), T12= (control +NAA)

Agronomic characteristics

The results in *Table 2* show that the maximum germination rate, leaf area, root activity and leaf area index (LAI) had been measured or calculated of (LAI = leaf area / ground area, m^2/ m^2) were recorded in the two varieties treated T12 (control + NAA), while the minimum germination rate was reported of T4 (80 mM + NAA). The results in *Table 3* indicate that the T12 treatment of the two varieties reported maximum shoot length, shoot fresh and dry weight, and moisture content, while T5 (60 mM) reported the minimum shoot length, indicating that NAA increased the shoot length under salt stress. The results in *Table 4* indicate that the maximum root length and root fresh and dry weight were recorded of T3 (40 mM) treatment in Iqbal, while the minimum values were reported in T5 and T6 (60 mM + NAA). The data in *Table 5* shows that treatment of T12 in both varieties reported maximum leaf number and leaf fresh and dry weight, and reported the smallest in T3 (60 mM) and T4. A detailed physiological and biochemical evaluation of these varieties revealed significant differences in these varieties under salt stress.

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

Table 2. *Effect of salinity on root length, root freshness and dryness, and moisture content (Zea mays L.)*

Treatments	Root length (cm)		Root Fresh weight (g)		Root Dry weight (g)		Moisture content (%)	
	VI	V2	VI	V2	VI	V2	VI	V2
Tl	4.4±0.007	2.5±0.008	0.2015±0.05	0.143±0.06	0.1115±0.06	0.078±0.07	0.09±0.02	0.065±0.03
T2	5.9±0.017	3.5±0.018	0.235±0.035	0.24±0.036	0.115±0.015	0.129±0.016	0.12±0.06	0.111±0.07
T3	2±0.013	3.05±0.014	0.148±0.027	0.1275±0.028	0.0915±0.06	0.0425±0.07	0.0565±0.06	0.085±0.07
T4	5.5±0.01	2.5±0.02	0.141±0.02	0.09±0.03	0.099±0.011	0.082±0.012	0.042±0.06	0.008±0.07
T5	3±0.02	3.5±0.03	0.226±0.02	0.122±0.03	0.0855±1.01	0.0545±1.02	0.1405±0.02	0.0675±0.03
T6	6±0.034	2±0.035	0.213±0.045	0.414±0.046	0.12±0.013	0.321±0.014	0.093±0.05	0.093±0.06
T7	3.25±0.034	3±0.036	0.2085±0.02	0.234±0.03	0.138±0.011	0.162±0.012	0.0705±0.06	0.072±0.07
T8	3±0.001	2.5±0.002	0.244±0.027	0.13±0.028	0.138±0.01	0.05±0.02	0.106±0.04	0.08±0.05
T9	1.8±0.04	3±0.05	0.274±0.05	0.125±0.06	0.116±0.011	0.0905±0.012	0.158±0.17	0.0345±0.18
T10	5±0.015	2.3±0.016	0.165±0.01	0.112±0.02	0.076±0.015	0.09±0.016	0.089±0.04	0.022±0.05
T11	3.4±0.05	3.25±0.06	0.384±0.035	0.143±0.036	0.2815±0.01	0.078±0.02	0.1025±0.02	0.065±0.03
T12	4.8±0.024	3.3s±0.025	0.432±0.027	0.148±0.028	0.394±0.034	0.05±0.035	0.038±0.06	0.098±0.07

V1= (Iqbal), V2= (Pahari), Tl concentration) T6= (60mM + NAA), T7= (Control + NAA).: (l00mM Nacl concentration), T2= (l00mM + NAA), T3= (80mM Nacl concentration), T4= (80mM + NAA), T5= (60mM Nacl-(40mM Nacl concentration) T8= (40mM + NAA) (T9= (20mM Nacl concentration) T10 (20mM + NAA), T11 = (Control), T12= (control+ NAA)

Table 3. *Effect of salinity on shoot length, shoot fresh weight, dry weight and moisture content of maize (Zea mays L)*

Treatments	Shoot length (cm)		Shoot Fresh Weight (g)		Shoot Dry Weight (g)		Moisture content (%)	
	VI	V2	VI	V2	VI	V2	VI	V2
Tl	10.1±0.026	13.9±0.026	0.038±0.06	0.0725±0.07	0.024±0.05	0.05±0.06	0.014±0.001	0.02±0.002
T2	12.3±0.04	15±0.04	0.056±0.012	0.069±0.013	0.02±0.037	0.06±0.038	0.036±0.05	0.009±0.06
T3	11.5±0.01	17±0.01	0.0615±0.08	0.108±0.09	0.045±0.01	0.0655±0.02	0.0165±0.042	0.228±0.03
T4	0.036±0.05	21.6±O.17	0.074±0.08	0.123±0.09	0.07i0.011	0.09±0.012	0.004±0.05	0.039±0.02
T5	9.15±0.04	18±0.04	0.0465±1.01	0.12±02	0.0265±0.08	0.0675±0.08	0.02i0.034	0.0525±0.035
T6	17.5±0.02	15±0.02	0.083±0.06	0.255±0.07	0.024±0.027	0.094±0.028	0.059±0.04	0.161±0.05
T7	17±0.02	20.15±0.02	0.1105±0.05	0.193±0.06	0.0305±0.035	0.0555±0.036	0.08±0.03	0.1375±0.04
T8	10.5±0.07	22.7±0.07	0.047±0.015	0.143±0.016	0.011i0.017	0.012±0.018	0.036±0.027	0.131±0.028
T9	9.75±0.02	18.75±0.02	0.1±0.034	0.1165±0.035	0.04±0.016	0.0775±0.017	0.06±0.01	0.039±0.02
T10	12.7±0.01	21±0.01	0.075±0.015	0.133±0.016	0.071±0.05	0.074±0.06	0.004±0.02	0.059±0.03
T11	18.2±0.06	34.25±0.06	0.3435±0.06	0.481±0.07	0.0545±0.033	0.061±0.034	0.289±0.034	0.42±0.035
T12	30±0.02	26.5±0.02	0.395±0.034	0.348±0.035	0.099±0.023	0.12±0.024	0.296±0.02	0.228±0.03

V1= (Iqbal), V2= (Pahari), Tl= concentration) T6= (60mM + NAA), (Control + NAA). = (l00mM Nacl concentration), T2= (l00mM + NAA), T3= (80mM Nacl concentration), T4= (80mM + NAA), T5= (60mM Nacl T7= (40mM Nacl concentration) T8= (40mM + NAA) T9= (20mM Nacl concentration) T10 (20mM + NAA), Tl1= (Control), T12= (control+ NAA)

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

Table 4. *Effect of salinity on leaf number, leaf fresh and dry weight, and moisture content (Zea mays L.)*

Treatments	No of leaves (cm)		Leaf Fresh Weight (g)		Leaf Dry Weight (g)		Moisture content (%)	
	VI	V2	VI	V2	VI	V2	VI	V2
Tl	2±0.06	3±0.07	0.011±0.012	0.0325±0.013	0.0065±0.01	0.029±0.02	0.0045±0.07	0.0035±0.08
T2	2±0.01	3±0.02	0.01±0.024	0.013±0.025	0.007±0.027	0.009±0.028	0.003±0.01	0.004±0.02
T3	2.5±1.03	3.5±1.04	0.01±0.001	0.03±0.002	0.0065±0.02	0.0205±0.03	0.0035±0.06	0.0095±0.07
T4	3±0.04	4±0.05	0.008±0.033	0.032±0.034	0.002±0.03	0.029±0.04	0.006±0.02	0.003±0.03
T5	2.5±0.05	4±0.06	0.0155±0.014	0.0205±0.015	0.0095±0.033	0.0175±0.034	0.006±0.071	0.003±0.072
T6	2±0.015	3±0.016	0.013±0.024	0.065±0.025	0.007±0.17	0.052±0.18	0.006±0.02	0.013±0.03
T7	3±0.015	3±0.016	0.045±0.04	0.056±0.05	0.0315±0.04	0.0495±0.05	0.0135±0.017	0.0065±0.018
T8	3±0.06	4±0.07	0.019±0.047	0.037±0.048	0.011±0.02	0.03±0.03	0.008±0.06	0.007±0.07
T9	2±0.06	3.5±0.07	0.0335±0.023	0.0295±0.024	0.00845±0.034	0.023±0.035	0.025±0.021	0.0065±0.022
T10	3±1.03	3±1.04	0.028±0.08	0.042±0.09	0.02±0.07	0.033±0.08	0.008±0.02	0.009±0.03
T11	3.5±0.06	4±0.07	0.0825±0.027	0.079±0.028	0.04775±0.02	0.0765±0.03	0.03475±0.06	0.0025±0.07
T12	4±0.015	4±0.016	0.07±0.023	0.098±0.024	0.067±0.06	0.09±0.07	0.003±0.043	0.008±0.044

V1= (Iqbal), V2= (Pahari), Tl= (l00mM Nacl concentration), T2= (1mM + NAA), T3= (80mM Nacl concentration), T4= (80mM + NAA), T5= (60mM Nacl concentration) T6= (60mM + NAA), T7= (40mM Nacl concentration) T8= (40mM + NAA) T9= (20mM Nacl concentration) T10 (20mM + NAA), Tl1= (Control), T12= (Control + NAA)

Table 5. *Effect of salinity on leaf area, germination rate, vigor index and the root-shoot ratio of maize (Zea mays L.)*

Treatments	Leaf area (cm)		Germination (%)		Vigor's index		Root Shoot ratio (%)	
	VI	V2	VI	V2	VI	V2	VI	V2
Tl	2.225±0.05	7.375±0.06	3.5±0.012	2±0.013	184.14±0.07	219.25±0.08	4.905±0.012	1.947±0.013
T2	2.25±0.01	7±0.02	4±0.06	4±0.07	290.28±0.01	101.25±0.02	4.197±0.06	3.479±0.07
T3	2.975±0.01	7.8±0.02	3.5±0.06	3.5±0.07	169.425±0.06	378.63±0.07	2.290±0.06	2.280±0.07
T4	2.88±0.07	7.49±0.08	3±0.01	3±0.02	278.85±0.02	265±0.03	1.906±0.01	0.732±0.02
T5	3.25±0.06	12.25±0.07	3±0.08	3.5±0.09	96.2±0.071	104.25±0.073	3.401±0.08	1.048±0.09
T6	2.75±0.04	10.2±0.05	3±0.015	3±0.016	315±0.02	180±0.03	2.567±0.015	1.624±0.016
T7	12.75±0.03	12.325±0.04	3.5±0.02	3±0.03	217.5±0.017	229.95±0.018	3.434±0.02	1.269±0.03
T8	5.04±0.027	7.02±0.028	3±0.06	3±0.07	94.5±0.06	132±0.07	5.192±0.06	0.901±0.07
T9	3.46±0.023	5.75±0.024	2.5±0.06	3.5±0.07	96.25±0.021	113.5±0.022	1.576±0.06	1.507±0.07
T10	6.4±0.037	7.5±0.038	3±0.011	3±0.012	254±0.02	232±0.03	2.2±0.011	0.843±0.012
T11	9.45±0.05	18.5±0.06	2.5±0.034	2.5±0.035	16±0.06	144±0.07	1.191±0.034	0.922±0.035
T12	27±0.023	20.5±0.024	2 ±0.015	2±0.016	576±0.043	96.6±0.044	1.094±0.015	0.426±0.016

V1= (Iqbal), V2= (Pahari), Tl= (l00mM Nacl concentration), T2= (l00mM + NAA), T3= (80mM Nacl concentration), T4= (80mM + NAA), T5= (60mM Nacl concentration) T6= (60mM + NAA), T7= (40mM Nacl concentration) T8= (40mM + NAA) T9= (20mM Nacl concentration) T10 (20mM + NAA), Tl 1= (Control), T12= (Control + NAA)

Chlorophyll a/b ratio

The chlorophyll a/b ratio (mg/g) of the selected maize varieties Iqbal and Pahari during the vegetative phase was evaluated (*Figure 1*). The results showed that the maximum chlorophyll a/b ratio was found for T6 (60 mM + NAA) in Iqbal and T12 (control + NAA) in Pahari. In Iqbal, the chlorophyll a/b ratio content of the vegetative phase of T12 was statistically similar to T9 (100 mM), T10 (100 mM + NAA) and T11

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

(control), at P < 0.05. The chlorophyll a/b ratios of the two varieties were the lowest, T1 (20 mM), T3 (40 mM), T4 (40 mM + NAA) and T8 (80 mM + NAA), at P < 0.05, indicating that NAA did not have a role in induction. The positive effect of improving chlorophyll a content under salt stress.

Total chlorophyll content

The results in *Figure 2* show that the total chlorophyll content (mg/g) is the largest of the T11 treated (control) out of the two varieties, while in Pahari, the value of T12 (control + NAA) is significantly higher than that of T9 (100 mM) and T10 (T10). Similarly, (100 mM + NAA) at P < 0.05 at the nutritional stage. The minimum total chlorophyll content in two varieties of T1 (20 mM), T2 (20 mM + NAA), T5 (40 mM) and T6 (40 mM + NAA) was reported, at P < 0.05.

Carotenoid

A comparative study of carotenoid content (μg/g) was carried out at the nutrient stage (*Figure 3*). The results showed that the maximum carotenoid content of the two varieties was reported in the T12 (control + NAA) treatment. In Iqbal, the carotenoid content of T12 (control + NAA) was significantly similar to the T11 (control) of the vegetative phase, at P < 0.05. The lowest carotenoid content in the two varieties was T1 (20 mM), T2 (20 mM + NAA), T3 (40 mM) and T5 (60 mM), at P < 0.05, indicating that NAA did not have a role in carotenoid content. The positive role enhanced and influence the content is induced under salt stress.

Sugar

The sugar content (μg/g) was evaluated at the nutrition stage (*Figure 4*). The results showed that the maximum sugar content was reported for both T12 (control + NAA) treatments, while in Iqbal, the carotenoid content of T12 was significantly similar to T5 (20 mM), T7 (80 mM) and T11, at P < 0.05 nutritional stage. The lowest sugar content of the two varieties was reported as T1 (20 mM), T2 (20 mM + NAA) and T3 (40 mM), at P < 0.05.

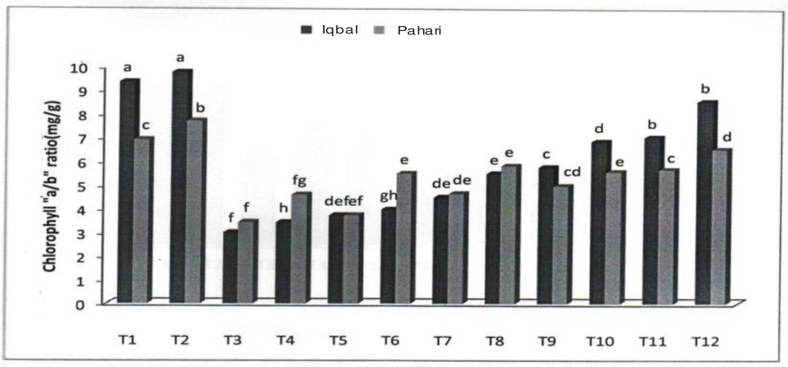

Figure 1. *Chlorophyll "a/b" ratio (mg/g) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage acid induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= (l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= (60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_18171834

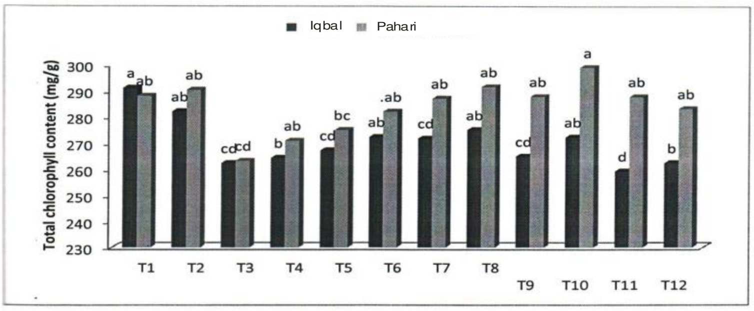

Figure 2. *Total Chlorophyll Content (mg/g) of the selected maize varities i.e Iqbal and Pahari at veget2: under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= (l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM+NAA) T5= (60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40nM NAA) T9= (20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control - NAA)*

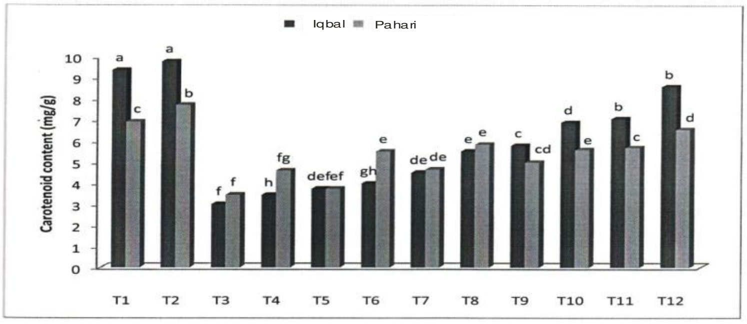

Figure 3. *Carotenoid content (mg/g) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= 60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= 20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

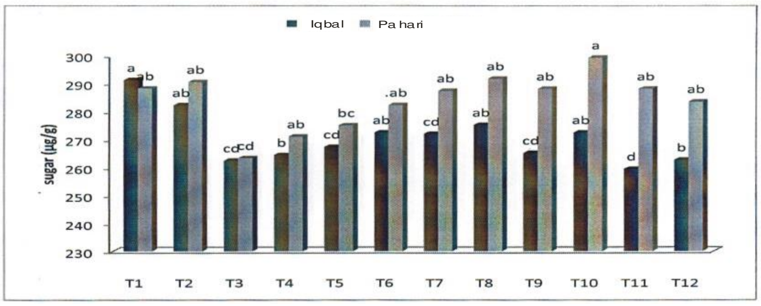

Figure 4. *Sugar content (ug/g) of the selected maize varities i.e Iqbal and Pahari at vegetative stage under - induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= 100mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= 60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mM + NaCl concentration) T10= (20mM + NAA) T11 = (control) T12= (Control + NAA)*

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Proline

A comparison of proline content (μg/g) was carried out at the nutrient stage (*Figure 5*). The results showed that the maximum proline content was detected of T10 treatment in both varieties, while the carotenoid content of T10 (100 mM + NAA) was reported in Pahari. In the vegetative phase, it was significantly similar to T4 (40 mM + NAA), T5 (60 mM) and T9 (100 mM), at P < 0.05. The lowest proline levels of T3 (40 mM), T4 (40 mM + NAA), T6 (60 mM + NAA) and T8 (80 mM + NAA) were reported, at P < 0.05, indicating that NAA is active in the enhancing effect of induction of sugar content changes under salt stress

Protein

Protein content (μg/g) was assessed during the nutrition phase (*Figure 6*). The results showed that the maximum protein content was reported in T10 (100 mM + NAA) treated Pahari and Iqbal. Pahari protein content T10 (100 mM + NAA) Iqbal is significantly similar to T2 (20 mM + NAA), T4 (60 mM + NAA), T7 (80 mM) and T12 (control + NAA) cultivars with the same vegetative phase at P < 0.05. The lowest protein content of T7 (80 mM), T3 (40 mM), T5 (60 mM) and T6 (60 mM + NAA) was reported, at P < 0.05, both of which indicated that NAA did not induce protein content under salt stress influences.

Anti-oxidant enzymes

Various biological processes in organisms result in reactive oxygen species (ROS) which cause oxidative stress. In response to such oxidative stress, organisms can deploy superoxide dismutase (SOD) and catalase (CAT) to scavenge ROS so as to protect the cellular homeostasis (Balaban et al., 2005). Plants have enzymatic and non-enzymatic antioxidant mechanisms that counteract the adverse effects of salinity. The former includes enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR). The latter includes compounds such as ascorbate, glutathione, flavonoids, and vitamins C and E (Noctor and Foyer, 1998). Thus, an imbalance between the production of free radical species and the cellular antioxidant defence system will produce an appearance of oxidative stress. Cell damage caused by excess free oxygen free radicals has been explained as a result of changes in the cell membrane produced by acid oxidation of the lipid bilayer, a process known as lipid peroxidation. This produces changes in chemical composition and deterioration of cell membrane ultrastructure, reduces their fluidity, alters their permeability, and inactivates enzyme and membrane channel receptors (Mansour and Salama, 2004).

Peroxidase

Comparison of POD (OD/mint/g) in the selected maize varieties including Iqbal and Pahari at the vegetative stage was made from the plant samples collected from the designed experiment (*Figures 7,8*). The results showed that the T12 (control + NAA) treatment of both varieties reported the maximum POD, while the POD activity of Pahari, T12 (control + NAA) was significantly similar to T2 (20 mM + NAA) and T4 (40 mM + NAA). T6 (60 mM + NAA) and T8 (80 mM + NAA) at P < 0.05 at the vegetative stage. The lowest POD activity among the two varieties was T1 (20 mM), T7

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(80 mM) and T9 (100 mM + NAA), at P < 0.05, indicating that NAA has a positive role in the improvement of POD activity under salt stress induction.

Superoxide dismutase

The SOD activity (OD/min/g fw) in the selected maize varieties including Iqbal and Pahari at the vegetative stage was made for the plant samples collected from the designed experiment (*Figure 9*). The results showed that the maximum SOD was reported for both T12 (control + NAA) treatments, while in Iqbal, the SOD activity of T12 (control + NAA) was significantly similar to T2 (20 mM + NAA) and T4 (40 mM + NAA) and T6 (60 mM + NAA) in the vegetative phase at P < 0.05. It was reported that T9 (100 mM + NAA), T7 (80 mM), T10 (100 mM + NAA) and T11 (100 mM + NAA) had the lowest POD activity in two varieties, at P < 0.05, indicating that NAA acts to induce SOD activity under salt stress.

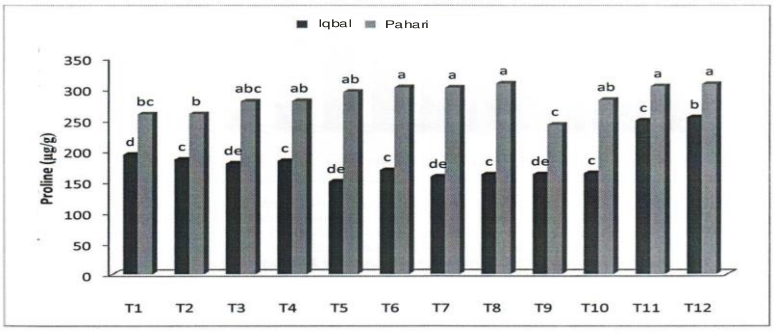

Figure 5. *Proline content (u.g/g) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl = 100mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= 60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= 20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

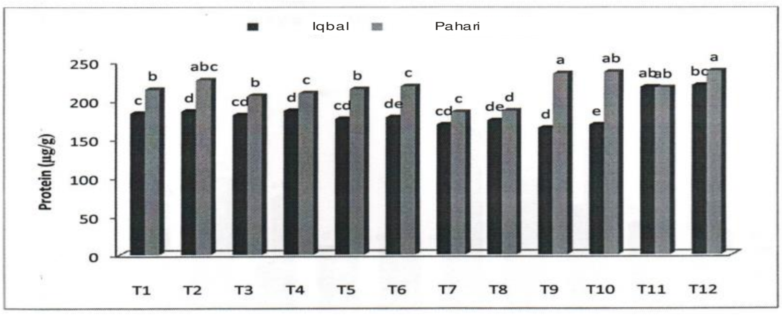

Figure 6. *Protein content (ig/g) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= I l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= (60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mMNaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

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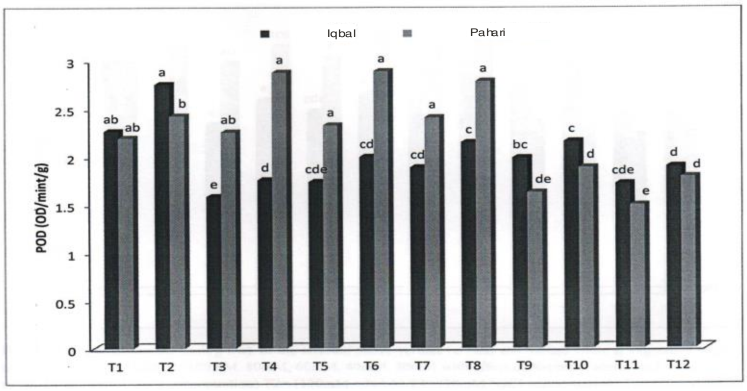

Figure 7. *Peroxidase (OD/mint/g) of the selected maize varities i.e Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= (l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= (60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mM NaCl concentration) T10= (20mM + NAA) Tl 1= (control) T12= (Control + NAA)*

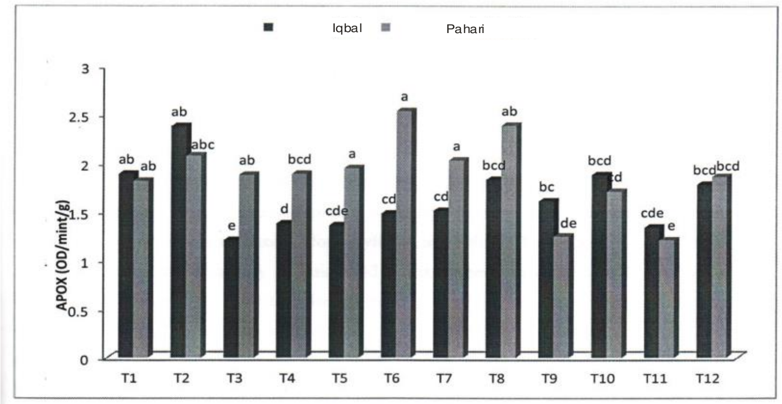

Figure 8. *Ascorbate peroxidase(OD/mint/g) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage under induced soil salinity of 100Mm,80Mm,60rnM,40Mm,20Mm with nephthyl acetic acid foliar spray. V1= Variety 1 (Iqbal) V2= Variety 2 (Pahari) Tl= (l00Mm Salt solution) T2= (l00mM solution sprayed) T3=(80mM Salt solution) T4=(80mMSalt solution sprayed) T5= (60Mm Salt solution) T6= (60Mm Salt solution sprayed) T7=(40Mm Salt solution) T8=(40Mm Salt solution sprayed) T9=(20Mm Salt solution) T10=(20Mm Salt solution sprayed) T11 = (control) T12=(Control spray)*

Catalase

A comparison of CAT activities (OD/min/g fw) in the selected maize varieties including Iqbal and Pahari at vegetative stage were made for the plant samples collected from the designed experiment (*Figure 10*). The results showed that the maximum CAT was reported in the T12 (control + NAA) treatment of the two varieties, while in Pahari, the CAT activity of T12 (control + NAA) was compared with T6 (60 mM + NAA), T11 (control) and T4 (40 mM). + NAA) at $P < 0.05$ in the nutritional phase. The lowest CAT activity of T9 (100 mM + NAA), T1 (20 mM), T3 (40 mM), T5 (60mM) and T10

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(100mM + NAA) was reported in two varieties, at P<0.05, indicating positive NAA effect of enhancing CAT activity under induced salt stress.

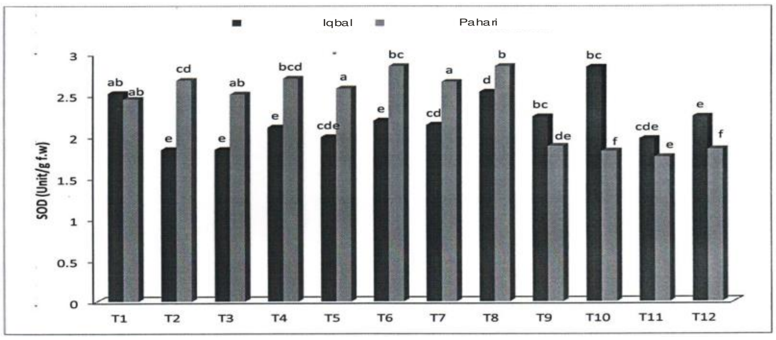

Figure 9. *Superoxide dismutase (unit/g f/w) of the selected maize varities i.e Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= (l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= (60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= (20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

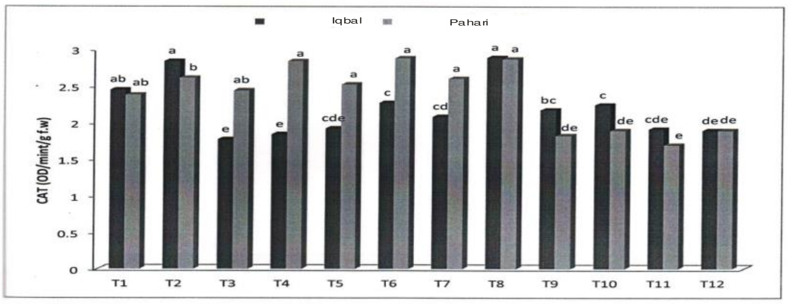

Figure 10. *Catalase (OD/mint/g f.w) of the selected maize varities i.e. Iqbal and Pahari at vegetative stage under induced soil salinity of l00mM, 80mM, 60mM, 40mM, 20mM with nephthyl acetic acid foliar spray. Tl= l00mM NaCl concentration) T2= (l00mM + NAA) T3= (80mM NaCl concentration) T4= (80mM + NAA) T5= 60mM NaCl concentration) T6= (60mM + NAA) T7= (40mM NaCl concentration) T8= (40mM + NAA) T9= 20mM NaCl concentration) T10= (20mM + NAA) Tl1= (control) T12= (Control + NAA)*

Discussion

Effect of nephthyl acetic acid foliar spray on the physiological character of maize under salt stress

The importance of this study shows significant changes in all physiological indicators of all maize genotypes. For both genotypes, all treatments showed significant changes in chlorophyll content under control and salinity conditions. During the vegetative growth phase, an increase in salt stress resulted in an increase in Pahari's chlorophyll "a", chlorophyll "b", chlorophyll "a/b" ratio and total chlorophyll content, and a decrease in registered Iqbal (*Figure 1*). The application of nephtyl acetic acid (NAA) showed a significant increase in both Iqbal and Pahari in both conditions (saline and control). Current surveys show a significant increase in the levels of chlorophyll "a"

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(*Figure 1*) and chlorophyll "b" (*Figure 2*) as the salinity of the two germplasm stages decreases. These results are consistent with the views of Jamil et al. (2012). They believe that the content of chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids in salt-stressed plants are significantly reduced, depending on the NaCl concentration. Salinity has a greater effect on chlorophyll "a" than chlorophyll "b". In Basmati 385, chlorophyll "a" is disturbed by salinity. In the current study, chlorophyll content was significantly reduced under salt stress because chlorophyll content was sensitive to salt experience, and salt stress caused a decrease in chlorophyll content (Ashraf et al., 2004; Elgamaal and Maswada, 2013).

Current studies indicate that chlorophyll content "a" (*Figure 1*), chlorophyll "b" (*Figure 2*), chlorophyll a/b ratio (*Figure 3*) and total chlorophyll content (*Figure 2*) are significant ($P < 0.05$) with all consent. The increase in duration of salt stress during the somatic cell phase. In all treatments, GAs (gibberellin 5 mM) showed the best result with increased chlorophyll content, and GA (gibberellic acid 10 mM) also showed a significant increase with increased chlorophyll content. These results are similar to those of Aldesuquy and Gaber (1993), who reported the use of gibberellic acid to increase plant growth and pigment content. Carotenoids act as unique pigments and activate the defense system, but the effects of SA are not significant under stress-free conditions. Current studies have shown a significant increase in carotenoid content (*Figure 5*) in both materials (*Figure 5*) ($P < 0.05$), increased salinity in the vegetative growth phase and decreased carotenoid content as saline conditions decrease. At the somatic stage, the extreme increase in carotenoid content is a consideration when added Iqbal variety in (*Figure 5*) initially increased but decreased at the minimum NaCl treatment, while the maximum increase in carotenoid content observed when Pahari was added (*Figure 5*). Increasing carotenoid levels under salt stress can express a tolerant genotype because it may be a mechanism to escape stress (*Figure 5*). Carotenoids successfully eliminated singlet oxygen from primary photochemical reactions, so there is a close relationship between leaf carotenoid content and leaf genotype production of tomato genotypes under salt stress (Juan et al., 2005; Sami et al., 2016).

The current results show that under salt stress, most of the treatments increased significantly, while under salt stress, the increase of Iqbal variety increased significantly, while Pahari increased significantly under the maximum and minimum salt stress. Under salinity and control conditions, all treatments showed the lowest proline content in Iqbal, but the greatest increase in proline content under minimal saline conditions (*Figure 7*). NAA foliar application in Iqbal showed its effect in both conditions, whereas, in foliar sprays, no effect on Pahari was observed under both conditions of vegetative phase ($P < 0.05$). These results are consistent (Cha-um et al., 2009; Tůmová et al., 2018). Salt-tolerant plant species may survive salt stress conditions using other defense mechanisms such as ionic homeostasis, anti-oxidation and hormonal systems (Sami et al., 2016; Zhang et al., 2006). Therefore, the evaluation of many parameters in salt-stressed plants will lead to the identification of some valid criteria for the classification of plants for salt tolerance.

Sugars are compatible solutes which accumulate in plant tissues that are exposed to abiotic stresses, such as water deficit, or dangerous salt stress. Addition of solutes especially proline, glycine-betaine, and sugar is a common observation under stress condition (Al-Temimi et al., 2013; Qasim et al., 2003; Tůmová et al., 2018). All treatments showed a significant increase in sugar content under both salinity and control conditions in all two genotypes Iqbal and Pahari, but initially sugar content was

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increased. Present investigation revealed that sugar content (*Figure 6*) significantly (P< 0.05) decreased but increased with the increase in the duration of water stress at vegetative stages in all accessions. At vegetative stage maximum increase in sugar content was observed in Pahari (*Figure 6*). Our result is similar to the work of those (Al-Temimi et al., 2013; Shah and Bano, 2012; Kareem et al., 2017), who suggested that salinity increased sugar contents, protein, proline and superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APOX) activities. Gemes et al. (2008) have described that SA application increased the soluble sugar content of tomato plants exposed to salt stress. Sugars are compatible solutes which accumulate in plant tissues that are exposed to abiotic stresses, such as water deficit, or dangerous salt stress (Morsy et al., 2007; Kareem et al., 2017).

The effect of the current examination showed that all treatments of Iqbal increased significantly under saline conditions, while Pahari was added, some treatments began decrease significantly, under minimal saline conditions and then increased (*Figure 8*). All treatments resulted in a significant increase in sugar content of Iqbal and Pahari under salinity compared to the control. Application of NAA foliar spray in Pahari did not show its effect in both conditions (salinity and control), whereas, in Iqbal, the foliar spray was significant for both conditions of the plant growth period (salinity and control) (P < (0.05). Our results are compared with Jamil et al., 2012. By increasing the salt concentration, a significant decrease in the protein content of rice plants under stress was observed. Khan and Srivastava (1998) and Shanker et al. (2014) also reported a decrease in chlorophyll and protein content with increasing NaCl.

The current analysis showed that all treatments of the Iqbal population were significantly increased under saline conditions, while some treatments initially decreased significantly and then decreased under minimal saline conditions when Pahari was added (*Figure 9*). All treatments resulted in a significant increase in peroxidase (POD) (*Figure 8*), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APOX), levels of Iqbal and Pahari under saline conditions compared to the control. Application of NAA foliar application in Iqbal did not affect both conditions (salinity and control), while foliar application in Pahari had significant effects on both vegetative conditions (salinity and control) (P < 05). According to Garratt et al. (2002) plants containing high concentrations of antioxidants showed considerable resistance to oxidative damage caused by reactive oxygen species.

Conclusion

The study was meant to evaluate the outcome of nephthyl acetic acid on chlorophyll content, protein, proline, sugar, and carotenoid along with certain enzyme activates (POD, SOD, CAT, and APOX). It was concluded that both varieties namely Iqbal and Pahari showed a different level of salt tolerance. All of the antioxidative enzyme activities (POD, SOD, CAT, and APOX), sugar, proline and protein contents reached maximum values in treatment T2, T4, T10, and T12 under saline condition Pahari. On physiological basis variety Pahari was found the most tolerant to the saline condition and responsive to the exogenous supply of NNA while variety Iqbal was more sensitive to salt stress. However, further studies into the best method of application of the bio-regulator to achieve optimum effect should be encouraged and the possiblity to combine treatment of bio-regulators to improve plant productivity should also be considered.

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Recommendations

The salinity stress is a major salt stress limiting factor affecting crop yield, much research has been conducted to develop plants with improved salt tolerance. Salinity stress affects many aspects of plant physiology, making it difficult to conduct comprehensive research. Instead, the plant's response is broken down into a trait that is assumed to be involved in the overall tolerance of the plant to salinity.

Acknowledgements. The authors gratefully acknowledge the support received from Department of Botany, Bacha Khan University, Charsadda, Pakistan. This research work received no specific grant from any donor agency in the public, commercial, or not-for-profit sectors, and these organizations have had no involvement in the analysis and interpretation of data, in the writing of the draft, and in the decision to submit the article for publication.

Conflict of interests. All authors declare to have no conflict of interests.

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LAND COVER MAPPING USING A NOVEL COMBINATION MODEL OF SATELLITE IMAGERIES: CASE STUDY OF A PART OF THE CAMERON HIGHLANDS, PAHANG, MALAYSIA

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(Received 19th Oct 2018; accepted 22nd Dec 2018)

Abstract. Information about land covers is essential for a variety of purposes, such as environmental studies, sustainable development, and regional managements. This study aims to use a novel combination model to generate a land cover map in a part of the Cameron Highlands, Malaysia, where there are different kind of land covers, including tea plantation, florification and forest. Because of the high similarity in land covers of the study area, only through satellite imageries with a high spatial and spectral resolution the land covers can be differentiated. We have combined satellite imageries of Sentinel-1 (S1A, GRD, IW) and Landsat-8 (Operational land imager) for the year 2017 as well as different algorithms of Maximum Likelihood (ML), Minimum Distance (MD), Support Vector Machine (SVM), Spectral Angle Mapper (SAM) and Artificial Neural Network (ANN). The results showed that the combination model is an applicable technique for extracting land covers in areas with high similarities in land covers. The overall accuracy of the confusion matrix and the Kappa Coefficient are 98.1984% and 0.9579, respectively, which indicate that it is a robust model for extracting land covers in areas like the Cameron Highlands. The obtained results can be useful for different purposes, including urban and environmental management, change detection, agriculture and many more purposes.
Keywords: *land use, satellite data, remote sensing, geographic information system, Cameron Highlands*

Introduction

Information about Land Covers (LC) and changes affecting them is essential for many purposes, including urban planning, vegetation and environmental issues (Shahabi et al., 2012; Chen et al., 2015; Gómez et al., 2016). Combination model refers to the ability of using two or more satellite imageries, in which we can improve the spectral resolution to detect the land covers more precisely. In recent years, database and methodology for creating a land cover map have undergone considerable changes (Tehrany et al., 2014; Megahed et al., 2015). Advancements in Remote Sensing (RS) technology and also a demand for using them for different issues have made it a the most used technology for gaining information about earth surface objects since 2 decades ago (Walker and Blaschke, 2008; Belward and Skøien, 2015; Ma et al., 2017).

Land cover has been undergoing considerable changes in either local or global scales (Megahed et al., 2015; Miettinen et al., 2016). Land cover mapping is not an easy issue and needs considerable attention regarding both data collection and model selection (Megahed et al., 2015; Khatami et al., 2016). Selecting data sets should be based on the

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DOI: http://dx.doi.org/10.15666/aeer/1702_18351848

study area and the kinds, shapes and color of the land covers, which help us to select appropriate satellite imagery (Moser et al., 2013; Tehrany et al., 2014; Hasmadi et al., 2017). Since the study area is one of the touristic destinations for both locals and foreigners, so its preservation for Malaysian government is vital.

One of the highlighted issues when generating a land cover map from satellite imagery is the spectral and the spatial resolutions (Griffiths et al., 2013; Poursanidis et al., 2015). However, the precise information about land cover is highly essential. Until about 15 years ago land cover data bases were in form of pre-existing maps, gathered from field survey and previous maps (Griffiths et al., 2013). From two decades ago onwards, RS and Geographical Information System (GIS) have drawn researcher’s attention toward using satellite imagery and machine learning algorithms (Loveland et al., 2000; Tehrany et al., 2014).

Many studies have been conducted with the land cover mapping using different models, techniques and satellite imageries, including global land cover map using Landsat satellite imageries and SVM algorithm (Gong et al., 2013); object-based and pixel-based approaches using SPOT-5 satellite imagery (Tehrany et al., 2014); airborne LiDAR data (Yan et al., 2015); different algorithms of Savitzky-Golay, asymmetric Gaussian, double-logistic, Whittaker smoother and discrete Fourier using MODIS imagery for Great Lake, USA (Shao et al., 2016); supervised and unsupervised algorithms using SPOT-5 satellite imagery (Hasmadi et al., 2017); hyperion data using SVMs classifier (Lamine et al., 2018).

The main objective of the current study was to use a novel combination model for extracting land covers in a part of the Cameron Highlands, Pahang, Malaysia. However, this study dealt with two significant combinations of satellite imageries (Landsat-8 and Sentinel-1), and also combination of different algorithms of ML, MD, SVM, SAM, and ANN by using a Decision Tree (DT) model.

Materials and methods

Description of the study area

The study area is a part of the Cameron Highlands, Pahang, Malaysia between longitudes 101° 20’ 00” E to 101° 27’ 10” E and latitudes 4° 23’ 30” N to 4° 31’ 10” N (*Fig. 1*). This scope with an area of 81.249 km^2 is located approximately in the south-western part of the Cameron Highlands (Tien Bui et al., 2018). The lowest and the highest area of the study area is 912 and 1960 m above the sea level, respectively. However, the average rainfall fluctuates between 1800 mm to 3000 mm annually (Jebur et al., 2015; Tien Bui et al., 2018).

Data collection

Sentinel-1 is a part of the Copernicus Programme conducted by the European Space Agency (ESA). This product consists of the two satellite platforms of 1A and 1B. It has four sensor modes (Strip Map (SM), Interferometric Wide Swath (IW), Wave (WV), and Extra Wide Swath (EW)), and three product types namely, Ground Range Detected (GRD), Single Look Complex (SLC), and Ocean (OCN) (Potin et al., 2012). In this research, we have acquired Landsat-8 and Sentinel-1 satellite imageries for the year 2017 online for free from www.earthexplorer.usgs.gov and www.scihub.copernicus.eu,

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respectively. *Table 1* and *Figure 2* illustrate the technical characteristics of satellite data and geographical position of the study area on the satellite imageries, respectively.

Research methodology

Using Sentinel Application Platform (SNAP) software and Environment for Visualizing Image (ENVI) software, either images radiometrically, spectrally and geometrically were corrected, co-registered and stacked. Using pan-sharpening command in ENVI software, the spatial resolution of Landsat-8 improved to 15 m by its panchromatic band, then via co-registration process the spatial resolution of Landsat-8 (Slave image) enhanced to 10 m by Sentinel-1 as the master image.

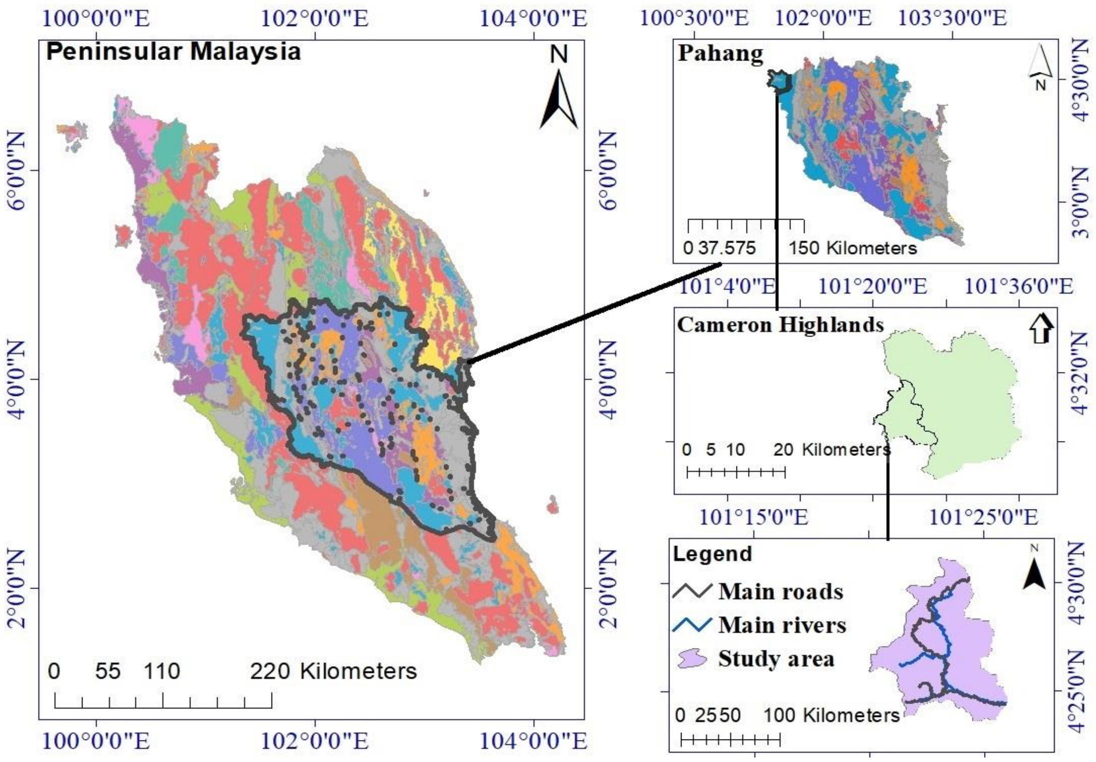

Figure 1. *Geographical position of the study area*

Table 1. *Show the technical attributes of Landsat-8 and Sentinel-1*

No.	Sentinel-1	Landsat-8
Sensor	Synthetic Aperture Radar (SAR)	Operational Land Imager (OLI)
Spatial resolution	10 m	-30 m (Multispectral) -15 m (Panchromatic)
Temporal resolution	12 days	16 days
Spectral resolution	Single band (C)	9 bands
Date	06/09/2017	23/08/2017
More information	- Interferometry Wide swath (IW) - Ground Range Detected (GRD)	

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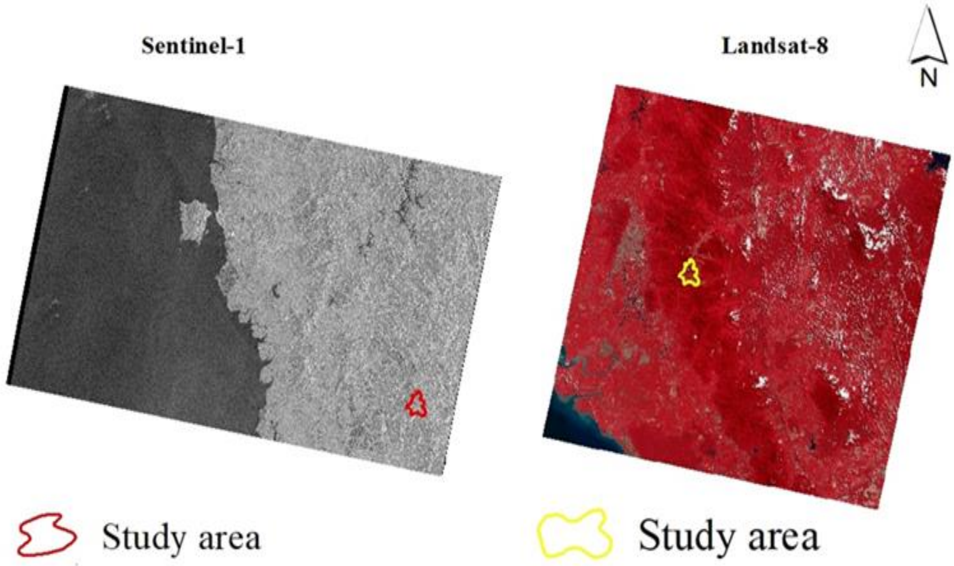

Figure 2. *Shows scope of the study on the satellite data*

After picking up the Region of Interests (ROIs) from co-registered image, different algorithms of maximum likelihood, minimum distance, support vector machine, spectral angle mapper and artificial neural network, were applied for classification. Each algorithm has shown a 1 or 2 land covers better, therefore in order to use the information of all algorithms, the DT method was used. Finally, after the post classification process, to evaluate the overall accuracy some Ground Control Points (GCPs) were extracted using Google Earth (For water body) and field checks for the other land covers (*Fig. 3*). Using ArcGIS the raster map was converted into a vector file and then was dissolved as the number of land covers. Moreover, after calculating the area for each land cover, the attribute table was imported into Microsoft Excel for calculating the total area and creating the final output table.

Description of the used algorithms

Maximum likelihood (ML)

Maximum likelihood model assumes that the statistics for each class are normally distributed and measures the probability that a given-pixel belongs to a certain class (Stamatakis, 2006). Each pixel is assigned to a class, which has the highest probability (Richards and Richards, 1999; Stamatakis, 2006). Once the highest probability has been smaller than the threshold, then the pixel remained unclassified (Richards and Richards, 1999).

Minimum distance (MD)

The minimum distance classification utilize the mean vector of every endmember and measures the Euclidean distance from unknown pixels to the mean vector for each class (Abtan et al., 2017; Kolesár, 2018). Based on the specified standard deviation, number of classes and a distance threshold, all pixels will be classified to the nearest class, however, some pixels may remain unclassified if they do not meet the selected criteria.

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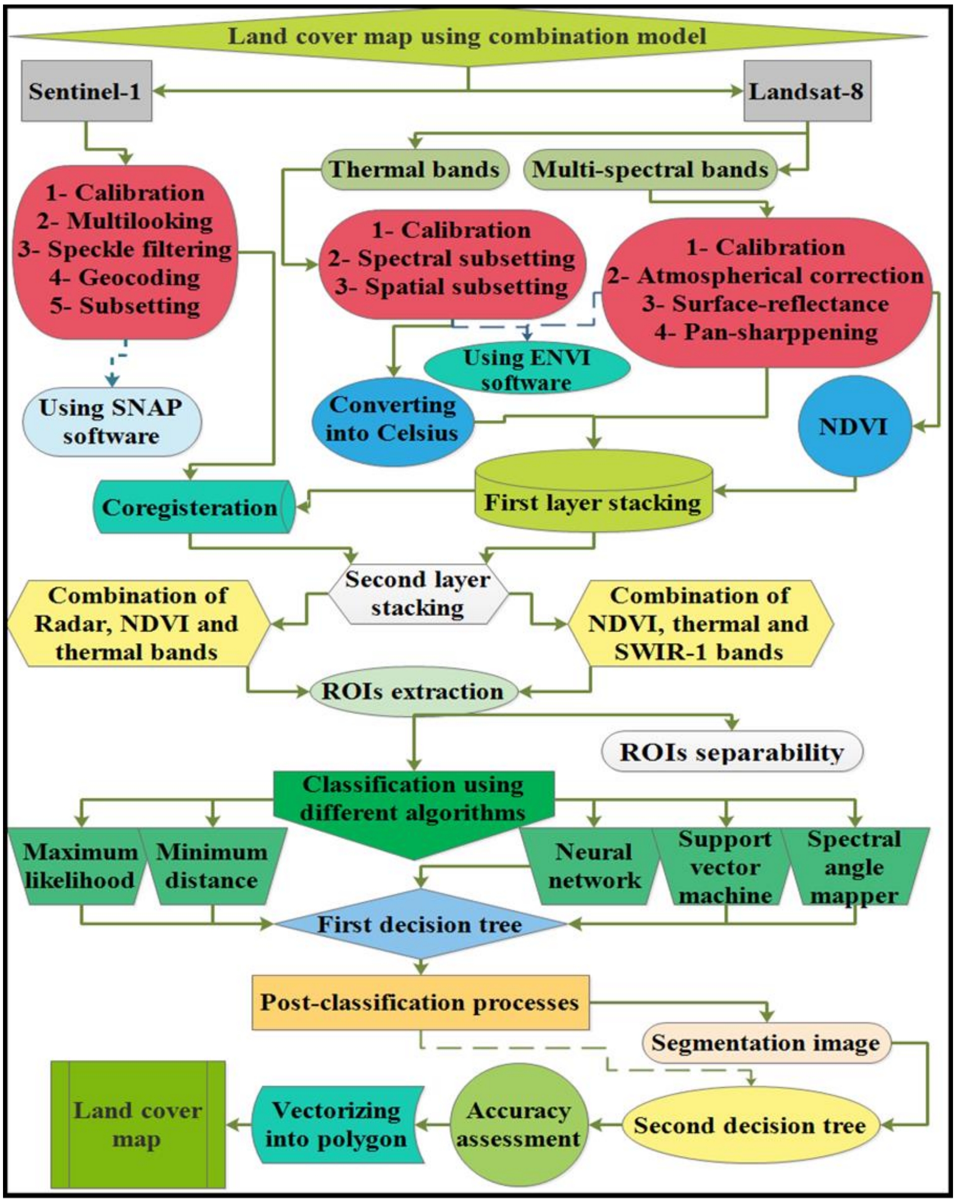

Figure 3. *Methodology of the study*

Neural network (NN)

The neural network technique implies standard back-propagation for a supervised learning (Yoshida and Omatu, 1994). You can choose a number of hidden layers to use and also you can select between hyperbolic or logistic activation functions (Zhang and Foody, 2001). The NN algorithm can be used to perform non-linear classification (Scott et al., 2017). In this model, the error is back-propagated through the network and weight adjustment is made by using a recursive method (Scott et al., 2017; Chakraborty and Roy, 2018).

Support vector machine (SVM)

The SVM is a fast and accurate model for the land cover/ land use studies (Sukawattanavijit et al., 2017). This model provides a good result from noisy and

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complex data (Gu and Sheng, 2017). It tries to separate the created classes with a decision surface, which maximizes the margin between the selected classes (Mohammadi et al., 2018). Despite this fact that the SVM is a binary classifier, it works as a multiclass classifier as well, provided that several binary SVM classifiers are combined (Gu and Sheng, 2017; Mohammadi et al., 2018). The classifier performs training at a lower resolution level, because retraining at each level provides higher accuracy for the resampled image, however, it considers the rule image values to identify those that exceed the probability threshold (Sukawattanavijit et al., 2017; Mohammadi et al., 2018).

Spectral angle mapper (SAM)

The SAM classifier is a physically-based classification that utilize an n-D angle to match pixels to reference spectra (Renza et al., 2017). The model determines the spectral similarity between two different spectra by calculating the angle among the spectra (Kumar et al., 2015). End-member spectra used by SAM model can come from ASCII files, spectral libraries, and you can extract them from Region of Interests (ROIs) (Renza et al., 2017; Yan and Roy, 2018). SAM compares the angles between the end-member spectrum vector and each pixel vector in n-D space (Yan and Roy, 2018). However, smaller angles represent a closer match to the reference spectrum.

Decision tree (DT)

A DT is a type of multistage classifier, which can be applied to a single imagery or a stack of images (Chasmer et al., 2014). It comprises of a series of binary decisions, which are applied to determine the correct group for each pixel (Hua et al., 2017). It can be based on the available characteristics of the dataset (Lu et al., 2014). As an example, you may have an elevation dataset and two multispectral imageries that collected at different times, and any of those images can contribute to decision within the same tree.

Validation

The confusion matrix is important to validate statistical classification (Stehman, 1997). Overall accuracy is calculated by measuring the number of corrected classified pixels then dividing them by the total number of pixels (*Eq. 1*) (Jensen and Lulla, 1987). Kappa coefficient is measured through multiplying the total pixel classes by the sum of the confusion matrix, then subtracting the sum of ground truth pixels in a class times the sum of classified pixels in that class summed over all classes (*Eq. 2*).

$$OA = \frac{1}{N}\sum P_{ii} \quad \text{(Eq.1)}$$

where: OA = total accuracy, N = total number of test pixels, and $\sum P_{ii}$ = total pixels that are correctly classified.

Equation for the Kappa coefficient is written as below:

$$K = (OA - \frac{1}{q})(1 - \frac{1}{q}) \quad \text{(Eq.2)}$$

where: K = kappa coefficient and q = unclassified pixels.

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Results and discussion

One of the most important steps in extracting ROIs is the separability assessment of them (Laurin et al., 2013; Megahed et al., 2015; Levin, 2016). However, the value for measuring the ROIs separability is between 1 and 2, the values less than 1.8 are not acceptable (Laurin et al., 2013). The higher the value the more precise the ROIs are (Levin, 2016). *Table 2* illustrates the least to most separability values of extracted ROIs for this study.

Table 2. *Pair separation of ROIs (least to most)*

Vegetation/florification and township = 1.99	Water body and cleared forest = 1.99
Vegetation/florification and tea plantation = 1.99	Township and tea plantation = 1.99
Road network & vegetation/florification = 1.99	Cleared forest & vegetation/florification = 2
Road network and township = 1.99	Forest and water body = 2
Forest and township = 1.99	Forest and road network = 2
Cleared forest and township = 1.99	Forest and cleared forest = 2
Water body and township = 1.99	Water body and tea plantation = 2
Water body and road network = 1.99	Cleared forest and tea plantation = 2
Forest and vegetation/florification = 1.99	Forest and tea plantation = 2
Water body & vegetation/florification = 1.99	Cleared forest and road network = 2
	Road network and tea plantation = 2

It is worth mentioning that the pair separation of the extracted ROIs from the combination of Sentinel-1 and Landsat-8 satellite imageries showed that this model is a robust way in extracting ROIs for generating land cover maps (*Fig. 4*).

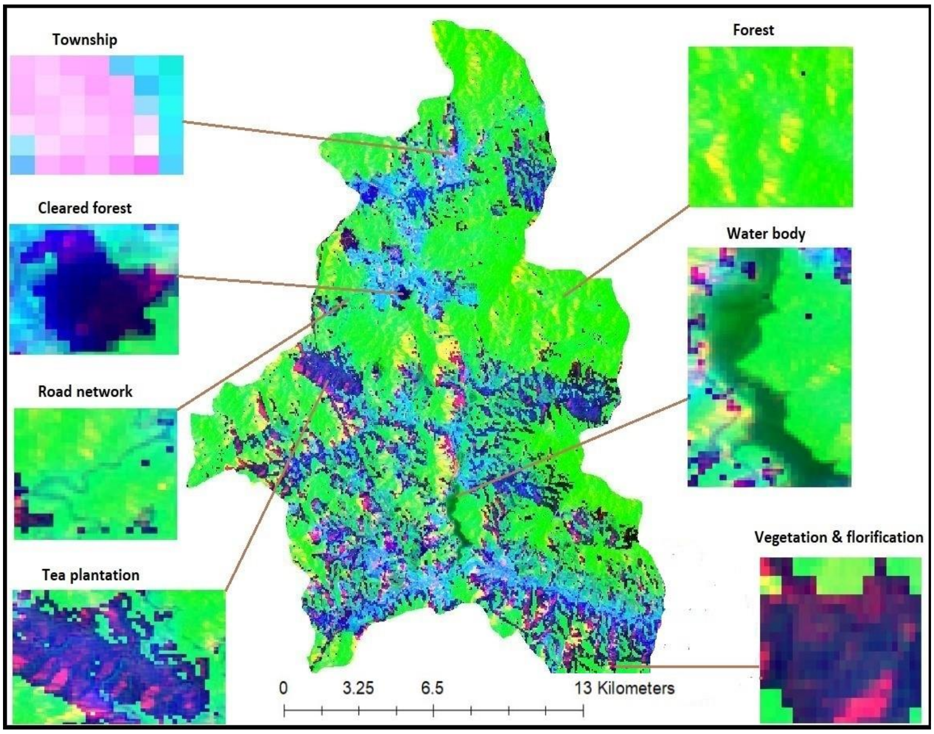

Figure 4. *Different land cover's tones using combination model*

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As a matter of fact, the land covers were extracted based on the ROIs extraction, where every land cover has its own reflection in the satellite imagery. If we do not extract the ROIs for one single land cover with a different reflection, it will remain unclassified. In this study, because of the different reflection in the satellite imagery, the tea plantation fields are separated from the vegetation coverage, spatially and statistically. Overall, a total number of 6 land covers were extracted for the study area (*Table 3* and *Fig. 5*). Using ArcGIS and Microsoft Excel the area of extracted land covers were calculated and finalized. The vegetation and florification fields as well as the forest areas have the biggest area among all of 39.13176 km^2 and 33.90341 km^2, respectively. However, water body has the smallest area of only 0.364556 km^2.

Table 3. *The area of extracted land covers*

No	Name	Area km^2
1	Forest	33.90341
2	Water body	0.364556
3	Tea plantation	3.024364
4	Township	4.139532
5	Vegetation and florification	39.13176
6	Cleared forest	0.436157

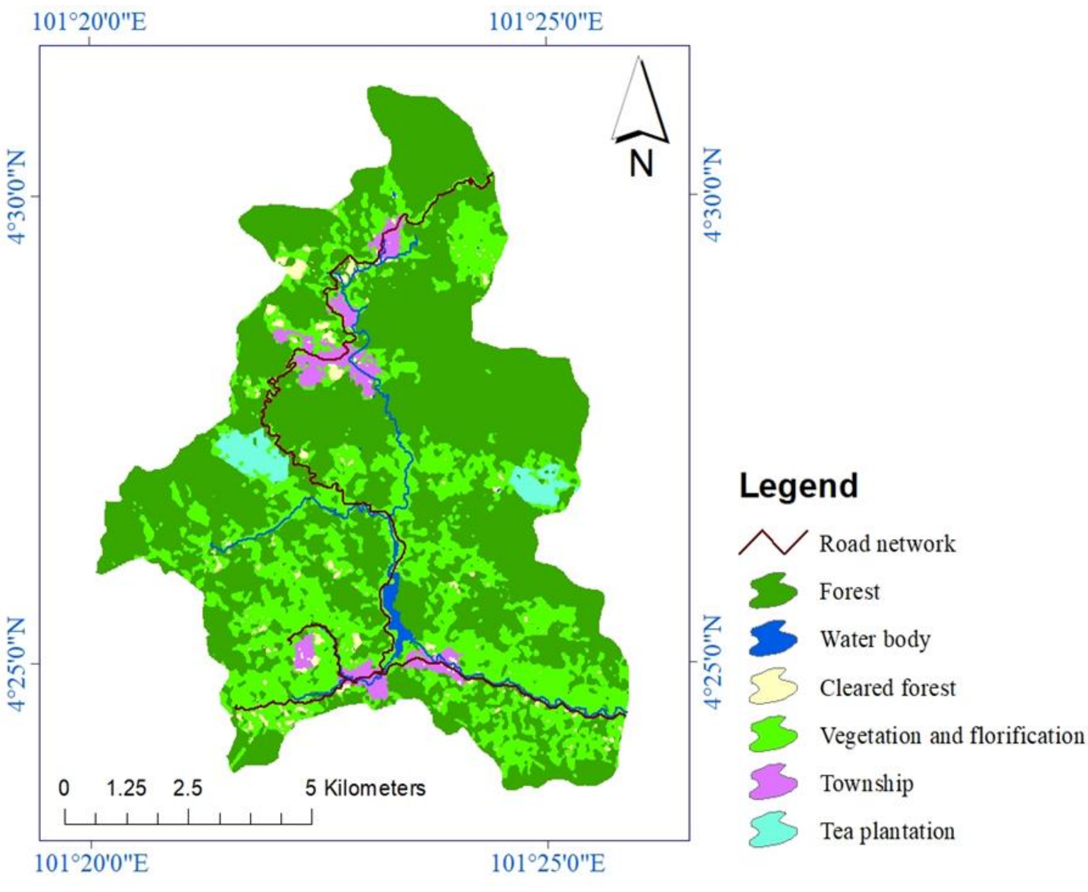

Figure 5. *Land cover map of the study area*

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Validation

Validation of land cover map is important to show the quality of used methods and techniques (Chen et al., 2017; Ma et al., 2017). Using a handheld Geographic Positioning System (GPS), a total number of 20 Ground Control Points (GCPs) were picked up for each land cover (Except the water body, where the Google Earth was employed for extracting GCPs for) by Ayub Mohammadi on Thursday 19 July, 2018 (*Fig. 6*). Because of the hilly situation of the Cameron Highlands, the GCPs were extracted near the main road and the accessible areas only. However, these GCPs were applied for validation purposes using the overall accuracy and Kappa statistic. *Table 4* lists the result of the validation.

Comparison of the combination model with a single model

Using Enhanced Thematic Mapper plus (ETM+) satellite imagery for the date 06/09/2017 and Support Vector Machine (SVM) model, the land covers of the study area were extracted to compare with the combination model. Based on the results there are not too much differences between the two models statistically. The most considerable change has occurred inside the tea plantation area, where in the single model about 0.600 km^2 was added to the tea fields. At the same time, roughly 1.80 km^2 was decreased from the vegetation and florification areas (*Table 5*).

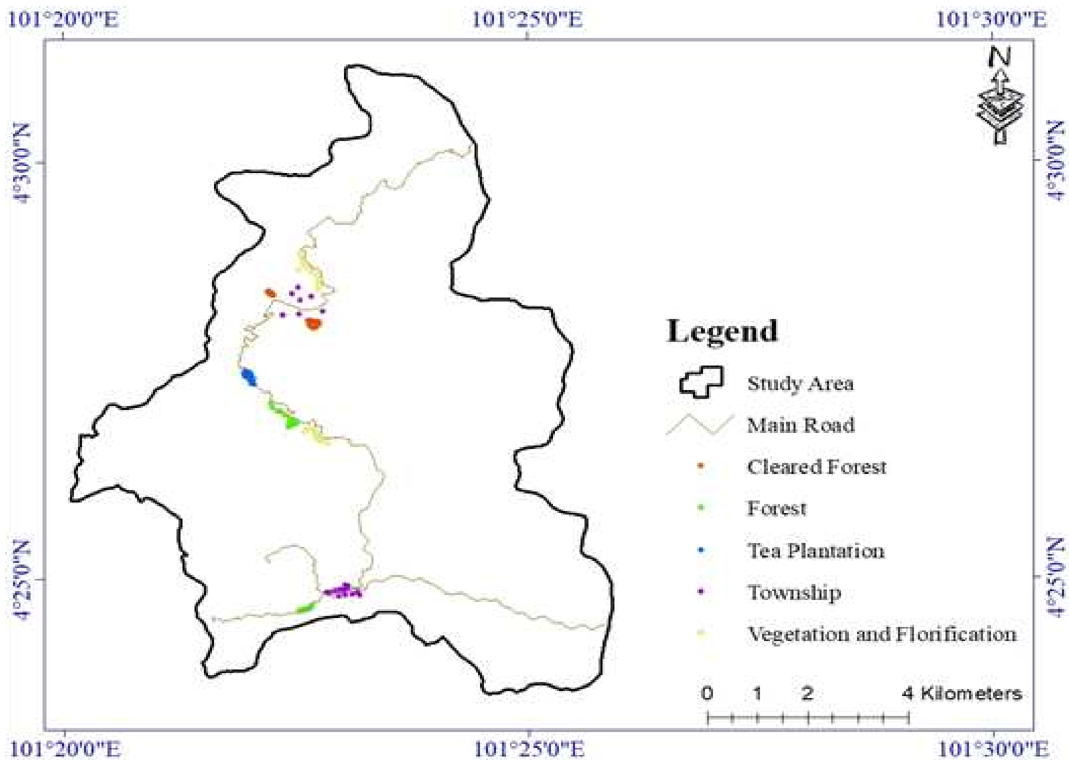

Figure 6. *Shows the geographical positions of the extracted GPS points*

Table 4. *Highlights the confusion matrix for this study*

Overall accuracy	**Kappa coefficient**
98.1984%	0.9579

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***Table 5.** Represents the area for extracted land covers from ETM+ and SVM model*

No.	Name	Area km^2
1	Cleared forest	0.429154
2	Forest	34.1433
3	Tea plantation	3.623
4	Township	4.63918
5	Vegetation and florification	38.0521
6	Water body	0.364556

As a matter of fact, compared to the Google Earth images (*Fig. 7*), the combination model has shown the final map more realistic. In the output map of the single model the land covers are seen more unrealistic (especially for the vegetation and florification fields; *Fig. 8*).

Conclusion

Information about land covers is essential for environmental issues. Combination model refers to using the ability of two or more satellite imagery or even different algorithms, in which we can improve the spectral resolution of them to detect the land covers more precisely. In this study, a novel combination model of satellite imageries of Sentinel-1 and Landsat-8 as well as the combination of different algorithms namely: maximum likelihood, minimum distance, support vector machine, spectral angle mapper, and artificial neural network were used for extracting land covers in a part of the Cameron Highlands, Pahang, Malaysia.

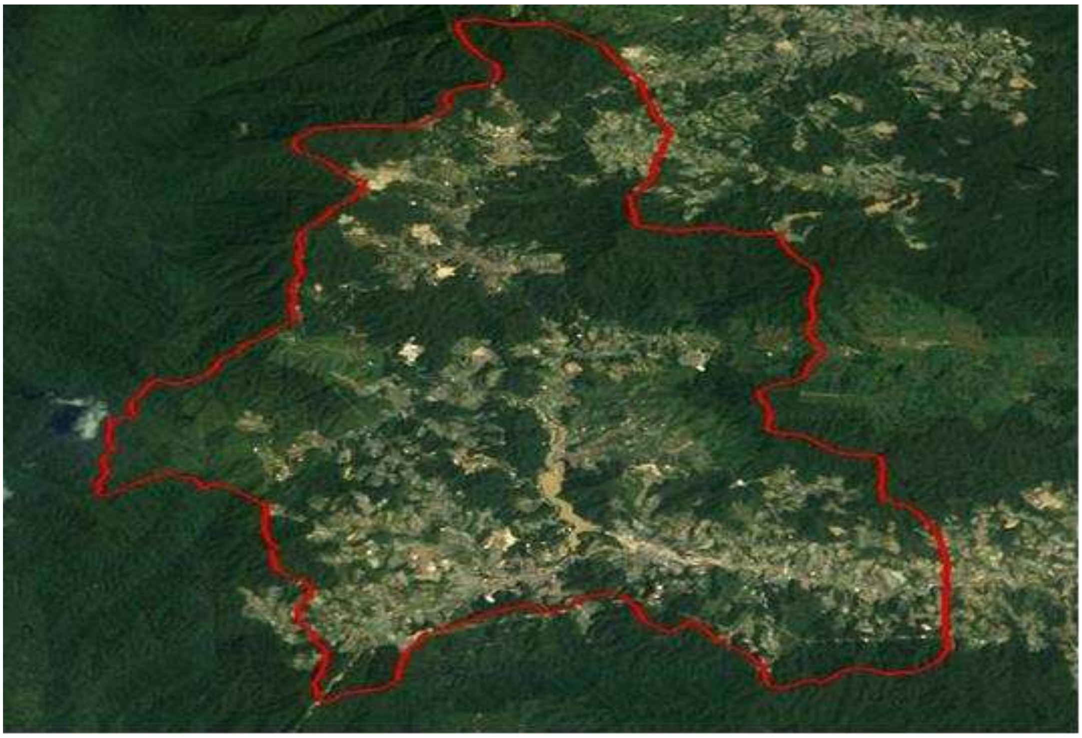

***Figure 7.** Shows the study area on the Google Earth image*

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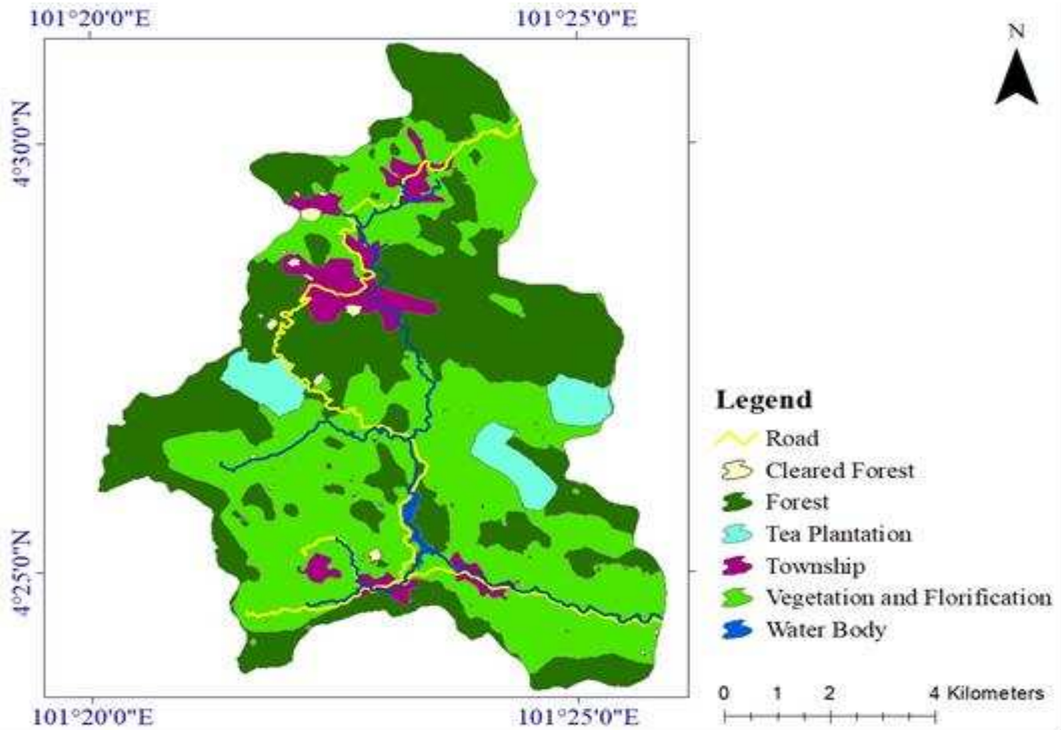

Figure 8. *Illustrations of the extracted land covers using ETM+ and SVM model*

However, a total number of 6 land covers were mapped, which using the Google earth and field survey all land covers were validated. Eventually, by a set of ground control points (GCPs), overall accuracy and Kappa statistic were measured of 98.1984% and 0.9579, respectively. It is noticed that the outputs of this study have promising results for multi-temporal monitoring of land covers in the Cameron Highlands for long term analysis. The obtained results showed that dual Sentinel-1 (S1A, GRD, IW) and Landsat-8 Operational Land Imager (OLI) are suitable satellite imagers for rapid land cover extraction in the geographical situation with high similarity in land covers.

Acknowledgements. The authors wish to express their sincere thanks to Universiti Teknologi Malaysia (UTM) based on Research University Grant (Q.J130000.2527.17H84) for their financial supports.

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AN EVALUATION OF VISUAL LANDSCAPE QUALITY OF COASTAL SETTLEMENTS: A CASE STUDY OF COASTAL AREAS IN THE VAN LAKE BASIN (TURKEY)

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(Received 22nd Oct 2018; accepted 7th Jan 2019)

Abstract. During the planning and design stages of visual resource management and landscape utilization, it is necessary to evaluate the visual quality of landscape elements, determining the requirement for their preservation, restoration, enhancement, and concealment. Turkey has the advantage of offering a wide range of visual resources to its visitors, shaped by its climatic and geographical features. The Van Lake Basin located in the Eastern Anatolia Region is of particular significance as it is one of the rare locations offering numerous visual resources to the visitors. Sustainable management of these resources is very important in terms of their contributions to the economic development of the region and its local population. Coastsal areas provide resources with limited landscape elements, that are hard to be reproduced. These elements play a critical role in recreation and tourism sectors. In respect of landscape architecture, coastal areas represent a combination of land and water, providing the visitors with the opportunity to enjoy the beauty of nature. The coastal areas around Van Lake have a high potential for recreation activities due to their richness of landscape resources. This study, therefore, aims to evaluate the landscape quality of Van Lake Basin by conducting a biophysical investigation of its coastal areas, containing considerable amount of landscape elements. The study determines the areas that should be prioritized for preservation or restoration, and reveals the most suitable precautions for their sustainability, taking into account their nature and identity.
Keywords: *visual resource, landscape quality, recreation activities, coastal area, Van Lake Basin (Turkey)*

Introduction

According to the European Landscape Convention, every country is obliged to identify, plan, preserve, and restore the landscape elements within their borders (Anonymous, 2000). Thus, it is necessary to evaluate the cultural and natural landscape properties of different regions in a country. The determination of human-environment interactions through the method known as “visual impact analysis” offers a solution for the challenge of improving a given environment in terms of its basic social and physical potential, and utilization of modern habitable areas in these areas (Bozhüyük, 2007). The coastal areas have been the most important habitable locations for numerous civilizations during history. The importance attributed to water as a visual landscape element dates back to the gardens of Mesopotamia and Egypt (Burmil et al., 1999). Many studies (Arriaza et al., 2004; Özhancı and Yılmaz, 2011) report that water aesthetically increases the quality of a given landscape.

Coşkun and Kaplan (2001) maintain that visual problems arising from changes in the urban landscapes lead to loss of prestige for the habitat and reduce the natural-cultural value of the location. Consequently, the studies evaluating the impact of such changes on the cultural properties, physical development, and loss of social life become extremely important (Hepcan, 2003). The cities with coastal areas suffer from certain limits due to interventions to the landscape. Generally, shorelines can provide a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

valuable opportunity for recreational activities and tourism sector; the coastal line of the Van Lake and nearby regions are quite rich in this respect.

Review of Literature

Coastal Landscape

Coastal areas are landscape resources with limited landscape elements that are hard to reproduce, and they can provide the opportunity to enjoy the beauty of nature to their visitors as they represent the combination of land and water.

The strips of land that lie beside seas, lakes, and rives are called “coastal areas”. Coasts are some of the most rapidly evolving geographical formations on the face of the earth (Karabey, 1978; Duru, 2001). Coastal areas and shores are important resources that can meet the ever-increasing recreational needs of urban dwellers. While deciding on the physical planning of shorelines, it is important to balance the needs of the society with the natural potential, and to underline the identity and culture of the shoreline while preserving the integration of the settlement (Clark, 1996; Duru, 2001; Kaya, 2006; Gülez et al., 2007; Yazici, 2018). The populations of the coastal regions keep increasing all around the world. As a result of this phenomenon, the utilization of the landscape elements for recreational and tourism demands has been rising. Resultantly, coastal areas suffer from environmental problems that are very difficult -and sometimes outright impossible- to fix (Yılmaz, 2006).

The interest of humans on water resources have not been limited to seas, but have also been directed towards lakes and rivers. Activities like swimming, fishing, bird-watching, kayaking, sailing, windsurfing, and canoeing are conducted not only on seas but on the lakes as well (Soykan, 2000). Van Lake is the largest lake in Turkey, the largest alkaline lake in the world, and represents one of the most significant visual landscape resources of the region. As the lake is located over the migration routes of birds, it also provides for an ornithological and recreational potential as an ecosystem.

Visual Landscape Quality

Evaluation of a visual landscape is an inquiry for the location with the aesthetical criteria and elements determined beforehand in mind (Parsons and Danial, 2000; Palmer, 2003). In that regard, a visual landscape consists of a series of interactions between abiotic, biotic, visual, and cultural elements (Kane, 1981; Bulut and Yılmaz, 2007; Gruehn and Roth, 2008; Yazici, 2018). Visual landscape evaluation is a method utilized while comparing the aesthetical values of study areas, determining priorities during preservation efforts, and identifying the changes in the landscape character. During the planning and design stages of visual resource management and landscape utilization, it is necessary to evaluate the quality of visual landscape elements of the study area to prioritize their preservation, restoration, enhancement, and concealment (Kane, 1981; Palmer and Hoffman, 2001).

There are three approaches used in the determination of the visual quality of a given piece of landscape. These are the physical (topographical aesthetics), psychological, and psychophysical approaches (Daniel and Boster, 1976; Lothian, 1999; Daniel, 2001; Kaptanoğlu, 2006; Çakcı, 2007). In the perception-based evaluation method, a group of observers are asked to quantify the quality of the landscape they are observing or seeing through pictures (Daniel, 2001). Making use of photographs is a valid approach when

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

determining the visual status of a landscape (Hall, 2001; Clay and Smidt, 2004; Çakcı, 2007; Dupont et al., 2017; Palmer, 2019).

As the visual quality of the landscape is naturally an extension and result of its physical properties, the evaluation in this method is based on the objective or physical approaches. According to Palmer (2019), bio-physical influences of a landscape should always be considered in the evaluations. The morphological-aesthetic approach where the landscape qualities and elements that are believed to have influence over the beauty of the sight -and have been analyzed in terms of their contents- shall be conducted by experts only. Since the evaluations of a single expert might be questionable, it is reasonable to conduct evaluations with at least five experts (Wherrett, 1996; Çakcı, 2007; SNH, 2013; Palmer, 2019). According to literature (BCMF, 1997; Clay and Daniel, 2000; Wu et al., 2006; Arriaza et al., 2004; Sevenant and Antrop, 2009; Uzun and Müderrisoğlu, 2011; Jahany et al., 2012; SNH, 2013; Huang, 2014; BLM, 2016) the biophysical parameters are the angle of visibility, the configuration and silhouette of visible borders, the silhouette of the land, the expanse, inclination, impression, land formations, surface relief, land cover, neighboring vistas, cultural properties, vegetation, richness in terms of species, and the presence of water.

Various studies have been conducted on the evaluation of visual qualities of landscapes. Smardon (1979) have counted color, texture, line, shape, and proportion properties with influence levels of low, medium, high, and irrelevant; and further quantized each element and created a numerical system. He grouped landscape elements as vegetation, water surface, and structure, and evaluated them numerically.

Ayad (2005) has researched the potential of GIS and distant perception methods in a coastal region in Egypt. He transferred the landscape properties of the study area into a GIS environment with numerical values and determined the utilization of land, distance to coast strip, cover, and topographical variations in the digital environment.

Huang (2014) has analyzed the landscape elements of Washtenaw County, Michigan, and evaluated the visual quality. In his study, Huang presented the participants with 50 photographs of various vistas from the study area, and asked them to sort the locations out based on their preferences and intuitions. He also conducted map-making analyses and visual quality evaluations. He found out that participants were inclined towards the vistas with natural properties.

Naspetti et al. (2016) used a selection of landscape images containing photovoltaic elements and conducted a Q-sort study. 34 participants, some of which were landscape and lighting experts, were presented with 54 landscape images. They were asked to sort them in three different categories to determine the best way of integrating photovoltaic systems into the urban and rural landscapes.

Keleş et al (2018) studied Edirne / Turkey, representing one of the most interesting cities of the region in terms of tangible and intangible cultural landscape elements. They presented a questionnaire and photographs of the landscape elements to a group of experts and asked them to evaluate the structures, textures, and characters. The results of the study were used in rational planning and preservation of the cultural resources by various authorities.

The Van Lake Basin, rich in physical geographical formations, contains various types of geomorphic elements. It also contains ancient settlements developed by numerous civilizations in its long history. Thus, the area contains various population and settlement types that are quite different from each other. As it is a closed basin, the area contains idiosyncratic elements and properties each of which should be inspected

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

from different perspectives. This is a novel study since the evaluation of visual landscape qualities of settlements in the coastal lines of the Van Lake has never been carried out before. In the present study, significant settlement areas in the coastal lines of the cities of Van and Bitlis are inspected in terms of their visual landscape qualities using Q-Sort analysis. Q- sort analysis is a relatively new tool not only as approach but particularly following the quite recent rediscovery of its usefulness in those fields where psychometric knowledge of individuals have thorough implications (Kramer et al., 2003). Various biophysical parameters (general interest, naturalness, the presence of water, vegetation, topographic variation, natural and cultural elements, and color variety) are also investigated. The aim of the study is to present a reference point for future studies and efforts to remove the negative impact of various factors on the natural and visual landscape elements and to act as a guide for visual resource management, decision making/strategy, and restoration studies.

Materials and Methods

Material

The coastal strips of significant settlements on the shorelines of Van Lake Basin form the material of the study (*Figure 1*). These settlements belong to district of Erciş, Adilcevaz, Ahlat, Tatvan, Gevaş and Edremit, the most significant district in the region.

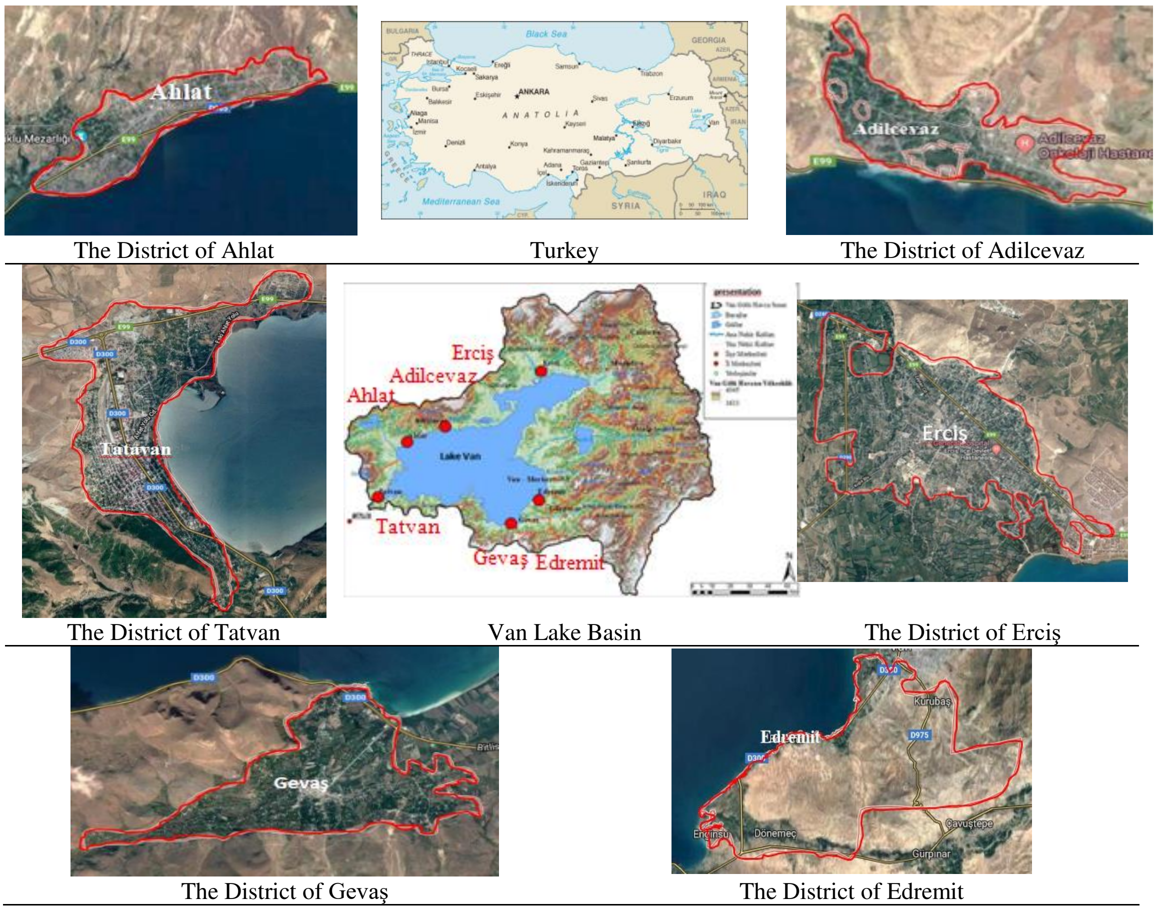

Figure 1. *Satellite images of settlements on the coastal regions of the Van Lake Basin*

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The photographs of the coastal locations were taken between 09.00 and 17.00 in summer months using a digital camera. Areas open to the public were preferred, taking into consideration the active recreational use of the locations by the community. A total of 186 photographs were taken in 6 different coastal locations, 24 of them selected (4 from each location) for the final inquiry. The experts were then asked to specify a preference ratio for each location (*Table 1*), and each individual picture.

Van Lake is believed to have formed as a result of water gathering inside a crater formed by the explosion of the Nemrut volcanic mountain. The surface area of the lake is 3,713 km^2, and the lake contains numerous bays. It represents an aquatic ecosystem, different from both the fresh and saltwater ecosystems. With an elevation of 1646 meters, it is surrounded by volcanic mountain masses to the west and north, mountain ranges with steep slopes to the south, and a relatively lower morphological field towards east and northeast, creating a closed basin. The shores are grainy and pebbly, and contain numerous rocky or cliffy areas, presenting various areas of interest in terms of views and vistas.

The district of Erciş is approximately 100 km to the Van city center and is built over the Erciş plains. The district has an elevation of 1690 meters, influenced positively by the well-watered terrain, large number of streams and watercourses, and its coastal position. The area has numerous natural catchment areas. Many landscape design studies were conducted on the shorelines by municipalities in 2017 to provide recreational opportunities for the public (Anonymous, 2018a).

The district of Adilcevaz is positioned inside a valley surrounded by the skirts of the Süphan Mountain, positioned 71 km distance to the city center of Bitlis towards the northwest of the lake with an elevation of 1690 meters. Most of the district lacks a soil ground cover; thus, it is weak in vegetation. Local authorities have conducted various coastal landscape studies in order to increase the recreational use of the landscapes (Anonymous, 2018b).

The district of Ahlat is within the borders of Bitlis, with an elevation of 1545 meters, 44 km north of Tatvan district. The district is built over a plateau between Süphan and Nemrut Mountains and East Anatolia Region. Ahlat is located in the northwest shores of the Van Lake, and the coastal landscapes are actively used by the community, especially during summer (Anonymous, 2018b).

The district of Tatvan is also within the borders of Bitlis, approximately 25 km to the city center. With an elevation of 1671 meters and its position on a high elevation location towards the southwest of Van Lake, most of the district area is covered with mountains. Tatvan also has a historical importance as it is positioned over the Silk Road (Anonymous, 2018b).

The district of Gevaş is 35 km distant to the Van city center, built at an elevation of 1750 meters on the shorelines of the Van Lake. The district is quite rich in historical artworks and natural vistas. Gevaş is the favorite location of many tourists and local populations, particularly during the summer. Tatvan is located in the skirts of the Artos Mountain, the highest of the Kavuşahap Mountain range and the extension of the Southeastern Toros Mountains (Anonymous, 2018a).

Finally, the district of Edremit is only 15 km to the city center of Van within elevation of 1846 meters. With a south-southeastern orientation, the district is quite rich in natural and cultural landscape elements. Edremit has always been popular due to its beautiful shoreline, having a high value in terms of visual landscape (Anonymous, 2018a).

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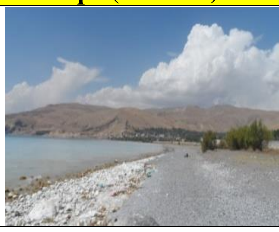
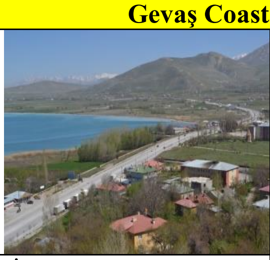
Table 1. *Selected photographs of coastal landscapes, and the preference ratios by experts*

Erciş Coastal Landscape (E.C.L.)			
			
İmage 1 E.C.L. %7,2	İmage 2 E.C.L. %50,5	İmage 3 E.C.L. 3 %34,2	İmage 4 E.C.L.%8,1
Adilcevaz Coastal Landscape (Ad.C.L.)			
			
İmage 1 Ad.C.L. %49,7	İmage 2Ad.C.L.%26,3	İmage 3 Ad.C.L.%12,1	İmage 4Ad.C.L. %11,9
Ahlat Coastal Landscape (A.C.L.)			
			
İmage 1 A.C.L. % 26,7	İmage 2A.C.L. %9,3	İmage3 A.C.L.% 56,6	İmage 4 A.C.L. % 7,4
Tatvan Coastal Landscape			
			
İmage 1 T.C.L.%12,4	İmage2 T.C.L. %20.2	İmage 3T.C.L. %58.3	İmage 4T.C.L. %9.1
Gevaş Coastal Landscape			
			
İmage 1G.C.L. %20,2	İmage 2G.C.L. %10,3	İmage 3 G.C.L. %60,7	İmage 4 G.C.L. % 8,8
Edremit Coastal Landscape			
			
İmage 1Ed.C.L. %59,8	İmage 2Ed.C.L. %7,3	İmage 3Ed.C.L.%11,2	İmage 4Ed.C.L. %19,7

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The touristic data regarding the provinces of Bitlis and Van where these settlements are located are as follows: According to Bitlis Province Directorate of Culture and Tourism 2018 data, the native and foreign tourist count that visited touristic facilities of Bitlis in 2017 is 179,639, of which 176,073 were natives and 3,566 were foreigners. According to Van Province Directorate of Culture and Tourism 2018 data, the native and foreign tourist count that visited touristic facilities of Bitlis in 2017 is 1,070.260, of which 584,040 were natives and 486,220 were foreigners.

Method

The method chosen for the evaluation of the visual landscape quality was a combination of methods in the literature by BCMF (1997), Wu et al. (2006), Çakcı (2007), Uzun and Müderrisoğlu (2011), Jahany et al. (2012), SNH (2013), BLM (2016), and Huang (2014), developed by Aşur (2017) (*Table 2*). The main criteria evaluated by the experts by looking at the photographs were their personal opinion of the general view, naturalness, presence (and clarity) of water, vegetation, topographical variation, man-made constructs with a positive contribution, and color variety.

Table 2. *The evaluation chart according to visual landscape biophysical parameters that developed by Aşur (2017)*

Landscape qualifications	Points				
	+2(Very beautiful n1)	+1(Beautiful n2)	0(Ordinary n3)	-1(Ugly n4)	-2(Very Ugly n5)
General view	I liked it very much	I like	normal	I do not like	I did not like at all
Degree of naturalness	Natural elements are very dominant	Natural elements less dominant	Natural elements half dominant	Natural elements are not dominant	Non-natural elements dominant
Vegetation presence	%80-100	%60-80	%40-60	%20-40	%0-20
Water view	Very dominant and clear water surface	Water surface is very visible but non-clear water	Water surface obvious	Water surface is obvious and dirty	appearance of the water is very bad, dirty
Topographic diversity	Very clear	Clear	Little clear	Not clear	Not at all clear
Presence of human-made positive elements / historical buildings and typical houses	%80-100	%60-80	%40-60	%20-40	%0-20
Variety of colors	Color variety is very and compatible	Color diversity less and compatible	Color variety less	Very little variety of colors	No variety of colors

In this study, because of the biophysical evaluation of visual landscape quality, expert opinions were consulted. Biophysical properties of the visual landscapes were quantized numerically (very beautiful as +2, beautiful as +1, ordinary as 0, ugly as -1, and very ugly as -2). 35 experts, all of whom had previously seen the locations in person, were presented with the 24 photographs from the 6 locations (4 from each location) with an online questionnaire. All the evaluations were recorded. Each of the 4 pictures for a given location was given a preference ratio by the experts. The value of a given piece of the landscape was calculated based on the scores given to them by the experts. A high score indicates a potentially desirable view and a high preference rate

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by the community. The results are listed in *Tables 3 to 8*. The Likert-scale scores explained above were then Q-sorted, revealing the meaningfully preferred locations over the others. In order to compute achieved points for each photo, the formula below is used (Kramer et al., 2003; Golchin et al., 2012).

$$N = \sum_{i=1}^{5} n_i (3 - i) \qquad \text{(Eq.1)}$$

N = Total points for each photo.
n1 = Number of users which choose very beautiful quality photo.
n2 = Number of users which choose beautiful quality photo.
n3 = Number of users which choose normal quality photo.
n4 = Number of users which choose ugly quality photo.
n5 = Number of users which choose very ugly quality photo.

Results and Discussion

As can be seen from the results (*Tables 3 to 8*), the image 2 E.C.L. was the most liked location in the Erciş coast (50.5% ratio), while the 1 Ad.C.L from the Adilcevaz coast (49.7%), the image 3 A.C.L from the Ahlat (56.6%), the image 3 T.C.L from the Tatvan (58.3%), image 3 G.C.L from the Gevaş coast (60.7%), and image 1 Ed.C.L. from the Edremit (59.8%) were the ones for every location earning the highest ratios. One common theme detected in these selections was the inclination towards natural beauties of shores, while the artificial landscape elements were mostly avoided. The assessment of study outcomes according to the 7 parameters of visual landscape quality; Q-Sort analysis of the expert opinions revealed that the coastal of the Gevaş district was the most preferred one amongst all the locations in terms of the beauty of the general view. Edremit and Tatvan coastal area follow closely.

Table 3. *The evaluation results of the Erciş's coastal area*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	3	10	14	7	1	**7**	0,96406
Veg. presence	1	12	13	7	2	**3**	0,95090
Water view	5	13	14	2	1	**19**	0,91853
Top. div.	4	9	16	5	1	**10**	0,93144
P. of hum. m.pos. elem.	2	6	16	9	2	**-3**	0,95090
Var. of colors	4	6	18	5	2	**5**	1,00419

Table 4. *The evaluation results of the Adilcevaz's coastal area*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	1	12	13	7	2	**3**	0,95090
Veg. presence	2	7	15	6	5	**-5**	1,08852
Water view	4	9	17	4	1	**11**	0,98731
Top. div.	2	9	16	7	1	**4**	0,90005
P. of hum. m.pos. elem.	0	8	18	6	3	**-4**	0,86675
Var. of colors	2	5	19	6	3	**-3**	0,95090

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

Table 5. *The evaluation results of the Ahlat's coastal area*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	2	9	16	7	1	**4**	0,90005
Veg. presence	0	8	18	6	3	**-4**	0,86675
Water view	4	15	10	5	1	**16**	0,98048
Top. div.	3	10	14	7	1	**7**	0,96406
P. of hum. m.pos. elem.	2	5	18	8	2	**-3**	0,90090
Var. of colors	3	8	18	5	1	**7**	0,90098

Table 6. *The evaluation results of the Tatvan's coastal area*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	4	9	14	7	1	**8**	1,00252
Veg. presence	3	8	18	5	1	**7**	0,90098
Water view	9	11	13	1	1	**26**	0,90005
Top. div.	4	9	17	4	1	**11**	0,98731
P. of hum. m.pos. elem.	2	6	16	9	2	**-3**	0,95090
Var. of colors	6	10	15	4	0	**18**	0,90563

Table 7. *The evaluation results of the Gevaş's coastal area according to visual landscape biophysical parameters*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	6	10	15	4	0	**18**	0,90563
Veg. presence	10	11	11	2	1	**27**	0,98144
Water view	11	16	6	2	0	**36**	0,85700
Top. div.	9	11	12	3	0	**26**	0,95001
P. of hum. m.pos. elem.	4	7	13	7	4	**0**	1,16316
Var. of colors	6	12	14	2	1	**20**	0,94824

Table 8. *The evaluation results of the Edremit's coastal area*

Landscape qualifications	+2 (n1)	+1 (n2)	0 (n3)	-2 (n4)	-1 (n5)	Photo score N	Standart dev
Deg. of nat.	4	15	10	5	1	**16**	0,98048
Veg. presence	6	12	14	2	1	**20**	0,94824
Water view	7	17	9	2	0	**29**	0,82197
Top. div.	4	15	10	5	1	**16**	0,98048
P. of hum. m.pos. elem.	0	8	18	6	3	**-4**	0,86675
Var. of colors	10	11	11	3	0	**28**	0,96406

As can be observed from *Table 9*, half of the 6 coastal locations have received higher view scores compared to the other 3. Adilcevaz shores were given the lowest score.

The standard deviation for the general view property of the expert evaluations of visual landscape value of Erciş, Adilcevaz, Ahlat, Tatvan, Gevaş and Edremit coastal areas are given in *Figures 2, 3, and 4*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

Table 9. *The results of the evaluation of visual landscape elements in terms of beauty of the general view*

Photo name	+2 (Very Beautiful n1)	+1(Beautiful n2)	0(Ordinary n3)	-1(Ugly n4)	-2(Very Ugly n5)	Photo score N
E.C.L.	4	9	17	4	1	**11**
Ad.C.L.	4	10	14	6	1	**10**
A.C.L.	7	17	9	2	0	**29**
T.C.L.	11	15	8	1	0	**36**
G.C.L.	17	13	4	0	1	**45**
Ed.C.L.	12	18	3	1	1	**39**

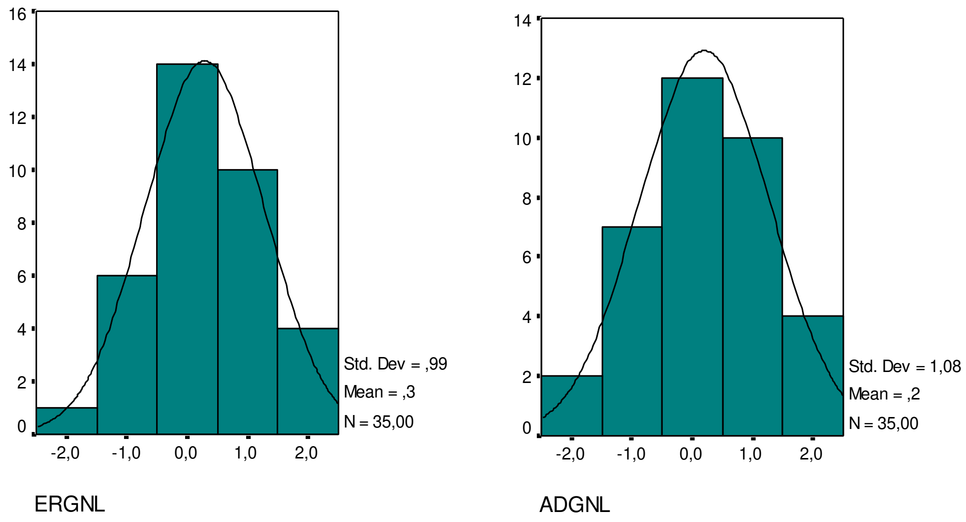

Figure 2. *Standard deviation values of general opinion evaluation by experts of visual landscape value of Erciş and Adilcevaz Coastal areas*

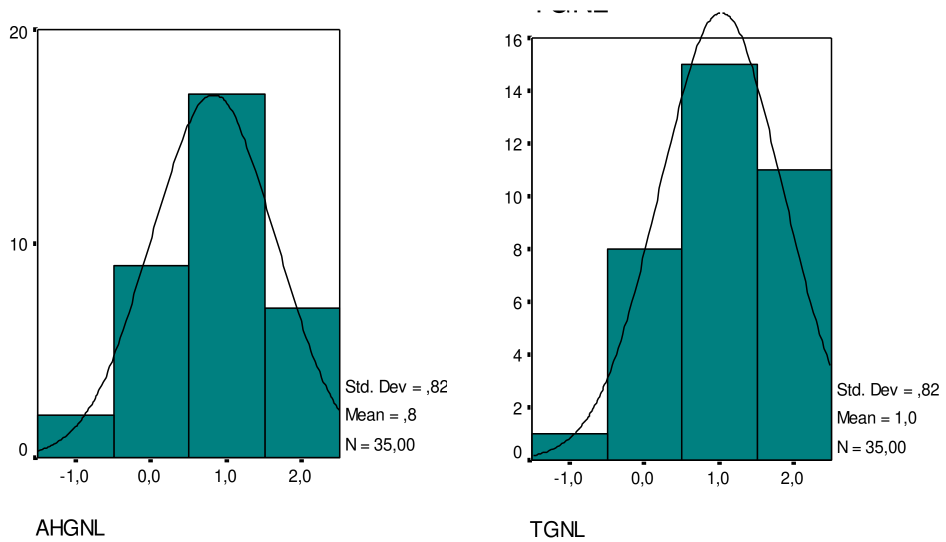

Figure 3. *Standard deviation values of general opinion evaluation by experts of visual landscape value of Ahlat and Tatvan Coastal areas*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

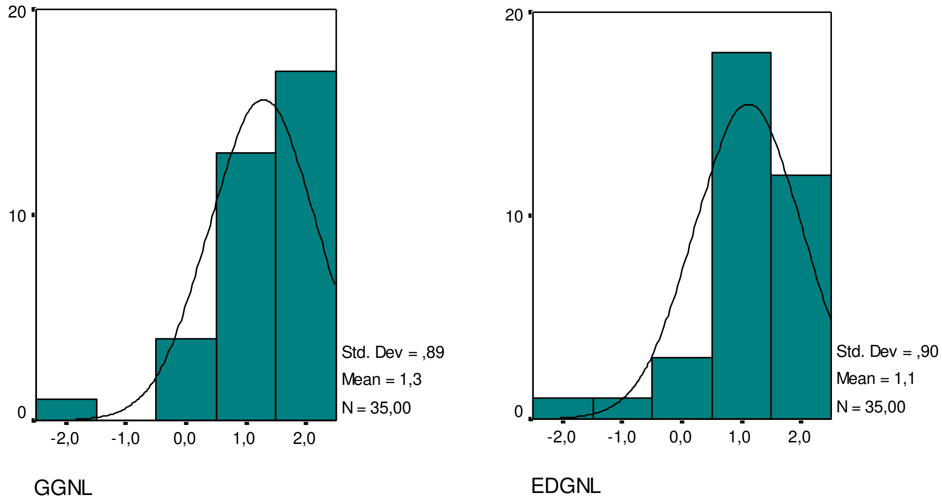

Figure 4. *Standard deviation values of general opinion evaluation by experts of visual landscape value of Gevaş and Edremit Coastal areas*

When the evaluation results of the Erciş's coastal area is investigated (the one with high level of flat topography) according to the visual landscape biophysical parameters it is found that: the lack of man-made elements contribute to the attraction of the locations in the coastal areas of the Erciş based on inspection of positive artificial structures parameter for the landscape elements. The results are in accordance with the actual vegetation levels of the districts.

Coastal area of the Erciş has received low scores in terms of protection of naturalness. In terms of water presence, the district of Erciş achieved the highest score for its waterscape beauty, as can be seen in *Table 3*.

As can be observed from *Table 4*, Adilcevaz's coastal area received the lowest score in terms of vegetation presence, presence of human-made positive elements, and variety of colors. The results also parallel the vegetation presence of the district.

When the assessment results of the Ahlat's coastal area is analysed against the biophysical parameters of the visual Ahlat's coastal area received the lowest score in terms of vegetation presence and presence of human-made positive elements. On the other hand, as can be seen from *Table 5*, Ahlat's coastal area received the highest score in terms of water view.

When the assessment results of the Edermi's coastal area according to visual landscape biophysical parameters as can be observed from *Table 6*, the interpretation of this result indicates that positive artificial structures parameter for the landscape elements reveals the lack of man-made elements contributing to the attraction of the locations in the coastal areas of the Tatvan. While as can be seen from *Table 6*, Tatvan's coastal area received the highest score in terms of water view and variety of colors.

When the biophysical property of naturalness parameter is inspected, the Q-sort analysis supports the fact that Gevaş's coastal area has a lower amount of settlements. When the parameter of vegetation presence is inspected, it can be realized that although being quite small in size, the coastal area of the Gevaş district were given the highest scores. This is probably due to the presence of the only forest in the region. The outcomes of the Q-sort analysis also parallel the vegetation presence of the district. In terms of water presence, the district of Gevaş achieved the highest score for its

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

waterscape beauty, as can be seen in *Table 7.* Since this coastal area is farther than the city center compared to other locations’ shorelines, it has a clearer water overall, also reflected in the results of the questionnaire.

When the assessment results of the Edermi’s coastal area according to visual landscape biophysical parameters is inspected, it is found that: Edremit’s coastal area received a high score in terms of water view and variety of colors. Being called the “Green Edremit” in the region, Edremit is rich in terms of vegetation presence. The results are in accordance with the actual vegetation levels of the districts (*Table 8*).

Towards the north and west of the Van Lake lie the volcanic mountain masses, while its southern side is dotted with high-slope mountain ranges; the east and northeast are relatively lower in terms of morphological formations. This configuration has also influenced the results of the questionnaire. When the topographical variety parameter of the study area is inspected, it can be understood that the presence of formations of various heights contributing to the vistas was reflected into the results of the questionnaire as higher scores. Results of the analysis reveal that the Gevaş coastal area received the first position in terms of morphological variation, while Edremit coasts were placed second with a close score.

These results show that the balance between natural and man-made elements was lost for all the locations. Since the region lacks the understanding of sustainable landscaping, suitable for the nature and identity of its elements, the problematic urbanization of the coastals seems inevitable. This is also reflected in the result of the questionnaire.

Displays the results for the color variation biophysical parameter where Edremit coastal landscape earns the highest score. The interpretation of this result indicates that while the coastal of Gevaş district had indeed preserved its naturalness, it is somewhat lacking in color variety, while the coastal landscaping conducted by local authorities in the Edremit district had indeed increased the color variety and attractiveness of the location. Adilcevaz coastal landscaping, on the other hand, was given the lowest score in terms of color variety.

Conclusion and Suggestions

As a result of the ongoing population increase, it becomes inevitable that humankind disturbs the nature more and more every day. Presenting a source for beautiful visual resources, coastal areas are being repurposed through landscaping so they can provide for the physical and recreational/touristic requirements of the population. When carried out improperly, such landscaping results in serious environmental problems in these coastal regions. Evaluation of these areas in terms of their visual landscaping impact enables the decision makers to determine the priority for preservation and restoration studies, offering solutions for improving and preserving modern settlement areas in terms of sustainable landscaping.

In the present study, the coastal areas of 6 significant locations commonly visited by the local population in the Van Lake Basin, namely Erciş, Adilcevaz, Ahlat, Tatvan, Gevaş, and Edremit districts, are evaluated in terms of their visual landscape qualities. These locations are very popular recreational areas for visitors, especially during summer months and weekends. Considering the fact that visual landscape quality has a strong influence on the recreational attraction, the landscaping of these locations are conducted, heeding some of the most important criteria. The outcomes of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18491864

evaluations revealed that the highest scores were achieved by Gevaş, Edremit, and Tatvan districts' coastal regions, respectively (*Figure 5*). The general view of the coastal areas, their naturalness, and the presence of vegetation were determined to be the most influential parameters.

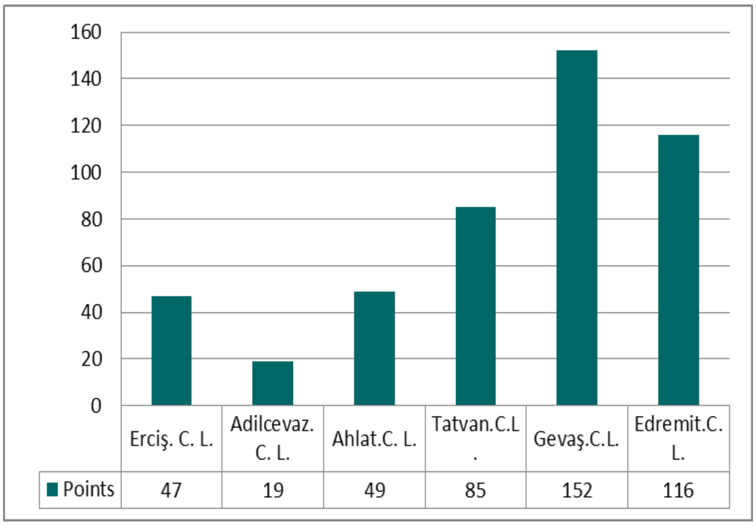

Figure 5. *Results of the visual landscape quality evaluation of the coastal area*

The analysis conducted in this study reveals that the highest score in terms of general attractiveness was achieved by the coastal area of the Gevaş district, followed closely by the coastal strips of Edremit district. Among all the locations under study, the Gevaş coastal area maintained its naturalness to the highest level; thus, it received the best score in terms of biophysical parameters of the visual landscape. While these two locations achieved high scores due to their vegetation presence, dominating water presence and beauty, and topographical variety, they received no scores for the man-made positive landscape elements. This shows that the landscaping conducted in the region by the authorities was not approved by the experts. Similar results were also achieved for Ahlat, Erciş, Tatvan and particularly for Adilcevaz coastal areas, as the landscape designs applied in these locations are unrelated to the identities of the locations, looking like ordinary parks.

The lowest scores for the visual landscape evaluation were obtained by the coastal areas of Adilcevaz district, presenting an urgency and priority for restoration and improvement studies for the location. To achieve this, the weak vegetation presence in the coastal areas of Adilcevaz could be initially enhanced, using ample amounts of plants appropriate for the ecological conditions of the region. Meanwhile, prevention of the waste being dumped into the lake should be sufficient to improve the visual quality of the water itself. All of the coastal areas included in the study suffered from the lack of positive contribution of man-made landscape elements, receiving low scores for this particular parameter. Considering this, future artificial landscape elements should be designed considering the history of the locations. A strategy should also be employed to choose appropriate construction materials, accessories, and local plants.

The coastal areas of Gevaş and Edremit districts were highly praised by the experts in the study; thus, they can be the focus of future preservation efforts and utilization of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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sustainable landscape strategies. These strategies should be developed based on most influential biophysical parameters discussed in this study.

The coastal areas of the Van Lake are subject to the Shore Protection Law (no 3621) (Anonymous, 2018c). Implementation and execution of preservation and protection laws is essential for prevention of constructions breaching the Construction Zoning Law and the damages caused by solid and liquid wastes. Construction of facilities that aid community life by focusing on recreation, entertainment, culture, sports, and social aspects are of paramount importance, but they should also adhere to determined constructions of the selected strategy and preserve natural habitats and vistas. The landscaping studies should be reinforced by the contribution of decision makers, communities, and their engagement in consciousness-raising efforts, taking into consideration the vistas and views into the plans. Cooperation between the institutions in various scales is also necessary to properly manage the visual resources in the preservation and restoration of coastal landscape regions.

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THE EFFECTS OF GYTTJA ON SOIL PROPERTIES IN NICKEL-CONTAMINATED SOILS

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(Received 25th Oct 2018; accepted 7th Jan 2019)

Abstract. In this study, the effects of gyttja on some soil properties in two different nickel-contaminated soils (Inceptisol and Entisol) were investigated. This study was conducted with the application of three levels of gyttja (0, 5 and 10%) in two different soils (Inceptisol and Entisol) contaminated with Ni at three levels of concentration (0, 40 and 80 mg kg^{-1}) in a factorial experimental design with three replications. Some soil properties showed differences in different nickel and gyttja applications. Soil nickel content significantly ($P < 0.01$) increased in nickel-contaminated soils. Soil organic matter and phosphorus contents in gyttja applicated soils were found to reach higher levels compared to those without gyttja application.
Keywords: *organic material, heavy metal, soil properties, Inceptisol, Entisol*

Introduction

Heavy metals natural components of the Earth's crust, the concentration of several heavy metals can reach toxic levels due to consequence of anthropogenic activities. Heavy metals are recognized as important environmental contaminants in industrialized societies. Heavy metal contamination is originated from local sources, mostly industry, agriculture, waste incineration, combustion of fossil fuels and road traffic (Farid et al., 2015). Fifty three elements are in the heavy metal category and defined as the group of elements whose densities are higher than 5 g cm^3 (Özbolat and Tuli, 2016).

Nickel is one of the most important heavy metal in terms of its potential toxicity to plants and animals (Kabata-Pendias and Pendias, 2001). Although nickel has been recognized as essential micronutrient in plant cells (Ankel-Fuchs and Thaver, 1988), its excess has destructive effect on plant growth, causing disturbances in photosynthesis, destruction of cell membranes and damages of cells (Pandolfini et al., 1992). Baranowska-Morek (2003) reported that the presence of heavy metals can induce deficiency of the elements necessary for proper plant development similarly. It was reported that high concentrations of heavy metals cause an imbalance of nutrients and have an adverse effect on the synthesis and functioning of many enzymes, vitamins and hormones (Luo and Rimmer, 1995).

It was reported that mobility and availability of heavy metals are controlled by adsorption and desorption characteristics of soils (Krishnamurti et al., 1999). The soil properties including pH, cation exchange capacity, organic matter content, clay minerals contents, lime and Fe and Mn oxides play important role in adsorption and desorption of heavy metals (Antoniadis et al., 2008; Usman et al., 2008).

Organic matter has been implicated for alleviating bioavailability of heavy metals in soils recently soil organic matter has been of particular interest in researches of heavy metal sorption by soils, because of transformation of metal cations to stable complexes

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18651873

form with organic ligands (Elliott et al., 1986). Organic matter makes strong complexes with heavy metals (Bloom, 1981). Khan et al. (2000) reported that amendment of contaminated soils with organic matter decreased bioavailability of heavy metals.

The humic substances, the major component of soil organic matter, have effects on various biochemical outcomes in plant tissue and soil properties such as aggregation, aeration, permeability, water holding capacity, micronutrient transport and availability (Tan, 2003). Among the deposits of humic substances, gyttja, which is a semi-formed lignite coal cover layer and its content of organic matter, can change from 6 to 40% by weight (Demirkıran et al., 2008). In the other researchers reported that gyttja which low-cost and locally available natural materials is a mixture organic and inorganic materials as calcareous and clay, its content of organic matter generally varied from 40 to 50% by weight, and located on lignite deposits. The gyttja which is not used in the coal-fired power plant due to its low quality must be removed for the lignite layer can be taken for mining (Saltalı et al., 2015).

In this study, the effects of gyttja on some soil properties and nickel content in nickel-contaminated two different soils (Inceptisol and Entisol) were investigated.

Material and methods

This study was conducted with the pots having 2 kg different soils (Inceptisol and Entisol) in a plant growth room. Experimental soils were taken from depth of 0-20 cm in Van province, east of Turkey. The Inceptisol soil used in this study had a clay loam texture, non saline, and neutral, limely, insufficient in organic matter (OM), sufficient in phosphorus (P) and potassium (K) contents. The Entisol soil used in this study had a silty loam texture, non saline, slightly alkaline, limely, insufficient in organic matter and P contents and sufficient in K content (*Table 1*).

Table 1. *Some properties of Inceptisol and Entisol soil*

Analyses		Soil types	
		Inceptisol	**Entisol**
pH		7.41	7.64
Texture		Clay loamy	Silt loamy
Lime	%	2.03	2.27
Organic matter	%	1.03	1.70
Salinity	µS cm^{-1}	120	118
Phosphorus (P)	mg kg^{-1}	7.20	4.40
Potassium (K)	mg kg^{-1}	35	35
Calcium (Ca)	mg kg^{-1}	696	594
Magnesium (Mg)	mg kg^{-1}	70	24
Iron (Fe)	mg kg^{-1}	1.96	1.05
Manganese (Mn)	mg kg^{-1}	3.53	4.64
Zinc (Zn)	mg kg^{-1}	0.15	0.16
Copper (Cu)	mg kg^{-1}	0.42	0.33

After filling each pot without drainage holes with two different soils, 54 pots were autoclaved. Three levels of gyttja (0, 5, 10%) were applied into pots including two

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DOI: http://dx.doi.org/10.15666/aeer/1702_18651873

different soils contaminated with three levels of nickel (Ni) (0, 40 and 80 mg kg^{-1}) in factorial experimental design with three replications. The planned doses of gyttja were directly mixed the each pot. Nickel doses were applied as their solutions prepared by using distilled water. Yedikule lettuce cultivar was used as experimental plant. The lettuce seedling having three leaves planted to each pot. This experiment was carried out within 65 days. At the end of experiment firstly it was determined that increasing gyttja doses had the positive effects on plant growth criteria of lettuce in Ni contaminated soils. Conversely, Ni applications decreased the means of plant growth criteria (Gülser et al., 2017). Secondly effects of gyttja on some soil properties in nickel-contaminated soils were determined by using following soil analyses methods.

The gyttja used in this study had neutral pH (7.28), 0.71 dS m^{-1} in salinity, 51.3% in organic matter (OM) content, 1.88% in nitrogen (N), 0.11% total P content and 55.12% in humic acids.

Some soil physical and chemical properties of soil were determined as follows; pH in 1:1 soil water suspension by pH meter (Jenway, 3310), salt content in the same suspension by EC meter (YSI 3100), organic matter by modified Walkey-Black, lime content by Scheibler calcimeter, phosphorus with Olsen bicarbonate method by spectrophotometer (Shimadzu UV mini 1240), potassium (K), calcium (Ca) and magnesium (Mg) by the extraction with 1 N neutral ammonium acetate and heavy metal by extraction with a mixture of nitric acid and perchloric acid in 2:1 ratio by using atomic absorption spectrophotometer (Thermo 1CE 3000 series) according to Kacar and İnal (2008).

Statistical analyses was done using SAS package program (SAS Institute, 1989) to show difference among the mean values of some soil properties and some nutrient contents from the different applications.

Result and discussion

The results of variance analyses for the some soil properties are given in *Table 2*.

Table 2. *F values of the variance analyses for the some soil properties*

V.S.	DF	pH	EC	Lime	O.M.	P	K	Ca	Mg	Ni
Soil (A)	1	18.94**	0.26ns	9.73*	8.36*	0.02ns	4.03ns	66.58**	1001.06**	907.22**
Gyttja(B)	2	14.89**	7.47*	15.23**	107.60**	0.90ns	2.80ns	92.76**	0.14ns	10.10**
Ni (C)	2	4.12ns	1.51ns	0.32ns	2.46ns	1.24ns	3.58ns	4.24ns	1.65ns	6.07*
AxB	2	3.75ns	0.08ns	7.23ns	9.09**	1.91ns	8.80**	1.04ns	0.87ns	5.74*
AxC	2	1.02ns	0.10ns	1.92ns	0.37ns	6.36*	2.77ns	1.81ns	4.52*	1.91ns
BxC	4	0.64ns	0.25ns	1.37ns	0.72ns	0.82ns	5.73*	1.35ns	2.86ns	3.80*
AxBxC	4	5.42*	0.85ns	2.07ns	0.84ns	4.27*	2.74ns	1.66ns	1.19ns	1.51ns

*significant at 0.05 level, **significant at 0.01 level, ns: non significant

Soil types had significant (P < 0.01) effects on pH, Ca, Mg, Ni contents. Lime, OM contents were also significantly (P < 0.05) influenced by the soil types. Gyttja treatments significantly affected pH, lime, OM, Ca, Ni contents (P < 0.01), EC (P < 0.05) contents. Ni applications had no effects soil properties expect Ni contents (P < 0.01). Interactions of soil type and gyttja had significantly (P < 0.01) effects on OM, K and (P < 0.05) Ni contents. The effects of soil type and Ni interactions were found significant at 5% level in P and Mg contents; Ni and gyttja interactions had

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significantly ($P < 0.05$) effects on K and Ni contents. The effects of soil type, gyttja and Ni had significantly ($P < 0.05$) effects on pH level and P contents (*Table 2*).

EC, OM, Ca and Ni contents increased by increasing gyttja doses though pH and lime decreased. These increases were significant statistically. The highest means of EC, OM, Ca, Mg and Ni contents were obtained as 1607.63 µS cm^{-1}, 6.49%, 8618 mg kg^{-1}, 541.38 mg kg^{-1}, 63.59 mg kg^{-1} in 10% gyttja treatment respectively (*Figs. 1–6*). Gyttja used in this study had lower pH value than those in experimental soils. Therefore gyttja applications may be decrease pH value of soils of growing media.

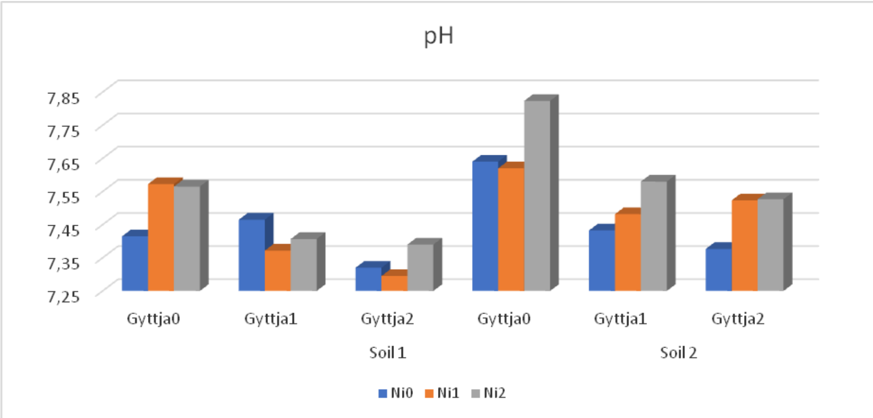

***Figure 1.** Effects of different gyttja level ($P < 0.01$) and nickel doses (ns) on pH. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Ni0: 0 mg Ni kg^{-1}, Ni1: 40 mg Ni kg^{-1}, Ni2: 80 mg Ni kg^{-1}, LSDsoil ($P < 0.05$): 0.05, LSDgyttja ($P < 0.05$): 0.05 LSDnickel ($P < 0.05$): 0.04*

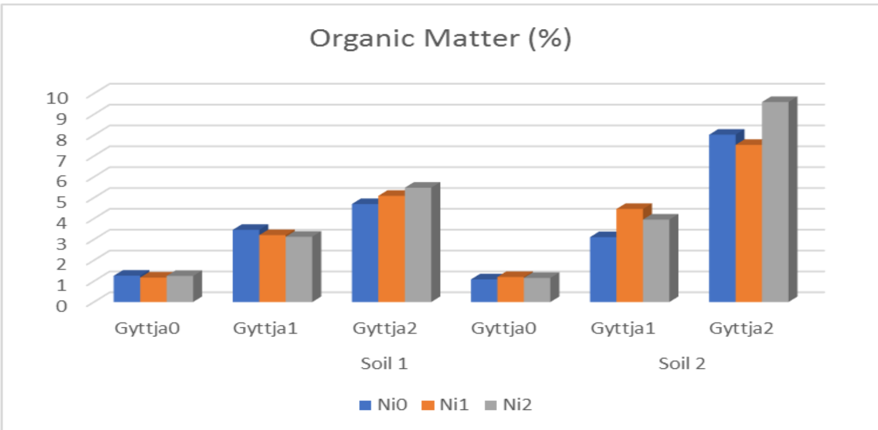

***Figure 2.** Effects of different gyttja level ($P < 0.01$) and nickel doses (ns) on organic matter. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Soil1: Inceptisol, Soil2: Entisol, Ni0: 0 mg Ni kg^{-1}, Ni1: 40 mg Ni kg^{-1}, Ni2: 80 mg Ni kg^{-1}, LSDsoil ($P < 0.05$): 0.38, LSDgyttja ($P < 0.05$): 0.38, LSDnickel ($P < 0.05$): 0.31*

The means of some soil properties determined in Entisol soil were significantly higher than those in Inceptisol soil (*Figs. 1–6*). Ni contents means obtained in Entisol soil were also higher than those in Inceptisol soil in ratio of 22.67%. It was thought that differences in Ni contents among two soil types caused by differences in soil properties. The pH value and OM content of Entisol soil were in higher level than those in

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Inceptisol soils. Zhao et al. (2010), reported that soil pH was found to play the most important role in determining metal speciation, solubility from mineral surfaces, movement and bioavailability of metals in the soil solution. The Entisol soil used in this study had higher pH and OM content according to Inceptisol soil. Ramachandran and D'Souza (2013) determinate that Ni adsorption capacity of the soil increased with an increase in pH of the soil. On the other hand OM is a major factor to the availability of soils for retaining heavy metals in exchangeable form (Mc Cauley et al., 2009).

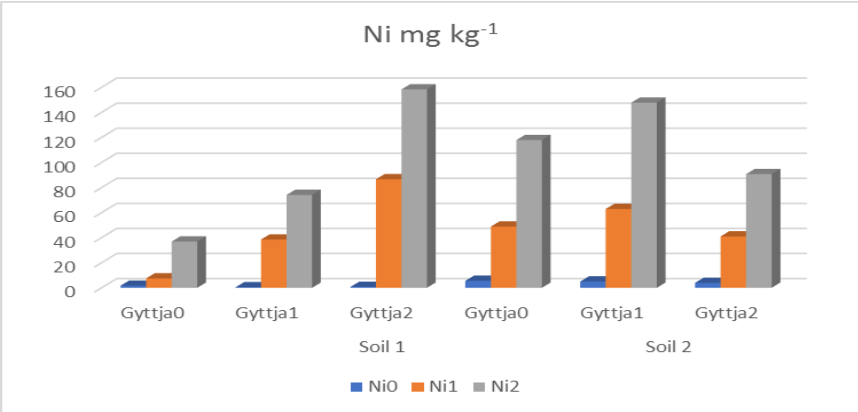

***Figure 3.** Effects of different gyttja level (P < 0.01) and nickel doses (P < 0.01) on nickel content. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Soil1: Inceptisol, Soil2: Entisol, Ni0: 0 mg Ni kg^{-1}, Ni1: 40 mg Ni kg^{-1}, Ni2: 80 mg Ni kg^{-1}, LSDsoil (P < 0.05): 7.85 LSDgyttja (P < 0.05): 7.85, LSDnickel (P < 0.05): 6.41*

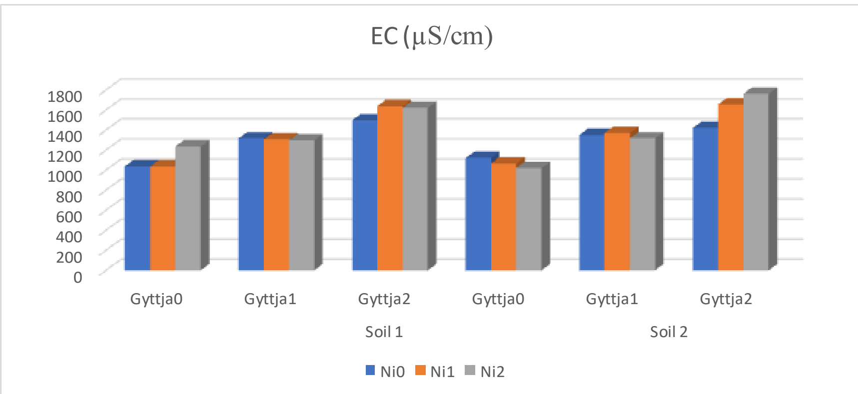

***Figure 4.** Effects of different gyttja level (P < 0.05) and nickel doses (ns) on EC. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Soil1: Inceptisol, Soil2: Entisol, Ni0: 0 mg Ni kg^{-1}, Ni1: 40 mg Ni kg^{-1}, Ni2: 80 mg Ni kg^{-1}, LSDsoil (P < 0.05): 0.6, LSDgyttja (P < 0.05): 0.6, LSDnickel (P < 0.05): 0.5*

Similarly, high EC level of Gyttja used in this study may be lead to increase in EC levels of growing media. Numerous studies demonstrated that humic substances such as gyttja and leonardite increased soil organic matter content. Ece et al. (2007) reported that leonardit applications did not changed pH, lime content and EC significantly while OM content of growth media soils added leonardit increased significantly. Similarly, it was also reported that gyttja applications to soil significantly increased soil organic

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matter content (Karaca et al., 2006; Turgay et al., 2011; Gülser et al., 2014; Saltalı and Eryiğit, 2015). Turgay et al. (2011) reported that gyttja as humic substances added to the soil did not affected soil parameters and macro nutrient contents. Applying of organic materials (barnyard manure, poultry manure, sewage sludge, leonardite, gyttja etc.) is a commend practice to increase OM content (Stevenson, 1994). Decomposition of such organic materials added to soil enhance energy source and this stimulate soil microbial activity (Flieβbach and Mäder, 2000) which finally accelerates the conversions of added organic materials to the background soil organic matter. It was known that humic substances play a critical role in increasing soil organic matter content having useful effects on soil structure, water-holding capacity, adsorption and retention complexes and are responsible for increasing stable carbon levels in soil (Paulin and Q'Malley, 2008).

Generally, nickel contents of soil were higher than critical level (50 mg kg^{-1}) reported by Bergman (1992) in 80 mg kg^{-1} gyttja applications in both soil types. It was thought that high organic matter contents gyttja applied soils caused retention of Ni.

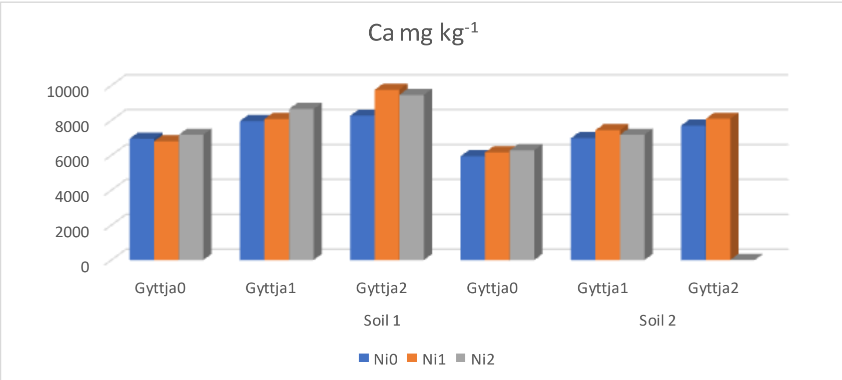

Figure 5. *Effects of different gyttja level (P < 0.01) and nickel doses (ns) on Ca. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Soil1: Inceptisol, Soil2: Entisol, Ni0: 0 mg Ni kg*$^{-1}$*, Ni1: 40 mg Ni kg*$^{-1}$*, Ni2: 80 mg Ni kg*$^{-1}$*, LSDsoil (P < 0.05): 220, LSDgyttja (P < 0.05): 220, LSDnickel (P < 0.05): 179*

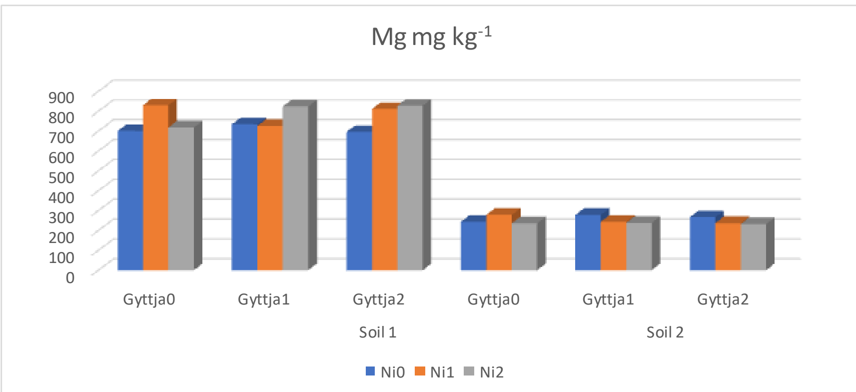

Figure 6. *Effects of different gyttja level (ns) and nickel doses (ns) on Mg. Gyttja0: 0%, Gyttja1: 5%, Gyttja2: 10%, Soil1: Inceptisol, Soil2: Entisol, Ni0: 0 mg Ni kg*$^{-1}$*, Ni1: 40 mg Ni kg*$^{-1}$*, Ni2: 80 mg Ni kg*$^{-1}$*, LSDsoil (P < 0.05): 22, LSDgyttja (P < 0.05): 22, LSDnickel (P < 0.05): 18*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18651873

The Ni applications increased significantly Ni contents of soils. Nickel contents of control soils were determined at low levels in both soil types. The doses of gytjja 10% had more positive effect to prevent nickel contamination in Entisol soil according to those in Inceptisol soil (*Fig. 3*). Apart from soil pH, organic matter content in soils is also one of the most important soil properties affecting heavy metal availability (Mc Cauley et al., 2009).

Antoniadis et al. (2008) and Mc'Cauley et al. (2009), Bai et al. (2008), reported that organic matter is a major factor to the ability of soils for retaining heavy metals in exchangeable form. They also determined that heavy metal adsorption onto soil constituents declined while soil organic matter content decreased. Organic matter supplies organic chemicals to the soil solution. These organic chemicals can serve as chelate and increase metal availability to plants.

The lowest and highest Ni means of Inceptisol soil were obtained as 2.92 mg kg^{-1} and 158 mg kg^{-1} control and G2, in while the lowest and highest Ni means of Entisol soil were in control and G1 application as 8 mg kg^{-1} and 142 mg kg^{-1}, respectively (*Fig. 3*).

In this study, increasing gyttja doses decreased Ni contents according to control in Entisol soil with 10% gyttja applications in Inceptisol soils. Karaca et al. (2006) reported that gyttja application decreased Ni concentrations of soil among 90th-180th day following applying time. In this regard, applying gyttja to soil contaminated with Ni can decrease the availability of nickel.

Conclusion

As a result, 10% gyttja application decreased Ni contents according to control in Inceptisol and Entisol soils. The organic matter contents of experimental soils increased by increasing gyttja doses. These increases were found at higher level in Entisol soils than those in Inceptisol soils. Similarly increasing gyttja doses increased EC values in both soil types without causing of salinity problem. According to results of this study 10% gyttja application was suggested to increase the soil matter contents and to decrease damages of heavy metals in plants growing nickel polluted soils. On the other hand it was thought that gyttja doses using to prevent heavy metal damages may be show the changes according to soil type, plant variety and kind of heavy metal and degree of heavy metal pollution in soil. So, determination of the beneficial effects of gyttja in heavy metal contaminated soils needs the more researches in long-term experiments.

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DOI: http://dx.doi.org/10.15666/aeer/1702_18651873

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF ITALIAN, IRANIAN AND SPANISH SAFFRON (*CROCUS SATIVUS* L.) ACCESSIONS

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(Received 27th Oct 2018; accepted 11th Jan 2019)

Abstract. The use of saffron dates back to ancient times. It is commonly used in medicine, as well as the function of dye and spice in the food industry. The oldest documents about the edible use of saffron are recorded by Polyen (in the 2nd century BC), a Greek military writer who, in his graduation thesis entitled “Stratagemes” reports a list of foods consumed by the court of the Persian Achemenide dynasty (550-330 BC), which had been carved on a bronze column in front of the kitchen (Abrishami, 2004). Its cost is variable according to the production vintage and drying methodologies, it can reach up to 20,000 euros per kilo. The reason for the very limited genetic diversity in cultivated saffron is explained by its asexual mode of reproduction. The AFLP technique could be a good choice to investigate the genetic diversity because of its high-multiplex-ratio which allows detecting differences even between samples sharing most of the genetic material, and it is still one of the most used tools for the molecular characterization of genetic variability in cultivated plants. The aim of this study was to assess the variability of morpho-physiological traits and to evaluate the genetic differences among different proveniences of *C. sativus* with AFLP. The results of this paper in part confirm those obtained by previous studies. Despite morphological differences found between the accessions, molecular markers have revealed limited genetic differences for samples with different origin.
Keywords: *AFLP, Crocus sativus L., morphology characterization, genetic diversity, molecular markers, genetic pool*

Introduction

Saffron (*Crocus sativus* L.), with a basic chromosome number of x = 8, is a triploid and sterile plant, is propagated exclusively by vegetative means (Petersen et al., 2008; Agayev et al., 2009) via its corms which undergo a period of dormancy (Fernandez, 2004).

Saffron is one of the most expensive spices in the world because of the high demand and the different uses, but also for the high costs of cultivation and production. Saffron consists of the dried dark red “stigmas” of the *Crocus sativus,* flower (Chryssanthi et al., 2007), which on average weighs about 6 mg. Therefore, approximately 150,000 to 200,000 flowers are required to yield 1 kg of saffron spice. It is worth nothing that its high price reflects the time required for its cultivation, harvesting and handling (separation of stigma from other parts of the flower parts). In order to gather the saffron, the stigmas of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

the saffron flower must be handpicked. This becomes very time consuming, considering that in order to produce just 1 g of saffron, need to collect an average of 150 flowers (Hosseinzadeh Gonabad, 2013).

In addition to its culinary uses, saffron has a long history as a potent component of traditional medical systems (Gutheil et al., 2012; Premkumar et al., 2006; Abdullaev, 2002; Kim et al., 2014).

Like most plant derivatives, saffron contains a multitude of bioactive molecules. Chief among these molecules are *crocin*, *crocetin*, and *safranal*, members of the carotenoid family of molecules that also includes forms of vitamin A (Aung et al., 2007).

Commercial saffron (dried stigmata of Crocus sativus L.) contains: crocin and crocetin (colors) Crocin is the principle coloring pigment, at nearly 10% (Pfander and Wittwer, 1975; Tsimidou and Tsatsaroni, 1993), picrocrocin (bitter principle), the glycoside picrocrocin which is a precursor of safranal and responsible for its distinctly bitter flavour safranal (flavor), safranal, is the deglycosylated form of picrocrocin and the major organoleptic principle of the stigmas.

Its cultivation has long been concentrated on a broad belt of Eurasia, bounded by the Mediterranean Sea in the Southwest, to Kashmir and China in the northeast. Iran is the main producer of saffron, accounting for more than 90% of world production (about 300 tonnes in the year 2017), followed by India (5%) and countries of the Mediterranean basin (Greece, Morocco, Spain, Italy and Turkey) (Gresta et al., 2008a). However, the Mediterranean region is recognized worldwide as producing the best quality of saffron, which is attributed to many factors (Gresta et al., 2008b; Lage and Cantrell, 2009).

About crocus cultivation in Europe one theory states that Moors reintroduced saffron corms to the region around Poitiers in AD 732 (Pearce, 2005). Two centuries after their conquest of Spain, Moors planted saffron throughout the southern provinces of Andalucía, Castile, La Mancha, and Valencia. Later the proliferation of saffron began to spread throughout Europe (Abrishami, 1987, 2004). Today Spain is the biggest exporter of saffron in the world. Every year, a saffron festival (Fiesta de la Rosa del Azafran) takes place in Spain, consisting of competition for the best saffron dish and saffron picking contest. In Spain there are many dishes which need saffron; the most important one is Paella (Abrishami, 1987; Winterhalter and Straubinger, 2000; Dadkhah et al., 2003).

Archaeological records indicate that saffron was cultivated and used as a spice or medicinal plant in the Mediterranean basin as early as the late Bronze Age. However, there is no consensus on where the first saffron plants were domesticated and grown (Grilli Caiola and Canini, 2010; Molina et al., 2015).

Saffron is one of the oldest crops, which, despite its long history of cultivation compared to conventional crops, is still mainly grown in marginal lands and in low-input farming systems. Therefore, it can be considered an alternative crop in sustainable agricultural systems and marginal land with low efficiency. Several years of studies have demonstrated that differences in saffron quality can be due to environmental effects, post-harvest processing the stigmas, and any genetic variation (Agayev et al., 2006; Nehvi et al., 2007; Ghaffari and Bagheri, 2009; Fluch et al., 2010; Siracusa et al., 2013; Babaei et al., 2014).

Recent studies carried out with molecular techniques have demonstrated that genotype could also play a very important role. The high price of saffron makes it frequent subject of adulteration of various types, i.e. including the mixing authentic Saffron stigmas with other plants flowers colored with natural or artificial dyers. Therefore, the development of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

molecular tools could be fundamental not only to control the purity of saffron products, avoiding adulteration but also for tracing the origin of pure saffron products.

DNA markers have been used in Crocus spp. to characterize germplasm collections, inform breeding programs, and facilitate genetic diversity studies and taxonomic analysis. Over the last decade some technological advances in DNA marker techniques were made. The methods include: random amplified polymorphic DNA (RAPD) (Grilli Caiola et al., 2004; Beiki et al., 2010; Rubio Moraga et al., 2010); ii) inter-retrotransposon amplified polymorphism (IRAP) (Alavi Kia et al., 2008); iii) amplified fragment length polymorphism (AFLP) (Zubor et al., 2004; Erol et al., 2011; Nazzal et al., 2011; Siracusa et al., 2013); iv) intersimple sequence repeat (ISSR) (Rubio Moraga et al., 2010); v) simple sequence repeat (SSR) (Rubio Moraga et al., 2010; Nemati et al., 2012); and vi) single-nucleotide polymorphism (SNP) (Syvanen, 2001; Singh et al., 2010).

Among these molecular markers, AFLPs, based on the selective PCR amplification of restricted total DNA fragments (Vos et al., 1995; Powell et al., 1996) were found to show high levels of polymorphisms per primer combination and yielded high resolution and reproducibility (Meudt and Clarke, 2007; Erol et al., 2014). AFLPs could then be a good choice for investigating genetic variability among saffron accessions because of its high-multiplex-ratio which allows detecting differences even between samples sharing most of the genetic material (Vos et al., 1995). In recent years we successfully have applied AFLP for dissecting genetic variability in faba bean (Gresta et al., 2010), in lentil, (Torricelli et al., 2012), celery (Torricelli et al., 2013), olive (Albertini et al., 2011) and saffron (Siracusa et al., 2013).

The aim of this study was to assess the variability of morpho-physiological traits in *C. sativus* populations coming from Iran and Italy and to evaluate the genetic differences among different origins (Iran, Italy and Spain) with AFLP markers. here are many advantages to AFLP when compared to other marker technologies including RAPD, RFLP, SSRs and SNPs. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt and Clarke, 2007).

Materials and methods

Morphological analysis

Corms coming from Iran (6 accessions) and from Italy (2 accessions) (*Table 1*) were planted in September 2012 on a sandy-clay soil in Umbria, Central Italy (220 m a.s.l.). Sowing was executed in raised beds at 20 cm depth 50 cm between rows and 20 cm between corms in rows. A complete randomized block design with two replications was adopted. Fifteen corms per accession per replication were used; no fertilizer and no irrigation were applied. Weeds were managed by hand. Seventeen morpho-physiological traits were recorded per single plant; detailed information about the recorded traits is reported in *Table 2*.

DNA extraction and AFLP analysis

About eighty days after transplant, leaf tissues were collected for DNA extraction, which was carried out on a single plant basis using the DNeasy 96 Plant Kit and an MM300 Mixer Mill (Qiagen GmbH, Hilden, Germany). In addition to the saffron

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accessions used for the morphological characterization, two Italian populations (Cascia – PG and S. Anatolia di Narco – PG) and one population coming from Spain (Mancha) for molecular analysis were used (*Table 1*). For each population 3 plants were used as biological replicas. DNA was concentrated and quality was assessed using a Thermo Scientific NanoDrop 1000 ultraviolet spectrophotometer and confirmed by agarose gel electrophoresis.

Table 1. *List of saffron accessions used in morpho-physiological and genetic analysis altitude, rainfall and geographical coordinates*

Accessions		m a.s.l.	Average annual rainfall (mm)	Geographic coordinates
1	Citta della Pieve - Italy	509	805	42°57’N 12°E
2	Rahn - Iran	1100	140	43°21’N 58°41’E
3	Tapeh Righ - Iran	1100	140	43°21’N 58°41’E
4	Ghassabbeh - Iran	1100	140	43°21’N 58°41’E
5	Torbat Heidarieh - Iran	1340	152	35°17’N 59°12’E
6	Ghaien - Iran	1450	180	33°43’N 59°11’E
7	Torbat - Market - Iran	1340	152	35°17’N 59°12’E
8	Barisciano - Italy	940	631	42°19’N 13°35’E
*9	Cascia - Italy	653	1050	42°43’N 13°1’E
*10	S. Anatolia di Narco - Italy	328	1080	42°44’N 12° 50’E
*11	Mancha - Spain	700	370	39°51’N 4°1’O

*Supplementary accessions used in genetic analysis

Table 2. *Morpho-physiological traits recorded and their codes*

Traits	Code
1. Days to emergence (days from October 15)	DEME
2. Buds number in vegetation	BUNU
3. Leaves number per bud	LENU
4. Sheath height (mm)	SHE
5. Main leaf length (cm)	MLL
6. Main leaf width (cm)	MLW
7. Leaf area (cm2)	LEA
8. Dry flower weight (mg)	DFW
9. Dry weight of stigmas (mg)	DWS
10. Tepals length (mm)	TEL
11. Tepals width (mm)	TEW
12. Anther length (mm)	ANTL
13. Stigma length (mm)	STIL
14. Corms number per plant	CORN
15. Corms diameter (mm)	CORD
16. Flowers number per plot	FNPLO
17. Flowers number per plant	FNPLA

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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All amplifications were performed on Gene Amp PCR System 9600 (Perkin Elmer, USA). One microliter of each sample was combined with 10 µl of Hi-Di formamide, 0.25 µl size standard Liz 500 (Applied Biosystems), denaturated and fractionated on an ABI 3130xl capillary sequencer (Applied Biosystems).

AFLP analysis was carried out according to (Vos et al., 1995) with minor modification as reported by (Albertini et al., 2011). Genomic DNA (300 ng) was restricted with EcoRI /MseI enzyme combination and double-strand adaptors were ligated to fragment ends. Pre-amplification of the diluted (10-fold) ligated DNA was performed using primers carrying one selective nucleotide (EcoRI + C, MseI + A). The pre-amplification products were diluted 10-fold and used as a template for the selective amplification. Selective amplifications were performed by using fluorescent-labeled EcoRI+3 primer and unlabeled MseI+3 primer (*Table 6*).

Statistical data analysis (morpho-physiological data)

For the morpho-physiological data, ANOVA (Analysis of variance) was carried out using PROC GLM of SAS (SAS, 2008). Means were compared using Duncan's multiple range test. Finally, Pearson's correlation between each couple of morpho-physiological was also calculated. In order to summarize all the information from the collected data, a Principal Component Analysis (PCA) was performed.

Molecular data

5 AFLP fragments were scored dominantly and recorded in a 0/1-matrix for the peak absence/presence along with their sizes using the Genemapper 4.0 software (Applied Biosystems). Molecular data were arranged in a data matrix. The number and percentage of polymorphic loci were calculated for each marker and in total on the entire data set. Number of private bands within an accession (i.e. bands belonging only to a certain farmer population or variety) were also counted. AFLP fragments were scored as 1 or 0 for presence or absence of the band, respectively, using the Genescan software (Applied biosystem). Genetic similarity (GS) was estimated using the similarity coefficient of Jaccard (1908): $SGij = a / (a + b + c)$, where GSij is the genetic similarity between individual i and j; a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. Genetic similarities among samples were clustered by the unweighted pair-group method of arithmetic average (UPGMA; Sneath and Sokal, 1973). The statistical analyses were worked out using the SAS Institute Inc. (SAS, 2008) and NTSYS-PC Version Q 2.11 (Rohlf, 1993) software.

Results

Morpho-physiological analysis

Based on results of ANOVA the accessions of *C. sativus* differed in most characteristics. The average values of the evaluated traits, compared by using the Duncan's multiple range test, are shown in *Table 3*. There were no significant differences for following traits: sheath height (SHE), main leaf length (MLW), tepals length (TEL), tepals width (TEW) and corms number produced per plant (CORN). Significant difference was detected among accessions for all other traits (value $P < 0.05$). In particular regarding days to emergence (DEME), Barisciano and Torbat

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Market were the earliest populations, while Ghaien and Rahn were the latest. Città della Pieve accession shows the highest buds number in vegetation (BUNU) per plant (5.27) while Rahn has the lowest buds number (2.36). Barisciano and Città della Pieve have the highest number leaves per bud (LENU), 10.00 and 9.54 respectively. Barisciano and Città della Pieve populations highlighted the highest corms diameter (CORD), 31.35 and 23.34. Regards the flowers production (per plot and per plant), the Italian saffron populations were the most yielded. The correlation matrix between morphological traits was summarized in *Table 4*. Pearson's coefficients were calculated, significant differences were found and will be taken into account only those traits related to the spice and corms production. Days to emergence (DEME) is negatively correlated with flower number per plot (FNPLO) (r= -0.54) and flower number per plant (FNPLA) (r = -0.59). Buds number in vegetation (BUNU) is positively correlated with corms number per plant (CORN) and flower number per plot (FNPLO) (r = 0.70). Leaves number per bud (LENU) is positively correlated with dry weight of stigmas (DWS), corms diameter (CORD) and flower number per plant (FNPLA) (r = 0.65, 0.75 and 0.94, respectively). Stigma length (STIL) is positively correlated with dry flower weight (DFW), dry weight of stigmas (DWS), corms diameter (CORD) and flower number per plant (FNPLA) (r = 0.80, 0.88, 0.65 and 0.78 respectively). Anther length (ANTL) is positively correlated with flower number per plant (FNPLA) (r = 0.83). Corms diameter (CORD) is highly correlated with flower number per plot (FNPLO) (r = 0.72) and flower number per plant (FNPLA) (r = 0.80). Finally, dry weight of stigmas (DWS) is positively correlated with anther length (ANTL), corms diameter (CORD) and flower number per plant (FNPLA) (r = 0.76, 0.66 and 0.74 respectively). To summarize all the variability among the saffron accessions a principal component analysis (PCA) was worked out by using averages of data recorded per accession. The first three components (PC1, PC2 and PC3) of the PCA accounted for 46.15%, 26.82% and 12.03% of the total variation, respectively (85.00% of total variation) (*Table 5*). Eigenvectors (*Table 5*) showed that: i) PC1 was highly correlated with flowers number produced per plot (FNPLO) (0.35), flowers number produced per plant (FNPLA) (0.34), leaves number per bud (LENU) (0.34), anther length (ANTL) (0.33) and stigma length (STIL) (0.32); ii) PC2 was highly correlated with main leaf length (MLL) (0.45), leaf area (LEA) 0.44), main leaf width MLW (0.35) and tepals length (TEL) (0.34); iii) PC3 was highly correlated with tepals width (TEW) (0.53) and Dry flower weight DFW (0.47). The plot of PC 1 and PC2 is shown in *Figure 1*. From the scatter plot it is clear that the Italian saffron populations are separated clearly from the Iranian accessions. This separation is mainly due to PC1. The saffron accession which comes from the market (Torbat Market) is separated from all other Iranian accessions especially thanks to the second main component (PC2). The cultivated saffron populations coming from Iran are subdivided into two main groups, the first group comprises Rhan and Ghaien; the second group includes Ghassabeh, Tapehrigh and Torbat. With regard to the morpho-physiological traits the Iranian accessions highlight among them some phenotypic diversity.

Molecular analysis

A total of 22 samples (2 samples per accession) were genotyped using 8 AFLP primer combinations and a total of 318 amplification products were scored (*Table 6*). Detected polymorphisms range from 0% (Eco-CCA/Mse-ATC and Eco-CCA/Mse-AGC) to 8.51% (Eco-CCA/Mse-AGC) depending on the primer combination, with an

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DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

average of 4.13%. The molecular weight of the amplification products ranged from 51 to 389 bp. and the average number of bands scored per reaction was 39.75 with a variation from 24 (Eco-CAA/Mse-ACA) to 61 (Eco-CCA/Mse-AGC).

Table 3. *Saffron accessions, average values of the evaluated traits and the Duncan's multiple range test*

Traits	Accessions							
	Città della Pieve	**Rahn**	**Tapeh Righ**	**Ghassabbeh**	**Torbat Heidarieh**	**Ghaien**	**Torbat Market**	**Barisciano**
DEME	11.20 AB	14.30 AB	11.25 AB	9.77 B	13.24 AB	15.60 A	4.10 C	3.45 C
BUNU	5.27 A	2.36 D	3.14 CD	2.60 CD	2.68 CD	2.43 D	4.30 B	3.45 C
LENU	9.54 A	4.72 C	5.03 C	4.34 C	4.55 C	4.79 C	6.44 B	10.00 A
SHE	17.90	14.00	14.56	16.60	18.09	16.17	18.20	15.30
MLL	21.84 BC	21.23 C	23.45 ABC	25.23 AB	22.82 ABC	20.87 C	25.70 A	23.10 ABC
MLW	0.26	0.26	0.28	0.27	0.25	0.23	0.30	0.28
LEA	2.89 ABC	2.76 BC	3.31 ABC	3.54 AB	2.89 ABC	2.49 C	3.69 A	3.18 ABC
DFW	87.00 AB	79.00 AB	77.00 B	90.00 A	87.00 AB	83.00 AB	84.00 AB	89.00 AB
DSW	7.70 AB	6.70 BC	6.40 C	7.20 BC	6.50 BC	7.60 ABC	6.60 BC	8.70 A
TEL	38.15	38.57	38.45	39.26	38.57	37.94	38.67	37.88
TEW	16.55	16.34	16.61	17.29	16.76	17.11	17.10	18.82
ANTL	14.65 A	13.39 BC	13.10 C	14.18 AB	13.55 BC	13.62 BC	13.55 BC	14.98 A
STIL	31.39 AB	29.23 BC	28.62 C	30.53 ABC	29.71 BC	30.00 ABC	29.49 BC	32.23 A
CORN	6.33	2.95	4.17	4.17	4.00	2.50	6.34	3.84
CORD	23.34 AB	19.09 B	18.48 B	17.24 B	20.55 B	20.89 B	19.40 B	31.35 A
FNPLO	122.00 A	12.00 C	9.50 C	22.50 C	24.50 C	22.50 C	54.00 B	122.50 A
FNPLA	8.14 B	1.20 D	1.12 D	1.50 D	1.64 D	1.56 D	3.60 C	13.35 A

Average values followed by the same letter are not significantly different at P ≤ 0.05, Duncan test

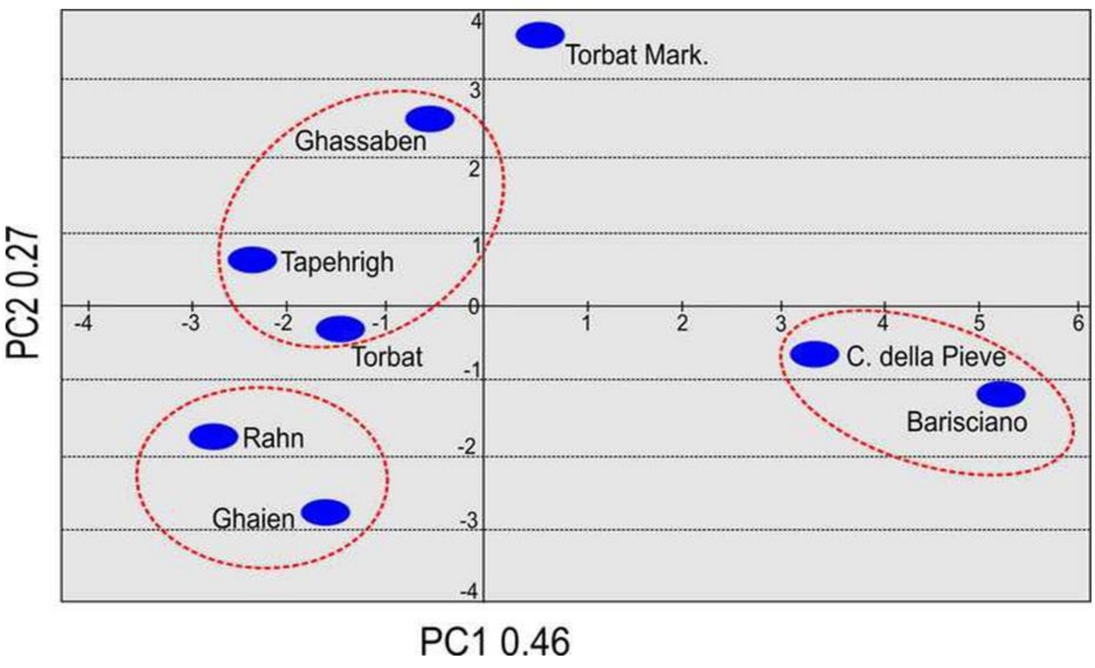

Figure 1. *Principal component analysis based on the morpho-physiological traits recorded in the saffron populations*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

Table 4. *Pearson's correlation coefficients among 17 morpho-physiological traits*

TRAITS	DEME	BUNU	LENU	SHE	MLL	MLW	LEA	DFW	DWS	TEL	TEW	ANTL	STIL	CORN	CORD	FNPLO	FNPLA
DEME	1.00																
BUNU	-0.41	1.00															
LENU	-0.57*	0.69**	1.00														
SHE	-0.24	0.40	0.14	1.00													
MLL	-0.70**	0.11	0.00	0.31	1.00												
MLW	-0.73**	0.35	0.27	0.07	0.67**	1.00											
LEA	-0.73**	0.22	0.08	0.17	0.92**	0.89**	1.00										
DFW	-0.24	0.16	0.31	0.29	0.31	0.10	0.23	1.00									
DWS	-0.22	0.18	0.65**	-0.09	-0.10	0.00	-0.09	0.68**	1.00								
TEL	-0.13	-0.12	-0.20	0.28	0.55*	0.25	0.42*	0.50*	0.07	1.00							
TEW	-0.21	-0.06	-0.06	0.34	0.48*	0.04	0.26	0.63**	0.35	0.79**	1.00						
ANTL	-0.51*	0.42*	0.77**	0.21	0.15	0.21	0.14	0.62**	0.76**	0.11	0.30	1.00					
STIL	-0.37	0.30	0.72**	0.23	0.07	0.10	0.04	0.80**	0.88**	0.26	0.41	0.86**	1.00				
CORN	-0.37	0.70**	0.28	0.30	0.40	0.48*	0.47*	0.29	0.00	0.21	0.26	0.29	0.12	1.00			
CORD	-0.36	0.18	0.75**	0.09	-0.16	-0.02	-0.16	0.26	0.66**	-0.26	-0.08	0.55*	0.65**	-0.30	1.00		
FNPLO	-0.54*	0.70**	0.97**	0.22	0.00	0.20	0.04	0.45*	0.70**	-0.18	0.04	0.84**	0.78**	0.36	0.72**	1.00	
FNPLA	-0.59*	0.53*	0.94**	0.06	0.00	0.21	0.05	0.42*	0.74**	-0.20	0.04	0.83**	0.78**	0.22	0.80**	0.96**	1.00

Days to emergence (DEME), Buds number in vegetation (BUNU), Leaves number per bud (LENU), sheath height (SHE), main leaf length (MLL), main leaf width (MLW), leaf area (LEA), Dry flower weight (DFW), Dry weight of stigmas (DWS), Tepals length (TEL), Tepals width (TEW), Anther length (ANTL), Stigma length (STIL), Corms number per plant (CORN), Corms diameter (CORD), Flowers number per plot (FNPLO), Flowers number per plant (FNPLA)

Table 5. *Principal component analysis based on morpho-physiological traits of 8 saffron accessions, and significant loadings (in bold) of the first three principal components*

	PC1	PC2	PC3
Eigenvalue	7.8453	4.5590	2.0457
Percent of total variation	0.4615	0.2682	0.1203
Cumulative	0.4615	0.7297	0.8500
DEME	-0.2484	-0.2618	0.0746
BUNU	0.23669	0.1325	-0.3196
LENU	0.3378	-0.0630	-0.2014
SHE	0.1011	0.1998	0.1999
MLL	0.0459	0.4483	0.0924
MLW	0.1326	0.3467	-0.2711
LEA	0.0681	0.4365	-0.0456
DFW	0.2319	0.0836	0.4714
DWS	0.2893	-0.1971	0.1968
TEL	-0.1573	0.3400	0.1771
TEW	0.0271	0.2153	0.5262
ANTL	0.3280	-0.0595	0.1800
STIL	0.3237	-0.1008	0.2219
CORN	0.1668	0.3008	-0.2492
CORD	0.2999	-0.1914	-0.0237
FNPLO	0.3498	-0.0535	-0.0918
FNPLA	0.3437	-0.0822	-0.0755

A graphic representation of saffron accession genetic similarity level, based on a cluster analysis, is showed in *Figure 2*. With the exception of the saffron population coming from Spain, which showed to be well differentiated from all others, all the accessions are grouped into two main clusters (A and B). Cluster A consists of two sub-

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DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

groups: the first includes all Umbrian accessions (Cascia, Città della Pieve, and S. Anatolia di Narco). In particular, with the primer combinations used, accessions from Città della Pieve and S. Anatolia di Narco showed no molecular differences. The second sub-group consists of two Iranian populations (Rahn and Ghaien) and the population belonging to Abruzzo Region (Barisciano). Cluster B grouped all the remaining Iranian populations (Ghassabbeh, Torbat, Tapehrig, and Torbat_Market). However, in cluster B it is also possible to distinguish two sub-clusters: the first includes the accession collected both in Tapehrigh and in the market (Torbat Market), the second sub-cluster gathers one accession from Torbat and one from Ghassabeh.

Table 6. *Primer combinations and level of AFLP polymorphisms*

Primer combinations	Fragment size (bp)	Number of fragments	Monomorphic fragments	Polymorphic fragments	Polymorphic %
EcoCCA/MseACC	54-347	56	52	4	7.14
EcoCCA/MseAGC	55-389	47	43	4	8.51
EcoCCA/MseACA	52-268	30	29	1	3.33
EcoCCA/MseAAC	51-311	28	27	1	3.57
EcoCAA/MseACC	51-338	47	46	1	2.13
EcoCAA/MseAGC	52-351	61	61	0	0.00
EcoCAA/MseACA	60-281	24	22	2	8.33
EcoCAA/MseATC	51-309	25	25	0	0.00
Total	-	318	305	13	-
Mean	-	39.75	38.13	1.63	4.13

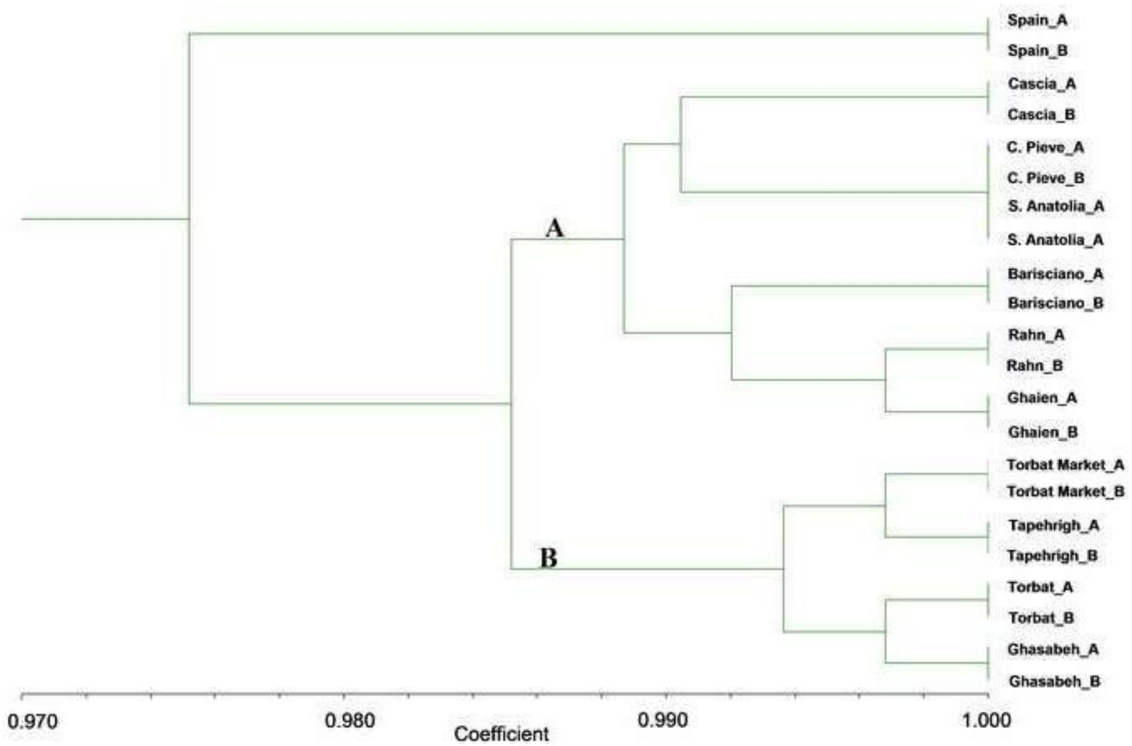

Figure 2. *UPGMA clustering of the saffron populations based on the Jaccard's genetic similarity matrix*

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DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

Discussion

In this study, a morpho-physiological and molecular characterization was developed for investigating the existing diversity among different populations of *C. sativus* L. Analysis of variance of the morpho-physiological data shows that the Italian accessions of saffron yielded a greater number of flowers than the Iranian accessions. Regarding to the diameter of the corms, highly correlated with the production of flowers, the Italian accessions (in particular Barisciano) showed higher values than those collected in Iran. This is probably due to the traditional practice of the annual selection of bigger corms carried out in Abruzzo (Gresta et al., 2008a), and the increased availability of water and nutrients. Barisciano accession was also characterized by the earliest emergence of leaves and by the highest length of the stigmas. In the present work, the morpho-physiological characteristics of the leaves, flower and corms allowed to discriminate saffron populations. This result highlights the usefulness of the morphological characterization of local germplasm, especially in the early stages of the genetic characterization. Indeed, the morpho-physiological traits have allowed a clear discrimination of the Italian accessions from those collected in Iran and in the Torbat market.

For the genetic characterization the AFLP markers were chosen among other classes of molecular markers because they do not require a priori knowledge of the genome of the species, further more they are highly informative and reproducible. The eight AFLP primer combinations discriminated the Spanish accession from all other saffron populations. In addition, this study showed that populations belonging to Umbria (Cascia, Città della Pieve, S. Anatolia di Narco) may have the same genetic origin, as they show no or very little molecular differences. Probably the original saffron was spread through Umbria by exchange of corms between farmers and this is particularly true for saffron belonging to Città della Pieve and S. Anatolia di Narco. The Abruzzo population (Barisciano), although belonging to the same cluster (cluster A) of other Italian populations, seems to be genetically closer to two Iranian populations (Rhan and Ghaien). The other four Iranian populations were grouped apart from other accessions in a separate cluster (cluster B) (*Fig. 2*).

Conclusion

The results of this study partially confirm obtained in previous studies carried out in saffron (Grilli Caiola and Canini, 2004). However, according to (Fernandez, 2007) it is possible to find different saffron products on the market. This may be due to the growing environment, to the collection and processing of spice, but probably also to genetic differences between the different clonal populations. For instance in Navelli (Abruzzo Region – Italy) the selection of corms carried out each year has produced a significant “genetic pool” with respect to corm dimensions and disease resistance (Gresta et al., 2008a). The same results eas shown in comparison the results of this study, with other saffron research’s (Yousefi Javan and Gharari, 2018), using the other molecular markers for prove genetic diversity. The main difference between the two studies is use of different markers and different regions.

Particularly in this study, despite several morphological differences found between accessions, molecular markers (AFLP) have revealed limited genetic differences in particular for Italy and Iran (Genetic Similarity = 0.985) samples. Also in another research with the different markers, *C. sativus* demonstrated minimal genetic variation,

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DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

in contrast to the intraspecific variability seen in other *Crocus* species, and it is concluded that the triploid hybrid species has most probably happened only once. The data show that saffron is an allotriploid species, with the IRAP analysis indicating that the most likely ancestors are *C. cartwrightianus* and *C. pallasii* subsp. *Pallasii* (or close relatives). The results may facilitate resynthesizing saffron with improved characteristics, and show the need for conservation and collection of wild *Crocus*.

The vegetative multiplication limits for this crop the application of conventional breeding approaches for its further improvement. The selection of the best lines among natural or cultivated populations is restricted to searching for a quote of variability deriving from some rare mutations.

Therefore greater efforts must be made to investigate the genetic variability in this crop that is having an increasing economic value. Looking for saffron populations, that have retained some level of sexual reproduction, with the aim of crossing and selecting new types, could be a further challenge.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18751887

EXCESSIVE FERTILIZATION RESULTED IN DECREASED ANTIOXIDANT PERFORMANCE OF THREE VARIETIES OF SUPER RICE

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(Received 28th Oct 2018; accepted 11th Jan 2019)

Abstract. Fertilizer application is an important part in super rice production. In order to investigate the effect of fertilizer amounts on the antioxidant performance of the super rice, three super hybrid rice cultivars, *YLiangyou-1173*, *YLiangyou-911* and *Chaoyou-1000*, were used in the experiment. The fertilizer amounts were set as: (F1)1050 kg N ha^{-1}; (F2) 1200 kg N ha^{-1}; (F3) 1350 kg N ha^{-1}; (F4) 1500 kg N ha^{-1}; (F5) 1650 kg N ha^{-1}. The results showed that the activities of antioxidant enzymes were decreased when excessive fertilizers such as F4 and F5 were applied. Compared with F1, F4 and F5 significantly reduced the activity of superoxide (SOD, EC 1.15.1.1), guaiacol type peroxidase (POD EC1.11.1.7) and catalase (CAT, EC1.11.1.6). Furthermore, there was no remarkable significant difference related to the yield among F2, F3, F4 and F5.
Keywords: *rice, superoxide, catalase, peroxidase, yield, fertilizer*

Introduction

Rice (*Oryza sativa* L.) has been cultivated in China for a very long time and its production has played an important role in Chinese food security (Ren et al., 2017). Many experts had done many researches in different fields to improve to improve rice yield and quality (Wang and Zhang, 2017; Wu et al., 2018; Jiang et al., 2018). Generating rice varieties with high yield potential is a main method to improve rice yield. In 1996, China's ministry of agriculture proposed the super hybrid rice cultivation program which was led by Yuan Longping, the "father of hybrid rice", in 2000, the super hybrid rice varieties reached the first-stage single rice yield standard, that is, the yield per hectare exceeded 10.5 tons and in 2004, the output of super hybrid rice reached the second stage (Schmalzer, 2017). Normally, in order to improve the potential of yield and increase the final yield of super rice, researchers and farmers would apply as much fertilizer as possible and try to found the correlation between rice yield and fertilizing amount. However, there was no much report about the effect of fertilizer amount on rice physiological property especially antioxidant property.

The antioxidant system is very important when rice faces the environment stress such as heat stress, chilling, heavy metal stress and it also has relationship with rice yield and quality. When rice faces the environmental stress including UV stress and pathogen invasion, the reactive oxygen species (ROS) will be produced and accumulated while oxidative stress is induced. Then, a lot of cellular reactions will be produced in by various enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). The formation of ROS is prevented and decreased by an antioxidant system which including ROS-interacting enzymes such as SOD, CAT and POD (Blokhina, 2003). The study of Kong (Kong et al., 2017) showed that the malondialdehyde (MDA) content would increase when rice suffered from high temperature stress at filling stage and the soluble protein, POD, SOD, CAT and free proline would work together to quench the ROS and decrease the MDA concentration. Pan (Pan et al., 2013) demonstrated that spraying paclobutrazol (PBZ) and 6-Benzylaminopurine (6-BA) at heading stage could not only increase POD and SOD activities but also improve the rice yield and grain quality.

Many researches had proved that fertilizer application is a key part in rice production (Shakoor et al., 2018; Liu et al., 2018). The study of Li et al. (2016) showed that manganese (Mn) fertilizer could enhance enzyme activity involved in 2-acetyl-1-pyrroline (2-AP) biosynthesis in fragrant rice while regulating the grain yield. The investigation of Pan et al. (2017) revealed that mechanized deep placement of nitrogen fertilizer in direct-seeded rice improved rice yield by promoting the fertilizer uptake of rice and increasing the utilization rate. Fertilizer application is the other source of rice nutrition beside photosynthesis and it also could affect the rice growth environment such as soil properties (Sun et al., 2018; Ghaley et al., 2018). However, the relationship between fertilizer amount and rice antioxidant property is still unknown to us.

Therefore, the study was conducted in Guangzhou, Guangdong (major rice producing province in South China) in early season in 2018 in order to investigate the effect of different amount of fertilizer on antioxidant performance of three super rice cultivars.

Materials and methods

Plant materials and growing condition

Three super hybrid rice cultivars, *YLiangyou-1173*, *YLiangyou-911* and *Chaoyou-1000*, well-known and widely grown in South China, were in planted in the early season in Guangzhou (23°16’ N, 113°23’ E) during 2018. Before sowing, the seeds were soaked in water for 24 h, germinated in manual climatic box for the next 24 h, shade dried and the germinated seeds were sown in PVC trays for nursery raising. 20-day-old seedlings were transplanted to the field at the planting distance (30 cm × 12 cm). The experimental soil in Guangzhou was sandy loam with of 24.65% organic matter content, 1.560% total N, 0.956% total P, and 18.460% total K.

Treatments and plant sampling

Five amounts of commercial compound fertilizer were applied at the experiment and set as below:

F1: 1050 kg ha^{-1} commercial compound fertilizer (pure nitrogen 131.25 kg ha^{-1}, pure phosphate 63 kg ha^{-1}, pure potassium 105 kg ha^{-1}, organic fertilizer 210 kg ha^{-1});
F2: 1200 kg ha^{-1} commercial compound fertilizer (pure nitrogen 150 kg ha^{-1}, pure phosphate 72 kg ha^{-1}, pure potassium 120 kg ha^{-1}, organic fertilizer 240 kg ha^{-1});
F3: 1350 kg ha^{-1} commercial compound fertilizer (pure nitrogen 168.75 kg ha^{-1}, pure phosphate 81 kg ha^{-1}, pure potassium 135 kg ha^{-1}, organic fertilizer 270 kg ha^{-1});

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F4: 1500 kg ha^{-1} commercial compound fertilizer (pure nitrogen 187.50 kg ha^{-1}, pure phosphate 90 kg ha^{-1}, pure potassium 150 kg ha^{-1}, organic fertilizer 300 kg ha^{-1});
F5: 1650 kg ha^{-1} commercial compound fertilizer (pure nitrogen 206.25 kg ha^{-1}, pure phosphate 99 kg ha^{-1}, pure potassium 165 kg ha^{-1}, organic fertilizer 330 kg ha^{-1}).

The fertilizer was invented by Tang Xiangru (CN 101955394 A). The special fertilizer is a mixture of nitrogen (N) fertilizer, phosphate (P) fertilizer, potassium (K) fertilizer and biological organic fertilizer (12.50% N, 6.00% P, 10.00% K and 20.00% organic matter content). Normally, the local farmers in Guangdong were used to applied 1500 kg ha^{-1}. The treatments were arranged in randomized complete block design (RCBD) in triplicate in each year with net plot size of 20 m^2.

The fresh flag leaves were separated and collected from the rice plants at tillering stage, heading stage and maturity stage, respectively. The sample was washed with double distilled water and stored at -80 °C for physio-biochemical analysis.

Measurement of malondialdehyde (MDA) and anti-oxidants responses

The MDA content and activities of POD, SOD and CAT were determined according to the methods described by Kong et al. (2017). MDA reacted with thiobarbituric acid (TBA) and the absorbance was read at the 532 nm, 600 nm, and 450 nm. The content of the reaction solution was calculated as: MDA content (μmol/L) = 6.45(OD532 −OD600) − 0.56OD450, while the final result was expressed as μmol/g FW.

Guaiacol type peroxidase (POD EC1.11.1.7) activity was estimated after the reaction which the solution was including enzyme extract (50 μl), 1 ml of 0.3% H2O2, 0.95 ml of 0.2% guaiacol, and 1 ml of 50 mM l−1 sodium phosphate buffer (pH 7.0) while One POD unit of enzyme activity was expressed as the absorbance increase because of guaiacol oxidation by 0.01 (U/g FW). The superoxide (SOD, EC 1.15.1.1) activity was measured by using nitro blue tetrazolium (NBT). 0.05 ml of enzyme extract was added into the reaction mixture which contained 1.75 ml of sodium phosphatebuffer (pH 7.8), 0.3 ml of 130 mM methionine buffer, 0.3 ml of 750 μmol L^{-1} NBT buffer, 0.3 ml of 100 μmol L^{-1} EDTA-Na 2 buffer and 0.3 ml of 20 μmol L^{-1} lactoflavin. After reaction, the absorbance was recorded at 560 nm. One unit of SOD activity is equal to the volume of extract needed to cause 50% inhibition of the color reaction. Catalase (CAT, EC1.11.1.6) activity was estimated by adding an aliquot of enzyme extract (50 μl) to the reaction solution containing 1 ml of 0.3% H2O2 and 1.95 ml of sodium phosphate buffer and then the absorbance was read at 240 nm. One CAT unit of enzyme activity was defined as the absorbance decrease by 0.01 (U/g FW).

Estimation of rice yield

At maturity stage, the rice grains were harvested from ten unit sampling area (1.00 m^2) in each plot and then threshed by machine. The harvested grains were sundried and weighed in order to determinate the grain yield.

Statistical analysis

Data were analyzed using statistical software ‘Statistix 8.1’(Analytical Software, Tallahassee, FL, USA) while differences amongst means were separated by using least significant difference (LSD) test at 5% probability level. Graphical representation was conducted via Sigma Plot 14.0 (Systat Software Inc., California, USA).

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

Results

SOD activity

As shown in *Figure 1,* there were some differences on SOD activity among different fertilizer amounts. For *YLiangyou-1173*, there was no remarkable difference between F1 and F2. However, F4 and F5 had lower SOD activity than F1 at tillering stage, heading stage and maturity stage. The trend of SOD activity at tillering stage and maturity stage was recorded as: F1 = F2 > F3 > F4 > F5. For *YLiangyou-911*, the activity decreased with the increase of fertilizer amount. The lowest activity was recorded in F5 while the highest was recorded in F1 at tillering stage and heading stage. The trend at maturity was recorded as: F1 > F2 = F3 > F4 = F5. For *Chaoyou-1000*, at tillering stage, the trend of SOD activity was recorded as: F1 = F2 > F3 > F4 = F5, at heading stage, highest activity was recorded in F1 and the lowest was observed in F5 while in maturity stage, there was no significant difference among F1, F2 and F3 while F4 and F5 were significantly lower than F1.

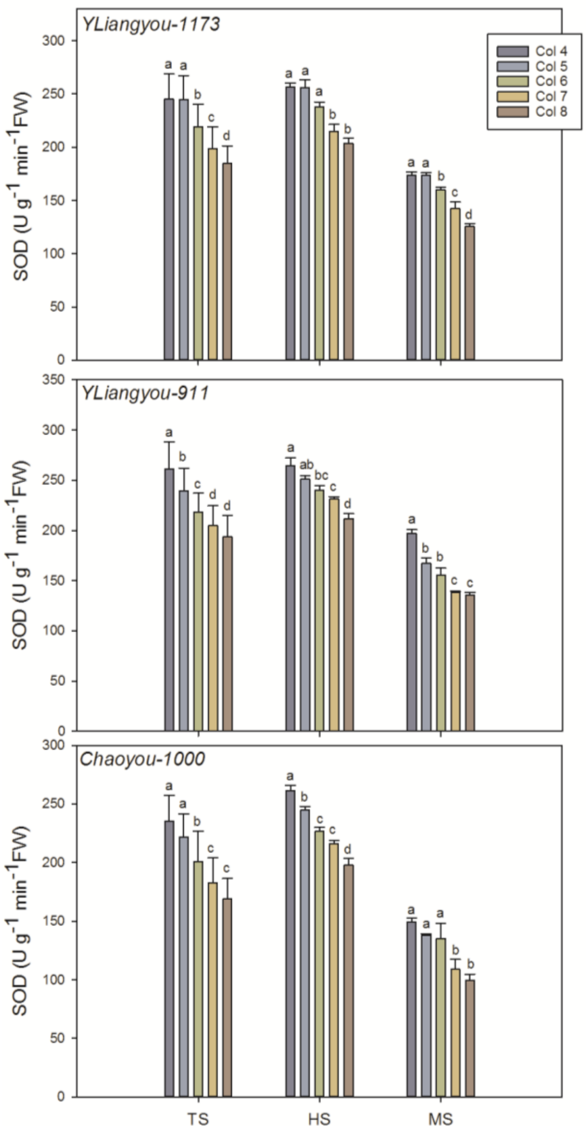

***Figure 1.** Effect of fertilizer amounts on SOD activity. TS: tillering stage; HS: heading stage; MS: maturity stage. Means sharing a common letter do not differ significantly (at P < 0.05) according to least significant difference (LSD) test for both the seasons*

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

CAT activity

As shown in *Figure 2,* fertilizer amounts affected CAT activity significantly. For *YLiangyou-1173*, the trends of CAT activity at tillering stage, heading stage and maturity stage were recorded as: F1 = F2 > F3 > F4 = F5, F1 = F2 ≥ F3 ≥ F4 = F5, F1 = F2 = F3 ≥ F4 ≥ F5, respectively. For *YLiangyou-911*, at tillering stage, the highest CAT activity was recorded in F1 and there was no remarkable difference among F3, F4 and F5. At heading stage, there was no significant difference among F1, F2, F3 and F4 while F5 had lower activity than F1. At maturity stage, compared with F1, F2, F3, F4 and F5 all significantly reduced the CAT activity while there was no remarkable difference among F2, F3, F4 and F5. For *Chaoyou-1000*, F3, F4 and F5 all had lower activity than F1 at tillering stage and heading stage, however, at maturity, there was no significant difference among all fertilizer treatments.

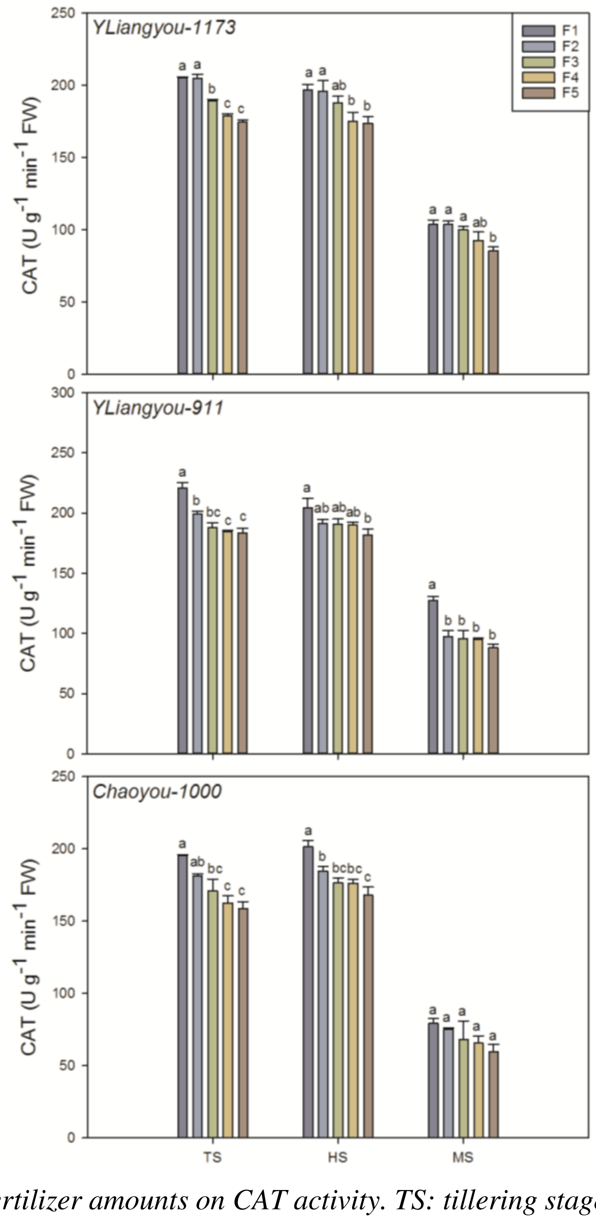

Figure 2. *Effect of fertilizer amounts on CAT activity. TS: tillering stage; HS: heading stage; MS: maturity stage. Means sharing a common letter do not differ significantly (at P < 0.05) according to least significant difference (LSD) test for both the seasons*

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POD activity

As shown in *Figure 3,* fertilizer amounts affected POD activity significantly. For *YLiangyou-1173*, compared with F1, F4 and F5 reduced the POD activity significantly and there was no significant difference between F2 and F1 at heading stage and maturity stage. For *YLiangyou-911*, there was no remarkable difference between F1 and F2 at all three growth stage. However, F3, F4, F5 had lower activity than F1 at all three growth stage. For *Chaoyou-1000*, compared with F1, F4 and F5 significantly decreased the CAT activity at all growth stage. Furthermore, there was no remarkable difference among F1, F2 and F3 at maturity stage.

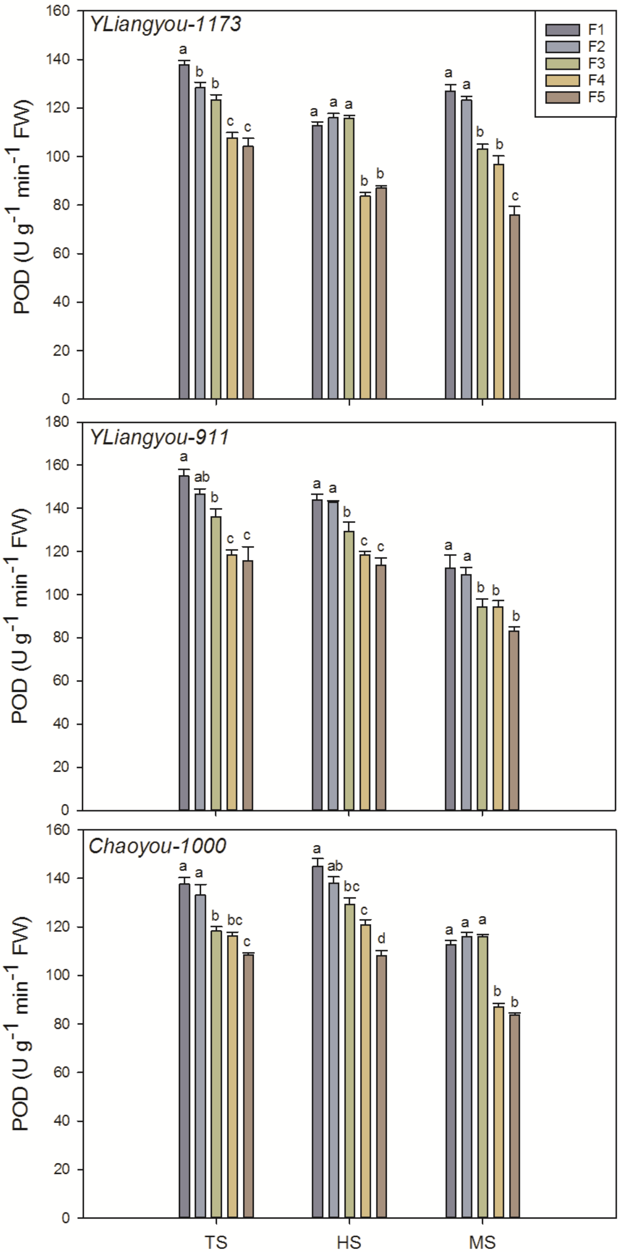

Figure 3. *Effect of fertilizer amounts on POD activity. TS: tillering stage; HS: heading stage; MS: maturity stage. Means sharing a common letter do not differ significantly (at P < 0.05) according to least significant difference (LSD) test for both the seasons*

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

MDA content

As shown in *Figure 4,* fertilizer amounts affected MDA concentration significantly. For *YLiangyou-1173*, the highest MDA content was recorded in F5 and the lowest was recorded in F2. For *YLiangyou-911*, at tillering stage, there was no significant difference among F1, F2, F3 and F4, at heading stage, the highest content was recorded in F5 while there was no remarkable difference among F1, F2 and F3, at maturity stage, there was no significant difference among all fertilizer treatments. For *Chaoyou-1000*, the trend of MDA content was recorded as: F5 > F1 > F4 > F3 > F2 at tillering stage, at heading stage, the highest content was recorded in F5 while the lowest was recorded in F2 and F3. At maturity, the highest concentration was recorded in both F5 and F4 while the lowest was recorded in F1.

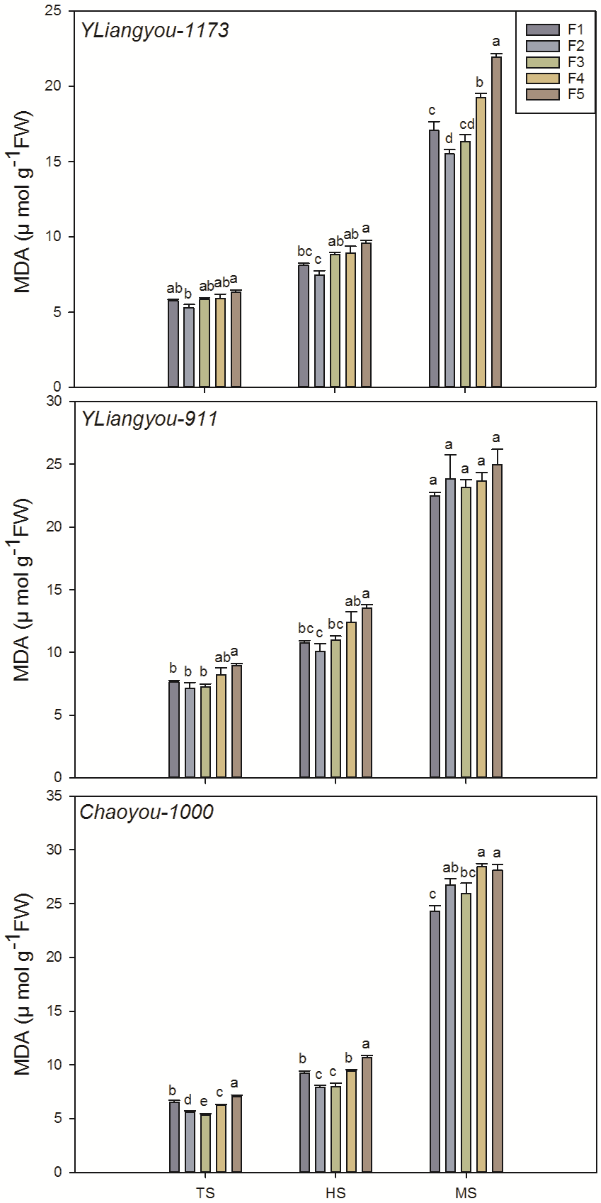

Figure 4. *Effect of fertilizer amounts on MDA content. TS: tillering stage; HS: heading stage; MS: maturity stage. Means sharing a common letter do not differ significantly (at P < 0.05) according to least significant difference (LSD) test for both the seasons*

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

Yield

As shown in *Figure 5,* fertilizer amounts affected grain yield significantly. For *YLiangyou-1173*, F2, F3, F4 and F5 had higher yield than F1 but there was no remarkable difference among F2, F3, F4 and F5. For *YLiangyou-911*, with the increase of fertilizer application, the yield increased first and then decreased but there was no significant difference between two adjacent fertilizer gradients. For *Chaoyou-1000*, the trend of was recorded as: F5 = F4 = F3 ≥ F2 ≥ F1.

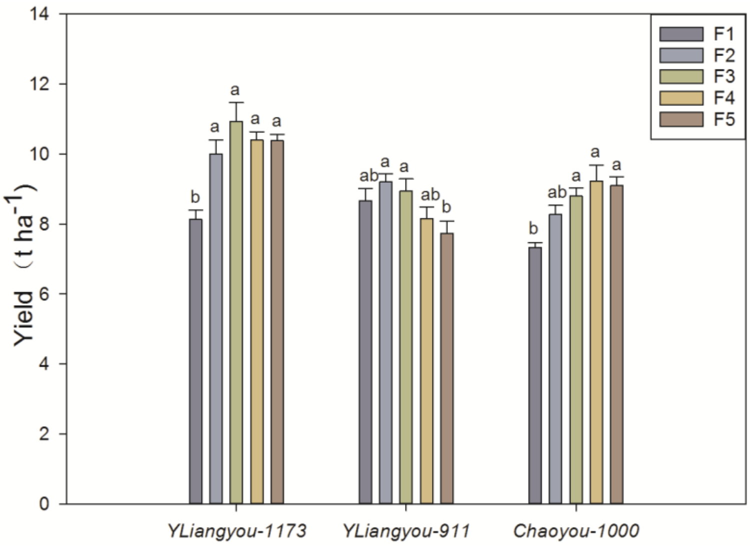

Figure 5. *Effect of fertilizer amounts on yield. Means sharing a common letter do not differ significantly (at P < 0.05) according to least significant difference (LSD) test for both the seasons*

Discussion

The effect of fertilizer on rice had been reported by many researchers. Mo et al. (2017) demonstrated that Silicon (Si) fertilizer had ability to regulate the fragrant rice yield and the content of 2-AP. The research of Sikar et al. (2008) showed that 80 kg hm^{-2} nitrogen improved grain quality significantly and the nitrogen utilization efficiency is connected to the locations and rice genotypes. Previous study (Mahajan et al., 2010) also revealed that high nitrogen application could cause the lodging in rice. In our study, we observed that different amounts of fertilizer influenced the super rice antioxidant system in terms of POD, SOD, CAT and MDA. The result showed that F4 and F5 treatments reduced the enzymes activity significantly which indicated excessive fertilization might improve rice growth, but would reduce the antioxidant capacity.

Antioxidant capacity which involves antioxidant enzymes such as POD, SOD and CAT is a key indicator to measure the ability of rice to resist environmental stress (Almeselmani et al., 2006). Normally, when rice faces environmental stress such as heat damage, chilling, heavy metal stress or extreme weather, the reactive oxygen species (ROS) concentration would increase quickly and cause a number of reactions (ZhangWei and Peng, 2018). For example, the peroxidation of unsaturated fatty acid in plant membranes induced by ROS, resulting in a decrease in the content of unsaturated fatty acid, an increase in MDA concentration and a decreased in membrane fluidity (El-Shintinawy, 2000), then, POD, SOD and CAT would play an important part in

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

quenching the ROS and lowering the MDA content. In our study, the results showed that the activity of antioxidant enzymes including SOD, CAT and POD would decrease if too much fertilizer was applied in super rice production. The decrease in antioxidant enzymes activity meant that the reduction of rice ability to quench the ROS and decreased MDA. Then, the risk of environmental stress and disease would be increased.

The harm of excessive fertilization had been reported in previous studies. For example, the study of Zhong and Zu (1997) revealed that supplying with excessive phosphate (P) fertilizer in early stage could not increase yield more than that applied with adequate P, due to the reduction in the valid grain percentage and weight per thousand grains. Similar phenomenon also was found in present study by showing that both F4 and F5 did not have a higher yield than F3. The investigation of Peng et al. (2010) showed that excessive nitrogen application to rice in China could cause environmental pollution, increase the cost of rice farming, reduce grain yield and contribute to global warming. From the perspective of antioxidant performance of rice, this experiment proved again that the importance of moderate fertilization and the harm of excessive fertilization.

Conclusion

Excessive fertilization could cause the decrement of activity of antioxidant enzymes such as SOD, POD and CAT, thus reduced antioxidant capacity of super rice. In rice production, people should pay more attention on rational application of fertilizer and for revealing the mechanism of how fertilizer amounts affecting rice antioxidant system, much work should be done at molecular and physiological level.

Acknowledgements. This study was supported by National Natural Science Foundation of China (31271646), National Key R&D Program of China (2016YFD0700301), the World Bank Loan Agricultural Pollution Control Project in Guangdong (0724-1510A08N3684), the Technology System of Modern Agricultural Industry in Guangdong (2017LM1098) and Student's Platform for Innovation and Entrepreneurship Training Program (201810564029). The authors declare no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_18891898

GENETIC DIVERSITY AND POPULATION STRUCTURE OF QUINOA (*CHENOPODIUM QUINOA* WILLD.) USING IPBS-RETROTRANSPOSONS MARKERS

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(Received 30th Oct 2018; accepted 16th Jan 2019)

Abstract. Quinoa (*Chenopodium quinoa* Willd.) is a nutritionally important plant with a good protein quality and a high concentration of vitamins and minerals. It has been cultured for several thousand years in South America. In this study, we investigated the use of inter-primer binding site (iPBS) for the molecular characterization of 17 quinoa genotypes (*Chenopodium quinoa* Willd.) cultivated in Turkey. For this purpose, 25 iPBS markers were employed, and six primers provided sufficient polymorphic data generating a total of 19 alleles with an average of 2.83 bands/primer. The number of iPBS bands per individual was calculated as 1.12. The rate of polymorphism information content ranged from 0.02 to 0.49 with an average of 0.20. Genetic associations were assessed using the Dice dissimilarity coefficient between different pairs of accessions and revealed an average value of 0.84 for the French population and Q-52 genotypes. Cluster analysis on the unweighted pair-group mean average divided the 17 quinoa genotypes into two major clusters. The results of the principal component analysis were in agreement with those of the cluster analysis. The highest number of alleles, Nei's genetic diversity, and Shannon's information index were obtained from the French Vanilla genotype at 1.99, 0.50 and 0.69, respectively, whereas the lowest values were observed in the Q–52 genotype at 1.10, 0.09 and 0.20, respectively. The expected heterozygosity ranged from 0.398 in the first sub-population to 0.140 in the second sub-population with an average of 0.269. The mean population differentiation measurement (F_{st}) values of the sub-populations were 0.048 and 0.676 for the first and second sub-populations, respectively. The results of this study provide useful information for the management of the quinoa germplasm and contribute to the improvement of existing breeding approaches. They also presented the iPBS marker system as a suitable tool for identification and genetic diversity analysis of quinoa genotypes.
Keywords: *Bayesian clustering, Chenopodium quinoa, genetic diversity, iPBS*

Introduction

In the region of Andean-South America, quinoa (*Chenopodium quinoa* Willd.) is considered to be one of the essential food crops. It is known as a member of the amaranth family (formerly *Chenopodiaceae*), which also contains other frugally essential species, such as spinach (*Spinacea oleracea* L.) and sugar beet (*Beta vulgaris* L.). Quinoa usually grows in saline and arid soils, frequent forests, and high altitudes of the Altiplano (Prado et al., 2000). Quinoa is traditionally widely consumed as a food crop by the people of the Altiplano region as part of their daily diet. Quinoa contains a perfect balance of lipids, protein, and carbohydrates, as well as amino acids essential for human nutrition (Chauhan et al., 1999). It is an allotetraploid (2n = 4x = 36) which has a domestic inheritance of most quality characters (Ward, 2000).

To simplify the application of molecular tools and enhance basic knowledge concerning quinoa, Fairbanks et al. (1990) and Ruas et al. (1999) reported DNA-based markers for this species based on the random amplified polymorphic DNA (RAPD) method. Microsatellite markers (SSR) have been developed to characterize the quinoa germplasm by Mason et al. (2005), Christensen (2007), Fuentes et al. (2009), Costa

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(2012) and Lu et al. (2015). Ana-Cruz et al. (2017) and Al-Naggar et al. (2017) also attempted to characterize the genetic diversity of a collection of quinoa using inter-simple sequence repeats. Employing markers, such as amplified fragment length polymorphism (AFLP), RAPD, and SSR, a genetic linkage map was established for quinoa (*C. quinoa*) (Maughan et al., 2004); furthermore, single nucleotide polymorphism (SNP) markers were developed (Coles et al., 2005; Maughan et al., 2012). Anabalon-Rodriguez and Thomet-Isla (2009) detected the level of polymorphism and the genetic relationships using the AFLP technique in quinoa (*C. quinoa*).

The fundamentally predominant existence of the tRNA complement as a converse transcriptase primer binding site (PBS) in LTR retrotransposons is a source of polymerase chain reaction (PCR)-based iPBS amplification (inter-primer binding site - iPBS). Specifically, the iPBS amplification technique was developed as a remarkable DNA fingerprinting technology that does not require any primary sequence data. Therefore, for controlling the changes in the DNA profile of plants, the iPBS marker system is an easy and fast process. This technique has been successfully used in flax (Smykal et al., 2011), apricot (Baránek et al., 2012), latvia (*Saussurea esthonica* L.) (Gailite and Rungis, 2012), chickpea (Andeden et al., 2013), guava (Mehmood et al., 2016), grape (Guo et al., 2014), okra (Yildiz et al., 2015), rice (Comertpay et al., 2015), lentil and pea (Baloch et al., 2015), tea (Phong et al., 2016), saffron (Gedik et al., 2017) and motherwort (*Leonurus cardiaca* L.) (Borna et al., 2017). In this study, to simplify the application of molecular tools and offer a better understanding of the genetic diversity of quinoa genotypes, we utilized iPBS molecular markers for these species for the first time in the literature.

The purpose of this experiment was to investigate the genetic diversity of 17 quinoas genotypes using the iPBS marker system, to evaluate the structure of the diversity in the germplasm, and to generate useful information for future breeding programs on quinoa.

Materials and methods

Genetic material

The quinoa (*C. quinoa* Willd.) genotypes were collected from different countries as a work package of a project supported by The Scientific and Technological Research Council of Turkey (TUBITAK) (TOVAG 214 O 232). A total of 17 quinoas genotypes were used in the experiment (*Table 1*).

Genomic DNA isolation

Total genomic DNA from the quinoa genotypes was isolated from 300 mg young leaf tissue using the method described by Zeinalzadeh-Tabrizi et al. (2015). For determination of the concentration and quality of genomic DNA, a spectrophotometer (Thermo Fisher Scientific) was used and electrophoresis was performed in 0.8% (w/v) agarose gel.

iPBS-PCR amplification

Twenty-five primers were used in iPBS-PCR reactions (*Table 2*). The PCR amplifications were carried out in a thermal cycler (Labcycler). The PCR mixture consisted of 10x buffer, 2 µM (20 pmol) primer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 U *Taq* polymerase, and 50 ng/µl DNA template in a total volume of 20 µl. The

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amplification conditions were; an initial denaturation step of five min at 95 °C, 38 cycles of 60 s at 95 °C, 60 s at 44-60 °C and 60 s at 72 °C, and a final extension step of 10 min at 72 °C. The amplification products were resolved in 1.5% agarose gel in 1X SB buffer at 100 V/cm for 120 min, stained with ethidium bromide (0.2 ug/ml), and visualized under a UV light in a Universal Hood II (Bio-Rad, Hercules, CA, USA). The sizes of the base pairs were determined based on a DNA ladder between 50 and 1000 bp (Vivantis Product No: NM2421).

Table 1. *List of quinoa (Chenopodium quinoa Willd.) accessions used in the experiment*

Number	Genotype name	Country
1	Ecuador 7	Ecuador
2	Q haslala Blanca	Peru
3	Red population	Peru
4	Q-52	Denmark
5	White Population	United Kingdom
6	Titicaca	Peru
7	UK6	USA
8	French Vanilla	USA
9	Red Head	USA
10	Sandoval Mix	United Kingdom
11	Mint Valle	USA
12	Oro de Valle	USA
13	Chinese Population	China
14	France Population	France
15	Chery Vanilla	USA
16	Moqu Arrochilla	Peru
17	Rainbow	USA

Data analysis

For each primer, the presence and absence of a strong and sharp polymorphic band were scored as 1 and 0, respectively using TotalLab TL120 software package (Germany). The association between the genetic dissimilarity was evaluated with the Numerical Taxonomy and Multiware Analysis System (NTSYSpc version 2.0) according to the Dice similarity matrix (Dice, 1945). Using the same software, an unweighted pair-group mean average (UPGMA) tree was constructed and a principle component analysis (PCA) was undertaken (Rohlf, 1998). The diversity of each iPBS marker was calculated using polymorphism information content (PIC) according to the following equation: $PIC = 1 - \Sigma pi2$, where P_{ij} is the frequency of the patterns (j) for each marker (i) (Anderson et al., 1993). To determine the genetic parameters, the number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were calculated by POPGEN 1.32 (Yeh et al., 1997).

The genetic structure datasets were formed to be determined by a model-based cluster analysis using STRUCTURE software version 2.2 (Pritchard et al., 2000a, b). The number of populations (K) was presented for a run of ten times for each population, which varied from 2 to 10, characterized by a set of distinctive allele frequencies at each locus, and individuals were situated in K clusters. In this process, the posterior

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probabilities were estimated using the Markov Chain Monte Carlo (MCMC) method. The MCMC chains were run with a 100,000-iteration burn-in period, followed by 100.000 iterations using a model allowing for admixture and correlated allele frequencies. The most expected value for K was predicted using Evanno's ΔK method (Evanno et al., 2005) using STRUCTURE HARVESTER. Furthermore, the expected heterozygosity (gene diversity) and population differentiation (Fst) between the individuals in a sub-population were determined using STRUCTURE (Earl, 2012).

Table 2. *Primers used in iPBS -PCR and retrotransposon, amplified fragment length*

Primer no.	Primer name	Primer sequence (5′→3′)
1	iPBS-2074	GCTCTGATACCA
2	iPBS-2075	CTCATGATGCCA
3	iPBS-2076	GCTCCGATGCCA
4	iPBS-2077	CTCACGATGCCA
5	iPBS-2080	CAGACGGCGCCA
6	iPBS-2221	ACCTAGCTCACGATGCCA
7	iPBS-2222	ACTTGGATGCCGATACCA
8	iPBS-2224	ATCCTGGCAATGGAACCA
9	iPBS-2225	AGCATAGCTTTGATACCA
10	iPBS-2270	ACCTGGCGTGCCA
11	iPBS-2279	AATGAAAGCACCA
12	iPBS-2375	TCGCATCAACCA
13	iPBS-2376	TAGATGGCACCA
14	iPBS-2377	ACGAAGGGACCA
15	iPBS-2378	GGTCCTCATCCA
16	iPBS-2379	TCCAGAGATCCA
17	iPBS-2380	CAACCTGATCCA
18	iPBS-2381	GTCCATCTTCCA
19	iPBS-2390	GCAACAACCCCA
20	iPBS-2391	ATCTGTCAGCCA
21	iPBS-2392	TAGATGGTGCCA
22	iPBS-2400	CCCCTCCTTCTAGCGCCA
23	iPBS-2401	AGTTAAGCTTTGATACCA
24	iPBS-2402	TCTAAGCTCTTGATACCA
25	iPBS-2415	CATCGTAGGTGGGCGCCA

Results and discussion

Polymorphism revealed by iPBS primers

In this experiment, 25 iPBS primers were used to evaluate 17 quinoa genotypes, and only six primers generated a sufficient number of polymorphisms (*Fig. 1*) with four primers providing more than one polymorphic band. The average of polymorphic bands was calculated as 2.83, and that of monomorphic bands was 0.33. Based on these values, the number of iPBS bands per individual was calculated as 1.12 (*Table 3*).

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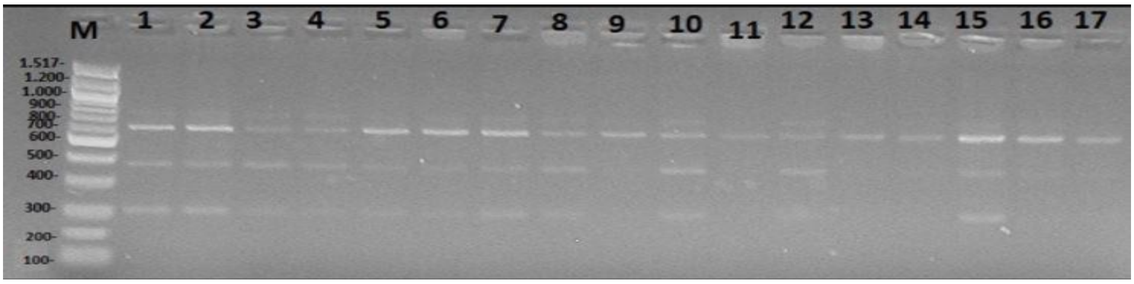

Figure 1. *iPBS profiles of 17 quinoa accessions obtained with the primer iPBS 2391*

Table 3. *Diversity statistics for Quinoa (Chenopodium quinoa Willd.) with iPBS*

Primer name	**Allele number**	**Percentage of polymorphism**	**PIC value**
iPBS-2080	7	100.00%	0.49
iPBS-2270	1	100.00%	0.03
iPBS-2279	3	66.66%	0.14
iPBS-2390	1	100.00%	0.02
iPBS-2391	4	75.00%	0.23
iPBS-2392	3	100.00%	0.18

Similar to our results, Mehmood et al. (2013) selected 6 primers out of 83 iPBS primer set in guava genotypes and Guo et al. (2014) selected 15 primers out of 41 iPBS primers in grape varieties, for further analysis. This is also in agreement with reports on other plants, such as grape (Guo et al., 2014), guava (Mehmood et al., 2016), and those investigated by Kalendar et al. (2010). The total number of polymorphic bands was 19 for iPBS markers, of which 17 were polymorphic and the remainder were considered as monomorphic. In this research, the number of alleles/polymorphic loci ranged from one to seven with an average of 3.16 for the iPBS analysis. Furthermore, the highest number of polymorphisms was obtained from iPBS-2080 with seven bands. In a study that aimed to develop molecular markers for the Fusarium wilt resistance gene in eggplant, Mutlu et al. (2008) found an average number of 1.5 bands per primer. The PIC value varied between 0.02 (iPBS 2390) and 0.49 (iPBS 2080), with an average of 0.20 (*Table 3*). These results are in agreement with earlier research into different plants, such as guava (*Psidium guajava* L.) cultivars with an average PIC of 0.28 (Mehmood et al., 2016) and tea (*Camellia sinensis*) cultivars with an average PIC of 0.30 (Phong et al., 2016). The percentage of polymorphism (%) value ranged from 66% (iPBS 2279) to 100% (iPBS 2080, 2390 and 2392) with an average of 90.27% (*Table 3*).

Genetic diversity in quinoa

Table 4 presents a summary of the statistical results for each of the 17 quinoa genotypes. The highest number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were obtained from the French Vanilla genotype at 1.99, 0.50 and 0.69, respectively, whereas the lowest values were observed in the Q–52 genotype at 1.10, 0.09 and 0.20, respectively. In addition, the total average number of alleles (ne), Nei's genetic diversity (h), and Shannon's information index (I) were 1.52, 0.032 and 0.49, respectively. Fuentes et al. (2009) used 20 SSR loci and calculated

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DOI: http://dx.doi.org/10.15666/aeer/1702_18991911

Shannon's index as 2.582 for the Highland genotype and 3.714 for the coastal genotypes Furthermore, the average value of Shannon's information index was reported as 0.12 in the Turkish okra germplasm (Yildiz et al., 2015), 0.29 for the *Crocus sativus* genotypes (Gedik et al., 2017), and 0.27 for guava (Mehmood et al., 2013) using iPBS-retrotransposon markers.

Table 4. *Summary statistics for 17 for Quinoa genotypes assessed with 10 iPBS primers*

Number	Genotype name	(ne)*	(h)	(I)
1	Ecuador 7	1.36	0.27	0.44
2	Q haslala Blanca	1.63	0.39	0.58
3	Red Population	1.63	0.39	0.58
4	Q - 52	1.10	0.09	0.20
5	White Population	1.63	0.39	0.58
6	Titicaca	1.23	0.19	0.34
7	UK6	1.23	0.19	0.34
8	French Vanilla	1.99	0.50	0.69
9	Red Head	1.50	0.33	0.51
10	Sandoval Mix	1.11	0.10	0.21
11	Mint Valle	1.50	0.33	0.51
12	Oro de Valle	1.36	0.27	0.44
13	Chinese Population	1.95	0.49	0.68
14	France Population	1.36	0.27	0.44
15	Chery Vanilla	1.50	0.33	0.51
16	Moqu Arrochilla	1.87	0.47	0.66
17	Rainbow	1.87	0.47	0.66
Mean		**1.52**	**0.32**	**0.49**

*ne = Effective number of alleles, h = Nei gene diversity, I = Shanon information index

Cluster analysis and principal component analysis for iPBS-retrotransposon markers

The Dice genetic similarity coefficient was used for the diversity estimation of the genotypes. This coefficient is commonly utilized to estimate genetic distances. The genetic similarity between the accessions based on the iPBS markers ranged from 11% to 100% with an average value of 55.5%. In this study, the 100% similarity value was found between the two most closely related accessions, Titicaca and UK6, and Q haslala Blanca and Red (*Table 5*). Nemli et al. (2014) reported similar findings in a study that explored the genetic similarity between the accessions of common bean (*Phaseolus vulgaris* L.) by peroxidase gene-based markers. The authors found the similarity coefficients to vary between 0.7 and 1.

The UPGMA tree constructed using the Jaccard genetic distance coefficient is presented in *Figure 2*. The analysis divided the quinoa genotypes into two main groups: Group A containing three genotypes (17.65%) and group B containing 14 genotypes (82.35%). Group A had two sub-groups: the first containing French Population and the second comprising the genotypes Chinese Population and Mint Valle. Similar to group A, group B had two sub-groups; the first containing Rainbow, Moqu Arochilla, and Red Head and the second comprising French Vanilla, USA 4, Red Population, Q Blanca, Sandoval Mix, Q-52, Chery Vanilla, Oro de Volle, UK6, Titicaca, and Ikwadur 7 (*Fig. 2*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_18991911

Table 5. *Dice genetic similarity coefficient among 17 quinoas genotypes based iPBS*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
(1) Ecuador 7	1																
(2) Qhaslala Blanca	0.11	1															
(3) Red Population	0.11	1.00	1														
(4) Q-52	0.16	0.26	0.26	1													
(5) White Population	0.11	0.21	0.21	0.26	1												
(6) Titicaca	0.05	0.16	0.16	0.11	0.16	1											
(7) UK6	0.05	0.16	0.16	0.11	0.16	1.00	1										
(8) French Vanilla	0.32	0.21	0.21	0.47	0.32	0.37	0.37	1									
(9) Red Head	0.37	0.47	0.47	0.21	0.37	0.32	0.32	0.58	1								
(10) Sandoval Mix	0.11	0.21	0.21	0.05	0.21	0.16	0.16	0.42	0.26	1							
(11) Mint Valle	0.63	0.53	0.53	0.79	0.53	0.68	0.68	0.32	0.58	0.74	1						
(12) Oro de Valle	0.11	0.21	0.21	0.16	0.21	0.05	0.05	0.42	0.37	0.21	0.63	1					
(13) Chinese Population	0.42	0.42	0.42	0.58	0.32	0.47	0.47	0.32	0.37	0.53	0.21	0.42	1				
(14) France Population	0.68	0.58	0.58	0.84	0.58	0.74	0.74	0.37	0.63	0.79	0.16	0.68	0.26	1			
(15) Chery Vanilla	0.16	0.26	0.26	0.21	0.26	0.11	0.11	0.47	0.42	0.26	0.68	0.05	0.47	0.63	1		
(16) Moqu Arrochilla	0.42	0.53	0.53	0.37	0.42	0.47	0.47	0.63	0.26	0.32	0.53	0.42	0.32	0.47	0.37	1	
(17) Rainbow	0.53	0.63	0.63	0.37	0.53	0.47	0.47	0.74	0.26	0.42	0.63	0.42	0.42	0.47	0.37	0.11	1

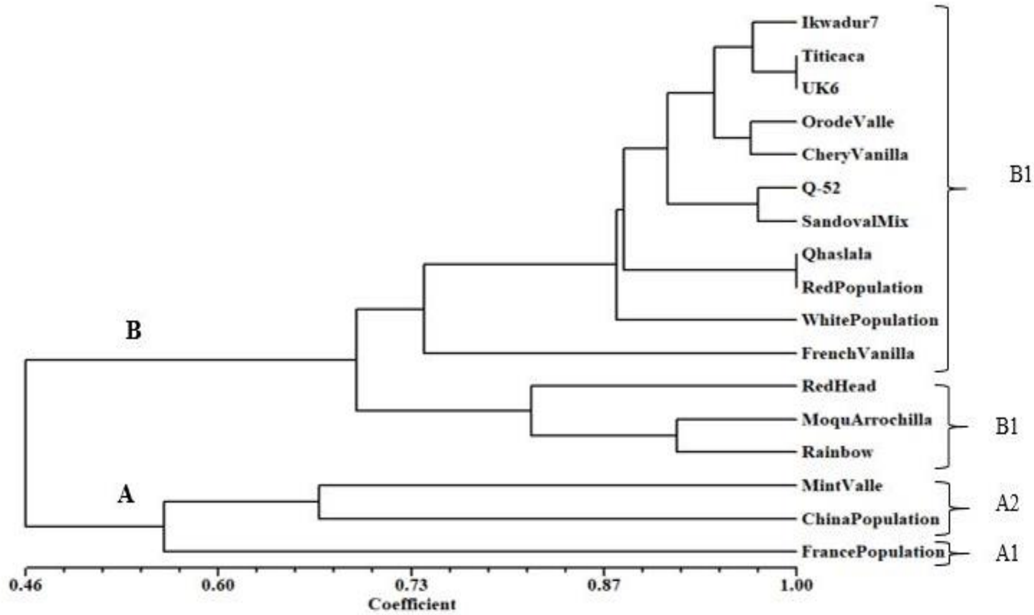

Figure 2. *Dendrogram of 17 Quinoa genotypes based on iPBS markers according to UPGMA with the Dice similarity index*

As a one-dimensional reduction technique, PCA can be used to review molecular marker profiles into some uncorrelated components (Hotelling, 1933). The pattern of cluster analysis was further confirmed by PCA because this method provides a better-defined structure than a dendrogram. In the current study, PCA was used to determine the variations between the two populations (*Fig. 3*). The clustering of varieties based on a dendrogram and a PCA plot was similar with no discrepancy being observed. This result is supported by Belaj et al. (2002) and Zargar et al. (2014), who reported similar

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findings using RAPD and SSR markers obtained similar findings related to the diversity of 32 olive cultivars based on the dendrogram topologies.

Christensen et al. (2007) evaluated the hereditary mutation in the USDA and CIP-FAO international nursery collections of quinoa (*Chenopodium quinoa* Willd.) by means of microsatellite markers and found that both UPGMA and PCA analyses divided the quinoa accessions into two key groups. Fuentes et al. (2009) investigated the genetic variety patterns in the Chilean quinoa (*Chenopodium quinoa* Willd.) germplasm using multiplex fluorescent microsatellite markers and reported that PCA divided PC1 into two major branches, conforming the clusters and groups of the highland and coastal quinoa accessions of the UPGMA analysis.

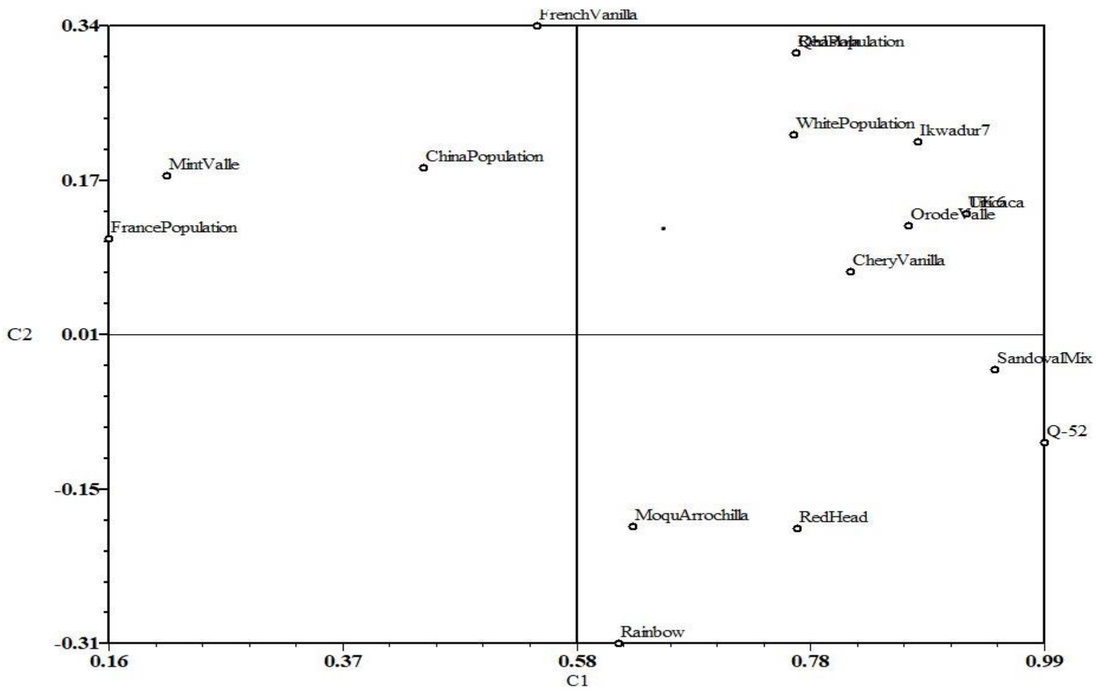

Figure 3. *PCA of 17 Quinoa genotypes based on 10 iPBS markers*

Population genetic structure analysis for iPBS-retrotransposon markers

Crop improvement is based on the understanding of the population assembly of germplasm collections. Before performing an association mapping study, it is crucial to first set the population structure within the germplasm to avoid spurious associations (Flint-Garcia et al., 2005). In this research, the population structure of the 17 quinoa accessions was categorized according to the iPBS data using STRUCTURE version 2.2 (*Fig. 4*) and two sub populations were found. Population 1 (POP 1) contained three accessions (French Population, Chinese Population and Mint Valle) and Population 2 (POP 2) consisted of the following 14 accessions (82.35% with membership probability of <0.7): Rainbow, Moqu Arochilla, Red Head, French Vanilla, USA 4, Red Population, Q Blanca, Sandoval Mix, Q-52, Chery Vanilla, Oro de Volle, UK6, Titicaca, and Ikwadur 7). The membership coefficient of the genotypes to specific sub-populations was very high and no possible admixture was detected in a reduced number of landraces. This may be due to the high rate of self-pollination of quinoa. Similar results were reported by Zhang et al. (2017) investigating the development of novel InDel

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DOI: http://dx.doi.org/10.15666/aeer/1702_18991911

markers and genetic diversity in *Chenopodium quinoa* through whole-genome re-sequencing. The authors found that according to both L (K) and △K values, the two groups presented the optimal classification for these quinoa accessions. Parallel results were reported with high degrees of genetic variation as detected by AFLP in *Sideritis tmolea* by Nemli et al. (2014), who revealed the presence of a model-based structural analysis of two populations. Yoon et al. (2012) reported genetic diversity and population structure analysis of two populations of strawberry (Fragaria x ananassa Duch.) using SSR markers.

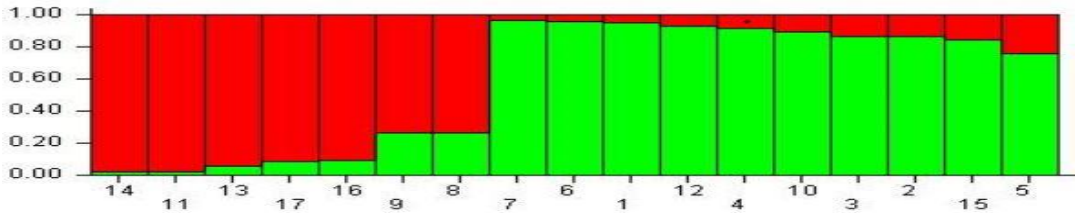

Figure 4. *Genetic structure of 17 quinoas genotypes as inferred by STRUCTURE software with 10 iPBS marker data sets. Single vertical line represents an individual accession and different colors represent genetic stocks/gene pools. Segments of each vertical line show extent of admixture in an individual*

In the current study, the population structure analysis confirmed the grouping of the genotypes, as observed by PCA and UPGMA clustering analyses. Similarly, Chen et al. (2015), who developed SSR markers to assess the genetic diversity of adzuki bean in the Chinese germplasm collection, showed that the structure and cluster analyses were usually consistent.

These results support the idea that dissimilar elevations in a topographical area might result in various levels of selection pressure for modification and could increase the differences within a population (Lopez-Gartner et al., 2009). The variation between gene pools suggests that cross breeding among these diverse areas will accelerate the process of diversifying germplasm creation and widen germplasm resources of quinoa. Meanwhile, efforts are being made to gather samples from different regions and produce the most effective markers to clarify the genetic diversity, population structure, and other details of population changeability in this ergonomically important genotype.

The expected heterozygosity which measures the probability of two randomly chosen individuals being different (heterozygous) in a given locus ranged from 0.398 in population 1 to 0.140 in the population 2 with an average of 0.269. The mean population differentiation measurement (F_{st}) values of the sub-populations were 0.048 and 0.676 (*Table 6*) for the first and second sub-populations, which was relatively high confirming the separation of the two sub-populations and their diversity in iPBS alleles. Similar results were reported by Zargar et al. (2016) who found that according to the population differentiation measurements (Fst), there were two distinct clusters or populations with an average Fst of 0.3301, indicating a clear separation of the sub-populations and their diversity in RAPD and SSR alleles. Blair et al. (2012) analyzed 108 common bean genotypes using 36 fluorescently labeled SSRs and also observed a high Fst value (0.203) for the genetic differentiation between all the five populations. In the current study, we obtained an even higher Fst value as a result of using the iPBS marker system.

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DOI: http://dx.doi.org/10.15666/aeer/1702_18991911

Table 6. *Heterozygosity and Fst value calculated for 2 quinoas sub-populations*

Sub-population (K)	Expected heterozygosity	Fst value
1	0.398	0.048
2	0.140	0.676
Average	0.269	0.362

Conclusion

Molecular markers are effectively used to explore genetic variation to enhance breeding efficiency. This study was undertaken using iPBS molecular markers for quinoa genotypes to simplify the application of this method and provide essential data on these genotypes. We also effectively categorized the population structure of 17 quinoa genotypes cultivated in Turkey using iPBS markers and model-based clustering. Moreover, the data obtained from the population structure analysis is valuable to perform association mapping on quinoa genotypes for various traits. The results obtained during the study can assist in the decision-making process concerning the selecting of markers for future experiments, as well as further characterization, breeding and management of the quinoa germplasm.

Acknowledgements. We greatly appreciate our grant from the Scientific and Technological Research Council of Turkey for project “TUBITAK 214 O 232”.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_18991911

Editorial Communication

RETRACTED

POST-ANTHESIS CHANGES IN CHLOROPHYLL AND AGRONOMIC TRAITS OF BREAD WHEAT GENOTYPES

This article has been retracted by the Editorial Board of Applied Ecology and Environmental Research, with the agreement of the author.

EFFECTS OF REAPING TIME ON VOLATILE COMPONENTS OF NATURAL *PHLOMIS RIGIDA* LABILL. AND *PHLOMIS MONOCEPHALA* P.H.DAVIS IN TURKEY

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(Received 3rd Nov 2018; accepted 16th Jan 2019)

Abstract. In this study, conducted between 2015 and 2017, 49 different volatile components were identified from *Phlomis rigida* Labill that was reaped from Gölcük highland located on Konya Seydişehir-Bozkır road and *Phlomis monocephala* P.H.Davis that was reaped from Mersin Silifke Bahçederesi Village in Turkey at three different periods (pre-flowering, flowering and post-flowering) through gas chromatography mass spectroscopy (GC-MS) after solid phase micro extraction (SPME). The main components of *Phlomis rigida* were found to be (E)-2-Hexenal (9.21%), β-Caryophyllene (60.23%) and Germacrene D (9.76%). The main components of *Phlomis monocephala* were found to be α-Pinene (15.59%), (E)-β-Farnesene (17.69%) and Germacrene D (18.92%). It was concluded that reaping *P. rigida* and *P. monocephala* species during the flowering period was important for the yield of volatile components.
Keywords: *Phlomis, GC-MS, Germacrene D, α-Pinene, Turkey*

Introduction

Turkey has a rich diversity of plant and animal species thanks to its geographical location, geological, geomorphological and climatic features. Turkey is a bridge between Asia and Europe; therefore, it has three different flora zones. For that reason, several species of Asian and European origin are distributed in Turkey (Durmuşkahya, 2005). Located at the intersection of three phytogeographical zones, Turkey has a high species endemism due to the high species richness and endemism by ecological and phytogeographical differentiation as a result of several factors such as its function as a bridge between the floras of South Europe and Southeast Asia, and since Anatolia is the hub of origin and differentiation for several genera and sections (Tan, 1992). Flora of Turkey has around 11,466 plant taxa, whereas European continent has around 12,000 plant taxa. Almost 3649 plant taxa out of those that are naturally distributed in Turkey are endemic (Güner et al., 2012). Hundreds of plant species naturally distributed in Turkey including the endemic species have a very high medical and aromatic value (Baydar, 2009).

Volatile oils have been used as therapeutic medicine since ancient times (Kubecka, 1973). Volatile oils are volatile lipoid mixtures with strong smell that are derived from plants or herbal drugs through water or steam distillation, and that are liquid under normal conditions but sometimes can be frozen (Tanker and Tanker, 1990). In particular, those medical and aromatic plants with a rich content of volatile oil are especially important. Volatile oils (extracts, etheric oils) and their aromatic extracts are

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19231928

commonly used in the fragrance and flavour industries as the source for perfumes, food additives, cleaning substances, cosmetic and medicine preparations, aroma-chemicals or initial material for synthesis of nature-identical and semi-synthetic beneficial aroma chemicals. Particularly, there has been a tremendous increase in the demand for volatile oils to be used in aromatherapy practices that have become popular in recent years (Weiss, 1997).

Across the world, Lamiaceae family that can grow nearly everywhere regardless of habitat type and elevation is one of the largest families considering the number of its members (Watson and Dallwitz, 1978). Its widest distribution area is located in the Mediterranean Basin, while the taxa in this family are mainly the open field plants except some of the genera growing in the tropical rain forests (Watson and Dallwitz, 1978; Morgaris et al., 1982; Davis, 1988). With 200 genera and around 3200 species, Lamiaceae family has 45 genera and 546 species in Turkey (Davis, 1982; Baytop, 1997).

Phlomis that is one of the genera of Lamiaceae family has around 100 species around the world. It is represented by a total of 52 taxa including 39 taxa and 13 hybrids in The checklist of the Flora of Turkey (Güner et al., 2012). Its appetising leaves and flowers are used as anti-allergic, diuretic, anti-diarrheal, gas relieving, stomach relieving, pain-killing, antidiabetic herbal tea and tonic. Furthermore, it s known to be commonly used by people for respiratory diseases and haemorrhoid (Harput et al., 2006).

In the last decade, there have been important developments with respect to the discovery of pharmacological mechanisms of *Phlomis* species and associated different components. The phytochemical studies on *Phlomis* showed that the species contained iridoid, flavonoid, phenylpropanoid, phenylethanoid, lignan, neolignan, diterpenoid, alkoloid and volatile oils (Kamel et al., 2000; Zhang and Wan, 2008).

Phlomis rigida Labill. is a perennial herbaceous plant that can grow as high as 125 cm with glandular hairs. Its leaves are large, oblong, elliptic or cuneate and have a size of 5-30 x 2-10 cm with pale greenish colour and tomentose hairs. It is verticillate, has 5-8- 18 flowers, numerous bracteoles, subulate 20-25 mm, calyx is 15-23 mm and dense hispid stellate-tomentose hairs, corolla is pink-purple. On the other hand, *Phlomis monocephala* P.H.Davis is a bush species that can grow as high as 150 cm with glandular hairs. Its leaves are ovate-lanceolate or ovate-oblong, cuneate or truncate, under leaf surface is yellowish and densely stellate-tomentose, upper hairs are greenish stellate-tomentose, and its size is around 2-6,5 x 1-3,5 cm. It is verticillate with 6-12 flowers, bracteole lanceolate is 5-8x1,5-2 mm long, calyx is 10-14 mm and has stellate-tomentose-lanate hairs, corolla is yellow (Davis, 1982). The purpose of this study was to identify the impact of different reaping times of *Phlomis rigida* and *P. monocephala* taxa on volatile oil components and determine the appropriate reaping time.

Materials and Methods

This study was conducted between 2015 and 2017. The research materials that were *Phlomis rigida* Labill. and *Phlomis monocephala* P.H.Davis were reaped from Gölcük Highland located on Konya Seydişehir-Bozkır road at an elevation of 1732 m (37°13′21′′N, 32°00′28′′E) and Mersin Silifke Bahçederesi Village in Turkey at an elevation of 527 m (36°21′17′′N, 33°48′28′′E), respectively (*Figure 1*). The samples were collected from the pre-determined sampling plots in 3 different periods, which were pre-flowering, flowering and post-flowering periods.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19231928

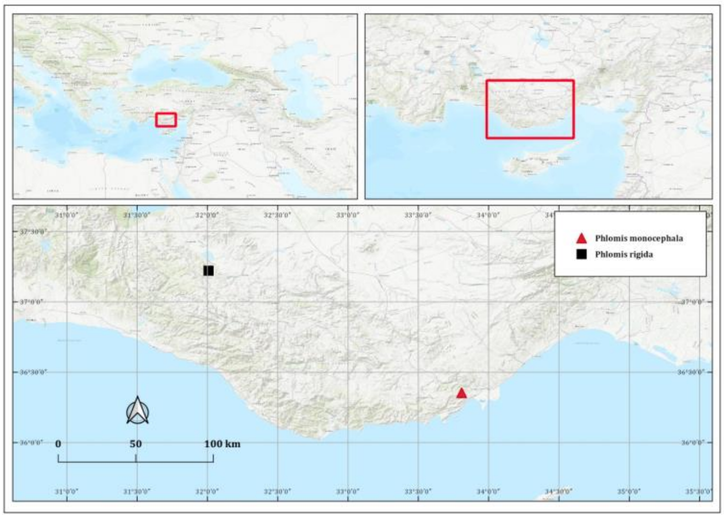

Figure 1. *Stands of collecting samples*

The leaf and flower samples of *Phlomis rigida* and *P. monocephala* collected from the sampling plots were placed in paper packages and transported to the laboratory without delay and avoiding their exposure to sunlight by private car in same day.

Flower and leaf samples were dried in room temperature (25ºC) and processed for solid phase microextraction (SPME). Samples were put into 10 mL vials from each after incubation at 60°C during 30 min. Then, GC-MS (Shimadzu 2010 Plus) process were applied at a temperature of 250°C for desorption (5 min) of the adsorbed volatile compounds for analysis. Constituents were identified by using libraries.

Results

The volatile components in the leaves and flowers of *Phlomis rigida* Labill. and *P. monocephala* P.H.Davis collected from the sampling plots were identified through gas chromatography mass spectroscopy (GC-MS) after solid phase micro extraction (SPME).

SPME analysis revealed 49 different volatile components *Phlomis r*igida and *P. monocephala*. The results of these samples are presented in *Table 1*.

(E)-2-Hexenal, β-Caryophyllene and Germacrene D were found to be the main components in *Phlomis rigida*. The rates in the pre-flowering period were as follows: (E)-2-Hexenal (8.23%), β-Caryophyllene (46.65%) and Germacrene D (8.74%); while the rates during the flowering period were as follows: (E)-2-Hexenal (9.21%), β-Caryophyllene (60.23%) and Germacrene D (9.76%); and the rates in the post-flowering period were as follows: (E)-2-Hexenal (8.85%), β-Caryophyllene (56.01%) and Germacrene D (9.29%) (*Table 1*).

α-Pinene, (E)-β-Farnesene and Germacrene D were found to be the main components of *Phlomis monocephala*. The rates in the pre-flowering period were as follows: α-Pinene (14.90%), (E)-β-Farnesene (16.47%) and Germacrene D (17.78%); while the rates during the flowering period were as follows: α-Pinene (15.59%), (E)-β-Farnesene (17.69%) and Germacrene D (18.92%); and the rates in the post-flowering period were as follows: α-Pinene (14.63%), (E)-β-Farnesene (15.69%) and Germacrene D (16.16%) (*Table 1*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19231928

Table 1. *Volatile components of Phlomis rigida Labill. and P. monocephala P.H.Davis in different vegetation periods*

			P. rigida			***P. monocephala***		
	Rt	**Components**	**Pre flowering**	**Flowering**	**Post flowering**	**Pre flowering**	**Flowering**	**Post flowering**
1.	1.403	Dimethyl sulphide	1.04	0.44	0.67	-	-	-
2.	1.440	2-Methyl-propenal	0.35	0.18	0.29	-	-	-
3.	1.517	3-Methyl-2-butanone	0.30	0.10	0.19	-	-	-
4.	1.887	Crotonaldehyde	0.37	0.23	0.39	0.77	0.80	0.58
5.	1.929	3-Methylbutanal	1.16	0.21	0.46	0.37	0.10	0.84
6.	2.009	2-Methylbutanal	1.08	0.33	0.85	0.06	0.10	0.09
7.	2.197	Ethyl vinyl ketone	0.32	0.09	0.15	-	-	-
8.	2.325	Pentanal	0.51	0.40	2.05	0.45	1.27	0.76
9.	2.357	Furan, 2-ethyl-	3.38	0.63	0.13	-	-	0.32
10.	3.181	(E)-2-Pentenal	0.30	0.12	0.30	0.25	0.73	0.62
11.	4.101	n-Hexanal	2.52	2.53	2.93	1.97	0.77	3.88
12.	5.514	(E)-2-Hexenal	8.23	9.21	8.85	0.78	1.40	1.85
13.	5.589	cis-3-Hexene-1-ol	1.71	0.34	1.13	0.11	0.13	0.35
14.	6.028	n-Hexanol	0.25	0.16	0.20	0.15	0.11	0.09
15.	7.025	Heptanal	1.62	0.91	0.82	0.15	0.39	0.24
16.	7.837	α-Thujene	0.20	0.17	0.20	0.18	1.01	1.73
17.	8.069	α-Pinene	1.37	0.56	0.86	14.90	15.59	14.63
18.	8.944	2-Heptenal	0.31	0.34	0.10	0.67	0.15	0.79
19.	9.043	Benzaldehyde	1.31	0.74	1.03	0.50	0.17	0.40
20.	9.647	2-.β.-Pinene	0.76	0.30	0.49	0.60	0.59	0.50
21.	9.909	Vinyl amyl carbinol	2.00	1.09	0.51	0.50	0.36	0.35
22.	10.023	6-Methyl-5-hepten-2-one	1.18	0.32	0.49	-	-	-
23.	10.202	Myrcene	0.65	0.20	0.26	1.86	1.20	1.07
24.	10.208	2-Pentylfuran	0.50	0.57	0.29	-	-	-
25.	10.527	2-[(2E)-2-Pentenyl]furan	0.31	0.41	0.30	-	-	-
26.	10.705	Octanal	0.47	0.40	0.21	0.41	0.18	0.78
27.	10744	α-Phellandrene	-	-	-	0.56	0.28	0.18
28.	10.968	2,4-Heptadienal	0.42	0.09	0.22	-	-	-
29.	11.458	Cymene	-	-	-	0.33	0.34	0.60
30.	11.649	Limonene	1.89	1.00	1.28	2.70	2.36	3.65
31.	12.005	3-Octen-2-one	0.09	0.10	0.20	6.79	6.97	5.72
32.	12.134	Benzeneacetaldehyde	0.57	0.38	0.55	-	-	-
33.	12,370	Trans-.β.-Ocimene	-	-	-	0.68	0.42	0.11
34.	12.763	(E)-2-Octenal	0.20	0.28	0.55	0.45	0.32	0.58
35.	14.417	Linalool	0.97	0.57	0.62	-	-	-
36.	14.590	n-Nonanal	0.65	0.98	0.52	0.70	0.29	1.24
37.	15.501	(4E,6Z)-Alloocimene	-	-	-	0.73	0.82	0.23
38.	17.824	Methyl salicylate	0.30	0.28	0.19	-	-	-
39.	18.452	Decanal	0.70	0.41	0.16	1.02	0.38	0.93
40.	22.975	Cyclohexane	-	-	-	0.22	0.23	0.17
41.	23.086	δ-Elemene	-	-	-	0.41	0.43	0.21
42.	23.506	.α.-Cubebene	0.23	0.22	0.16	5.16	5.13	5.38
43.	24.264	Ylangene	-	-	-	0.45	0.70	0.34
44.	24.479	.α.-Copaene	0.66	0.70	0.55	1.93	1.77	1.21
45.	24.744	.β.-Bourbonene	0.39	0.23	0.15	0.25	1.97	0.24
46.	24.949	.β.-Elemene	-	-	-	0.23	0.68	0.15
47.	25.546	.α.-Gurjunene	-	-	-	0.34	0.48	0.36
48.	26.076	β-Caryophyllene	46.65	60.23	56.01	1.07	1.01	1.26
49.	26.238	10,10-dimethyl-2,6-bis(methylene)-	0.43	0.44	0.25	0.30	0.50	0.70
50.	26.565	γ.-Muurolene	-	-	-	1.56	1.84	1.76
51.	26.570	(+)-Aromadendrene	0.26	0.17	0.51	-	-	-
52.	27.108	(E)- β-Farnesene	-	-	-	16.47	17.69	15.69
53.	27.124	.α.-Humulene	1.50	1.25	1.55	-	-	-
54.	27.359	Epi-bicyclosesquiphellandrene	-	-	-	0.30	0.52	0.50
55.	27.730	Cadina-1(6),4-diene	-	-	-	0.46	0.36	0.94
56.	27.971	Germacrene-D	8.74	9.76	9.29	17.78	18.92	16.16
57.	28.305	α.-Copaene	0.40	0.23	0.29	2.50	1.57	2.76
58.	28.438	Bicyclogermacrene	1.09	0.62	0.74	3.46	3.04	3.17
59.	28.548	α-Muurolene	0.21	0.17	0.50	0.25	0.62	0.20
60.	28.998	.γ.-Cadinene	0.39	0.21	0.50	2.80	0.41	0.71
61.	29.176	δ-Cadinene	0.73	0.40	0.54	3.83	4.27	3.62
62.	30.409	Germacrene B	-	-	-	1.38	0.38	1.08
63.	30.954	Spathulenol	-	-	-	0.21	0.25	0.28
64.	31.105	Caryophyllene oxide	0.33	0.30	0.52	-	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19231928

Discussion and Conclusions

SPME analysis revealed that *Phlomis rigida* Labill. and *P. monocephala* P.H.Davis had 49 different volatile components. Main components of *Phlomis rigida* were (E)-2-Hexenal (9.21%), β-Caryophyllene (60.23%) and Germacrene D (9.76%). Demirci et al. (2006) found through GC-MS analysis that the main components of *Phlomis rigida* Labill. were β-Caryophyllene (31.2% - 38.7%) and β-selinene (13.1% - 15.1%). β-Caryophyllene was found to be the dominant component in that study. This finding is consistent with our result. Contrary to the abovementioned study, (E)-2-Hexenal and Germacrene D were also identified as dominant components in our study.

α-Pinene (15.59%), (E)-β-Farnesene (17.69%) and Germacrene D (18.92%) were found to be the main components of *Phlomis monocephala*. In a study conducted by Demirci et al. (2009), they aimed at exploring the antimicrobial effects and phytochemical profile of *Phlomis lunariifolia* Sm., *Phlomis amanica* Vierh., *Phlomis monocephala* P.H. Davis, *Phlomis sieheana* Rech. fil., *Phlomis armeniaca* Willd. species in Turkey. They applied GC and MS for the analysis of the volatile oils that they derived through hydrodistillation. They identified 143 components in the volatile oils of *Phlomis amanica* Vierh. and *Phlomis monocephala* P.H.Davis. They found that 8(14), 15-Isopimaradien-11α-ol (1) were the main components in the volatile oils of *Phlomis monocephala* P.H.Davis. This finding is different from our result. Contrary to that study, Germacrene D, (E)-β-Farnesene and α-Pinene were identified as the dominant components in our study.

In conclusion, the volatile components β-Caryophyllene, (E)-2-Hexenal, Germacrene D, (E)-β-Farnesene and α-Pinene obtained in this study may be considered as potential sources and materials for the pharmacology and cosmetic industry thanks to their antimicrobial activities. It was found that reaping *P. rigida* and *P. monocephala* species, which were collected from the field in the form of leaves and flowers, during the flowering period was important with respect to the yield of volatile components. These findings are considered to help preventing the haphazard collection of plants by traders and local people and economic losses that may arise due to misinformation and raise awareness for the collection of the plants. Although *Phlomis* taxa are used in many areas, there are limited numbers of studies conducted on *Phlomis* taxa in Turkey. There is a need for further studies in this field.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19231928

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF THREE ECOTYPES OF CAROB (*CERATONIA SILIQUA* L.) AGAINST DROUGHT STRESS IN ALGERIA

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(Received 9th Sep 2018; accepted 2nd Jan 2019)

Abstract. The greatest failure rate of reforestation programs in Algeria is related to the water deficit especially at young plant stage. Hence, the objective of this paper is to study the responses to drought stress generated by three different water regimes 100% (control), 60% (moderate stress) and 40% (severe stress) of field capacity (FC) on 3 young populations of *Ceratonia siliqua* L. in Algeria with different eco-geographical origins (*Zeralda, Tissemsilt* and *Ain Sefra*). The germination seeds of these populations were conducted in February 2013. The results exhibit a significant decrease in stomata conductance (cs) and in relative water content (RWC) with the increase in the intensity of drought stress. Negative and significant correlations were recorded between the RWC and the intensity of drought stress in the studied plants compared to the control plants. A considerable increase of proline and soluble sugar was positively correlated with drought stress severity for the three ecotypes of *Ceratonia siliqua* L. Significant differences in the chlorophyll and carotenoid content were detected among the three ecotypes in three water regimes. According to the canonical discriminant analysis of our data, the three ecotypes were separated by the physiological and biochemical parameters studied. It is clear that the ability of drought tolerance in the three studied contrasting ecotypes is different. We suggest the following order with respect to the ability of drought tolerance: *Ain Sefra - Tissmesilt - Zeralda.*
Keywords: *water regimes, proline, soluble sugar, young plant, relative water content*

Introduction

Most of Mediterranean natural plants are exposed to persistent and severe drought stress (Nogués and Baker, 2000). Drought, the most important abiotic stress, affects the physiological and biochemical process in plants leading to a reduction of growth and productivity (Yoon et al., 2014; Lambers et al., 2008).

Drought significantly reduces germination mainly due to low water absorption during the imbibition phase of germination, to the low energy supply and to the decreased enzyme activity (Okcu et al., 2005; Taiz and Zeiger, 2010). It also reduces leaf size, stem elongation and root proliferation, and disturbed stomata variations, plant water and nutrient relations with diminished crop productivity (Li et al., 2009).

Responses of plants to drought stress in the arid and semi-arid areas are complex and different mechanisms (morpho-anatomical, physiological, biochemical and molecular) involve allowing the plant to survive (Rodziewicz et al., 2014). There are often the main mechanisms of tolerance to water deficit (Tardieu, 2005); (i) maintenance of leaf water

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potential (RWC, stomatal conductance), (ii) the biosynthesis and accumulation of various osmolytes (proline, soluble sugars), (iii) and the activation of different resistant genes (Chaves et al., 2003; Reddy et al., 2004).

In Algeria, a gradual rise in aridity towards the north was observed over the past thirty years. This aridity is marked by a pronounced increase in the degradation of all components of the ecosystem (Ait Chitt et al., 2007).

This desertification, which reaches the most advanced stage of land degradation resulted in the reduction of biological potential, the breakdown of ecological and socio-economic equilibrium, and a remarkable regression of plant genetic resources (Le Houérou, 1985). Central and north-western regions of Algeria are characterized by rare and irregular rainfall, and long dry summer periods (Batlle and Tous, 1997). Forests are in a perpetual decline due to anthropogenic activities such as forest fires, overgrazing, and uncontrolled urbanization (Batlle and Tous, 1997). As a result, several forest development programs have been implemented in many areas of Algeria. To that end, the government uses the multipurpose tree and drought resistant species that exhibit morpho- physiological traits and genetic adaptation to climatic variation (Ait Chitt et al., 2007). Among these species is *Ceratonia siliqua* L. which is a perennial evergreen tree. It is an agro-sylvo-pastoral species with enormous socio-economic and ecological interests (Batlle and Tous, 1997; Gharnit et al., 2001). Carob tree is considered one of the most interesting forest trees since all its parts (leaves, flowers, fruits, wood, bark, and roots) are exploited (Aafi, 1996).

Although carob tree is a non-nodulable species, endophytic associative bacteria can be observed in their root systems (Nautiyal et al., 2000; Reva et al., 2002).

The presence of these bacteria inside vegetative tissues would probably contribute significantly to the palliation of nutritional deficiencies of carob, which settles favorably on poor soils. They have a great ability to solubilize and release certain mineral or organic elements such as phosphorus and iron origins essential for plant nutrition (Konate, 2007).

The carob tree is mainly concentrated in coastal, semi-arid and arid zones, owing to its great ability to develop adaptation strategies by reducing its leaf area, leaf curling (Rejeb, 1995; Batlle and Tous, 1997) and enhancing the growth of its root system. Often, the response of a plant with a water deficit results in a preferential allocation of biomass to the roots expressed by an increase in the dry matter ratio between the underground part and the aerial part (Gales, 1979; Benbelkacem et al., 2000; Albouchi et al., 2003).

The greatest failure rate of revegetation programs faced by carob trees is at the seedling stage. The installation of the seedling root system requires adequate soil hydration and will be more difficult in heavy areas characterized by a high saturation deficit (Letreuch, 1991). Vegetation with weak rooting can disappear; also, we propose the study of physiological mechanisms of resistance to drought stress on three ecotypes of *Ceratonia siliqua* L. aged 18 months, from three different bioclimatic regions of Algeria.

Materials and methods

Plant material

Populations of *Ceratonia siliqua* L. were collected from three different bioclimatic stages in the Centre and North-Western of Algeria respectively in Zeralda (wet

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bioclimatic stage), Tissemsilt (upper semi-arid bioclimatic stage) and Ain Sefra (arid bioclimatic stage). Climatic, geographical and hydrological conditions of these three regions are markedly different (*Table 1*). Sapling locations are illustrated in (*Fig. 1*).

Table 1. *Geographical and climatic characteristics of the different sites of Ceratonia siliqua L. ecotypes. (ANAT, 2004)*

Ecotype	Latitude	Longitude	Altitude (m)	Pluviométrie	T min (°C)	T max (°C)
Zeralda	36.7040 N	2.8672 W	30	600-900	0-9	28-31
Tissemsilt	35.9049 N	1.5248 W	866	400-600	2-4	33-38
Ain Sefra	32.7439 N	-0.8801 W	1078	100-300	-10	35-42

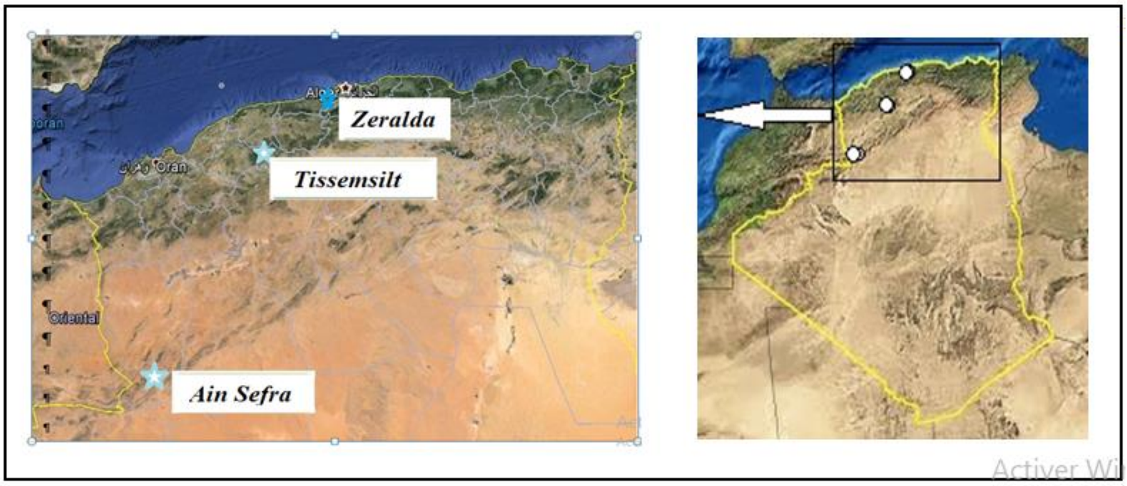

Figure 1. *Repartition map of the different sites of Ceratonia siliqua L. ecotypes of Algerian carob populations*

Culture conditions

After being scarred with a sharp instrument at the opposite side of the embryo, seeds were disinfected using bleach diluted for 10 min and finally rinsed twice with distilled water and deposited in Petri dishes on filter paper a reason of 10 seeds per petri dish. Petri dishes were put in an oven 48 h at 27 °C in the dark. Germination rate are 54.5%, 100%and 74.4%for *Zeralda, Tissemsilt* and *Ain Sefra,* respectively.

Plants were transferred into pots (15 × 27 cm) filled with a mixture of peat and soil 2/3 (v/v) with one seedling per pot and watered daily with tap water. The experiment was conducted at 28 ± 1°C and the range of relative humidity is 60% to 70% over 18 months.

The experiments were performed during July 2015, on plants at the same stage of development. 162 young plants of *Ceratonia siliqua* L., 18 months old have been divided into three groups which undergo three different water regimes:100% (control), 60% (moderate stress) and 40% (severe stress) of field capacity (FC).

The treatments were applied during two months and each treatment included eighteen plants for each ecotype. Leaves were collected after seventeen days of treatment. Three plants per treatment were used for each experiment.

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Physiological parameters

Relative water content

The water status of the plants is measured by the Relative Water Content (RWC) according to Ladiges (1975), used by Clarke et al. (1982) and Rascio et al. (1988). RWC calculations were made according to *Equation 1*:

$$RWC (\%) = [(FW - DW) / (TW - DW)] \times 100, \qquad (Eq.1)$$

where FW, TW and DW refer to fresh weight, Turgid weight (after 24 h rehydration on distilled water), and dry weight after oven drying for 48 h at 70 °C respectively.

Stomata conductance

Stomata conductance (CS) was measured using a porometer (AP4DELTA-T Devices, Cambridge, UK) and on 18 month old plants. Measurements were made on fully exposed leaves between 10 h and 12 h on the lower face of three young leaves per plant. Stomata conductance has been expressed in mmol $H_2O/m^2\ s^{-1}$.

Biochemical parameters

Proline content

The determination of the proline content is carried out according to the colorimetric method of Troll and Lindsley (1955) later developed by Magné and Larher (1992).

The determination of the proline content is carried out according to the colorimetric method of Troll and Lindsley (1955) later developed by Magné and Larher (1992). Leaf samples (150 mg) are extracted by using 3 mL of methanol placed in batch at 90 ° C for 1 h. After cooling, extract (1 mL) was treated with 2 mL of ninhydrin solution (1% in glacial acetic acid). After addition of 2 mL toluene, the mixture is stirred vigorously and the upper phase is removed. The optical density is read at 520 nm with a spectrophotometer (U.V/visible Shimadzu Modèle V630). The results are referred to a standard curve made of increasing quantities of proline solution. The results are expressed in µg. g-1 MS. Three repetitions are planned by treatment between 08 h and 10 h (one plant per replicate).

Soluble sugar

The soluble sugar content (SS) was measured according to Schields and Burnet (1960). 5.25 ml of 80% ethanol was added to leaf tissues (100 mg) in a test tube at room temperature in the dark. After 20-h extraction, ethanol was evaporated in a water bath at 70 °C. Then, 2 mL of this solution previously diluted 10 times with ethanol 80% was transferred into another tube, adding 4 ml of reagent (prepared 4 h before the test) composed of 2 mg pure anthrone added to 100 mL of sulfuric acid and vortexing to homogenize the solution. After 10 min, tubes were placed again in the water bath for 08 min at 92 °C. The absorbance is read at the spectrophotometer (U.V/visible Modèle V630) at a wave length of 585 nm. The results are referred to a standard curve made of increasing quantities of a solution of glucose and the concentration is expressed in $\mu g.g^{-1}$ of MS. Three repetitions are planned by treatment (one plant per replicate).

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Chlorophyll pigments

The levels of chlorophyll (a + b) and carotenoids are determined according to Lichtenthaler (1987). The extraction is performed through cold acetone. A measure of the absorbance is made of 470, 662 and 645 nm using a spectrophotometer Shimadzu (UV-1605). The levels in pigments, expressed in $\mu g.ml^{-1}$ of MS, are calculated in *Equations 2* and *3:*

$$C\ (a + b) = 7.05\ A662 - 18.09\ A645 \quad \text{(Eq.2)}$$

$$C\ (x + c) = (1000\ A470 - 1.90Ca - 63.14Cb)\ 214 \quad \text{(Eq.3)}$$

where C(a + b) is total chlorophyll concentration of and C(x + c) is caroténoid concentration.

Statistical analysis

All statistical analyses are performed using SAS 9. On 162 observations, the ANOVA procedure of SAS was used to adjust the generalized linear model (GLM). The fixed factors are the accessions and the different water regimes. The variable factors chosen for this analysis are the various physiological and biochemical parameters.

The signification is chosen for a value of $p = 0.05$. In the declaration of the model, the options of Statistics Type 1 and Type 3 are used.

To differentiate between groups of accessions and water regimes which are homogeneous on the statistical plan, we used the test of Waller-Duncan. The meaning $\chi = 0.05$ in ANOVA was chosen, with the selection of the ANOVA model means of SAS 9.

All statistical analyses were performed using the Database Software SAS, version 9 for Windows, Version 7. The graphical extrapolation of the results was performed with Microsoft Excel software.

Results

Physiological parameters

The relative water content (RWC) of control seedlings of Zeralda ecotype (87.08%±5.16) was higher than Tissemsilt and Ain Sefra ecotypes with 83.22%±1.23 and 72.32%±7.92 respectively (*Fig. 2A*).

Moderate and severe drought stresses cause a decrease in RWC proportional to the severity of stress. However, Zeralda ecotype still has the largest RWC and Ain Sefra ecotype has the lowest regardless of water regime (*Fig. 2A*).

Highly significant differences between the three water regimes in Tissemsilt (ddl = 2; value $F = 9.38$; $P = 0.0035$) and Zeralda ecotypes (ddl = 2; Value $F = 20.25$; $P < 0.0001$) with $R2 = 60.99\%$, and no significant difference between the three water regimes of Ain Sefra ecotype were observed for RWC.

In control plants, the highest values of stomata conductance were noted in *Ain Sefra* ecotype with 11.16 ± 1.12 mmol $H_2O/m^2/s$. Moderate and severe water stress cause a slight decrease in stomata conductance in *Zeralda* and *Tissemsilt* ecotypes. The most significant decrease was, however, recorded in *Ain Sefra* ecotype (*Fig. 2B*). We have noted that drought stress (moderate and severe stress) significantly reduced ($P < 0.001$)

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stomata conductance of *Ain Sefra* ecotype (ddl = 2; F = 76.74; P < 0.0001). There is no significant variation in stomatal conductance in moderate and severe stress of *Zeralda* and *Tissemsilt* ecotypes.

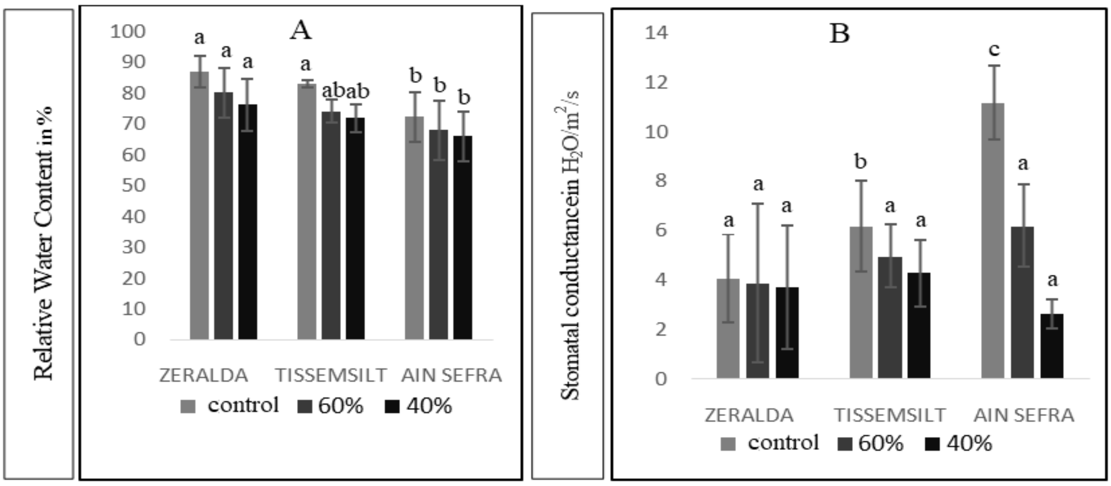

Figure 2. *Effect of drought stress on leaf water content (A) and stomatal conductance (B) of three Ceratonia siliqua L. ecotypes. Eighteen-month-old young plants were exposed to three water regimes (control, 60 and 40% of field capacity (FC)) during 17 days. Mean ± SE (n = 3) with distinct letters are significantly different at 5% (Waller-Duncan test)*

The statistical analysis shows that RWC and CS are strongly influenced by ecotype and water regimes (p < 0.05). The water regime (WR) has a significant effect on the expression of RWC (p < 0.0001) and a significant variation (p < 0.05) on CS. The interaction of the two factors (type of ecotype and water regime) also exerts significant variations on RWC and CS (p < 0.05). Therefore, the ecotypes tested reaction distinctly with respect to the intensity of drought stress.

This study shows also that RWC is the first parameter affected by the progression of water stress severity of *Zeralda* and *Tissemsilt* ecotypes (P < 0.001). Under drought stress, especially in severe water stress (40% of field capacity), we noticed a significant decrease in RWC compared to the control (P < 0.001). However, we did not observe a significant difference in the different water regimes *of Ain Sefra* ecotype (*Table 2*).

Table 2. *Statistical analysis of the variance of water regime (WR), ecotype (ECO) and ECO×WR interactions in Ceratonia siliqua L.*

	ECO		**WR**		**ECO×WR**	
RWC	29.88	<.0001	18.82	<.0001	2.72	0.0319
CS	16.8	<.0001	7.12	0.0011	5.48	0.0004

Biochemical parameters

Stressed plants showed significantly higher concentrations of soluble sugars (*Fig. 3A)* and proline than control plants (*Fig. 3B*). An increase in the level of proline and soluble sugar, which is a function of the severity of stress in the studied ecotypes, is

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observed. In severe drought stress, the highest concentrations of proline and soluble sugars were found in the *Ain Sefra* ecotype (*Fig. 3*).

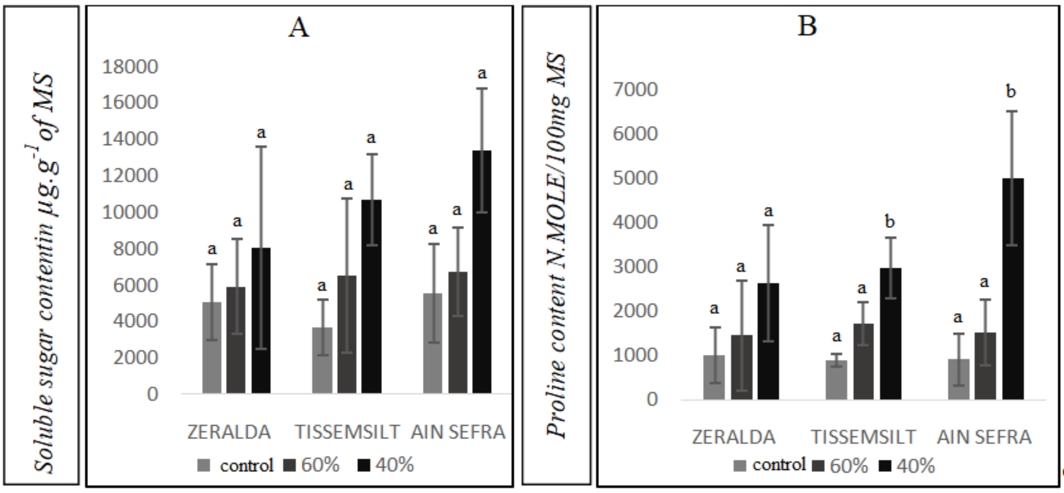

***Figure 3.** Effect of drought stress on prolin content and soluble sugar of three Ceratonia siliqua L. ecotypes. Eighteen-month-old young plants were exposed to three water regimes (control, 60 and 40% of field capacity (FC)). Mean ± SE (n = 3) with distinct letters are significantly different at 5% (Waller-Duncan test)*

A very high significant difference was detected in the concentration of the proline between the different water regime of *Zeralda*, *Tissemsilt* and *Ain Sefra* (*Table 3*).

Water Regime has a tremendous effect on proline and soluble sugar accumulation ($p < .0001$). The interaction of the two factors exerts also significant variations ($p < 0.05$) while the ecotype factor has no significant effect (*Table 4*).

***Table 3.** Analysis of variance of the proline and soluble sugars of the plants of Ceratonia siliqua Lunder three water regimes (control, 60 and 40% of field capacity (FC))*

	Zeralda		Tissemsilt		Ain Sefra	
	F	**P**	**F**	**P**	**F**	**P**
Proline	22.33	<0.0001	16.76	0.0003	45.34	<0.0001
Soluble sugars	6.33	0.0025	5.33	0.0221	17.12	<0.0001

The relationship between the accumulation of proline and the water regime was studied using the regression procedure of SAS 9. We have found a strong positive correlation between the accumulation of proline and the water regime (ddl = 1; value of the test t = 8.87; $p < 0.0001$).

***Table 4.** Statistical analysis of the variance of water regime (WR), ecotype (ECO) and ECO×WR interactions in Ceratonia siliqua L.*

	ECO		WR		ECO(WR)	
	F	**Pr>F**	**F**	**Pr>F**	**F**	**Pr>F**
Proline	1.06	0.3502	49.74	<.0001	6.26	0.0001
Soluble sugar	1.77	0.1737	15.86	<.0001	2.7	0.0329

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Our results show a notable difference in soluble sugars of *Ain Sefra's* ecotype. In contrast, there has been a significant difference between *Zeralda* and *Tissemsilt* (*Table 3*).

In control plants, the highest values of total chlorophyll were observed in *Zeralda*'s ecotype with 4.70 ± 2.23 µg.mL^{-1}. For the three ecotypes, total chlorophyll contents are lower in stressed plants compared to control plants (*Fig. 4A*).

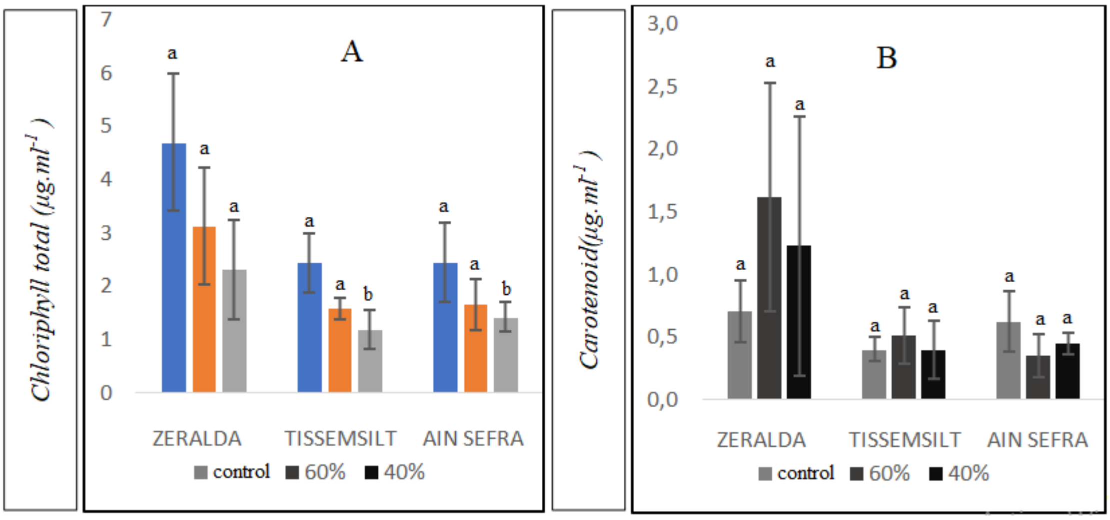

Figure 4. *Effect of drought stress on chl T and carotenoids of three Ceratonia siliqua L. ecotypes. Eighteen-month-old young plants were exposed to three water regimes (control, 60 and 40% of field capacity). Mean ± SE (n = 3) with distinct letters are significantly different at 5% (Waller-Duncan test)*

Significant differences in the total chlorophyll content between the three water regimes (F = 4.60, P = 0.0119), (F = 12.64, P = 0.0011) and (F = 8.71, P = 0.0012) are recorded in *Zeralda, Tissemsilt* and *Ain Sefra* ecotypes respectively (*Table 5*).

Table5. *Statistical analysis of the variance of water regime (WR), ecotype (ECO) and ECOxWR interactions in Ceratonia siliqua L.*

	Zeralda		***Tissemsilt***		***Ain Sefra***	
	F	**Pr>F**	**F**	**Pr>F**	**F**	**Pr>F**
Chlorophyll	4.60	0.0119	12.64	0.0011	8.71	0.0012
Caroténoid	4.62	0.0118	0.51	0.6138	6.08	0.0066
Raportchl a/b	3.90	0.0230	1.11	0.3637	13.21	0.0001

Water regimes altered the content of carotenoids among the ecotypes of *Ain Sefra* (F = 6.08; P = 0.0066) and *Zeralda* (F = 4.62; P = 0.0118), but not for the ecotype of *Tissemsilt.* The greatest value of the carotenoid has been recorded in the ecotype of *Zeralda* under moderate stress with 1.65 ± 0.92 *µg.ml*$^{-1}$ (*Fig. 4B*).

The highest Chl a/b ratio for the control plants was noted in *Ain Sefra's* ecotype. It significantly decreased in stressed plants of *Ain Sefra* (F = 13.21, P = 0.0001) and

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Zeralda (F = 3.90, P = 0.023) ecotypes (*Table 6*). Conversely, WR has no significant effect on the Chl a/b ratio Tissemsilt's ecotype (p < .0001) (*Table 5*).

Table 6. *Effect of drought stress on ratio Chlorophyll a/b of three Ceratonia siliqua L. ecotypes. Eighteen-month-old young plants were exposed to three water regimes (100, 60 and 40% of FC) during 17 days. Mean ± SE (n = 3)*

	Chlorophyll a/b		
	WR		
	100%	**60%**	**40%**
Zeralda	1.40 ± 0.78	1.79 ± 0.93	2.02 ± 1.42
Tissemsilt	1.06 ± 0.36	1.36 ± 0.42	1.66 ± 0.33
Ain Sefra	2.20 ± 1.06	0.78 ± 0.43	0.72 ± 0.43

Canonical analysis

The analysis of principal components (APC) of osmolytes shows two separate groups (*Fig. 5*; *Tables 7* and *8*). The first group includes the stands in the region of *Zeralda*. In the second group, there are still two sub-groups; one constituted of the whole samples of *Tissemsilt* and the other formed by the samples of *Ain Sefra.*

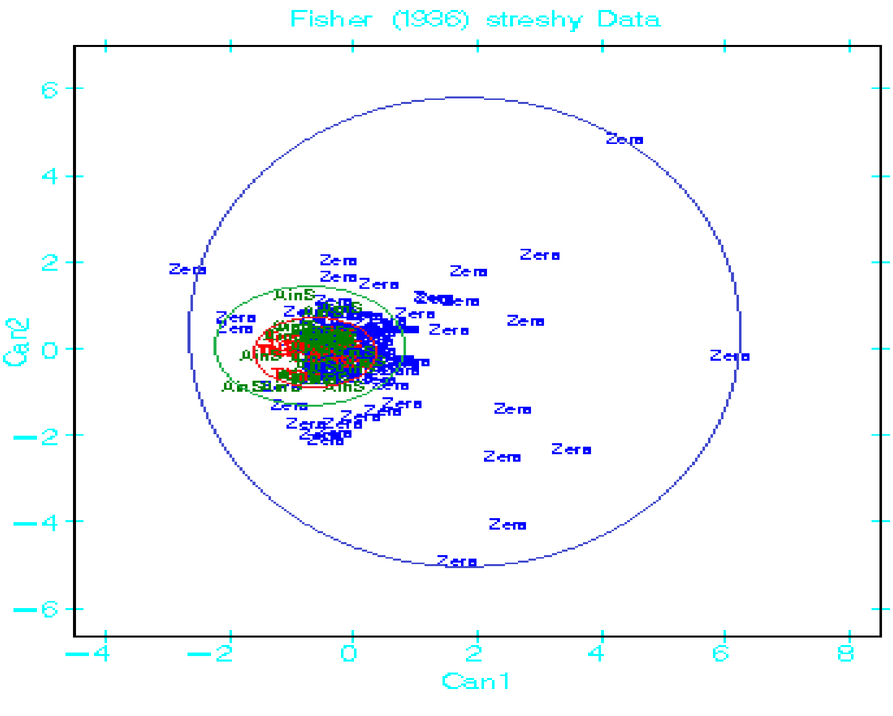

Figure 5. *Projection of points means of the regions studied on the first plan factorial of a principal component analysis*

Table 7 shows the point's means of the regions on the canonical variables. Group one is represented by *Zeralda* ecotype (means points (0.22/-0.0007)) and the group two is represented by ecotypes of *Tissemsilt* (means points (−0.63/0.061)) and *Ain Sefra* (means points (-0.55/0.033)) ecotypes.

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Table 7. *Medium of classes on the canonical variables*

Class meanings on canonical variables		
Ecotype	Can1	Can2
Ain Sefra	-0.5528	0.0338
Tissemsilt	-0.6304	-0.0616
Zeralda	0.2225	-0.0007

This interpretation is performed according to the plan 1-2 because it provides the maximum of information with 100% contribution to the total variation (99.56% of contribution for the axis 1 and 0.44% for the axis 2).

The values of squared distance, estimated between ecotypes studied two by two, do not exceed 3.002, indicating an overlap between the ecotypes (*Table 8*).

The first group includes the ecotypes of the locality of *Zeralda*. The second group brings together the stands of the locality of *Tissemsilt*. Finally, the third group is represented by the stands from the locality of *Ain Sefra*.

According to *Table 8*, squared distance between ecotype is less than 50 (maximum value 35 between *Zeralda* and *Ain Sefra* ecotype), which shows an overlap between the three studied ecotypes.

Table 8. *Distance square, value of F for the distance square and value of the probability of square distance between the different ecotypes*

Squared distance to ecotype			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	0	0.01513	0.60256
Tissemsilt	0.01513	0	0.73136
zeralda	0.60256	0.73136	0
F statistics			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	0	0.02950	2.80515
Tissemsilt	0.02950	0	1.89583
zeralda	2.80515	1.89583	0
Prob>			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	1.0000	0.9996	0.0187
Tissemsilt	0.9996	1.0000	0.0981
zeralda	0.0187	0.0981	1.0000

With respect to the parameters of RWC and CS, the graphical interpretation of the APC results is performed mainly in function of the plan 1-2 because it provides the maximum information with 100% contribution to the total variation (99.41% contribution of the axis 1 and 0.59% for axis 2) (*Fig. 5*)

Figure 6 shows the projection of the point's means of colonies on the first plan of a CPA. It is to show a clear differentiation in 3 distinct groups (*Fig. 6; Table 9*).

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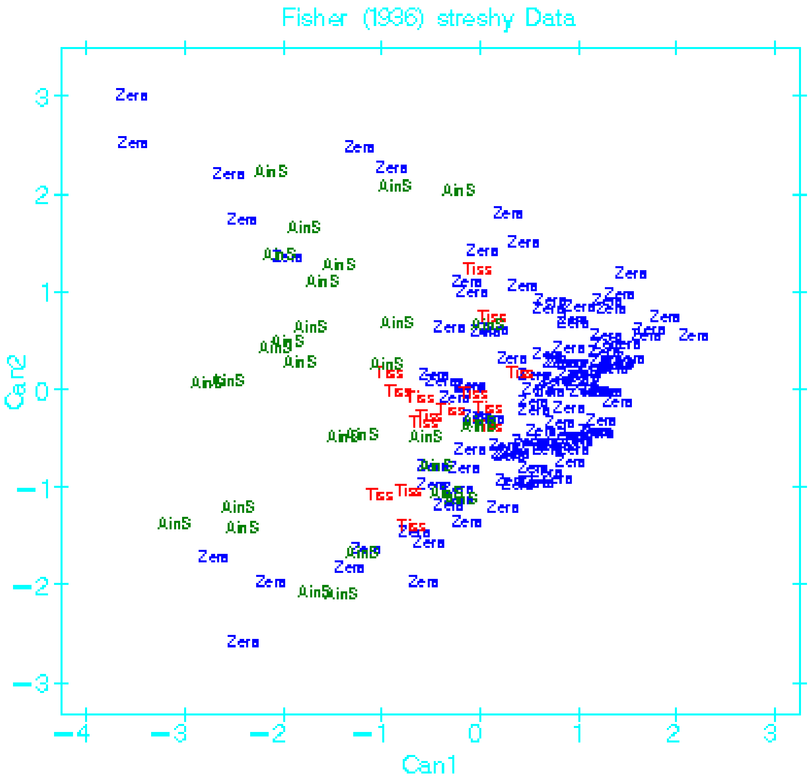

Figure 6. *Projection of the points means the regions studied on the first plan factorial of a principal component analysis*

Table 9. *Distance square, value of F for the distance square and value of the probability of square distance between the different ecotypes*

Squared distance to ecotype			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	0	1.02765	3.00215
Tissemsilt	1.02765	0	0.57412
Zeralda	3.00215	0.57412	0
F statistics			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	0	5.10594	35.61659
Tissemsilt	5.10594	0	3.79260
Zeralda	35.61659	3.79260	0
prob>			
	Ain Sefra	*Tissemsilt*	*Zeralda*
Ain Sefra	1.0000	0.0071	<,0001
Tissemsilt	0.0071	1.0000	0.0246
Zeralda	<,0001	0.0246	1.0000

Table 10 shows the points means of the regions on the canonical variables *Zeralda* (0.38/0.011), *Tissemsilt* (−0.348/−0.160) and *Ain Sefra* (−1.343/0.034).

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Table 10. *Medium of classes on the canonical variables*

Class meanings on canonical variables		
Ecotype	Can1	Can2
Ain Sefra	-1.3433	0.0342
Tissemsilt	-0.3486	-0.1608
Zeralda	0.3891	0.0118

According to the canonical analysis, the ecotypes *Ain Sefra* and *Zeralda* are well differentiated for all parameters studied. But *Tissemsilt* is getting closer to *Zeralda* for proline and soluble sugar parameters and form a different group for rwc and cs parameters.

Discussion

Drought stress is the key factor influencing plant growth and development in arid and semi-arid areas (Passioura, 2007). The assessment of the impact of the drought on the behaviour of plants includes mainly the estimate of their level of hydration. Thus, the RWC is a physiological indicator that is often used to assess the water status of the plant (Teulat et al., 1997). It has also been proposed as an important physiological indicator of the state of hydration in the function of the water regime (Lawlorand Cornic, 2002; Mefti et al., 2002).

Our results show that the water deficit is accompanied with a net decrease in the relative water content and stomata conductance in relationship with the intensity of water stress. The decrease of relative water content and stomata conductance documented in our study was in accordance with previous data (Maamar et al., 2015; Lassouane et al., 2013; Chakhchar et al., 2015b). The decrease in RWC is greater in *Ain Sefra's* ecotype. These two parameters are considered as the main responses against stress which is expressed by the stomata closure to minimize the water loss (Pita et al., 2005). This agrees with the results of some studies in a wide variety of plants (Nayyar and Gupta, 2006; Shultz, 2003). Under drought stress, the stomata closure with the decrease of their conductance constitutes one factor of plants drought tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006).

A significant relationship between RWC increase and low stomata conductance was observed among the three contrasted ecotypes with a very significant stomata closure, which plays a very effective role in the prevention of the water loss (Medrano et al., 2002; Flexas et al., 2004).

The water deficit tolerance of plants is realized also through the maintenance of a low water potential (Blum, 2005). This strategy is mainly based on the osmotic adjustment, provided by the accumulation of a large diversity of osmolytes (Jalil et al., 2007; Sankar et al., 2007). Proline was considered as an osmolyte compatible which protects subcellular structures and macromolecule under osmotic stress (Kavi-Kishor et al., 2005). It may, also, increase the activity of many enzymes and stabilize protein integrity (Szabo and Savoure, 2010; Lipiec et al., 2013). In another context, the accumulation of the proline plays a very important role in the absorption of water (Ashraf and Foolad, 2007; Miller et al., 2010) to ensure osmotic adjustment which is the main physiological characteristics of tolerance (Manivannan et al., 2008).

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DOI: http://dx.doi.org/10.15666/aeer/1702_19291945

Our study results show a significant accumulation of proline and soluble sugars at the three studied ecotypes, with different degrees under water deficit. Hence, the ecotype *Ain Sefra* has the highest accumulation of proline and soluble sugars. We suggest that this ecotype is associated with a high tolerance to drought compared to other ecotypes. A significant increase of proline content in drought stress has been recorded in several species such as Olive (Sofo et al., 2005; Boughallleb and Mhamdi, 2011), poplar (Yin et al., 2005), and rice (Mostajeran and Rahimi-Eichi, 2009). The accumulation of proline in dehydrated seedlings is the consequence of the activation of the proline biosynthesis and inhibition of its degradation (Nakashima et al., 1998). Furthermore, the accumulation of soluble sugars allows to protect the membranes and proteins in cells exposed to a water deficit and reduce the aggregation of denatured proteins (Ashraf and Harris, 2003). The involvement of these two osmoticumin the osmotic adjustment allows the maintenance of cell turgorat the highest level possible for low water potentials, thus maintaining the photosynthetic activity, membranes structure and growth (Lawlor and Cornic, 2002; Farooq et al., 2008).

The chlorophyll pigment content in the leaves is a good indicator to the detection of stress and the tolerance of the plants subjected to stress (Chakhchar et al., 2015a). Our results have shown that water deficit induced a significant decrease in the concentration of chlorophyll and carotenoids in the leaves of *Ceratonia siliqua* L., which is in agreement with the results of Smirnoff (1993). The decrease of chlorophylls documented in our study was in accordance with previous data (Cui et al., 2004; Lei et al., 2006). This diminution may be due to a low chlorophyll biosynthesis or their degradation (Bacelar et al., 2006). The stress response varies considerably between species, genotypes and even between the parts of the same plants (Kozlowski et Pallardy, 2002). There were clear intraspecific differences in stomatal sensitivity, proline content, RWC, soluble sugars and chlorophylls, suggesting different adaptations to drought related to the ecotype effect and significant genetic variability in carob populations in Algeria. The same results were obtained by (Chakhchar, 2015b; Maamar, 2015).

According to the APC (analysis of principal component), all three ecotypes separated into three different groups. Thus, we find that the ecotype of the arid zone (*AinSefra*) was the most tolerant ecotype; it can be used in future reforestation programs in the semi-arid area of Algeria.

Conclusions

An integrated study combining of physiological and biochemical analysis has been adopted to study the reactions and behavior of *Ceratonia siliqua* toward the water stress. Significant differences between ecotypes were recorded in leaves water status, the content of photosynthetic pigments, and the accumulation of osmoregulators.

This study shows that the water deficit is accompanied with a net decrease in the relative water content, stomata conductance, and a significant increase in the accumulation of proline and soluble sugars of the three studied ecotypes.

The canonical analysis classified of *Ain Sefra* ecotype as the most tolerant one from the arid zone and suggested the following order of the ecotypes with respect to the drought tolerance: the *Ain Sefra* (arid zone) - the *Tissemsilt* (semi-arid zone) - the *Zeralda* (sub-humid zone).

According to the analysis of principal components, the three ecotypes were distinguished on the basis of the RWC, CS, proline accumulation and soluble sugar. *Ain Sefra* ecotype is more likely to be the most droughts tolerant and very promising to the regeneration of carob tree cultivation in Algeria in arid and semi-arid areas.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19291945

APPLICATION OF ARTIFICIAL NEURAL NETWORK TECHNIQUES FOR PREDICTING THE WATER QUALITY INDEX IN THE PARAKAI LAKE, TAMIL NADU, INDIA

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(Received 10th Oct 2018; accepted 29th Nov 2018)

Abstract. Nowadays, the demand for forecasting techniques has increased due to conservation management of water resources. In this study, an artificial neural network model (ANN) was followed for determining the water quality index parameters. By the use of computer program, we can get a set of coefficients for a linear model in the calibration of ANN model. The Palayar River is one of the oldest rivers in Tamil Nadu originating from Western Ghats and flowing through Suchinthram area and Parakai Lake. Seven water quality parameters of Parakai Lake were chosen at four monitoring stations in the time period between December 2016 and March 2018. It is clear from the results that the water quality index (WQI) predicted with ANN model brings better output (correlation coefficient R = 0.9907) when compared with the Multiple regression model having R = 0.7908.
Keywords: *comprehensive pollution index (CPI), multiple linear regression, performance evaluation, water quality*

Introduction

Water is the vital resource for the existence of life. The ultimate use of water for various purposes such as drinking, irrigation and industrial needs has put forth an entire failure in the water quality standards. The excessive use of natural resources has developed a reduction in the water table apart from the significant increase in the concentration of pollutants (Yada et al., 2018). Because of disagreeable conservation management and expansion of population, the water resource has declined and existence of man has become threatened. Water quality indices (WQIs) are widely used to predict the eutrophication levels of lake waters by creating the data interpretation and gathering several parameters of water quality data into one single index. The parameter of all variables of this study is to observe the laboratory analysis and to calculate the pollutants of water body in place of the WQI.

Nowadays, a wide variety of applications of ANN is used to forecast the water quality (Kumar and Sharma, 2015) and to determine the water quality index by the use of independent variables such as DO, COD, BOD, SS, N-NO3 - and pH. By the use of customary water samples, the assessment of water quality and its laboratory analyses is exact, but very costly and more time utilization (Gorashi and Abdullah, 2012). Comprehensive pollution Index for the samples is also calculated. The pollution index method can give an assessable description of water quality which is low, heavy or extremely heavy but it cannot discriminate functional categories of water (Xing, 2011).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19471958

The total dissolved solids, dissolved oxygen and biochemical oxygen demands are forecasted using pH, TS, T-Hard, T-Alk, K+, Cl-, PO_4 -, Na+, N-NO_3 -, N-NH_4 +, and COD was done by means of a multi-layer perceptron neural network (Maier and Graeme Dandy, 1996). Similarly, Abyaneh (2014) employed the ANN model to forecast DO and BOD. Since the beginning of the 1990s, the applications of ANNs report was created by the areas of environmental sciences, ecological sciences and water engineering (Zali et al., 2011). The proposed model employed artificial neural networks (ANNs) with the back-propagation algorithm which can obtain a highly nonlinear relationship (Chine et al., 2009). The ANN model is constructed for fast calculation and predicting the selected water quality variables at any location. At other locations, particular remaining variables are used as the input parameters such are salinity, temperature, dissolved oxygen, and chlorophyll-alpha (Najah et al., 2013). The application of artificial neural network (ANN) models to simulate water quality parameters is cost-effective, quick, and reliable. This study provides two methods of mathematical and ANN modeling to simulate and forecast five important river water quality indicators (DO, TDS, SAR, BOD_5, HCO_3) correlated with variables such as EC, temperature, and pH which can be measured easily and almost with no cost (Salami et al., 2016; Spitz et al., 1999). The WQI is predicted by a simulative model using an ANN. This model has been developed for the assessment of the WQI and compared with the conventionally determined values of WQI. A Multilayer-Perceptron (MLP) network with a single hidden layer was used along with back-propagation algorithm. The results were found to be quite impressive. Thus, the ANN proved to be an efficient tool to assess the WQI of any sample (Hore et al., 2008). The most ordinarily used feed forward error back propagation neural network technique has been utilized. Monthly data sets on turbidity, total hardness, total dissolved solids, and electrical conductivity have been employed for the analysis. The results present the ability of suitable ANN models to predict the water quality parameters (Dawood et al., 2016). The Karoon River in Iran is selected to evaluate the capability of ANNs for water quality simulation. Several water quality variables including CO_3, HCO_3, SO_4, Cl, Na, Ca, Mg, K, EC, TDS and SAR has been simulated. Qnet 2000 ANN is selected for modeling purposes in the present research. Results show that Qnet 2000 is able to predict the water quality variables of the Karoon River very successfully with more than 90% accuracy (Musavi-Jahromi et al., 2008). The multiple regression models and artificial neural network models are used to predict the value of hardness with respect to the corresponding values of chloride, fluoride, and calcium contents of the groundwater sample based on the specific data. Novel techniques such as Artificial Neural Networks (ANNs) can be used to predict the output from the data set with better accuracy than that using Regression technique. This study will trigger DOE to use ANNs in order to predict WQI rather than using conventional method (WQI equation) that is currently being used by DOE. In addition, the ANNs managed to show remarkable prediction performance to predict the WQI in Juru River (Zali et al., 2011).

Many researchers have activated the artificial neural network technique in the satellite data in order to assess the water quality because of the limitations of linear regression methods. So, that the effort and computational time requirement can be reduced to develop the ANN water quality model. The main objective of this study is the variation of temporal and spatial surface water quality in the Parakai Lake is predicted by the use of multiple regression analysis and ANN model.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19471958

Materials and methods

Study area

The study area, Parakai Lake is Located at 8°09'46.2"N 77°26'59.9"E (*Fig. 1*). Parakai Lake is one of the lakes which fed water to irrigation field in southern parts of Kanyakumari district. The lake is fed by canals derived from Pazhayar River. The bound length of the lake extends to 4 m and water spread area of 1.684 km^2 and has an average storage depth of 3.2 m with holding capacity of 2.351 m^3. Kanyakumari district has four distinct seasons such as southwest monsoon (Jun. to Sep.), the northeast monsoon (Oct. to Dec.), the winter (Jan. and Feb.) and the hot weather (Mar. to May). Parakai Lake is located near the southern tip of India in Kanyakumari district in the state of Tamil Nadu, just to the north of Parakai village and west of the town Suchindram. According to the census, the total population of seven villages and two urban habitations along this river bank was 4, 76, 018. Calculation of water supply based on the 135 LPCD, the total quantity of water consuming in above area was 4, 55, 72, 200 l/day, and its sewage generation (80% of the consuming water) was 3, 64, 57, 832 l/day. The sewage has been discharged into the Parakai Lake without any treatment. The current study focuses the importance on the environmental and health risks subordinate with the use of lake water mixed with sewage fed from open sewerage in the system tank of Parakai Lake. The coordinates of sampling location are shown in *Table 1*.

Sample collection and analysis

The samples are collected from four locations such as 1) Pazhayar river inlet, 2) sewage water inlet, 3) irrigation channel (sluice gate no.2, outlet) and 4) irrigation channel (sluice gate no.5, outlet) (*Fig. 1*). The monthly water samples are collected from the four nodes by following the water sampling procedure IS 3025 (Part I) 1987. Totally 64 water samples are tested and analysed for the level of concentration of various parameters which are taken from IS 11624 (1986).

Figure 1. *Location of the sampling stations on the Parakai Lake*

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Table 1. *Water quality sampling stations along Parakai Lake*

Sampling station	Geographic coordinate	
	Latitude	**Longitude**
S1	8° 16.28'	77°44.99'
S2	8°14.62'	77°45.21'
S3	8°14.04'	77°45.39'
S4	8°14.58'	77°45.69'

According to which, the sodium, sodium adsorption ratio, total salt concentration, total dissolved solids, residual sodium carbonate, soluble sodium percentage and boron were considered for improving the water quality criteria for irrigation. The values of concentration are obtained by testing them under suitable conditions and the procedure as given in *Table 2*.

Table 2. *Water standard specifications given by IS 11624 (1986, reaffirmed 2009) and statistical information of ion concentrations*

Sl.No	Parameter	Max.	Min.	Mean	IS 11624 (2009)	Analytical method
1	Total salt concentration (µs/cm)	405	129	228.125	750	Electrometric conductivity meter
2	Total dissolved solids (mg/l)	520	195	329.813	1300	Filtration and gravimetric
3	Sodium (mg/l)	72	14	37.5625	215	Flame photometer
4	Sodium soluble percentage (%)	72.7	44.1	58.3175	60	By calculation
5	Sodium absorption ratio (mg/l)	17.23	3.75	10.2081	7	By calculation
6	Residual sodium carbonate (mg/l)	149.49	21.52	58.785	58	By calculation
7	Boron (mg/l)	1.52	0	0.42157	4	Annex H of IS 13428 4500- B B, APHA 22nd Edition 2012

Comprehensive pollution index (CPI)

Based on the assessment of single factor index and considering the combined effect of all factors evaluated, CPI was calculated through different mathematical models and determines the pollution degrees by the appropriate method (*Eqs. 1* and *2*).

$$CPI = \frac{1}{n}\sum_{i=1}^{n} \text{PI} \quad \text{(Eq.1)}$$

$$PI = \frac{measured\ value\ of\ individual\ parameter}{standard\ permissible\ value\ of\ parameter} \quad \text{(Eq.2)}$$

The CPI values of each sample are calculated and are analyzed by the use of limits stated in *Table 3*.

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Table 3. *CPI limits for water quality*

Sl.No	CPI Limits	Category
1	< 0.2	Clean water
2	0.2 - 0.4	Moderately polluted
3	0.4 - 0.6	Severely polluted
4	> 0.6	Purely sewage water

Modeling method

Multiple linear regressions

Multiple linear regressions (MLR) were functional as well in this work to validate the relationship between the water quality parameters and their impact on WQI. An MLR model takes the form (*Eq. 3*):

$$Y = \beta_p + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_{p-1} x_{p-1} + \varepsilon \quad \text{(Eq.3)}$$

where Y is the response variable, and there are $p - 1$ explanatory variable $x_1, x_2, \ldots, x_{p-1}$, with p parameters (regression coefficients) $\beta_0, \beta_1, \beta_2, \ldots, \beta_{p-1}$

In this study the adjusted R^2 value was calculated. This adjusted value indicates the loss of predictive power or shrinkage. While R^2 explains how much of the variance in Y is accounted for by the regression model, the adjusted value tells us how much variance in Y would be accounted for if the model is derived from the population from which the sample was taken. One version of R^2 that explains how well the models cross-validates is the Stein's formula below (*Eq. 4*):

$$adjusted\ R^2 = 1 - \left[\left(\frac{n-1}{n-k-1}\right)\left(\frac{n-2}{n-k-2}\right)\left(\frac{n+1}{n}\right)\right]\left(1 - R^2\right) \quad \text{(Eq.4)}$$

In Stein's equation, R^2 is the adjusted value, n is the number of subjects and k is the number of predictors in the model. It is useful to use the equation to cross validate a regression model.

Artificial neural network as WQI prediction model

In the Artificial Neural Network model, an output layer is water quality index and it is used according to the Indian standard. Here, the input variables are collected based on the correlation analysis. Based on the priority target, the forecaster variables are selected and separated where parameters can be analyzed in the field. The initial predictors contained seven parameters of water quality.

Pre-processing of data

The original testing sample data are collected and these data are converted into 64 segments such as 40 data are used for training and remaining 24 are used for testing. The net architecture for WQI comprises of input nodes and output node. The desired

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output delivered by the choices of input parameters used. In the present study, the input variables are sodium, soluble sodium percentage, sodium adsorption ratio, total salt concentration, total dissolved solids, residual sodium carbonate, and boron. The non-linear mapping structure comprises of natural networks of biological neurons in brain. It also contains simple calculated units called neurons. These are highly interrelated.

Progress of ANN model

This model is a multilayer ANNs and assembled with layers of units. The first layer is called as input units and the last layer is known as output units. In the statistical nomenclature the units are known as dependent variables or response variables and the arithmetical input units are called as independent variables remaining other units are called as hidden units and it having hidden layers. There are three basic layers or levels of data processing units viz., the input layer, the hidden layer and the output layer. Each of these layers consists of processing units called nodes of the neural network. The interconnections between the nodes of different layers are called weights of the neural network. These weights are updated or modified iteratively using the generalized delta rule or the steepest gradient descent principle (ASCE Task Committee, 2000). In the present study; the feed forward error back propagation algorithm (Rumelhart et al., 1986) is used for ANN training. Among most of the ANN applications studied in the literature, the feed forward error back propagation neural network is the most popular and widely used ANN architecture. The ANN network used for the present study is shown in *Figure 2*.

Training of ANN

An ANN stores the knowledge about the problem in terms of weights of inter-connections. The process of determining ANN weight is called learning or training. At the beginning of training, the initial value of weights can be assigned randomly or based on experience. The weights are systematically changed by the learning algorithm such that for a given input, the difference between the ANN output and the actual output is small. Many learning examples are repeatedly presented to the network, and the process is terminated when this difference is less than a specified value. At this stage, the ANN is considered trained. An ANN is better trained as more input data are used.

Performance evaluation

A large number of statistical criteria are available to compare the goodness/adequacy of any given model. The performance evaluation statistics used for ANN training in the present work are root mean square error (RMSE) and coefficient of correlation (R).

Some statistical analysis techniques are used to validate the result of prediction models. The root mean square error is used to measure the difference between actual value of water quality index and the value from prediction model (*Eq. 5*).

$$\boldsymbol{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(WQI_{act} - WQI_{Pre})_i^{2}}{n}} \quad \text{(Eq.5)}$$

where WQI_{act} = actual value of water quality index
WQI_{Pre} = predicted value of water quality index
n = number of monitoring data

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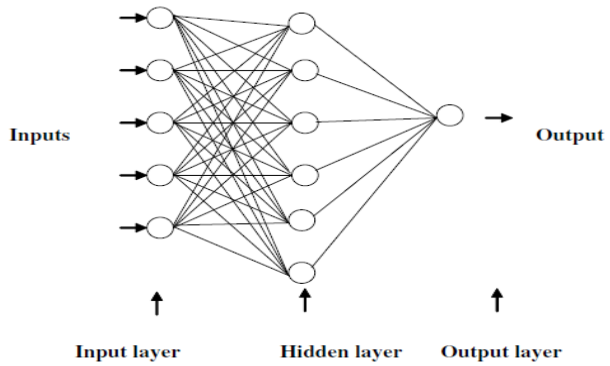

Figure 2. *Structure of a multi-layer feed forward artificial neural network model*

Results and discussion

Correlation between the water quality parameters

From the measured water quality parameters the Pearson's correlation matrix was drawn to find the possible causes of water pollution (Tyagi et al., 2013). The correlation coefficient of seven water quality parameters are categorized by choosing the bivariate significance level 0.05 and 0.01 (*Table 4)*. EC has significant positive correlation with TDS (r = 0.6952), sodium (r = 0.8373), SSP (r = 0.6733), SAR (r = 0.6991), RSR (r = 0.5765) and Boron (0.3379). It is observed that with increase or decrease in values of EC; TDS, Na, SSP, SAR, boron also exhibit decrease or increase in their values. It has been expressed that the increasing EC appear to be associated with the increasing use of alkaline detergents in residential areas effluent. TDS has significant positive correlation with sodium (r = 0.8498), SSP (r = 0.8215), SAR (r = 0.7916), RSR (r = 0.5621) and Boron (0.6519). This also shows that with increase or decrease in the values of TDS; Na, SSP, SAR, boron also show decrease or increase in their values. A significant positive correlation was found between Na and SSP (r = 0.9269), SAR (r = 0.9291), RSR (r = 0.7742) and boron (r = 0.7142). Further, study of boron (r = 0.7817) showed a positive correlation with sodium and therefore it can be assumed that B and Na originates from the same minerals, most likely from clay.

Table 4. *Correlation coefficient of water quality parameters*

	Total salt concentration	**Total dissolved solids**	**Sodium**	**Sodium soluble percentage**	**Sodium absorption ratio**	**Residual sodium ratio**	**Boron**
Total salt concentration	1						
Total dissolved solids	0.6952	1					
Sodium	0.8373	0.8498	1				
Sodium soluble percentage	0.6733	0.8215	0.9269	1			
Sodium absorption ratio	0.6991	0.7916	0.9291	0.9905	1		
Residual sodium carbonate	0.5765	0.5621	0.7742	0.8348	0.8642	1	
Boron	0.3379	0.6519	0.7142	0.7817	0.7803	0.7444	1

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Summary of regression model

The main objective of this work is to reduce the number of parameters needed to carry out the water quality prediction without much loss of information. To meet this objective, MLR is engaged to investigate the connection between the water quality parameters and the dependent variables (WQI). In *Equation 3,* four variables are meet the entry requirement and which is accounted for approximately 71% of the variance in WQI. Two variables did not meet the entry requirement, namely the EC and SSP, which is contributed only 8% and 2%, respectively. The multiple regression coefficient R is determined between the independent variables and the dependent variables.

The difference between the adjusted R^2 from the R^2 was only 7.5% (0.625–0.550) and the value of regression coefficient R = 0.7908. This shrinkage means that if the models were derived from December 2016 rather than a sample it would account for approximately 0.7% less variance in the outcome.

To gain clearer evidence, Stein's formula was applied to R^2 (*Eq. 4*) and it can be applied by replacing *n* with the sample size and *k* with the number of predictors. An adjusted R^2 is calculated and the value is equal to 0.6084 indicate that the cross-validity of this model is very good (*Table 5*).

Table 5. *Consequences of establishing prediction models*

Model	R	R-square	Adjusted R-square	Standard error	Observations	Intercept nodes	*t Stat*	*P-value*
1	0.428398	0.183525	0.02023	18.01	7	S1	2.335601574	0.066744
2	0.790836	0.625421	0.550506	11.4	7	S2	-0.541583643	0.611356
3	0.662754	0.439243	0.327092	5.01	7	S3	2.775151086	0.039126
4	0.583011	0.339902	0.207883	4.85	7	S4	2.099131502	0.089852

The model can be written in the following form (*Eq. 6*):

$$WQI = -338.127 + 1.50764\,\text{EC} + 0.741\,\text{TDS} + 7.082\,\text{Na} + 6.678\,\text{SSP} - 41.474\,\text{SAR} + 1.698\,\text{RSC} - 36.759\,\text{B} \quad \text{(Eq.6)}$$

A positive value indicates that, there is a positive relationship between predictor and the outcome whereas a negative coefficient represents a negative relationship. In this study two out of four predictors have negative β values. It is clear that when WQI decreases, the pollutant concentration in S1 and S4 decreases. Positive β values in S2 and S3 shows an increase of WQI values which is followed by the increase of pollution.

Predicting models for water quality index

In the neural network prediction model, 40 set of data are used for training and remaining 24 set of data are used for testing. Initially, the model was trained with the selected input and output variables under 8 numbers of neurons. After that the testing was performed by the use of remaining dataset. In order to obtain the accuracy of this model, the length of data sets and number of neurons are changed. The numbers of trials were performed when the value of correlation coefficient is high. This model stimulated the water quality index with great accuracy and the coefficient of correlation R is 0.9907 (*Fig. 3*). Numbers of observing data were used in *Equation 5* and received the

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root mean square value (RMSE) as 0.00617. The variations of original and predicted value of pollution index for all locations were identified.

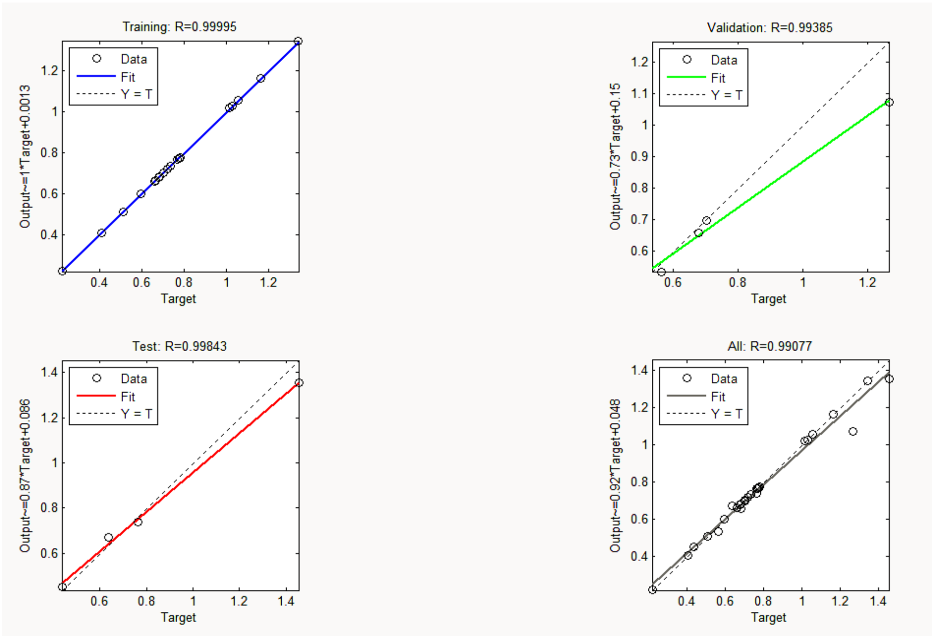

Figure 3. *Evaluation models for WQI for recurrent neural network*

It was detected (*Fig. 4*) that the range of CPI for inlet river water is under qualified category (CPI < 0.2) during December 2016 – March 2018. In the same way, the inlet river waste water, the parameters such as residual sodium carbonate and boron fall under severely polluted condition (CPI > 2) due to the flooding of waste water from the surroundings (*Fig. 5*). For outlet river waters S3 and S4, the parameters EC, TDS, Na and boron fall under qualified category (CPI < 0.8), the parameters such as SSP, SAR and RSC are affected moderately (*Figs. 6* and *7)*. The CPI value of each samples are calculated and are analyzed by the use of limits from *Table 3*. Based on these limits, we can identify the CPI value of sample 1 as 0.3126 indicates moderately polluted, sample 2 is 0.8789 indicates purely sewage water and sample 3 and 4 is 0.5468 and 0.5428 respectively indicates moderately polluted.

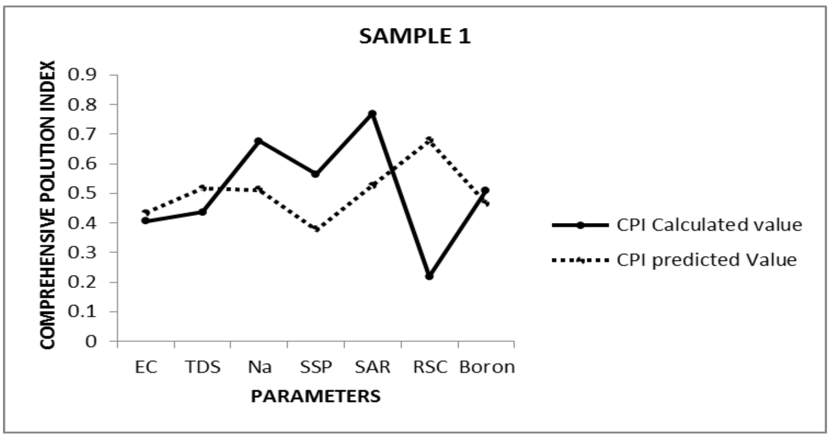

Figure 4. *Deviations in comprehensive pollution index for inlet river water*

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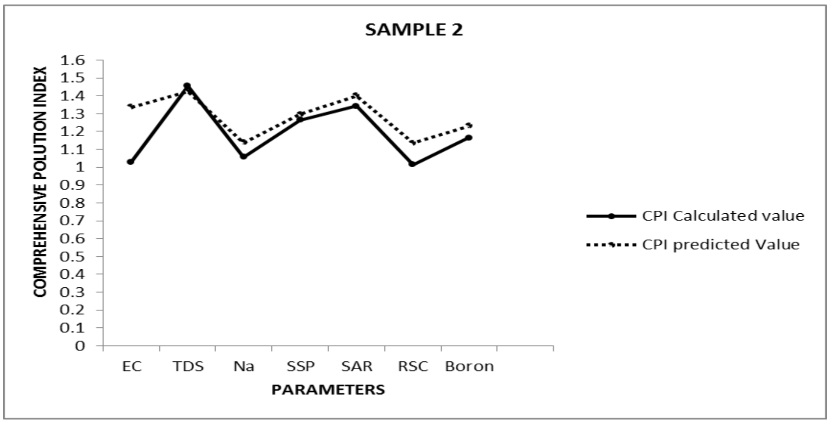

Figure 5. *Deviations in comprehensive pollution index for inlet waste water*

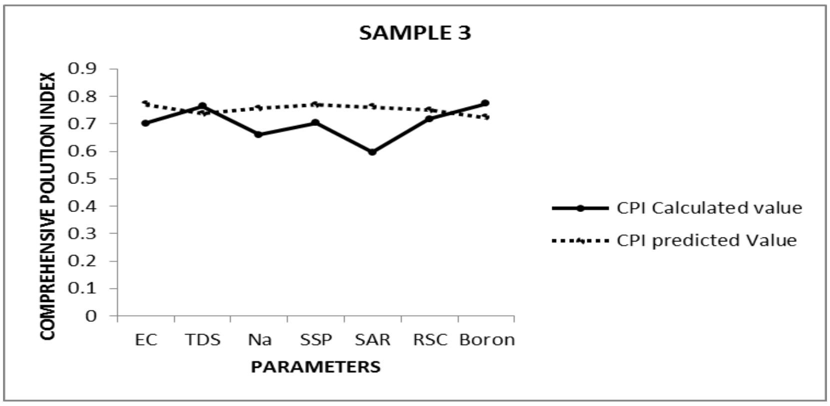

Figure 6. *Deviations of comprehensive pollution index for outlet river water*

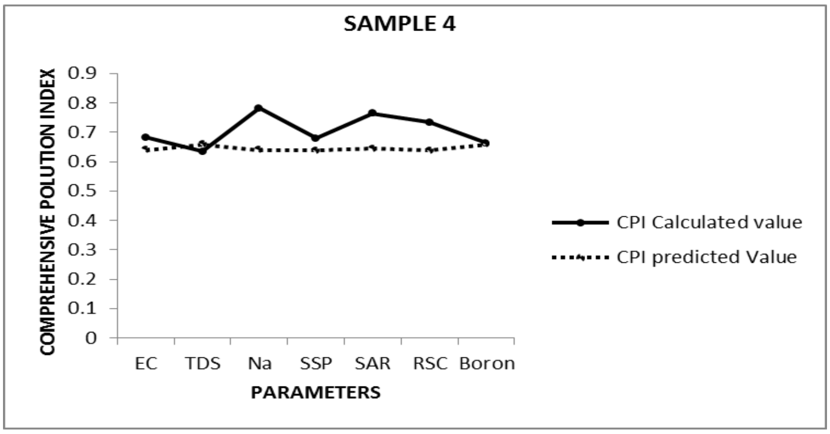

Figure 7. *Deviations of comprehensive pollution index for outlet river water*

The study proves that, in the ANN model, the coefficient of correlation (R = 0.9907) value is higher than the MLR model (R = 0.7908). Also, the MLR models are statistically significant; they contains higher errors than the ANN models. The results indicate that the ANN model is the wonderful prediction model compared with MLR model.

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Conclusions

The lake water outlet during the study period revealed that the water is suitable for Irrigation purpose. The measured water quality values are compared with the IS water quality standards of drinking water and it is found that the water is moderately polluted i.e. not suitable for drinking and other life supporting activities, but can be used for irrigation purposes. It is also clear that the increased concentration of sodium may be the root cause for the increase in pollution. Hence, it is summarized that the control of point source and non-point source pollution directly discharged into the lake and are vital to clean the Parakai Lake. From the study, it is understood that the ANN model predicts the water quality indices more accurately than the MLR and also ANN model is a wonderful realistic and forecasting tool. The ANN models explained outstandingly data as long span patterns and as long as good forecasting results.

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DEVELOPING AND CHARACTERIZATION OF AN ULTRAFINE FILTER MADE OF BANANA LEAF AND WATER HYACINTH TO REDUCE MOTORCYCLE EMISSION

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(Received 18th Oct 2018; accepted 2nd Jan 2019)

Abstract. $PM_{0.1}$ (ultrafine particles) emitted by motorcycles have been known as the major contributors to air pollution, and have a harmful impact on human health. Therefore, there is a need to decrease the concentration in the ambient air. In this study, we developed an ultrafine particle filter that was made of banana leaf powder (F_b) and water hyacinth powder (*Eichhornia crassipes,* F_{wh}). The powder was mixed to glutinous rice glue in the ratio of 80%:20%. The filter was characterized by the efficiency. The efficiency of the filter in reducing the ultrafine particles was determined by measuring the ultrafine particle concentration before and after passing through the filter. The ultrafine particle concentration was measured using the TSI P-Trak Ultrafine Particle Counter (Model 8525) for an hour with the sampling time interval of 5 min. The microstructure and the element contents of the filter were analyzed by an SEM and EDAX to determine the filter porosity and the filter element compound. The results show that the porosities were found 33% (F_b) and 21% (F_{wh}) and the filter contained organic and inorganic compounds. The filter efficiency was in the range of 55% to 64% for F_b and 35% to 59% for F_{wh}, respectively.
Keywords: *ultrafine particle filter, motorcycle emission, banana leaf, water hyacinth, efficiency*

Introduction

The usage of motorcycles has been increasing from year to year (Burke et al., 2017; Guerra, 2017). As a consequence, they produce more emissions that are classified into gas and particulate matter (PM) emissions (Grieshop et al., 2006). In terms of particulate matter with the diameter less than 0.1 μm (known as $PM_{0.1}$ or ultrafine particle), $PM_{0.1}$ deposits deeper into the lung (Clifford et al., 2018) and kidney (Wardoyo et al., 2018). Previous studies have showed the correlation between $PM_{0.1}$ and human health (Chen et al., 2016; Li et al., 2016; Liu et al., 2018; Solaimani et al., 2017). Therefore, there is a need to develop a particulate filtration technology in reducing the $PM_{0.1}$ concentration generated by motor vehicles.

The particulate filtration system has been developed, such as radiation based filter, electrostatic based filter, and porosity based filter (Barone et al., 2010; Budianto and Wardoyo, 2016; Wardoyo et al., 2016, 2017a). Meanwhile, the resulted filtration technology is not sufficient due to the high cost of the filter production. In this study, we developed low-cost $PM_{0.1}$ filters that were made of water hyacinth (F_{wh}) and banana leaf (F_b). Those two filter ingredients can be easily found in Indonesia. They also have a high cellulose content (25-39%) (Istirokhatun et al., 2015; Nada et al., 2010) that can adsorb ultrafine particles. Filter porosity can determine the level of adsorption (Ma et al., 2016). The aim of this study was to develop an ultrafine particle filter made of water hyacinth and banana leaf and to characterize the efficiency and the influence factors in terms of the porosity and the element compounds.

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Materials and methods

Motorcycle samples

Three motorcycles were used as $PM_{0.1}$ source. They were classified into M1 (model year: 2015), M2 (model year: 2011), and M3 (model year: 2013). All of them had automatic transmission engines with the same engine capacity (108 cm^3). They were chosen due to their prevalence in Indonesia.

$PM_{0.1}$ filter

Water hyacinth and banana leaves were collected from the rivers and farms of Sengonagung village, Pasuruan, Indonesia. They were cut into small pieces, blended (diameter ± 70 mesh) and dried in an oven for seven hours (controlled temperature: 100 °C). After that, the resulted powder (16 grams) was mixed with water (190 ml) and a tapioca glue containing glutinous rice (4 grams) which was heated with water (100 ml). The filter dough was printed into a layer.

Particle measurement

Each filter was installed on a filter frame (diameter = 50 mm) and mounted on the tip of the motorcycle exhaust. The $PM_{0.1}$ concentrations emitted by the motorcycle samples before (C_o) and after being equipped with the filters (C_i) were measured using a P-Trak Ultrafine Particle Counter (Model 8525) for an hour in an idle condition (sampling time = 5 min). All measurements were repeated three times (Wardoyo et al., 2015).

Filter characterization

The filter was characterized by the porosity and the element compounds. The porosity was analyzed by using a scanning electron microscope analyzer INSPECT S50 SEM EDAX to determine the filterability in capturing $PM_{0.1}$ emission and the structure of the morphological surface (Van der Zande et al., 2014). The element compounds were analyzed using an EDAX (Kedar et al., 2018).

Statistical analysis

Values are expressed as the means ± SD (standard deviation). The differences between the groups were evaluated using a Student's *t*-test. The relationship between $PM_{0.1}$ concentrations and time series was determined using R-square value (Wardoyo et al., 2017b). The filter efficiency (*%F*) was calculated using *Equation 1* (Mondal et al., 2015):

$$\%F = [(C_o - C_i) / C_o] \times 100\% \qquad \text{(Eq.1)}$$

Results and discussion

Emission measurement

Figure 1 represents the comparison of the $PM_{0.1}$ concentration measurement of all motorcycles without a filter and using a filter (F_{wh} and F_b). According to *Figure 1,* the $PM_{0.1}$ concentrations without being filtered are 19,020 ± 2,958 to 62,362 ± 3,664 particle/cm^3. These concentrations are higher than the $PM_{0.1}$ concentrations obtained from F_b and F_{wh}.

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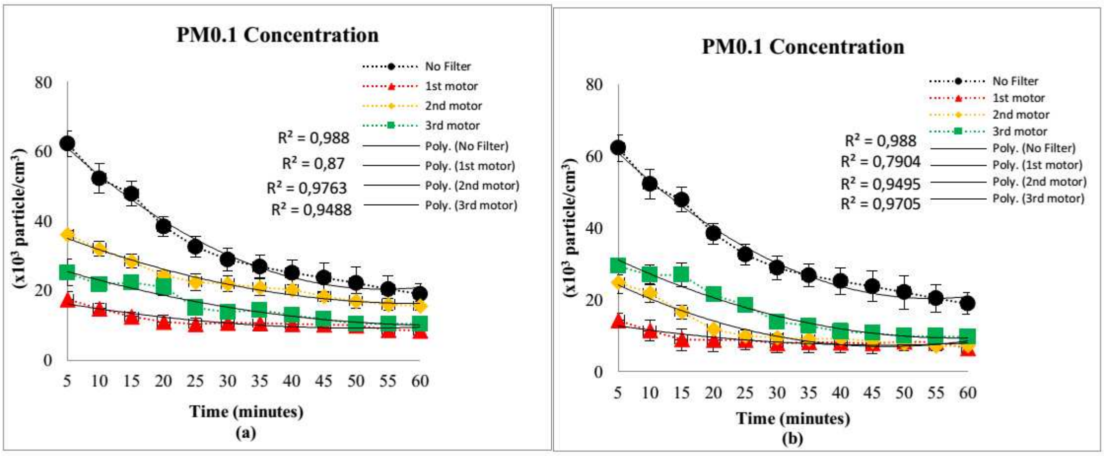

Figure 1. *The $PM_{0.1}$ concentrations of (a) F_{wh} and (b) F_b*

In contrast, the $PM_{0.1}$ concentrations obtained from using F_{wh} are 8,554 ± 389 to 36,136 ± 1,218 particles/cm^3. F_b has the lowest $PM_{0.1}$ concentration (6,565 ± 1,125 to 29,448 ± 2,307 particles/cm^3). The decreasing trend of the particle concentrations measured in all motorcycles is a polynomial trend with the order of 2. The R-square values of all motors using the filters are in the range of 0.79 to 0.98. If the R-square value is higher than 0.75 it presents a good relationship between the two variables on the graph (Duarte et al., 2014). *Figure 2* shows the average efficiency of F_{wh} and F_b on M1 and M2. According to *Figure 2,* the efficiency of F_b (55% to 63%) is higher than F_{wh} (35% to 59%) ($p < 0.05$).

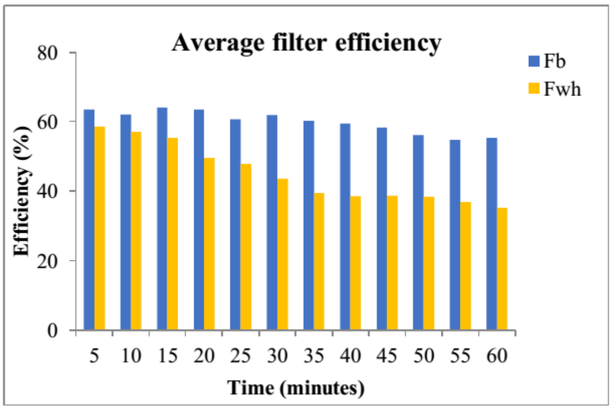

Figure 2. *Average efficiencies of F_{wh} and F_b*

The filter efficiencies were influenced by the content of cellulose in water hyacinth and banana leaf. Cellulose content in banana leaf is 39.2% (Nada et al., 2010) higher than cellulose content that is found in water hyacinth (25%) (Istirokhatun et al., 2015). Cellulose can bind the organic PAHs (Polycyclic Aromatic Hydrocarbons) and VOCs (Volatile Organic Compounds)) and inorganic content of $PM_{0.1}$ (Hokkanen et al., 2016; Liu et al., 2016). This is shown by the overall values of the weight percent of detected elements in F_{wh} and F_b after emission exposure as listed in *Table 1*. The most detected elements in *Table 1* are oxygen (O) and carbon (C). The spherical carbon was indicated

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as one of the elements of the particles emitted by a gasoline operated vehicle (Ritovski et al., 1999). Carbon and oxygen are also the part of VOC and PAH content in $PM_{0.1}$ (Charron and Harrison, 2003; Hong-li et al., 2017). VOC contains aldehydes, alkenes, and aromatic rings (Zhang et al., 2018). The aldehydes occur as C=O stretching vibration, alkenes and aromatic rings occur as C=C stretching vibration. While one of the PAH content in $PM_{0.1}$ is perylene ($C_{20}H_{12}$) (Charron and Harrison, 2003). So, thus elements C and O in *Table 1* may be a part of the VOC and PAH in $PM_{0.1}$.

Table 1. *Elemental analysis of F_{wh} and F_b after emission exposure*

Element	Weight (%)	
	F_{wh}	F_b
C	28.62	54.30
O	29.35	33.65
Mg	0.67	0.57
Si	0.42	0.63
Pb	2.44	1.22
K	20.22	5.17
Ca	1.40	-
Fe	0.76	0.73
Co	-	0.23

The SEM EDAX results also showed the presence of several inorganic elements, e.g. Ca (only detected on F_b), and Co (only detected in F_w). Other elements (Ca, Fe, and K) are related to the crustal matter (Zhang et al., 2014). Inorganic elements that have the highest weight percent are K then Pb.

In addition, the filter efficiency is also influenced by the filter porosity. Filter porosity can be known from the micrographic structure of the filter. *Figure 3* shows the micrographic structure of SEM result from the processing ImageJ software (Igathinathane et al., 2008) on F_{wh} and F_b before emissions exposure. *Figure 3* shows the surface pores of the filter (black). According to the figure, F_{wh} has pores sizes in the range of 1.019 μm^2 to 1.499 mm^2, with a total porosity of 21%. Meanwhile, F_b has pore sizes in the range of 1.038 μm^2 to 1.613 mm^2, with a total porosity of 33%. With the size area porosity, the filter has a potential to trap the particulate matters with a diameter of 0.1 μm.

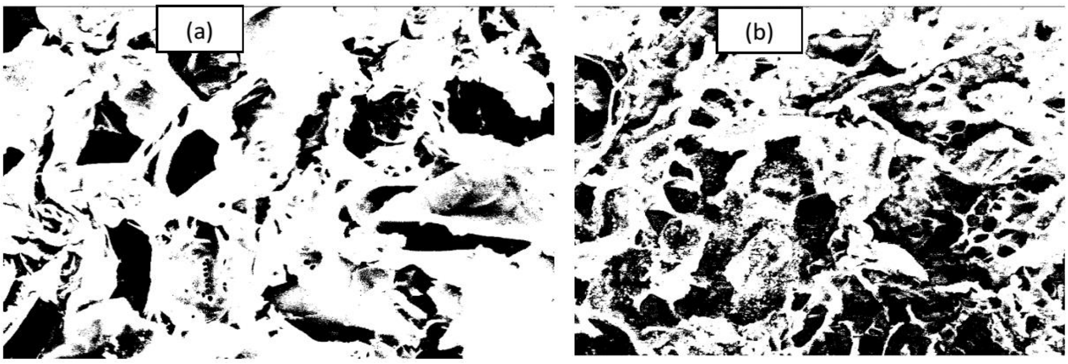

Figure 3. *Micrographic SEM of imageJ processing results of the filters before being tested: (a) F_{wh} and (b) F_b*

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DOI: http://dx.doi.org/10.15666/aeer/1702_19591965

The particles can be trapped in the filter pores with several mechanisms, i.e. inertial impaction, interception, and diffusion. Inertial impaction occurs when the particles pass through the filter with micrometer range and the particles hit the filter materials, so the particles trapped in the filter. Interception mechanism occurs when the center of the particle is shorter than the radius of the filter so the particle center does not touch the surface of the filter material to be deposited. Particles that move in the nanometer range generate Brownian motion, which causes the particles to collide with inertial or interception particles and diffuse on the surface and deposit during the time when they are passing the side of the filter (Liu et al., 2016).

Figure 4 shows the results of the water hyacinth filters before and after emissions exposure and banana leaf filters before and after emissions exposure. *Figure 4a* and *c* shows the cavities on the surface of the filters. Meanwhile, *Figure 4b* and *d* shows the invisible cavities and trapped particles on the surface of the filter. These results are similar to the results obtained in the Van der Zande et al. (2014).

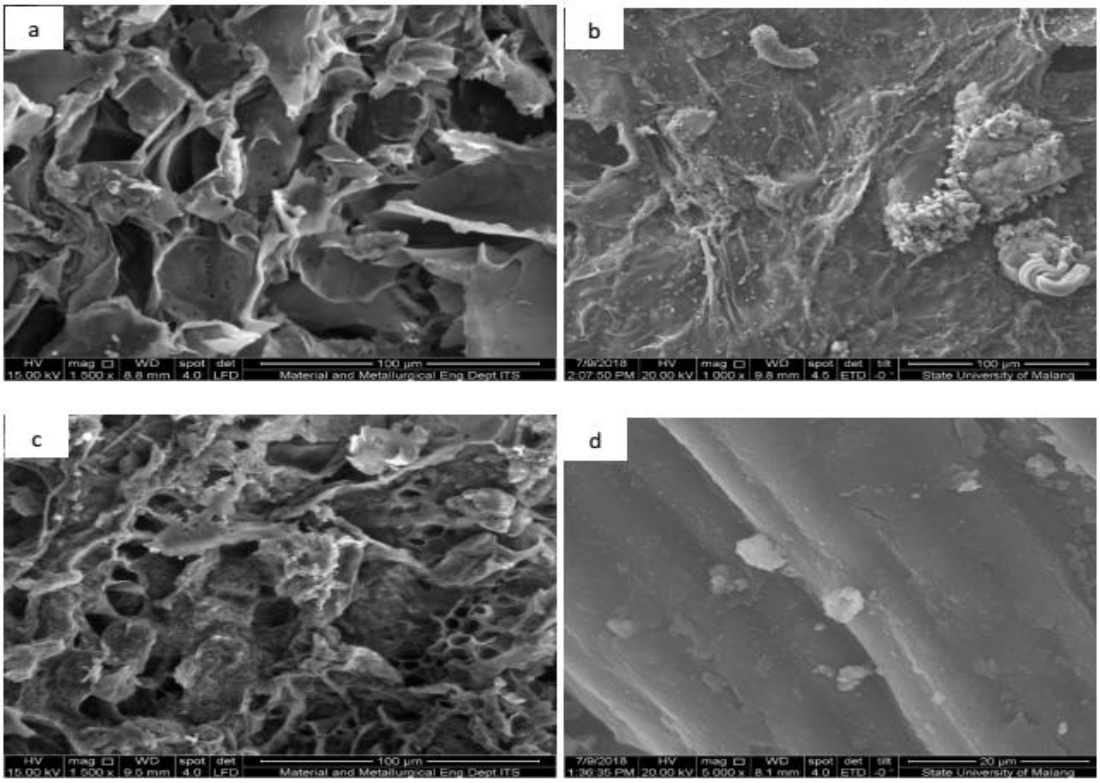

Figure 4. *SEM image of F_{wh} before (a) and after (b) being tested, and F_b before (c) and after (d) being tested*

Conclusion

In conclusion, the filters based on water hyacinth and banana leaf have a character to reduce the $PM_{0.1}$ concentrations with the efficiency of up to 59% and 64%, respectively. The filter efficiency is influenced by the cellulose content which can adsorb the organic and inorganic content of $PM_{0.1}$, which is proven by the presence of several $PM_{0.1}$ elements in the filter. Efficiency is also influenced by the filter porosity. F_b porosity is 33% higher than F_{wh} (21%), so the efficiency of F_b is also higher than F_{wh}.

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DOI: http://dx.doi.org/10.15666/aeer/1702_19591965

Acknowledgements. Authors extend their sincere thanks to the laboratory crews of Air Quality and Astro Imaging, Department of Physics Brawijaya University, for their kind cooperation and necessary instrumental help.

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DOI: http://dx.doi.org/10.15666/aeer/1702_19591965

BIOLOGICAL ACTIVITY OF SOME NATIVE *BACILLUS THURINGIENSIS* BERLINER STRAINS AGAINST *EUTETRANYCHUS ORIENTALIS* KLEIN (ACARI: TETRANYCHIDAE)

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(Received 18th Oct 2018; accepted 2nd Jan 2019)

Abstract. Ten *Bacillus thuringiensis* strains (Bt) isolated from Moroccan habitats were screened for their acaricidal proprieties against the Citrus brown mite, *Eutetranychus orientalis* Klein (Acarina: Tetranychidae) under laboratory conditions. Results of bioassay indicated that larvae are more susceptible to the tested Bt strains than adult females at different concentrations (0.5, 1, 2, 4 and 8 mg/ml). Among the Bt strains examined, Bt 26.4 (LC_{50} = 1.533 mg/ml) and *Bt* 13.4 (LC_{50} = 1.385 mg/ml) showed respectively the highest mortalities to adults and larvae when compared with others and with the commercial product of Bt. Concerning fecundity, the effect of all tested strains showed highly significant reduction within adult females, whereas no significant effect was observed in eggs hatching for all strains.
Keywords: *Bacillus thuringiensis, spore-crystal mixtures, Moroccan habitats, biological control, Eutetranychus orientalis, citrus*

Introduction

The Citrus brown mite, *Eutetranychus orientalis* (Klein), is principally a pest of citrus (Jeppson et al., 1975) that attacks a wide range of agricultural, ornamental, and medicinal plants (Rasmy, 1978). It has been found on about 70 host plants of important economic value (Jeppson et al., 1975; Smith-Meyer, 1987; EPPO, 1990). The damage engendered by *E. orientalis* can cause from one side direct effects, including change of leaves' color that become yellowish-brown following chlorophyll depletion, little webbing, leaf fall and branches dieback leading to trees defoliation. From another side, it set off indirect effects such as a decreased photosynthesis process and a retarded grow of plants (Yousef et al., 2006). Actually, the application of synthetic acaricides is commonly applied in a wide agricultural range to manage this invasive mite. Consequently, the use of these acaricides cause negative effects to human health and environment (Cavalcanti et al., 2010; Kumral et al., 2010). However, to achieve a sustainable management, alternative controls are necessary to reduce the use of conventional pesticides (Isman, 2001; Choi et al., 2003), one of which an

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

entomopathogenic bacterial toxins, *Bacillus thuringiensis,* may have interesting substitutive managerial effect.

Bacillus thuringiensis (Bt) preparations are one of the successful biological control products (van Frankenhuyzen, 2009) that can be used as alternative to achieve integrated pest management (IPM) program. Several forms of Bt preparations such as liquid and solid are now available on the market for application as biological control agents against many pests (George and Crickmore, 2012). Currently, over than 400 Bt preparations are used as bio-insecticide product which represents approximately 2% of the total insecticidal sold (Bravo et al., 2011).

The insecticidal activity characteristic of Bt is mainly due to its crystalline proteins (δ-endotoxins) produced concomitantly with endospore at the stationary phase of its growth cycle (Schnepf et al., 1998). Though δ-endotoxins are highly specific to their target, they are safe to humans, plants and vertebrates, and are completely biodegradable (IPSC-WHO, 1999). They are indeed toxic to several insects belonging to dipteran, lepidopeteran, and coleopeteran insects, as well as pests from other orders such as fluke, protozoa, nematodes, and mites (Jisha et al., 2013). To our knowledge, few studies have demonstrated the efficacy of Bt against mites, among which Hall et al. (1971) found that thuringiensin could be used to control the citrus red mite, *Pananychus citri* (McGregor). Similarly, the efficiency of thuringiensin was revealed on the adults of *T. urticae* (Royalty et al., 1990; Payne et al., 1993; Chapman and Hoy, 2009). Furthermore, some recent studies have suggested also that Bt preparations are effective to control *Tetranychus macfarlanei* (Neethu et al., 2015), *Aceria guerreronis* (Smitha et al., 2015) and *E. orientalis* (Jisha et al., 2017).

To our knowledge, no study has investigated the acaricidal effects of native Bt strains isolated from Moroccan habitats. Therefore, in the present study, we evaluate the biological activity of ten Bt strains isolated from Marrakech region and Argan field in Morocco. In addition, we test the effects of those Bt strains against eggs, larvae and adult females of *E. orientalis.*

Materials and methods

Rearing of E. orientalis

The Citrus brown mite, *E. orientalis*, was collected from citrus orchards of Agafay, latitude 31°30'01.6"N, longitude 8°14'55.4"W in May 2018. Collected mites were reared continuously on bean plants (*Phaseolus vulgaris* L.) at Laboratory of Molecular and Ecophysiology Modeling, Faculty of Sciences Semlalia, University Cadi Ayyad, Marrakech (Morocco). The rearing conditions used were 29 ± 2 °C and 70 ± 5% R.H. The photoperiod was 16 L: 8 D using fluorescent lamps.

Bacterial strains

10 Bt strains were selected from 83 Bt strains previously isolated from Moroccan habitats (Aboussaid et al., 2010, 2011). The selection of strains was based on the morphological, biochemical and genetic characters (*Table 1*).

Production of spore-crystal biomass

A loopful of bacterial from a colony of each selected strain grown in CCY agar medium were used to inoculate a small tube containing 4.5 ml of liquid CCY medium

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(pre-culture), then the pre-culture was left to grow for 48 h at 28°C and agitated at 200 rpm (Edmund Bühler GmbH KS-15, Shakers). An aliquot was taken from the pre-culture to verify the formation of spore and crystal (over 90% sporulation is optimum). To eliminate vegetative cells, the pre-culture was heating at 70°C for 20 min (synchronization). 40 ml of main culture was inoculated with 1/1000 volumes of synchronized pre-culture and incubated as mentioned above (Aboussaid et al., 2011). From this culture, serial dilutions10-fold (10^{-1} to 10^{-5}) has been realized to determine the total number of cells by plating 0.1 ml of each dilution on CCY plates. Then the whole culture was centrifuged for 10 min at 9 000 × g (Universal 320R, centrifuge Hettich).

Table 1. *Morphological characterization (phase-contrast microscopy), biochemical (SDS-PAGE) and genetic (SDS-PAGE and PCR) of the selected Bt Moroccan strains (Aboussaid et al., 2010, 2011)*

N°	Strains	Sampling site	Habitat	Crystal form	Gene *cry* (PCR)
1	Bt A1	Marrakech	Wastewater -Sludge of wastewater treatment system	Spherical	*cry 7/8 + cry 9*
2	Bt A4			Irregular	*cry 7/8 + cry 9*
3	Bt A10			Spherical	*cry 11*
4	Bt A14			Spherical	*cry 11*
5	Bt A-Mg Mg2.7	Taroudant	Argan soils	Spherical	*cry 11*
6	Bt 21.6	Essaouira	Argan soils	Irregular	*cry 11*
7	Bt 26.4			Irregular	*cry 4*
8	Bt 32.3			Crystal > 1	*cry 4*
9	Bt 13.4			Irregular	*cry 4*
10	Bt B9	Beni-mellal	Bean-cultivated soil	Spherical	*cry 4*

The supernatant was discarded and the pellet was washed one time with ice-cold 1 mol/l NaCl, 10 mmol/l EDTA solutions (Ethylenediaminetetraacetic acid). Then the pellet was concentrated by lyophilisation to express the mixtures spore and crystal in mg. Finally, the pellet lyophilized was suspended in 1 ml of 10 mmol/l KCl (Aboussaid et al., 2011). Optical Density (OD) was measured by spectrophotometry at 600 nm (Spectrophotometer “VR-2000”, P-Selecta) and the suspensions were stored at -20 °C until bioassay. To limit proteolysis after centrifugation, all steps were done on ice.

Doses preparation

Doses of spore-crystal of each Bt strain were prepared by mixing 10 microliters of Triton-X100 (G-Labrogos), with 80 mg of each Bt strain, then 10 ml of distillate water was added to obtain 8 mg/ml. A series of dilution were prepared from the stock solution (8 mg/ml) using distillate water.

Distillate water and Triton-X100 at a rate 0.01% were used as absolute control, and Bt commercial product (DELFIN®, *Bacillus thuringiensis* sp. *Kurstaki*) and Triton-X100 were used as positive control (Bt. sp. *Kurstaki,* 2 mg/ml + 0.01% Triton-X100).

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Acaricidal activities of Bt strains against E. orientalis

Acaricidal effect of Bt strains on adult females and larvae

Toxicity of spore-crystal mixture of Bt strains to *E. orientalis* was evaluated following the method of Royalty et al. (1990) with slight modifications. Bean plant leaf discs were immersed during seven seconds in five concentrations 0.5, 1, 2, 4 and 8 mg/ml of each selected Bt strains. We have followed the same method to prepare the absolute control and the positive control. Treated leaf discs were placed on the lower surface in a petri dish surrounded with moist cotton wool and then allowed to completely dry at ambient temperature. Twenty *E. orientalis* adult females were introduced by using fine brush into the center of each leaf discs. Five replicates were made for each concentration. The same protocol was conducted for the larvae; eggs were placed on treated leaf discs, then the newly emerged larvae were used for the bioassay.

For all assays, after being exposed to leaf discs treated with *B. thuringiensis*, the larval and the adult females' mortalities were daily evaluated within the 96 h following the treatment. Furthermore, the number of oviposited eggs on the leaf was recorded at the 96th post-treatment hour.

Ovicidal effects of Bt strains

To determine an ovicidal activity of spore-crystal mixture of Bt strains, 20 adult females of *E. orientalis* were introduced on bean plant discs for oviposition and kept overnight. These discs were placed, the lower surface in petri-dish lined with moist cotton wool. After 24 h, the adult females were removed and the leaf discs were kept with 20 eggs on it; the excess of eggs was removed using a fine brush. Eggs laid on leaf discs were sprayed with five concentrations of spore-crystal mixture of each Bt strains. The leaf discs of absolute control and positive control were also prepared. Hatchability was determined 9 days after treatment and the eggs that did not hatch after this time was considered dead.

Statistical analysis

Mortality observations were subjected to one-way ANOVA by using SPSS program, version 11.5. Tukey's test was used for comparisons of means mortality. Probit analysis was used to determine lethal concentrations (LC_{50}) and the control mortalities were corrected by using Abbott's formula (*Eq. 1*).

$$\text{Corrected mortality } \% = \frac{1 - \text{n in T after treatment}}{\text{n in Co after treatment}} \times 100 \qquad \text{(Eq.1)}$$

where: n = Insect population, T = treated, Co = control.

Results

Effect of spore-crystal mixture of Bt strains on adult females of E. orientalis

Acaricidal activity of spore-crystal mixture of 10 selected Bt strains in female adults of *E. orientalis*, is shown in *Table 2*. There were significant differences between the corrected mortality of *E. orientalis* adults treated with five concentrations of each Bt

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strains ($p < 0.05$). Furthermore, a positive correlation was observed revealing an increase of the corrected mortality with the increase of toxins concentrations.

The corrected mortality values varied significantly between strains and the acaricidal rates were ranging from 6.43 to 28.73% (df: 10, F = 17.96, p < 0.0001) at 0.5 mg/ml, from 21.84 to 43.67% (df: 10, F = 5.49, p < 0.0001) at 1 mg/ml, from 40.23 to 62.07% (df: 10, F = 4.23, p < 0.0001) at 2 mg/ml, from 45.97 to 71.26% (df: 10, F = 3.02, p < 0.005) at 4 mg/ml and from 56.32 to 83.91% (df: 10, F = 2.45, p < 0.020) at 8 mg/ml.

At 96 h after treatment within the screened strains, Bt 26.4 was found to be the most toxic with a caused mortality reaching 83.91% for the highest dose (LC_{50} = 1.533 mg/ml). The effect of Bt 13.4 was significantly lower compared to the others in every tested concentration (LC_{50} = 4.228 mg/ml). Moreover, significant difference was noted between corrected mortality caused by positive control (71.01%) and those caused by our strains with the concentrations 0.5, 1 and 2 mg/ml, whereas in higher concentrations, 4 and 8 mg/ml, no significant difference was observed (*Table 2*).

Table 2. *Effect of spore-crystal mixture of Bt strains on adult females of E. orientalis 96 h after treatment*

Bt strains	Corrected mortality ± standard error [a,b]					LC_{50}	95% confidence limits
	0.5 mg/ml	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml		
Bt A1	14.94±2.14abA	43.67±3.81cB	51.72±3.89abcB	54.02±1.81abB	67.81±2.92abC	2.457	1.611±3.652
Bt A4	8.73±2.53abA	29.88±6.65abcAB	52.87±6.13abcBC	55.17±8.79abBC	71.26±4.81abC	2.424	1.804±3.203
Bt A10	28.73±1.4bA	37.93±8.41abcAB	56.32±3.89abcB	66.66±2.81abC	77.01±1.81abC	1.703	1.122±2.385
Bt A14	11.72±7.25abA	21.84±6.94aAB	40.23±5.32aB	49.42±5.57abCD	65.51±3.63abCD	3.778	2.706±5.304
Bt A-Mg Mg2.7	6.43±2.42aA	25.28±2.56abB	41.38±4.94abBC	64.36±3.35abD	70.11±3.35abD	2.795	2.104±3.624
Bt 21.6	11.03±3.77abA	28.73±5.33abcA	62.07±4.66bcC	66.66±6.89abB	70.11±8.41abB	2.171	0.145±7.380
Bt 26.4	27.58±3.89bA	37.93±8.01abcAB	56.32±2.29abcBC	67.81±5.91abCD	83.91±4.22bD	1.533	1.078±2.039
Bt 32.3	14.94±4.94abA	41.38±1.15bcB	51.72±3.89abcB	71.26±5.45bC	79.31±5.01bC	1.768	1.293±2.300
Bt 13.4	19.54±7.26abA	31.03±3.14abcAB	43.67±5.86abB	45.97±4.31aC	56.32±7.83aC	4.228	2.541±8.545
Bt B9	25.28±4.06abA	40.23±2.93bcAB	45.97±4.66abB	67.81±5.33abC	75.86±2.81abC	1.889	1.279±2.623
Positive control*	71.01±1.87c	71.01±1.87d	71.01±1.87c	71.01±1.87b	71.01±1.87ab	_	_
_	df: 10, F=17.96;	df: 10, F=5.49;	df: 10, F=4.23;	df: 10, F=3.02;	df: 10, F=2.45;	_	_
_	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.005	p < 0.020	_	_

[a]Treatments followed by the same lowercase letter each column are not significantly different at $\alpha = 0.05$
[b]Treatments followed by the same uppercase letter each row are not significantly different at $\alpha = 0.05$
*Commercial product of *Bacillus thuringiensis.* Applied dose: 2 mg/ml and Triton-X100 at the rate of 0.01%

Effect of spore-crystal mixture of Bt strains on larvae of E. orientalis

Results of larvicidal effectiveness of 10 Bt strains are presented in *Table 3*. The difference of mite's corrected mortalities was significantly higher between those exposed to the lower concentrations of spore-crystal mixtures and those exposed to higher concentrations. The corrected mortality was dose dependent and varied significantly within all strains. Larvicidal rates were ranging from 17.01 to 35.10% (df: 10, F = 13.22, p < 0.0001) at 0.5 mg/ml, from 20.21 to 44.68% (df: 10, F = 8.29, p < 0.0001) at 1 mg/ml, from 39.36 to 67.02% (df: 10, F = 3.53, p < 0.002) at 2 mg/ml, from 57.45 to 71.27% (df: 10, F = 3.02, p < 0.842) at 4 mg/ml and from 72.34 to 85.11% (df: 10, F = 2.90, p < 0.007) at 8 mg/ml.

The lethal concentrations to kill 50% of larvae (LC_{50}) were determined after 96 h following treatment and summarized in *Table 2*. Results showed that Bt 13.4 at 8 mg/ml

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DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

cause maximum mortality (LC_{50} = 1.385 mg/ml) followed by Bt A-Mg Mg2.7 (LC_{50} = 1.472 mg/ml), Bt A10 (LC_{50} = 1.473 mg/ml), Bt 26.4 (LC_{50} = 2.191 mg/ml) and Bt A1 (LC_{50} = 2.263 mg/ml). Significant difference was noted between corrected mortality caused by positive control and those caused by our strains at lower concentrations 0.5 and 1 mg/ml, whereas no significant difference is revealed at the higher concentrations 2, 4 and 8 mg/ml (*Table 3*).

Table 3. *Effect of spore-crystal mixture of Bt strains on larvae of E. orientalis treated 96 h after treatment*

Bt strains	Corrected mortality ± standard error [a,b]					LC_{50}	95% confidence limits
	0.5 mg/ml	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml		
Bt A1	23.402±3.61abA	25.53±2.37abA	47.87±3.91abB	63.83±6.81aBC	77.65±2.61abC	2.263	1.737±2.907
Bt A4	28.72±2.71abA	34.04±2.71abA	52.13±4.75abB	63.83±5.42aBC	75.53±4.63abC	2.304	1.609±3.184
Bt A10	34.04±2.12abA	36.17±8.06abAB	54.25±4.93abBC	63.83±2.61aC	88.29±1.99bD	1.473	1.095±1.894
Bt A14	25.53±1.68abA	32.97±3.19abA	53.19±4.57abB	64.89±3.19aBC	72.34±3.91abC	2.286	1.627±3.129
Bt A-Mg Mg2.7	30.85±3.36abA	44.68±2.71bB	55.32±2.71abBC	63.83±6.15a C	80.85±3.61abD	1.472	1.028±1.976
Bt 21.6	26.59±4.87abA	34.04±7.25abA	39.36±5.72a A	65.95±7.99a B	85.11±2.61bB	2.419	1.794±3.130
Bt 26.4	25.53±7.89abA	44.68±2.71b B	52.13±2.37abBC	63.83±1.06aCD	74.46±2.61abD	2.191	1.482±3.097
Bt 32.3	22.338±2.71abA	34.04±1.31abAB	42.55±4.25a B	62.76±3.36aC	74.46±3.11abC	2.332	1.745±3.089
Bt 13.4	35.10±1.99bA	43.61±3.61bA	67.02±4.25b B	71.27±2.71aB	76.59±3.97abB	1.385	0.883±1.954
Bt B9	17.01±2.71aA	20.21±5.57aA	46.81±8.57abB	57.45±2.91aBC	73.41±4.75abC	2.829	2.195±3.648
Positive control*	68±2.54c	68±2.54c	68±2.54b	68±2.54a	68±2.54a	_	_
_	df: 10, F=13.22;	df: 10, F=8.29;	df: 10, F=3.53;	df: 10, F=3.02;	df: 10, F=2.90;	_	_
_	p < 0.0001	p < 0.0001	p < 0.002	p < 0.842	p < 0.007	_	_

[a]Treatments followed by the same lowercase letter each column are not significantly different at α = 0.05
[b]Treatments followed by the same uppercase letter each row are not significantly different at α = 0.05
*Commercial product of *Bacillus thuringiensis.* Applied dose: 2 mg/ml and Triton-X100 at the rate of 0.01%

Effect of spore-crystal mixture of Bt strains on fecundity of E. orientalis

The results of fecundity of treated adult females are presented in *Table 4*.

Table 4. *Fecundity of E. orientalis treated with Bt strains 96 h after treatment*

Bt strains	Mean fertility± standard error [a,b]				
	0.5 mg/ml	1 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml
Bt A1	2.01±0.19aB	0.56±0.06aA	0.49±0.09a A	0.42±0.11aA	0.11±0.04aA
Bt A4	5.54±0.55bB	1.44±0.39abcA	0.42±0.16a A	0.18±0.09aA	0.12±0.12aA
Bt A10	1.81±0.45aB	0.43±0.09aA	0.44±0.09a A	0.30±0.08aA	0.11±0.03aA
Bt A14	1.89±0.15aB	0.84±0.15abA	0.71±0.11a A	0.58±0.11aA	0.45±0.07aA
Bt A-Mg Mg2.7	1.83±0.32aA	1.00±0.17abA	0.69±0.13a A	0.39±0.09aA	0.53±0.04aA
Bt 21.6	3.91±0.56abA	2.48±0.65bcBC	1.44±0.38a AB	0.66±0.09aA	0.10±0.04aA
Bt 26.4	2.29±0.37aA	0.98±0.12abA	0.54±0.11aA	0.42±0.07aA	0.31±0.06aA
Bt 32.3	1.83±0.46aA	1.25±0.24abcA	0.68±0.11aA	0.51±0.06aA	0.25±0.03aA
Bt 13.4	2.32±0.31aB	1.21±0.22abcAB	0.78±0.06aA	0.32±0.07aA	0.28±0.08aA
Bt B9	2.11±0.35aA	0.35±0.11aA	0.51±0.07aA	0.48±0.27aA	0.08±0.03aA
Absolute control	9.63±0.61c	9.63±0.61d	9.63±0.61c	9.63±0.61c	9.63±0.61c
Positive control *	2.92±0.63a	2.92±0.63c	2.92±0.63b	2.92±0.63b	2.92±0.63b
_	df: 11, F=27.26;	df: 11, F=49.13;	df: 11, F=4.23;	df: 11, F=92.77;	df: 11, F=110.03;
_	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.00001	p < 0.0001

[a]Treatments followed by the same lowercase letter each column are not significantly different at α = 0.05
[b]Treatments followed by the same uppercase letter each row are not significantly different at α = 0.05
*Commercial product of *Bacillus thuringiensis.* Applied dose: 2 mg/ml and Triton-X100 at the rate of 0.01%

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DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

The bioassay has shown a decrease of oviposition within the treated females with increasing concentrations of selected Bt strains. In the absolute control, the mean of oviposited eggs reached 9.63 per female, a fecundity which is highly significantly superior to others of every Bt treated females ($p < 0.0001$). Whereas, the comparison between fecundities of females treated with the positive control and those treated with Bt strains has shown no significant difference at low doses (0.5 and 1 mg/ml), while at high doses, the fecundity values of Bt treated females decreased significantly.

Effects of spore-crystal mixture of Bt strains on eggs hatching

The observed hatch rates at 9 days after treatment are presented in *Table 5*. No concentration of all the ten Bt strains was able to avoid eggs hatching. There was no significant difference in eggs hatchability within tested Bt strains, absolute control and positive control ($P < 0.05$).

Table 5. *The percentage of eggs hatching 9 days after treatment with spores-crystal mixtures of Bt strains*

Bt strains	**Percentage of hatchability ± standard error[a]**				
	0.5 mg/ml	**1 mg/ml**	**2 mg/ml**	**4 mg/ml**	**8 mg/ml**
Bt A1	82±2.54ab	82±2.01ab	82±3.39ab	83±3.74abc	80±3.53a
Bt A4	89±1.87ab	83±2.54ab	77±2.54a	76±2.91ab	74±1.87a
Bt A10	87±4.06ab	79±2.91a	74±3.67a	74±1.87ab	73±2.54a
Bt A14	91±3.31ab	87±2.54ab	86±2.91ab	79±1.87ab	81±1.87ab
Bt A-Mg Mg2.7	91±2.91ab	85±4.47ab	79±3.67a	79±3.67ab	76±5.33a
Bt 21.6	84±3.67ab	83±2.54ab	79±3.67a	77±3.39ab	75±3.53a
Bt 26.4	88±2.54ab	84±2.91ab	82±3.74ab	81±4.01ab	79±4.31a
Bt 32.3	91±1.87ab	85±3.53ab	84±1.87ab	83±2.54abc	83±2ab
Bt 13.4	84±4.3ab	81±4.01ab	83±4.89ab	89±2.91abc	78±2.54a
Bt B9	83±2.54ab	79±3.31a	75±4.18a	72±2.54a	67±2a
Control	97±1.22b	97±1.22b	97±1.22b	97±1.22b	97±1.22b
Positive control[b]	77±6.04a	77±6.04a	77±6.04a	77±6.04ab	77±6.04a
_	df: 11, F=27.26;	df: 11, F=49.13;	df: 11, F=4.23;	df: 11, F=92.77;	df: 11, F=110.03;
_	p < 0.000	p < 0.000	p < 0.000	p < 0.000	p < 0.000

[a]Treatments followed by the same letter are not significantly different at $\alpha = 0.05$
[b]Commercial product of *Bacillus thuringiensis.* Applied dose: 2 mg/ml and Triton-X100 at the rate of 0.01%

Discussion

Bacillus thuringiensis is an entomopathogen able to product two proteins inclusions during sporulation. The parasporal crystalline inclusion containing Cry and Cyt (also known as δ-endotoxins) are toxic to different insect orders (Schnepf et al., 1998; Tsuchiya et al., 2002; Aboussaid et al., 2010). Many formulations of Bt toxins revealed high toxicity to mite (Vargas et al., 2001; Jisha et al., 2017). For instance, these toxins have been reported to have different influences on *Tetranychus urticae,* such as antifeedant effect and reduction of fecundity (Royalty et al., 1990, 1991). Such toxicity of Bts is principally due to the primary action of Cry toxins consisting of the lysis of the midgut epithelial cells in the target insect so that it forms pores in the apical microvilli membrane of the cells (Aronson and Shai, 2001; de Maagd et al., 2001; Bravo et al., 2007).

Our results showed a significant acaricidal effect of ten Bt strains spore-crystal mixtures against adult females and larvae of *E. orientalis.* For adult females, 100% of

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DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

tested Bt are toxic and at the higher concentration (8 mg/ml) the mortality ranged between 65.51% and 83.91% after four days. These are corresponding with the data of Neethu et al. (2015) who studied the Bt activity against adults of *Tetranychus macfarlanei* and have reported that the culture pellet, mixtures of δ-endotoxin and crystals, (1-10 mg/ml) mixed with artificial diet caused mortality ranged from 40% to 80% four days after the treatment. Moreover, Jisha et al. (2017) have demonstrated the efficacy of the crude pellet of Bt var. *Kurstaki* (Btk) to combat *E. orientalis*. The results of this study revealed that the crude Btk-toxin eliminate completely the mite of infested plants after 12 days of direct spraying, an effect that has been established without supplementing any adhesive or surfactant. For larvae, all tested Bt strains are highly toxic and the mortality exceeds 80% for some strains. Similar results were reported by Vargas et al. (2001) in a study where the larvae of *Tetranychus urticae* and *Pananychus ulmi* have been highly susceptible to thuringiensin (β- exotoxin). Nevertheless, the results found by Alper et al. (2013) are in disagreement with our data. These authors have studied the effects of spore-crystal mixtures of 31 native *B. thuringiensis* isolates against *T. urticae* nymphs where they have found that 42% of the isolates caused mortality ranged between 16% and 30%. In addition to this, the same study has reported that 58% of the isolates resulted in less than 15% mortality. The difference observed between our results and those of Alper et al. (2013) on the larvicidal effect of the mixture may be linked to variability on the genes coding the Cry proteins.

The larvae of *E. orientalis* are more susceptible to spore-crystal mixtures of our Bt strains than adult females. However, the mortality of both stages increased with increasing concentration of Bt strains. These results agreed with the findings of Royalty et al. (1990) and Vargas et al. (2001) who reported that the application of thuringiensin affect more the immature stages than adults of *Tetranychus urticae* and *Pananychus ulmi,* with an observed dose dependent mortality for the both stages. Therefore, the adult mortality might be a result of the disruption of a diverse biochemical mechanism to that in immature stages (Beebee and Bond, 1973a, b).

Furthermore, the results presented in *Table 4* indicate that the spore-crystal mixtures of our Bt strains caused a reduction in the fecundity of adult female of *E. orientalis.* These are consistent with the results found by Royalty et al. (1990) and Vargas et al. (2001) who noted that the number of eggs laid by treated females of *Tetranychus urticae* is lesser than untreated ones.

Moreover, our finding indicates that spore-crystal mixtures of our strains did not show any ovicidal effect to eggs of *E. orientalis,* which is in agreement with Reza et al. (2011) who postulated that all concentration of thuringiensin did not revealed significant difference in the percentage of eggs hatching of *Tetranychus urticae*.

Conclusions

Based on the present research, Moroccan Bt strains provide the evidence for their acaricidal properties. The most Bt strains tested revealed a great activity against adult females and especially towards larvae and adults, also they reduced greatly the fecundity of female adults. All these results confirm that most of our Bt strains can be used as a good alternative to conventional pesticides harmful to consumers and environment.

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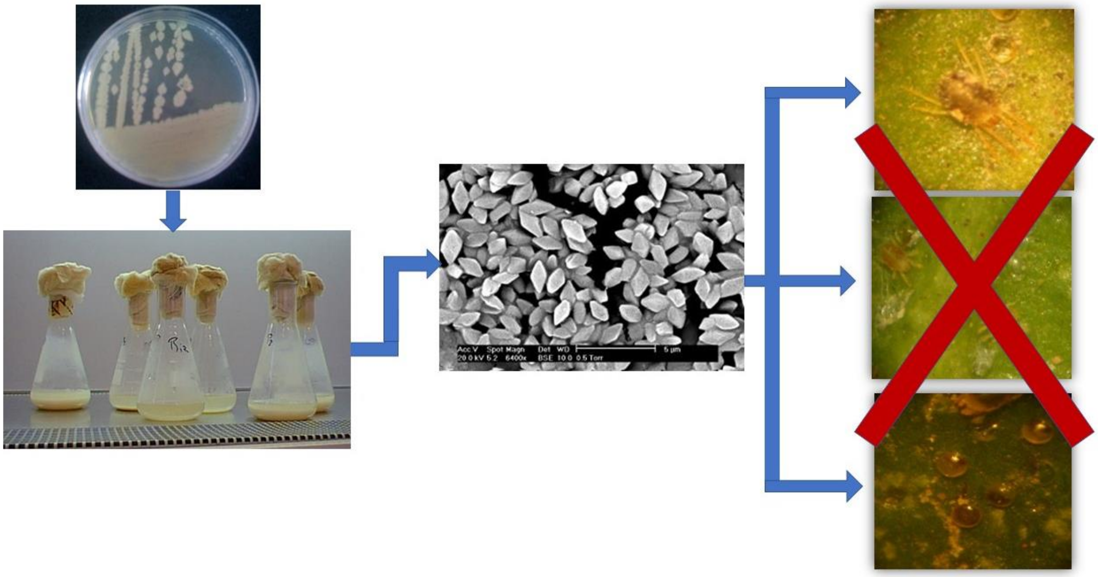
DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

APPENDIX

Graphical abstract



http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19671977

PHYSIOLOGICAL, AGRONOMICAL AND QUALITY RESPONSE OF BREAD WHEAT TO PHOSPHORUS APPLICATION UNDER DRYLAND CONDITION

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(Received 20th Oct 2018; accepted 21st Dec 2018)

Abstract. An understanding of physiological and agronomical traits associated with high grain yield and efficiency of phosphorus use is important to the improvement of genotypes under dryland conditions. An experiment was conducted to evaluate the influence of differentiated phosphorus fertilization rate on the yield and quality of wheat at the experimental farm, University of Dicle, Diyarbakir, Turkey during 2013-2014 and 2014-2015 growing seasons. The experiment was consisted of five treatments comprising of five phosphorus (P_2O_5) levels (0 kg ha^{-1}, 30 kg ha^{-1}, 60 kg ha^{-1}, 90 kg ha^{-1} and 120 kg ha^{-1}). It was observed that phosphorus levels exhibited significant differences to the grain numbers per m^2, grain weight and grain yield ha^{-1}, as well as to starch content and protein content in the first season. There was no significant effect of phosphorus on all studied grain yield and quality characters in the second season. The improvement in grain yield was significantly associated with the increase in the values of grain number, accordingly, the coefficients of grain number and grain yield showed that grain number had a positive and direct effect on grain yield suggesting a criteria trait to enhance yield. It was concluded that the highest grain yield of wheat crop was obtained with 120 kg ha^{-1} P_2O_5 and it showed comparatively better performance than another treatment under investigation.
Keywords: *wheat, grain yield and quality, protein yield, starch content, water use efficiency*

Introduction

Wheat (*Triticum aestivum* L.) is one of the main essential food and a vital source of energy in human diet. It is one of the major significant and widely cultivated crop of the entire world and it is a major source of nourishment (Aktas, 2016; Kizilgeci et al., 2017; Khaled et al., 2018; Hossain et al., 2018). It is a principal food for more than 35% of the world's population, and a source of food and livelihood for over one billion people in developing countries (Metwali et al., 2011). Wheat grains used in the making of flour, breads, biscuits, cakes, cookies, pasta, noodles and also for beer, and other alcoholic beverages or biofuel. Presently, wheat is grown on more than 200 million hectares of the world's cultivated land and is the most important agricultural commodity in international trade (FAO, 2010). The worldwide requirements of wheat production by 2020 reached about 950 million tons to achieve food needs imposed by the increase of population and further, this could be reached, if global wheat productivity is enriched by 2.5% yearly. (Singh et al., 2011; Barutcular et al., 2017; Yildirim et al., 2018). Consequently, to feed the rapidly growing population of the world, food production should be heightened.

Phosphorus is a necessary nutrient for the normal development of the plant (Kaleem et al., 2009). The quality and productivity of wheat crop may be affected by the management of primary fertilizers (NPK) but, it is also physiologically dependent on the different micronutrients along major nutrient management (Wiatrak et al., 2006). It plays an important role and has a number of functions in the growth of plant. It utilizes

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sugar and starch and involved in transfer of energy (Mumtaz et al., 2014). Balanced fertilization not only enhanced the yield of wheat, but also had a good influence on phosphorus uptake by plants (Rehman et al., 2006). While, deficiency of phosphorus is one of the factor of abiotic stresses that determine the productivity of cereal crops (Yu et al., 2013). Deficiencies regarding soil phosphorus (P) and zinc (Zn) noticed widely in numerous regions across the globe (Marschner, 1995). Keeping in view the importance of phosphorus in crop productivity, the aim of the field study was to evaluate the response of bread wheat in terms of growth, yield and quality properties to different rates of phosphorus fertilization.

Materials and methods

Plant material and growing conditions

Two field experiments were conducted under rainfed conditions at the experimental farm, University of Dicle, Diyarbakir (latitude 37°88′N, longitude 40°27′E, altitude 680 m above sea level), Turkey (*Figure 1*).

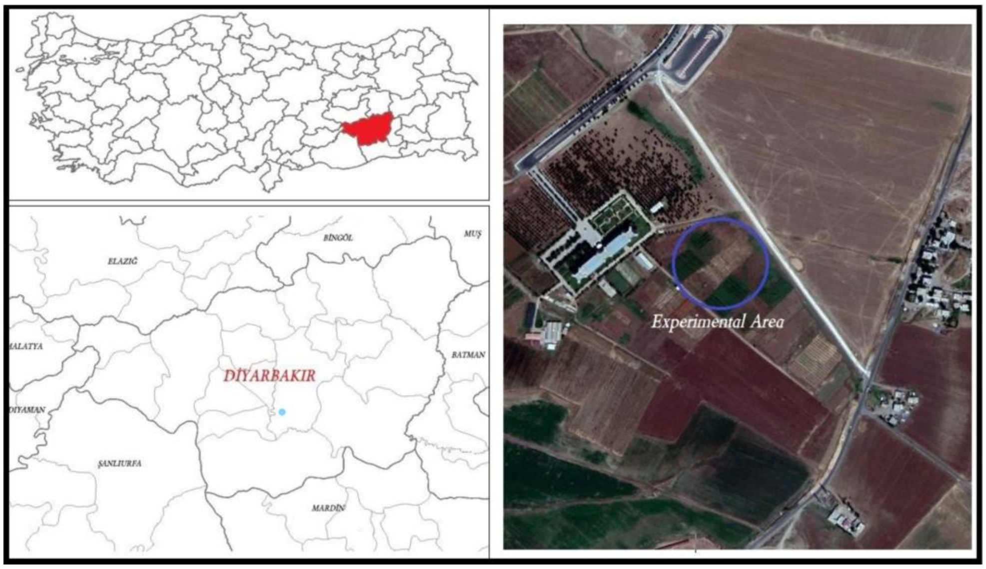

Figure 1. *Map of the experimental area*

Seeds of wheat cv: Pehlivan were sown on 12.12.2013 in the 1st year and on 26.12.2014 in the 2nd year. Pehlivan is one of the most important winter bread wheat variety registered in 1998. It has a medium early growth cycle. The spike is white, smooth, awnless and compact, and the plant height is 95-100 cm. The variety has high productive tillering with high winter hardiness to be grown in winter wheat areas of the Central Anatolian Plateau with a medium tolerance to drought. Pehlivan also has medium opposition to lodging. It is suitable for growing on fertile and poor fertile soils, and is grown in most wheat producing regions of Turkey. The grain is oval, hard-red, very large, and has good milling and baking qualities.

The physical and chemical properties of the soil used in the experiment were as follows: clay soil (71.6% clay), organic matter 1.25%, available phosphoros 16.3 kg ha^{-1}, salt 0.01%, calcareous 13.02%, pH 7.73. Meteorological data were given in *Table*

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1. The experiment was arranged in randomized complete block design with four replications. The plots were arranged in six rows with 4 m long, 0.2 m intra-row using an experimental sowing machine. Five phosphorus (P_2O_5) levels (0, 30, 60, 90 and 120 kg P_2O_5 ha^{-1}) were established in the form of triple super phosphate. The trial was conducted under rainfed conditions. Genotype were sown at a seed rate of 450 seed m^{-2}. Nitrogen fertilization was carried out 2 times in the form of ammonium nitrate (33%N). 60 kg ha^{-1} was applied at the sowing (basal dose) and tillering stage, in accordance with recommendations.

Table 1. *Meteorological data of experimental area for the season 2013-2014 and 2014-2015*

Years		Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	Total/ average
2013-2014	Precipitation (mm)	0.0	53.8	50.4	43.0	17.0	60.6	39.9	48.8	21.4	334.9
	Temperature (°C)	17.0	11.4	-3.4	3.4	5.0	10.8	14.7	19.7	26.5	11.7
2014-2015	Precipitation (mm)	84.2	10.4	31.6	77.4	69.2	55.6	29.0	41.4	18.4	417.2
	Temperature (°C)	18.4	9.9	3.9	1.1	7.9	9.7	15.7	19.9	26.8	12.6
Long-term (1950-2015)	Precipitation (mm)	33.0	52.8	71.8	69.0	67.7	69.1	68.4	44.4	8.8	485.0
	Temperature (°C)	17.2	9.3	3.9	1.7	3.6	8.4	13.8	19.2	26.2	11.7

Data collection and measurements

The chlorophyll content (SPAD) at the midpoint of the flag leaves of 10 randomly selected plants in each plot was measured by using a portable chlorophyll meter (Minolta SPAD-502, Osaka, Japan). Grain yield (kg ha^{-1}) was calculated in harvest plots. Grain yield in kilogram per hectare was calculated from the yield harvested from each plot, the plants collected with a harvester from the area were used for assessments of grain yield. The grain weight included a moisture content of 12%. The sampling and determination of grain quality traits were described according to AACC (2000).

Water use efficiency (WUE)

Water-use efficiency is normally described as the ratio of crop yield to the amount of water needed to achieve the yield according to total consumed water through wheat life circle, WUE was measured by referring to Gao (2000) and Gao et al. (2004).

Statistical analysis

The obtained data were subjected to analysis of variance as described by Gomez and Gomez (1984). The data were statistically analyzed by using MSTAT-C package program. The Least Significant Differences (LSD) test was used to compare the treatments at 0.05 levels.

Results and discussion

The statistical analysis of variance (*Table 2*) illustrated the influence of phosphorus on the yield and quality traits of wheat. It is evident from the table that the number of

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grains per m^2, thousand grain weight and grain yield were influenced significantly by the application of P fertilizer treatment in the first season. The analysis data showed that the analysis of variance of quality properties resulted in significant difference for phosphorus levels applications. Phosphorus fertilizer application also significantly infulenced the starch and protein content only in the 2014 growing season (*Table 3*). However, the maximum number of grains m^{-2} was observed with the application of 120 kg P_2O_5 ha^{-1}. While, the minimum value for number of grains was observed in control treatment. The results are in agreement with the findings of Ravnskov et al. (1995) and Reuter et al. (1995) who found that the application of phosphatic fertilizer significantly enhanced the number of grains per hill or per m^2 area.

Data regarding thousand grain weight influenced by various phosphorus rates are presented in (*Table 2*). Data after statistical analysis showed significant ($p \leq 0.05$) effect of various phosphorus rates on thousand grain weight. Data after statistical analysis revealed that in wheat crop thousand grain weight were significantly ($p \leq 0.05$) affected by phosphorus levels and maximum weight of thousand grains was recorded from plots applied with 120 kg P_2O_5 ha^{-1}, while the minimum thousand grain weight was observed from the control plot. Rahmatullah et al. (1994) found 90 kg P_2O_5 ha^{-1} the optimum rate for the greatest growth, development thousand grain weight of wheat. Similarly, Brennan (1992) found a progressive improvement in thousand grains weight with the increase in phosphorus dose from 60 to 120 kg P_2O_5 ha^{-1}, gradually.

It was intensively observed that, the data after statistical analysis showed significant ($p \leq 0.05$) effect of different levels of phosphorus on grain yield. Mean values for different phosphorus levels revealed that maximum grain yield were produced by the plots that received 120 kg P_2O_5 ha^{-1}, while less grain yield were produced by the control plots. The plot treated 120 kg P_2O_5 ha^{-1} achieved maximum yield (4510 kg ha^{-1}) which was statistically significant only in the first season, while the minimum grain yield (3100 kg ha^{-1}) was achieved in control plots in the first season. The main reason for the increase in grain yield with various rates of phosphatic fertilizer could be the higher thousand grain weight which might be due to higher rate of photosynthesis and better crop health which ultimately enhanced the final grain yield (Iqbal et al., 2003; Mehdi et al., 2003). Alam et al. (2003) has observed that the application of phosphorus fertilizer (SSP) to wheat has significantly improved the plant height, number of tillers $plant^{-1}$, straw and grain yield as well as P-uptake in grain over control. Regardless of the second year crop, phosphorus fertilizers shown no significant effect on the grain yield and attributes. This results are in agreement with the findings of Mihoub and Deraoui (2014), who observed that phosphorus fertilizer application did not effect significantly the P content in grain yield. The relationship of high grain yield, due to P fertilizer application with number of seed/plant have been reported elsewhere (Singh et al., 2012).

The analysis results of Protein yield (g m^{-2}) and Starch yield, (g m^{-2}) of wheat are presented in (*Table 3*), results showed the effect of phosphorus levels, the statistical analysis of variance indicated that the mean Protein yield (g m^{-2}) and Starch yield, (g m^{-2}) were significantly different. Among various factors, plant nutrition circumstances - usage of fertilizers, soil supply conditions - suit best to modify, and to enhance certain parameters (Sarkadi, 1981). According to views of several investigation variations in quality properties depend on fertilizing application (Tabl and Kiss, 1983). The yield traits and quality properties of wheat are usually affected by genotypes, environmental factors and their interactions (Barutcular et al., 2016).

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Table 2. *Mean squares of the variance analysis for studied character*

Source of Variance	DF	SPAD	PH	GY	TGW	GN	PC	SC	WG	ZS	Hardness	PY	SY
							2014						
Block	3	44.55	94.73	1331	1.91	670992.5	0.11	0.031	0.555	2.802	2.087	36.3	613.5
Phosphorus	4	12.19	12.55	11163.825**	2.234*	8666715.2*	0.26	0.104*	0.623	3.116	0.878	213.648*	4949.4
Error	12	7.71	11.82	1878	0.42	1731320	0.15	0.028	0.229	1.771	3.284	45.5	792.4
CV, %		5.53	3.67	11.66	2.03	11.29	2.52	0.25	1.59	2.66	1.53	11.77	11.59
							2015						
Replication	3	6.58	146.87	2355	5.41	2408949	0.39	0.170	2.337	27.829	5.753	38.2	1008.0
Phosphorus	4	3.33	47.08	3107	1.80	2843499	0.30	0.054	0.569	9.157	3.488	92.7	1319.9
Error	12	2.88	26.58	1139	2.27	1229514	0.27	0.075	1.041	13.623	3.702	38.4	496.1
CV, %		3.62	5.60	7.98	4.02	9.78	3.65	0.43	3.46	7.64	1.84	10.29	8.19

*, **, Significance level at 0.05 and 0.01 probability. DF: Degree of freedom.GY: Grain yield, PH: Plant height, TGW: Thousand grain weight, GN: Grain number m², PC: Protein content, SC: Starch content ZS: Zeleny sedimantation, WG: Wet gluten, PY: Protein yield, SY:Starch yield

Table 3. *Means of investigated traits in 2013-2014 and 2014-2015*

Phosphorus level	SPAD (unit)	Plant height (cm)	Grain yield (kg ha^{-1})	1000-Grain weight(g)	Grain number m²	Protein content (%)	Starch content (%)	Wet gluten (%)	Zeleny sedimentation (ml)	Hardness	Protein yield (g m^{-2})	Starch yield (g m^{-2})
							2014					
0	48.6	92	3100	30.6	10124	15.9	65.3	30.7	51.5	118	49.2	202
30	48.8	94	3400	32.2	10581	15.5	65.5	30.0	49.7	119	52.7	223
60	53.0	95	3750	31.8	11800	15.5	65.6	30.2	49.6	119	57.3	243
90	50.4	92	3830	32.3	11838	15.4	65.7	29.9	49.5	119	58.9	251
120	50.1	96	4510	32.3	13917	15.2	65.7	29.7	49.5	118	68.4	296
Mean	50.2	94	3720	31.8	11652	15.5	65.5	30.1	50.0	119	57.3	243
LSD 0.05	Ns	ns	668	1.0	2027.0	ns	0.15	ns	ns	ns	5.99	25.0
							2015					
0	45.4	88.5	3860	37.3	10355	14.0	64.3	29.0	46.5	105	54.1	248
30	46.6	89.0	4060	38.2	10622	13.9	64.2	29.4	46.8	103	56.4	261
60	47.6	96.8	4380	36.4	12044	14.3	64.5	29.6	49.1	105	62.7	282
90	47.2	92.5	4270	37.5	11403	14.6	64.2	30.0	49.9	106	62.2	274
120	47.5	93.8	4580	37.7	12266	14.3	64.4	29.6	49.0	104	65.7	295
Mean	46.9	92	4230	37.4	11338	14.2	64.3	29.5	48.3	105	60.2	272
LSD 0.05	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

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Correlation analysis

Correlation coefficients between all traits are illustrated in *Table 4* and grain yield of wheat crop show positive association with correlation coefficient values of grain number. Grain number was significant to high grain yield for both years. Under the low rainfall year (2014); Grain yield was positively and significantly influenced by 1000-grain weight. The SPAD value was positively and significantly associated with the grain yield in the 2nd year of 2015, which was a year with high rainfall. The extent of association of different traits also revealed the significant relationship with grain yield of the crop and this result was supported by Shah et al. (2003). In general, it is believed that grain quality improved with the increament of phosphorus rates, and phosphorus caused remarkable improvement in protein yield. The protein content was negatively associated with grain yield. The unit increase in grain yield was positively associated with the increased values of observed crop parameters by various yield attributes, grains spike^{-1}, seed index and harvest index (Noonari et al., 2016).

Table 4. *Correlation matrix of yield and quality traits under rainfed condition during the growing season since 2013-2014 and 2014-2015 in Diyarbakir*

Traits	GY	SPAD	PH	TGW	GN	PC	SC	WG	ZS	Hardness	PY	SY
2014												
GY	1.000											
SPAD	0.393	1.000										
PH	0.693	0.427	1.000									
TGW	0.695	0.278	0.472	1.000								
GN	**0.995****	0.402	0.699	0.618	1.000							
PC	-0.918	-0.351	-0.658	-0.922	-0.872	1.000						
SC	0.867	0.531	0.468	**0.884***	0.823	-0.938	1.000					
WGC	-0.836	-0.201	-0.551	-0.962	-0.775	**0.979****	-0.902	1.000				
ZS	-0.707	-0.518	-0.556	-0.964	-0.639	**0.907***	-0.912	**0.903***	1.000			
Hardness	-0.150	0.430	-0.102	0.492	-0.229	-0.179	0.327	-0.240	-0.570	1.000		
PY	**0.999****	0.362	0.675	0.675	**0.996****	-0.905	0.853	-0.825	-0.679	-0.188	1.000	
SY	**0.999****	0.363	0.683	0.704	**0.992****	-0.922	0.866	-0.847	-0.706	-0.156	**0.999****	1.000
2015												
GY	1.000											
SPAD	**0.923***	1.000										
PH	0.822	0.862	1.000									
TGW	-0.189	-0.241	-0.687	1.000								
GN	**0.975****	**0.905***	**0.917***	-0.402	1.000							
PC	0.616	0.642	0.639	-0.343	0.642	1.000						
SC	0.535	0.432	0.765	-0.760	0.680	0.124	1.000					
WGC	0.670	0.810	0.587	-0.054	0.620	0.863	-0.063	1.000				
ZS	0.798	0.840	0.799	-0.361	0.815	**0.954***	0.284	**0.911***	1.000			
Hardness	0.024	0.053	0.314	-0.584	0.148	0.743	0.067	0.386	0.553	1.000		
PY	**0.985****	**0.918***	0.839	-0.243	**0.971****	0.743	0.491	0.750	**0.885***	0.189	1.000	
SY	**1.000****	**0.920***	0.823	-0.193	**0.976****	0.603	0.547	0.657	0.788	0.012	**0.982****	1.000

*, **, %5 and %1 significant, respectively, GY: Grain yield, PH: Plant height, TGW: Thousand grain weight, GN: Grain number m², PC: Protein content, SC: Starch content ZS: Zeleny sedimantation, WG: Wet gluten, PY: Protein yield, SY:Starch yield

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Water use efficiency

Increasing phosphorus levels were positively and significantly improved water use efficiency (WUE) under low rainfall conditions (*Figure 2*). The WUE values are regulated, and the relationship is stronger. However, as reported by Gerloff (1977), when the performance of cultivars to P supply and their yield potential at low P supply. There are various considerations of P use efficiency in previous investigations of wheat, which have resulted in various definitions, such as agronomic efficiency (Alam et al., 2003).While, the non-significant inffluence of P application on crop water use might be that 90 kg P ha^{-1} was too high to influence root growth for superior water extraction (Lusiba et al., 2018). However, there is a deficiency of information on the interactive influence of phosphorus fertilizer on water use efficiency of wheat under dry conditions.

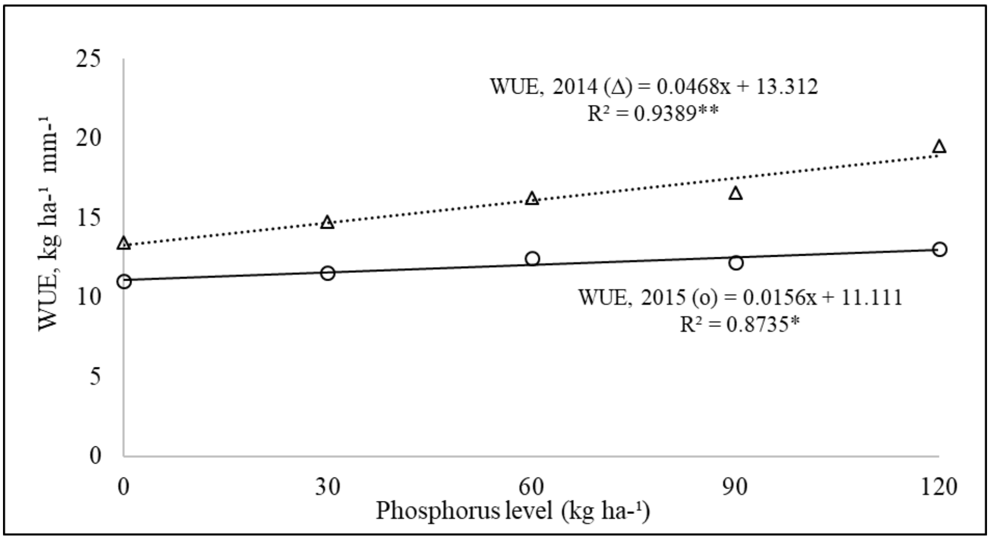

Figure 2. *Relationship between water use efficiency (WUE) and phosphorus level*

Conclusion

All these results indicate that phosphorus levels exhibited significant differences to the grain number per m^2, grain weight and grain yield ha^{-1}, as well as, for starch content and protein content in the first season. Evaluation of agronomical and quality traits in wheat showed that phosphurus application has significant effect on relationship between grain yield and quality. The grain yield increase was significantly associated with the improved values of noted plant traits like grain number and thousand grain weight. Accordingly, 120 kg P_2O_5 ha^{-1} was better rate of phosphorus for achieving reasonable productivity of wheat under investigation conditions.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19791987

THE EFFECTS OF GRAPE LEAVES EXTRACT ON HYPERHOMOCYSTEINEMIA INDUCED INFLAMMATORY ENDOTHELIAL DAMAGE IN CARDIOVASCULAR DISEASES

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(Received 23rd Oct 2018; accepted 7th Jan 2019)

Abstract. This study was designed to detect the anti-oxidant and anti-inflammatory effects of phenolic compounds in grape leaves Algerian variety (GLAV) on endothelial damage. The phenolics were identified by using HPLC/DAD/ESI-MS analysis. The research of the anti-oxidant and anti-inflammatory effects was conducted on mice through 15 days. Results showed high levels of phenols, anthocyanins, flavonols and *trans*-caftaric acid in GLAV. The plasma hs-CRP and homocysteine levels were elevated significantly ($p < 0.05$) however the glutathione reduced significantly ($p < 0.05$) after the administration of L-methionine in high doses to mice. This was associated with the desquamation of endothelium and muscular lysis with transformation of spindle nuclei to oval nuclei; this is due to the angiotoxic action of homocysteine on the aorta. These changes were not observed in mice treated with L-methionine plus the antioxidant and anti-inflammatory extract of GLAV. So, the study proved the antioxidant and anti-inflammatory effects of the GLAV on hyperhomocysteinemia induced inflammatory endothelial damage in cardiovascular diseases.
Keywords: *grape leaves, phenols, total Hyc, hs-CRP, GSH, endothelial damage*

Introduction

Hyperhomocysteinemia is defined as an abnormally high plasma homocysteine (Hcy) concentration after an oral Methionine load (Van Den Berg et al., 1995). It is a factor of risk for premature cardiovascular disease (Williams et al., 2002). Hence, it is one of the major pathogenic factors of atherosclerosis (Boldyrev et al., 2009). Besides its detection in all inflammatory diseases, hyperhomocysteinemia has been reported in other sicknesses like: type 2 diabetes, chronic kidney disease and cancer (Wu, 2008; Falvoa, 2007), and Alzheimer (Morris et al., 2001). It should be mentioned that hyperhomocysteinemia is not produced only by inflammation, but also by oxidative stress generated by high plasma homocysteine, which can cause a hyperhomocysteinemia induced inflammation (Jacobsen, 2000).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19892003

The relationship between hyperhomocysteinemia and cardiovascular disease is highlighted by the deficiency of the cystathionine beta-synthase (CβS) enzyme, which is deficient during homocysteinuria (Flemming et al., 2010). In most cases, hyperhomocysteinemia is a result of deficiency of the vitamins B6, B12, folate, or a combination of them (Chiang et al., 2005). These vitamins are essential co-factors of the key enzymes of the Homoysteine's metabolism. Moreover, some drugs such as fibrates, antiepileptic, methotrexate, theophylline, metformin, and other substances like nicotinic acid can also cause hyperhomocysteinemia (Stalder et al., 2010). Homocysteine acts directly on endothelial and damaged vessel wall through generating an oxidative stress, and stimulating a pro-coagulant and pro-inflammatory state of blood components (Bernardo et al., 2004) is the most accepted hypothesis about Hcy's action in cardiovascular disease.

Several studies have demonstrated that correcting the plasma deficiency of folic acid and vitamin B12 decreases or makes hyperhomocysteinemia disappear (Rigaud, 1999). But in this study, the focus is on natural antioxidants, especially plants traditionally used in folk medicine and precisely GLAV (*Vitis vinifera* L leaves).

Vitis vinifera L. is a widespread crop in Algeria. In 2000, following the Algerian agriculture ministry, vineyards occupied an area close to 56,500 ha. *Vitis vinifera* leaves have been traditionally used as food or as medications all over the world. The leaves are used to treat hypertension, diarrhea, hemorrhage and varicose veins, inflammatory disorders, and reduce blood glucose levels in diabetics (Dani et al., 2010).

The aims of the study is identifying the phenolic compounds (Anthocyanins and non anthocyanins) by using HPLC-DAD/ESI-MS, and measuring the total Hyc, the plasma hs-CRP and the concentration of the GSH to estimate the antioxidant and anti-inflammatory effect of the GLAV on the inflammation induced by Hyperhomocysteinemia. By the end, the aorta histology had been examined in order to confirm the angiotoxic action of homocysteine and the effect of GLAV on the aorta.

Materials

Plant material

The plant material was collected from the leaves of fully matured grape (*Vitis vinifera* L.) in August, from Media Algeria. Leaves were rinsed in tap water and freeze-dried. Afterwards, they were crushed with a blender for 5 min and the resulting powder was collected and stored in the dark at 20 °C until needed.

Chemicals

All the used solvents and the formic acid of HPLC-grade (from Merck, Darmstadt, Germany). The water was purified by a milli-Qplus system from Millipore (Milford, MA, USA). All the employed Reagents were of analytical grade, purchased from Carlo Erba (Milan, Italy). The standards of HPLC-grade[malvidin-3-glucoside (n° 04288)], the 5,5′-dithiobis-(2 nitrobenzoic acid) DTNB and all the rest of chemicals were purchased from Sigma-Aldrich, Inc. 0.45 µm Polytetrafluoroethylene (PTFE) syringe membrane filters was purchased From Waters Co. - Milford, MA, USA.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Methods

Analysis of anthocyanins and non-anthocyanin phenolic compounds of GLAV by reverse phase liquid chromatography-diode array detection/electrospray mass spectrometry (RPLC-DAD/ESI-MS)

Sample preparation

Three grams of powdered leaves were extracted three times at 1:2 (weight/volume) (w/v) ratio with cold methanol: HCl (1000:1 (volume/volume) (v/v) by using an ultra turrax (Ultra Turrax-Tube Drive, BM-G-ball-mill tube, IKA, Germany) with 10/CS glass balls, for 3 min of each sample. After the extraction, centrifugation took place (1600 *g*, 15 min, 4 °C), the supernatant was collected and stored on ice. The pellet was re-subjected to subsequent extraction, and a final volume of 14.5 ml was collected. The methanol/HCl extract was first filtered through the 0.45 µm membrane PTFE, and then it was exposed to Speed Vac concentration (SC250P1-250, Thermo Fisher Scientific Inc, Waltham, MA, USA) at 20 °C until dryness. Next, the residue was brought to a final volume of 10 ml by adding formic-acidified (pH 3.2) MQ water and kept at -20 °C until analysis. Discarding the pellets, the extraction was performed in triplicate (Kammerer et al., 2004).

HPLC-DAD-ESI-MS characteristics and protocols

HPLC-DAD and HPLC-MS apparatus

The reverse phase liquid chromatography (RPLC) was performed according to Villiers et al. (2004) and Kammerer et al. (2004) with slight changes using a High Performance Liquid Chromatography system consisting of Hewlett-Packard series 1100 L equipped with a Diode Array Detector (DAD) operated by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The HPLC-MS system was equiped with HPLC-DAD instrument coupled to a quadripole mass spectrometer HP 1100 MSD electrospray interface (ESI) (Agilent Technologies, Palo Alto, CA, USA). The Separation had been occurred on a reverse-phase Waters Nova-Pak C_{18} column [150 mm × 3.9 mm, 4 µm] for anthocyanins analysis and a reverse-phase Waters Nova-Pak C_{18} column [300 mm × 3.9 mm, 4 µm] for non-anthocyanin phenols, and both had been kept at 26 °C with a pre-column of the same phase.

DAD and ESI-MS parameters

The non-anthocyanin phenols DAD was performed according to Dobes et al. (2013) with slight changes from 220 to 380 nm and ESI-MS parameters were: drying gas (N_2) at 350 °C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from *m*/*z* 100 to 3000, employing a fragmentator voltage gradient of 100 V from 0 to 200 *m*/*z* and 200 V from 200 to 3000 min.

On the other hand, The anthocyanins DAD was performed from 260 to 600 nm, and ESI-MS parameters were: drying gas (N_2) at 350 °C with a 10 L/min flow, nebulizer pressure at 380 Pa (55 psi), and capillary voltage of 4000 V. The ESI scanned the mass from *m*/*z* 100 to 1500, employing a fragmentator voltage gradient of 100 V from 0 to 17 *m*/*z* and 120 V from 17 to 55 min.

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Elution parameters

Whereas, the elution of non-anthocyanin phenols was performed at a 0.7 mL/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/acetic acid (98:2, v/v), and the latter constituted of water/acetonitrile/acetic acid (78:20:2, v/v/v). A linear gradient started with 0% of 'B' and 100% of 'A', and then reached 80% of 'B' and 20% of 'A' after 55 min. After that, slight linear increase of 'B', from 80 to 90%, between 55 to 57 min, was observed, then it remained isocratic at 90% of 'B' and 10% of 'A'. Next, the process increased linearly from 90 to 95% of 'B' during 10 min. Finally, and still in a linear way, it reached 100% of 'B' at 90 min. The column was washed with Methanoland re-equilibrated from 90 to 120 min. The volume of the injected leaves extract was 15 μL.

Eluent of anthocyanins was performed at a 0.8 mL/min gradient flow of solvent 'A' and 'B'. The former was a combination of water/formic acid (90:10, v/v), and the latter constituted of water/methanol/formic acid (45:45:10, v/v/v). A linear gradient started with 15% of 'B' and 85% of 'A', then it reached 80% of 'B' and 20% of 'A' after 30 min.

After that, the process continued in an isocratic way, from 30 to 45 min, with 80% of 'B'. The column was then rinsed with methanol and re-equilibrated from 43 to 75 min. The injected volume of leaves extract was150 μL, and formic acid was employed as a pH modulating agent in order to optimise the anthocyanins detection by maximizing the absorption in the λ 520 nm region.

HPLC/DAD/ESI-MS identification of anthocyanins

The identification of malvidin 3-glucoside and cyanidin 3-glucoside was performed by comparing the results of commercial standards (Sigma-Aldrich) with the positive ion mass spectra achieved from the ESI-MS (retention time (t_R), UV λ_{max}, and MS^n) of the leaves extract, while other anthocyanins were identified by comparing the ESI-MS attained results against the ones available in the literature (*Table 1*). The flavonols were identified (glucose/galactose and glucuronide derivatives) under ESI-MS. This latter was achieved according to the molecular and fragment ions $[M\text{-H-}162]^-$ (quercetin-3-galactoside/glucoside) and $[M\text{-H-}176]^-$ (quercetin-3-glucuronide) (*Figs. 1* and *2*). In addition, the identity of all other constituents was validated by comparing the attained retention times (t_R), UV λ_{max}, and MS^n of peaks from the leaves extract with those reported in the literature (*Table 1*).

Compound quantitative analysis by HPLC/DAD

The quantification of anthocyanins in the leaves extract was obtained by measuring peak areas at 530 nm and taking into account the external standard calibration curve of malvidin-3-glucoside, which was measured at 524 nm. The concentrations were expressed as 'µg malvidin-3-glucoside equivalents/g of freeze-dried leaves'. The contribution of single anthocyanins was calculated and expressed in % of the total anthocyanins content.

The quantification of flavonols was performed according to the peaks attained at 340 nm and calculating the concentrations as 'µg quercetin or kaempherol equivalents/g of freeze-dried leaves' according to external standard calibration curve of quercetin and kaempherol. The contribution of single flavonols was calculated and expressed as % of the total flavonol content.

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DOI: http://dx.doi.org/10.15666/aeer/1702_19892003

Quantification of *trans*-caftaric acid was based on an external standard calibration curve carried out at 340 nm and expressed as 'µg *trans*-caftaric acid/g of freeze-dried leaves'.

The external standard calibration curves for malvidin-3-glucoside, quercetin, kaempherol and *trans*-caftaric acid, which were performed in duplicate by using five dilutions within linearity and an R^2 values, were 0.98, 0.97, 0.98 and 0.99 respectively.

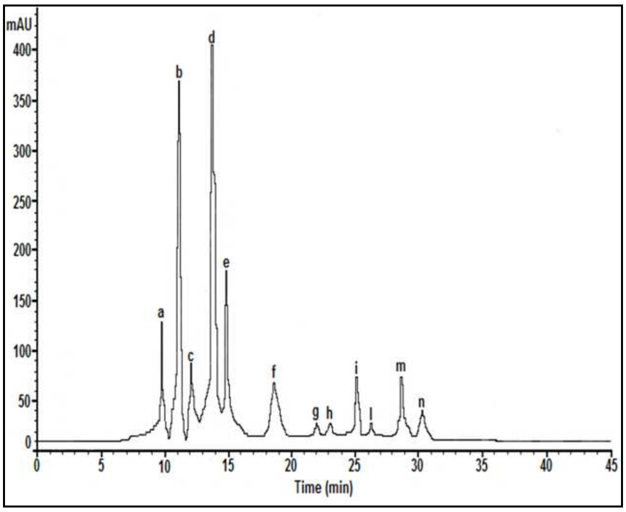

Figure 1. *HPLC-DAD Chromatogram at 530 nm of anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (a) delphinidin 3-glucoside; (b) cyanidin 3-glucoside; (c) petunidin 3-glucoside; (d) peonidin 3-glucoside; (e) malvidin 3-glucoside; (f) cyanidin 3-(6-p-acetyl) glucoside; (g) peonidin 3-(6-acetyl) glucoside; (h) delphinidin 3-(6-p-coumaroyl) glucoside; (i) cyanidin 3-(6-p-coumaroyl) glucoside; (i) petunidin 3-(6-p-coumaroyl) glucoside; (l) petunidin 3-(6-p-coumaroyl) glucoside; (m) peonidin 3-(6-p-coumaroyl) glucoside; (n) malvidin 3-(6-p-coumaroyl) glucoside*

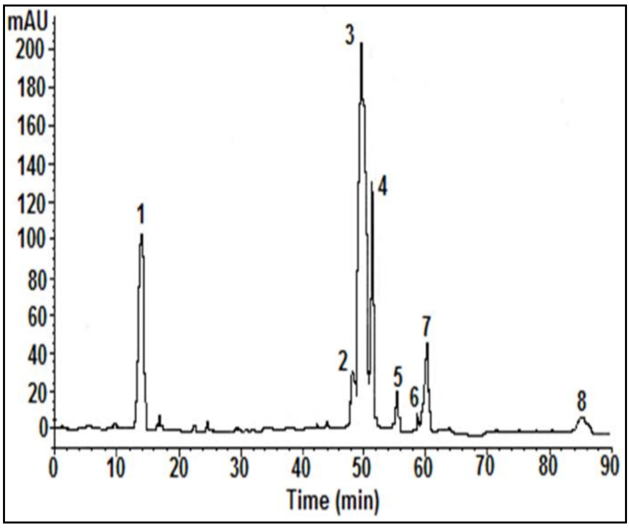

Figure 2. *HPLC-DAD Chromatogram at 340 nm of non-anthocyanin compounds from a Vitis vinifera L. leaves methanol extract. (1) trans-caftaric acid; (2) Quercetin-3-O-galactoside; (3) quercetin 3-0-glucuronide; (4) quercetin 3-0-glucoside; (5) kaempferol 3-0-galactoside; (6) kaempferol 3-0-glucuronide; (7) kaempferol 3-0-glucoside; (8) quercetin*

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Effect of GLAV on the inflammation induced by hyperhomocysteinemia

We are following the same protocol (the same conditions and diet) used by Benmbarek et al. (2013) but with a change in concentration of methionine and treatment period.

Animals and diets

Twenty eight Albino *Mus musculus* mice, 2 to 2.5 months old, weighed between 18 and 27 g, were used in this experiment. They were provided by the central pharmacy Constantine (Algeria). The mice were separated into four groups in four cages according to their body weight. The planned diet was given in the form of balls prepared with 0.5 mg of white flour and distilled water for 15 days. The first group (F) was fed with white flour (0.5 mg/kg/day), second group (M) was administered with with L-methionine (1 g/kg/day), third group (MP) was administerd with L-methionine and *Vitis vinifera* (1 g/kg + 500 mg/kg/day). The fourth group (P) was treated only with *Vitis vinifera* (500 mg/kg/day). Mice were housed at normal conditions of the animal house throughout the treatment period.

Blood biochemistry

At the end of experiment, mice were fasted overnight, and the blood samples were collected from the retro orbital vein into EDTA tubes by using glass capillaries. They were centrifuged immediately, and the plasma was stored at -30 °C. The values of plasma hs-CRP were measured by the immunoturbidimetric method on a Cobas integra 400 plus analyzer (Roche). Total homocysteine (t-Hcy) was estimated by competitive solid phase chemiluminescence immunoassay.

GSH glutathione assay (GSH)

We are following the same protocol mentioned by Houssem Eddine et al. (2014). After sacrificing the animals, the liver was dissected and washed with NaCl 0.9%. Then, the homogenate had been prepared with 0.5 g of the liver homogenized in 2 ml of TBS (Tris50 mM, NaCl 150 mM, pH 7.4). Next, it was centrifuged at 9000 g for 15 min at 4 °C. After that, the supernatant used for the determination of glutathione reduced (GSH), then it was measured spectrophotometrically by using 5,5′-dithiobis-(2 nitrobenzoic acid) (DTNB) as a coloring reagent, following the method of Rahman et al. (2006).

Histology

The animals were sacrificed and the aorta was removed for histological analysis. The tissues were then embedded in paraffin and cut in 5 µm thick sections and colored using heamatoxylineosin staining method.

Statistical analysis

Statistical analysis was carried out by one–way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests using statistics software package (SPSS for Windows, V. 20.0, Chicago, USA). P values < 0.05 were considered as statistically significant (common letter are not significantly different).

Results

Phenolic composition of extract GLAV (Vitis vinifera)

The HPLC/DAD/ESI-MS analysis was used to identify all compounds directly, or by comparing the results to literature, and the analysis of leaf ingredients were permitted the identification of cyanidin-3-glucosides, -3-(6-acetyl)glucosides, and -3-(6-p-coumaroyl)glucosides (*Table 1; Fig. 1*). Nonanthocyanin phenolic compounds identified in leaf ingredients included the flavonols quercetin-3-O-galactoside, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol- 3-O-glucuronide, kaempherol-3-O-glucoside, and quercetin (*Table 1; Fig. 2*).

Table 1. *Characteristics of anthocyanins (a-n) and flavonols (2-8) detected in a methanol extract of Vitis vinifera L. leaves, according to the retention time (tR), mass spectral details, UV data (λmax), corroborated by references*

Peaks	$^{x}t_R$ (min)	Compound	$[M+H]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)	λmax	Fragments (MS/MS)	Reference
A	9.8	Delphinidin 3-glucoside	465.2	-	522	303	Villiers et al. (2004); Kammerer et al. (2004)
B	11.1	Cyanidin 3-glucoside	449.3	-	514	287	
C	12.6	Petunidin 3-glucoside	479.4	-	522	317	
D	13.8	Peonidin 3-glucoside	463.4	-	515	301	
E	15.0	Malvidin 3-glucoside	493.1	-	524	331	
F	18.8	Cyanidin 3-(6-*p*-acetyl) glucoside	495.4	-	519	287	Villiers et al. (2004)
G	22.3	Peonidin 3-(6-acetyl) glucoside	505.0	-	516	301	
H	23.2	Delphinidin 3-(6-*p*-coumaroyl) glucoside	611.3	-	527	303	Villiers et al. (2004); Kammerer et al. (2004)
I	25.3	Cyanidin 3-(6-*p*-coumaroyl) glucoside	595.4	-	522	287	
L	26.3	Petunidin 3-(6-*p*-coumaroyl) glucoside	625.2	-	536	317	
M	28.8	Peonidin 3-(6-*p*-coumaroyl) glucoside	609.2	-	520	301	
N	30.5	Malvidin 3-(6-*p*-coumaroyl) glucoside	639.0	-	517	331	
1	13.9	*Trans*-caftaric acid	-	311.0	320	179	Kammerer et al. (2004)
2	49.2	Quercetin 3-*0*-galactoside	-	463.1	256	301	Kammerer et al. (2004); Flamini et al. (2015)
3	50.1	Quercetin 3-*0*-glucuronide	-	479.1	256	301	
4	51.7	Quercetin 3-*0*-glucoside	-	463.1	256	301	
5	55.6	Kaempferol 3-*0*-galactoside	-	447.3	262	285	Villiers et al. (2004); Flamini et al. (2015)
6	59.0	Kaempferol 3-*0*-glucuronide	-	463.1	262	287	Flamini et al. (2015
7	61.5	Kaempferol 3-*0*-glucoside	-	477.0	262	285	Kammerer et al. (2004); Flamini et al. (2015)
8	85.5	Quercetin	-	304.0	254	273	Kammerer et al. (2004)

$^{x}t_R$Mean retention time of 3 runs

Among the extracted polyphenols from the Algerian *Vitis vinifera* L. leaves, the anthocyanins are the main chemical group, about 80.34 µg in each 1 g of freeze-dried

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sample (*Table 2*). In this extract, 12 compounds were identified by HPLC-DAD as follows: the peonidins, as the main chemical group, with about half (46.50%) of the total anthocynins with 37.35 µg/g freeze-dried sample in each g of the predominant compound was peonidin 3-glucoside, the second abundant group was the cyanidins, with cyanidin-3-glucoside, of 78% per the total of cyanidins tailed by cyanidin-3-(6-acetyl)-glucoside (13%) and cyanidin-3-(6-*p*-coumaroyl)-glucoside (9%). The malvidins represented the third abundant group with 12.98 µg/g of sample and 2 compounds (malvidin 3-glucoside and malvidin 3-(6-*p*-coumaroyl) glucoside), the glucoside forms were the most abundant; the two remaining chemical classes, delphinidins and petunidins were contributed to total anthocyanins for 6.30% and 2.63%, respectively. Concerning the non-anthocyanins it is interesting to note the high concentrations of trans-caftaric acid and quercetin 3-*0*-glucuronide. Quercetins were the most abundant flavonols and were represented by quercetin (5.2%) and the glucuronide (76.5%), galactoside (8.3%) and glucoside (10%) forms. Kaempferols were presented 11.40% of flavons and the most abundant form was the galatoside (82.2%) one. For these compounds it is interesting to note the very high concentration of the glucuronide form and the notable concentration of quercetin.

Table 2. *Phenolic composition of Vitis vinifera L. leaves extract according to HPLC-DAD chromatography*

Anthocyanins[z]					
Cyanidins	**Delphinidins**	**Petunidins**	**Peonidins**	**Malvidins**	**Total anthocyanins**
22.83 µg 28.42%[y]	5.06 µg 6.30%	2.12 µg 2.63%	37.35 µg 46.50%	12.98 µg 16.15%	80.34 µg

Flavonols[z]		
Quercetins	**Kaempherols**	**Total flavanols**
39.70 µg 88.60%[w]	5.11 µg 11.40%	44.81 µg

Cinnamates
***Trans*-caftaric acid**
9.52 µg

[x]Expressed as µg malvidin-3-glucoside equivalents/g freeze-dried leaves
[y]% of total anthocyanins: Σ Cyandins = cyanidin-3-glucoside + cyanidin-3-(6-acetyl)-glucoside + cyanidin-3-(6-*p*-coumaroyl)-glucoside; Σdelphinidins = delphinidin-3-glucoside + delphinidin-3-(6-*p*-coumaroyl)-glucoside; Σ petunidins = petunidin-3-glucoside + petunidin 3-(6- *p*-coumaroyl)-glucoside; Σ peonidins = peonidin-3-(6-acetyl)-glucoside + peonidin-3-(6-*p*-coumaroyl)-glucoside; Σ malvidins = malvidin-3-glucoside + malvidin-3-(6-*p*-coumaroyl)-glucoside
[z]Expressed ad µg quercetin or keampherol equivalents/g freeze-dried leaves
[w]% of total flavonols: Σ Quercetins = quercetin-3-*O*-galactoside + quercetin-3-*O*-glucuronide + quercetin-3-*O*-glucoside + quercetin; Σ kaempherols = kaempherol-3-*O*-galactoside + kaempherol-3-*O*-glucuronide + kaempherol-3-*O*-glucoside
The anthocyanin/flavonol ratio = 1.79

Effect of Vitis vinifera extract on homocysteine induced inflammatory endothelial damage in cardio vascular diseases.

In this study, we have taken only 3 values from each group because some are outliers.

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As shown in *Figure 3,* the total Hcy levels in groups F (7.56 ± 0.21 µmol/l), M(12.20 ± 0.78 µmol/l), MP(9.06 ± 0.34 µmol/l) and P(7.24 ± 0.40 µmol/l) were showed a significant difference between groups in mice during 15 days of treatment $P \leq 0.05$. The Tukey test was showed that the homocysteine concentration in mice administered with L-methionine was increased highly significantly in group M when it was compared to the control group $P \leq 0.05$. However the homocysteine concentration was decreased significantly in the group of mice administered with L-methionine and treated with *Vitis vinifera L* when it was compared to the group (M) $P \leq 0.05$. Exceptionally, no significant changes were observed between the group treated only by *Vitis vinifera* leaves and the control group ($P > 0.05$).

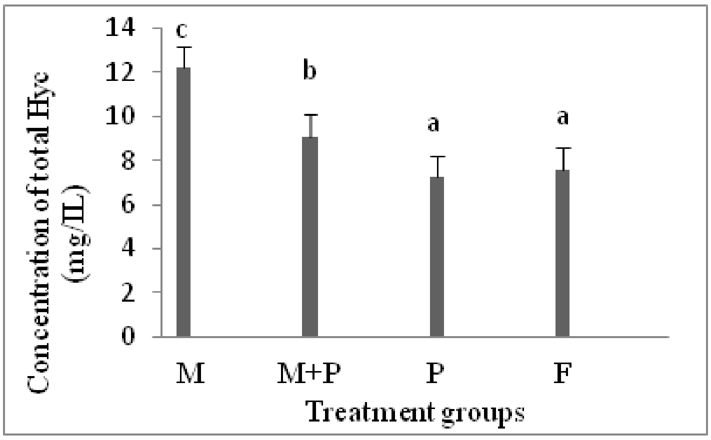

Figure 3. *Total Hcy level in the groups treated during 15 days. Values are the means ± SEM (n)*

The concentrations of hs-CRP in groups F (0.14 ± 0.017 mg/l), M (0, 28 ± 0,012 mg/l), MP (0.18 ± mg/l) and P (0.14 ± 0,012) were showed a significant difference between groups $P \leq 0.05$ (*Fig. 4*). The Tukey test was revealed that the hs-CRP concentration in the group (M) was increased highly significantly when it was compared to the groups (F) and (P) $P \leq 0.01$. However the concentration of hs-CRP was decreased significantly in the group (MP) when it is compared to the group (M).

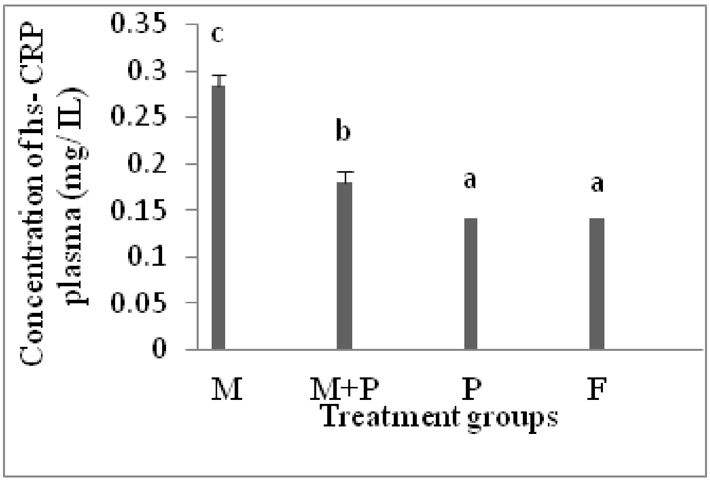

Figure 4. *Concentration of hs-CRP plasma in the groups treated during 15 days. Values are the means ± SEM (n)*

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Results demonstrated that the concentration of the glutathione reduced in groups F(29.28 ± 1.48 nmol/mg), M (19.38 ± 1.21 nmol/mg), MP(26.74 ± 1.06 nmol/mg) and group P(30.17 ± 1.37 were showed a significant difference between groups $P \leq 0.05$ (*Fig. 5*). The Tukey test showed that the concentration of the glutathione reduced in group M was decreased highly significantly when it is compared to the control group $P \leq 0.01$. However the concentration of GSH was increased in the groups treated with *Vitis vinifera L.*

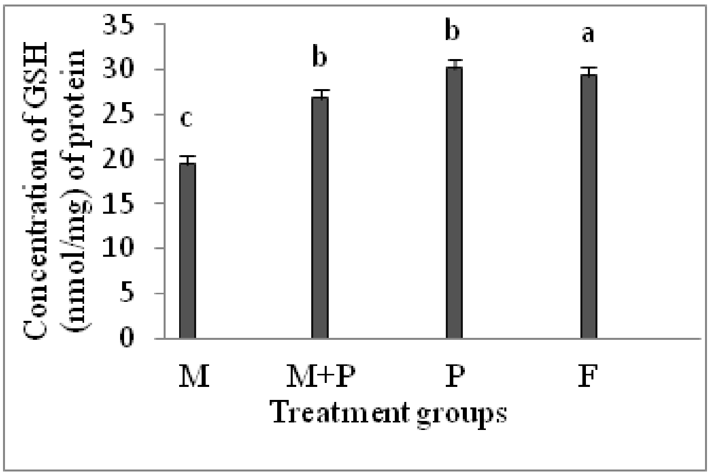

Figure 5. *Concentration of GSH in the groups treated during 15 days. Values are the means ± SEM (n)*

Aorta histological

The results of the histological investigation showed a clear modification in the aorta. The group (M) which was fed with 1 g/kg of L-methionine appeared with oval nuclei of muscular fiber, desquamation of endothelial cells. However, in the control group (F), the aortic sections were showed an intact endothelium. The group (MP) which was fed with leaves extracts of the *Vitis vinifera* and 1 g/kg of L-methionine was showed only slight modifications including some oval nuclei of muscular cells (*Figs. 6*, *7* and *8*).

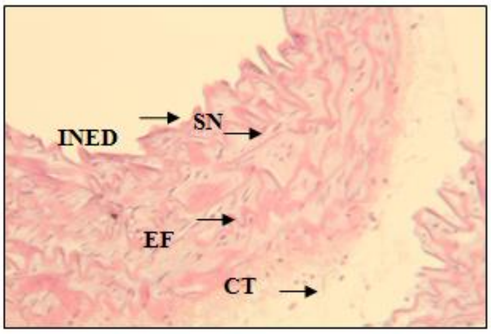

Figure 6. *Longitudinal section of arch aorta 15 days of flour application. Heamatoxylin Eosin staining (x100). EF: elastic fiber, SN: spindle nuclei, CT: connective tissue, INED: intact endothelium*

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DOI: http://dx.doi.org/10.15666/aeer/1702_19892003

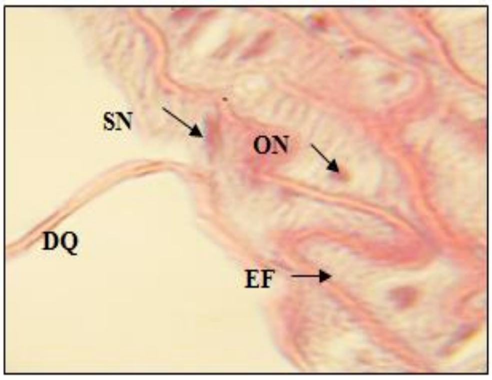

Figure 7. *Longitudinal section of arch aorta 15 days of L-methionine application. Heamatoxylin Eosin staining (x400). EF: elastic fiber, DQ: desquamation, ON: oval nuclei, SN: spindle nuclei*

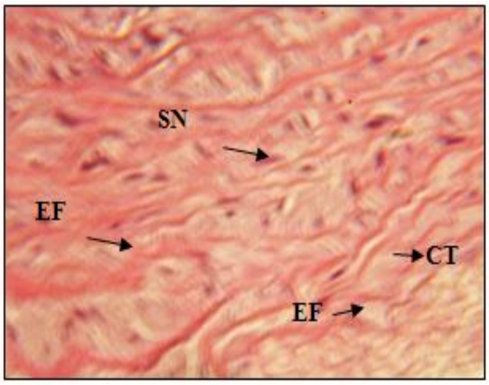

Figure 8. *Longitudinal section of arch aorta 15 daysof L-methionine and Vitis vinifera leaves application.Heamatoxylin Eosin staining (x100).EF: Elastic Fiber SN: Spindle nuclei CT: connective tissue*

Discussion

In this study, the High-performance liquid chromatography (HPLC) was used for the separation and quantification of polyphenols in leaves of *Vitis vinifera. L* species, the Algerian variety. The results showed that high levels of phenols; anthocyanins, flavonols and trans-caftaric acid. The results is agrees with the work of (Monagas et al., 2006) who reported that HPLC-DAD/ESI-MS analysis of *V. Vinifera* spp. leaves ingredient allowed the identification of anthocyanidin-3-glucosides,-3-(6-acetyl)glucosides, and -3-(6-p-coumaroyl) glucosides and the flavonols quercetin-3-O-glucoronide, quercetin-3-O-glucuronide, quercetin-3-O-glucoside, kaempherol-3-O-galactoside, kaempherol-3-O-glucuronide, kaempherol-3-O-glucoside, and quercetin like non-anthocyanin content. The chromatograph pattern and the compounds identified agree with other paper on the same subject (Monagas et al., 2006). Trans-Caffeoyltartaric acid (trans-caftaric acid) was the only hydroxycinnamic acid derivative identified in the studied leaf ingredients and results obtained were in agreement also with (Monagas et al., 2006). According of the results obtained, GLAV contain much higher concentration of peonidins, Trans-Caffeoyltartaric acid (trans-caftaric acid) and

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DOI: http://dx.doi.org/10.15666/aeer/1702_19892003

Quercetins but the concentration of Kaempherols is found inferior than values mentioned by the results of Monagas et al. (2006). The differences observed between the results may be attributed to the period of the plant growth cycle, variety, cultivar conditions, weather and finally to the processing and preparation because leaves used by Monagas et al. (2006) were cultivated using the commercial dietary technology.

Homocystein (Hyc) is considered as a risk marker and can be used for screening patients of high menace for cardiovascular events (Refsum et al., 1998). In this study, the oral administration of high dose of L-methionine 1 g/kg/day during 15 days showed a significant increase in the level of plasma tHyc compared to the control group. These results are in agreement with those found by (Zerizer, 2006), who showed that the high oral methionine load is the direct cause of the elevation of the total homocysteine (tHcy), the sum of all homocysteine forms that exist in plasma or serum, therefore having hyperhomocysteinemia means the elevation of total homocysteine (Bernardo et al., 2004). Two hypotheses were formulated to explain the atherogenicity of hyperhomocysteinemia. The lipid hypothesis stipulates that the alteration of lipoprotein metabolism secondarily induces an involvement of the vascular wall, and the inflammatory hypothesis is dominated by the direct aggression of the cells and vascular connective tissue (Demuth et al., 2000).

The levels of hs-CRP and GSH have been exploited to monitor the effects of GLAV on the inflammatory and oxidative effect caused by hyperhomocystemia.

Currently and in several scientific researches, the CRP is used as a marker of cardiovascular risk (Folsom et al., 2002). The group (M) showed significant high level of hs-CRP compared with the control one. Benmbarek et al. (2013) confirmed that methionine at dose of 200 mg/kg/day administered to mice, during the 21 days period, increased significantly the levels of plasma hs-CRP. This result was considered as an initiative of the inflammatory process, which was confirmed by the histological investigation of the aorta. Results are in agreement with the previous experimental studies of Benmbarek et al. (2013) who found that Hyperhomocysteinemia as angiotoxic and toxic activity explained by the loss and degeneration of the endothelium, formation of foam cells in the different sections of the aorta, change in the smooth muscle cells nuclei forms from a fusiform aspect to a rounded appearance, and the alteration of the cardiac muscle and liver necrosis. In addition, Zerizer and Naimi (2004) reported the structural alterations in the aorta, heart and liver caused by the administration of high doses of methionine.

Substantially reduced (GSH) involved in maintaining the redox potential of the cell cytoplasm and in a number of detoxification reactions and scavenging reactive oxygen species (Haleng et al., 2007). Result demonstrated that the group (M) showed a significant decrease in the level of GSH compared to the control group (F). These results confirmed an oxidative stress generated by the reactive sulfhydryl group (-SH) in the homocysteine (Jacobsen, 2000), which is quickly oxidized, leading to the formation of Hcy, mixed disulfides and Homocysteinethiolactone. The oxidation of the -SH group generates superoxide anion O2-, hydrogen peroxide H_2O_2 and hydroxyl radicals OH- (Zitoun, 1998). Zeng et al. (2004) confirmed that the Hcy induced the production of MCP1 and IL-8. Additionally, a recent study suggested that Hcy induced the production of O2- in vascular smooth muscle cells (Wang et al., 2001). Yalçinkaya et al. (2009) demonstrated that a high methionine diet induced oxidative stress in serum, heart, and aorta in rabbits.

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The group (MP) treated by leaf of *Vitis vinifera* and L-methionine (500 mg/kg and 1 g/kg) noted a significant decrease in the levels of tHyc compared to group (M). The same group rectified significantly the level of hs CRP compared with the control group and group (M). At the same time, the group (MP) re-established significantly the level of GSH compared to the group (M).

These significant relationships between the parameters can be explained as the effect of the existed phenols in the GLAV. Exactly, the group (MP) treated by the GLAV was able to restore the level of the hs- CRP and GSH and maintain the correlation between the three parameters and could correct the damaged cells in aorta.

The results confirmed that GLAV (500 mg/kg/day) has an antioxidant and anti-inflammatory effects induced by Hyperhomocysteinemia in mice fed by a high dose of L-methionine 1 g/1 kg/day during 15 days. Benmbarek et al. (2013) asserted that S. mialhesi extract lowered the plasma hs-CRP and corrected the damaged cells.

In addition, the phenols, which are ubiquitous in almost all plant foods could decrease the risk of the occurrence of considerable number of diseases, particularly those related to aging and oxidative injury (cancer, cardiovascular diseases and neurodegenerative) (Hennebelle et al., 2014). While, low circulating levels of polyphenols (maximum, few µmol/L) compared to those of other endogenous antioxidants (GSH and acid uric) or exogenous (vit E as well as vit C) do not allow to envisage a direct antioxidant action of polyphenols in the body. This, however, with the exception of the gastrointestinal tract where the polyphenols present in large quantities can act as scavengers of free radicals. Today at the level of organism, polyphenols are perceived as molecules "Signal" (Mornad et Milenkovic, 2014), that could stimulate multi-target modes of action. Many in-vitro studies showed that flavonoids could affect their biological targets by modulating some enzymatic activities, gene expression or cell signaling, interacting with membrane or cell receptors, or via epigenetic regulations (Farga et al., 2010). The diversity of these potential mechanisms of action explains the broad spectrum of activities flavonoids observed *in-vivo*, including anti-inflammatory activities, antioxidant, anti-angiogenic, anti-proliferative or pseudo-estrogenic (Mornad and Milenkovic, 2014).

Conclusion

The grape leaves have a good antioxidant and anti-inflammatory activities. Where, it could be directly related to the high content of active compounds like peonidins, Trans-Caffeoyl tartaric acid (trans-caftaric acid) and Quercetins. More studies in this area are required further to find new ways and new efficient molecules from *Vitis vinifera* leaves to treat degenerative diseases and to slow the aging process induced by oxidative intermediate products and other pro-inflammatory components.

Acknowledgements. The authors are grateful to ATRSS–DGRSDT (MESRS, Algeria), The Consiglio Nazionale delle Ricerche, an Italian public organization set up to support scientific and technological research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_19892003

THE EFFECTS OF NICKEL APPLICATIONS ON THE GROWTH OF COCKLEBUR (*XANTHIUM STRUMARIUM* L.) PLANT

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(Received 23rd Oct 2018; accepted 7th Jan 2019)

Abstract. Nowadays the environmental pollution caused by heavy metals is spreading all over the world, especially where the industry is developing rapidly, and soils are polluted very dramatically and hazardously at a high level. In this study, cocklebur-(*Xanthium strumarium* L.) plants were grown in nickel (Ni) contaminated soil (0, 50, 100, 200 and 400 mg Ni kg^{-1}) under greenhouse conditions for 6 weeks to study the ability of Ni uptake and accumulation of the plants. The Ni treated plants were compared with the control (0 mg Ni kg^{-1}) plant. As a result of that comparison, chlorophyll levels of old and young leaves, dry weights, reduced glutathione (GSH), macronutrient concentrations, such as nitrogen (N), phosphor (P), potassium (K), calcium (Ca) and magnesium (Mg)) and micronutrient concentrations, such as iron (Fe), zinc (Zn), and manganese (Mn) nutrient concentrations of the plants were decreased, whilst the concentrations of Ni and of copper (Cu) increased with increasing dose of Ni application. The results show that the studied plant (*Xanthium strumarium* L.) can be used for the cleaning up of Ni-contaminated soils and is suitable for phytoremediation.
Keywords: *chlorophyll, heavy metal, mineral element, phytoremediation, pollution*

Introduction

Soil pollution is one of the most important environmental problems nowadays. As stated in the previous studies, major soil pollutants, such as heavy metals, excessive use of fertilizers, pesticides, hormones, organic compounds, industrial wastes and accumulation of dangerous substances in the soil, etc. have become a rising problem as a consequence of human activities (Rahman et al., 2005; Daghan, 2007; Karaca and Turgay, 2012; Daghan and Ozturk, 2015).

Environmental pollution, especially caused by heavy metals such as Ni, lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), arsenic (As), zinc (Zn), copper (Cu), has been a research topic and attracted attention of many countries in the world.

Nickel is one of the most commonly found elements in the earth's crust and is located in the 22^{nd} order among the other elements (Kaviani et al., 2017). This element is an essential element for plant growth and development in small doses (Marschner, 1995; Yang et al., 1996; Gheibi et al., 2009). Nickel is a functional component of some enzymes, especially urease (urea amidohydrolase, EC 3.5.1.5) enzyme in the plant. Urease is a metalloenzyme and plays an important role together with nitrogen being the enzyme that catalyzes the hydrolysis of ammonia and carbon dioxide (Dos Reis et al., 2017). Urea is accumulating in the plant tissue under Ni deficiency condition and it causes the toxic effect (Gheibi et al., 2009). Nickel has also function to catalyze enzymes for nitrogen fixation in legumes plants and increased tolerance to plant disease (Gerendás et al., 1999). However, at high concentrations Ni exhibits a toxic effect, negatively affects photosynthesis and membrane functions, and also prevents seed germination, plant growth and development (Parlak, 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

Many researchers have focused on the effects of heavy metals on human health and the environment, on the detection of contaminated areas, on cleaning methods and facilities to minimize the damage that these areas have on human health and the environment (Chaney, 1988; Ebbs et al., 1997; Gupta and Gupta, 1998; Karenlampi et al., 2000; Yankov et al., 2000; Lombi et al., 2002; Prasad and Freitas, 2003; Murakami and Ae, 2009; Daghan et al., 2012; Syam et al., 2016; Rehman et al., 2018). Remediation of polluted soil is usually done by excavating the contaminated area and renewing the soil that has been cleaned. However, in recent years, technologies applied directly *in-situ* without digging the earth has gained more attention (Kocaer and Baskaya, 2003).

Phytoextraction is the removal of soil contaminants by plant roots, accumulation in the organs above the soil, and subsequent harvesting of the plants, respectively. This method is especially used on plants to clean up active elements, such as Zn and Cu, or metals not in the nutrient group like Ni, lead (Pb) and cadmium (Cd) (Hamutoglu et al., 2012).

In the literature, many plants such as *Helianthus annuus* L. (Ahmad et al., 2011), *Nicotiana tabacum* L. (Daghan et al., 2012), *Oryza sativa* L., *Zea mays* L., (Murakami and Ae, 2009), *Sebertia acuminate* (Jaffré et al., 2013), *Glycine max* L. (Syam et al., 2016), *Salicornia iranica* (Kaviani et al., 2017) have been tested and used for the phytoremediation of heavy metals from the contaminated soil. Likewise, the success of phytoextraction depends on the detection of suitable plant species that can tolerate and accumulate heavy metals and to produce large biomass using conventional agricultural techniques (Syam et al., 2016).

A plant that is selected to be used in phytoremediation methods, should have a great amount of metal accumulation in its shoots and harvested parts, be able to tolerate heavy metal that has accumulated, also has to be deep-rooted and fast growing (Daghan et al., 2012).

The aim of this study is to investigate the potential use of *Xanthium strumarium* L., which can grow very easily in nature, has a high amount of roots and shoots, also can be grown in Ni contaminated soil, for heavy metals accumulation and phytoextraction method.

Materials and methods

In this study, *Xanthium strumarium* L., which belongs to the *Asteraceae* family and the Xanthium species was used as plant material. *Xanthium strumarium* L. is an annual plant which has the ability of self-pollinating. Additionally, it can grow even in the most adverse conditions up to 1 m and having advanced pile roots and can be found in almost all regions in Turkey (Cesur and Senkal, 2016).

The Pasakoy soil series of Amik Plain was used in experiments as a soil material (Kılıc et al., 2008). Some physical and chemical properties of the soil were given in *Table 1*.

The air-dried soil sample was sieved from a 4 mm sieve and then 2 kg of soil was filled into each pot. In order to obtain a homogeneous distribution in the soil, increasing doses of (0, 50, 100, 200 and 400 mg kg^{-1}) Ni were applied in the form of $NiSO_4.6H_2O$ prior to planting, and incubated for 3 weeks. Before planting, each pot was fertilizer with the solutions of 200 mg kg^{-1} nitrogen (N) in the form of NH_4SO_4, 100 mg kg^{-1} phosphorus (P) in the form of KH_2PO_4 and 125 mg kg^{-1} potassium (K) with 2.5 mg kg^{-1} iron (Fe) in the form of Fe-EDTA. During the experiment (6 weeks) the pots were

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DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

irrigated with pure water in soil field capacity of 60-80%. The experimental design used was completely randomized design with 3 replications in a factorial arrangement.

Table 1. *The physical and chemical properties of the soil material*

Parameters	Results	References	Parameters	Results	References
Structure	Clay	Bouyoucos (1952)	N	1.12%	Bremner and Mulvaney (1982)
pH	7.56	Soil Survey Staff (1951)	P	19.1 P_2O_5	Olsen et al. (1954)
Salt	0.22%	Soil Survey Staff (1951)	K	77.3 K_2O	Richards (1954)
$CaCO_3$	45.1%	Loeppert et al. (1996)	Available Fe	24.4 mg kg^{-1}	Lindsay and Norvell (1978)
Organic matter	2.55%	Kacar (1995)	Available Cu	3.73 mg kg^{-1}	
Organic carbon	1.48%	Kacar (1995)	Available Mn	69.7 mg kg^{-1}	
Field capacity	32.4%	Alpaslan et al. (1998)	Available Zn	7.40 mg kg^{-1}	
			Available Ni	7.34 mg kg^{-1}	

Before harvesting the leaf chlorophyll contents were measured in 3 replicates by a chlorophyll meter (Konica-Minolta SPAD-502). At the end of the experiment, the plants were harvested about 1 cm above the soil surface and washed with pure water, and then dried in an oven until they reached a constant weight at 65 °C. Afterward, dry weights of the plants were taken and grounded in a grinding mill (Retsch RM 200) for analysis. Test results were evaluated for the whole plant.

The concentrations of Ni, P, K, Ca, Mg, Fe, Zn, Cu and Mn were measured by the inductively coupled plasma atomic emission spectroscopy (ICP-AES Varian Liberty Series II) instrument by dissolving the grounded plant samples in a microwave (MarsXpress CEM) with nitric acid (HNO_3). Nitrogen (N) analysis of plant samples was carried out according to the Kjeldahl method (Bremmer and Mulvaney, 1982).

Nickel content (uptake) of *Xanthium strumarium* L. was calculated as follows (*Eq. 1*):

$$\text{Ni content } (\mu g \text{ plant}^{-1}) = [\text{DW } (g \text{ plant}^{-1}) \times \text{Ni concentration } (\mu g\ g^{-1})] \quad (\text{Eq.1})$$

Reduced glutathione (GSH) analysis was performed according to Cakmak and Marschner (1992). The experimental data were evaluated according to the factorial design pattern using the SPSS 22.0 statistical analysis program to determine the significance of levels and grouped by Duncan test according to Bek (1986).

Results and discussion

The results of Ni applications effects on the chlorophyll contents of leaves, dry weight, reduced glutathione (GSH) and Ni concentration of *Xanthium strumarium* L. plant were given in *Table 2*.

As can be seen on *Table 2,* the Ni applications cause a reduction in dry weights of *Xanthium strumarium* L. plant and likewise, chlorophyll contents were also decreased with those applications ($p \leq 0.01$).

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DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

Table 2. *The effects of different doses of Ni on the chlorophyll content, dry weight, GSH and Ni concentrations of Xanthium strumarium L. (n = 3)*

	Ni (mg kg^{-1})	Chlorophyll (SPAD Unit)		Dry weight (g plant^{-1})	GSH (µg mL^{-1})	Ni concentration (mg kg^{-1})
		Old leaf	**Young leaf**			
Xanthium strumarium L.	0	30.7 a	32.5 a	6.61 a	266 b	5.03 e
	50	28.6 b	31.6 ab	6.50 a	281 ab	10.7 d
	100	28.5 b	30.3 b	5.88 b	269 b	21.7 c
	200	28.4 b	31.4 ab	5.61 bc	336 a	49.5 b
	400	22.4 c	32.4 a	5.25 c	225 b	101 a
Dose	F	53.2**	5.92**	21.0**	4.82*	3411**

**$p \leq 0.01$ statistically significant within error bounds
*$p \leq 0.05$ statistically significant within error bounds

The chlorophyll content showed a decrease in the old and young leaves of *Xanthium strumarium* L. plant. The lowest chlorophyll contents were measured at 400 mg kg^{-1} Ni application dose of old and young leaves (respectively 22.4 and 32.4 SPAD unit). On the contrary, a decrease was observed with increasing Ni doses according to control groups. The dry weights of the plant were varied between 6.61-5.25 g plant^{-1}. Nickel applications were decreased plant dry weights compared to the control plant ($p \leq 0.01$). Similar results were reported by Parlak (2016). The researcher has investigated the effect of Ni treatment (0, 25 and 50 µM) on the growth and biochemical properties of wheat *(Triticum aestivum* L.). He found that the chlorophyll content of plants decreased with Ni applications. Rahman et al. (2005) were investigated the effect of increased doses of Ni (0, 1.0, 10 and 100 µM) on the growth and composition of barley plants. They reported that the barley plant was generally adversely affected Ni application and the lowest chlorophyll content was measured at 100 µM Ni treatment.

The increased application doses of Ni on the plant resulted with the lowest GSH concentration in the control group as 266 µg mL^{-1} and the highest as 336 µg mL^{-1} at 200 mg Ni kg^{-1} dose in the plant (*Table 2*). The GSH concentrations were decreased with Ni applications (*Table 2*). The lowest GSH concentration (266 µg mL^{-1}) was obtained at the control group while the highest GSH concentration (336 µg mL^{-1}) was obtained at 200 mg Ni kg^{-1} application. Daghan et al. (2012) reported an enhancement of glutathione concentrations in transgenic and non-transgenic tobacco plants, which different Ni doses were applied to the soil.

The Ni concentration of the *Xanthium strumarium* L. plant increased (5.03-101 mg kg^{-1}) with increasing Ni doses (*Table 2*). According to Alloway (1995), the critical concentration of Ni was obtained in tissues of the plant in the range of 10-100 mg kg^{-1}. As Kaviani (2017) stated in a previous research by using *Salicornia iranica* plant, augmented Ni (0, 50, 250, and 500 mg Ni kg^{-1}) doses in the soil resulted in increased Ni concentration in the plants.

The Ni contents were increased with Ni application ($p \leq 0.01$) and the highest Ni content (530 µg plant^{-1}) was obtained at 400 mg Ni kg^{-1} application (*Fig. 1*). Khan and Khan (2010) studying the effects of increased Ni application (0, 10, 50, 100, 200 and 400 mg kg^{-1}) on growth and yield of chickpea plant. They found that the Ni content of the root, stem, and leaf of plants was increased with Ni applications.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

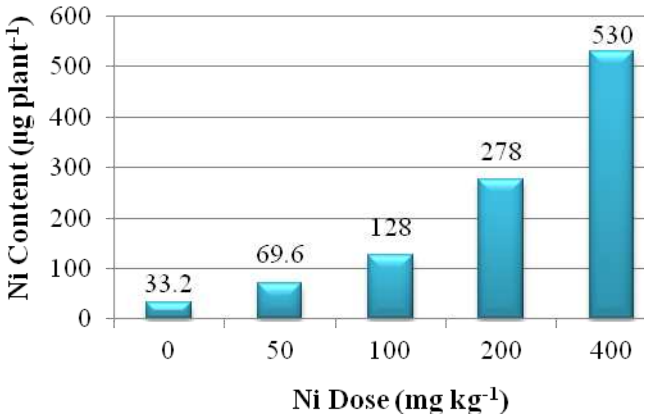

***Figure 1.** The effect of increasing Ni application on plant Ni content (n = 3)*

The effects of increasing doses of Ni on N, K, Ca and Mg concentrations of the plant were found statistically significant ($p \leq 0.01$). Compared to the control application the macro (N, P, K, Ca and Mg) element concentrations of plants were reduced with Ni applications (*Table 3*).

***Table 3.** The effect of different doses of Ni on N, P, K, Ca and Mg concentration of Xanthium strumarium L. (n = 3)*

	Ni	N	P	K	Ca	Mg
	(mg kg^{-1})	(g kg^{-1})				
Xantium strumarium L.	0	32.2 a	10.3 a	57.5 a	33.9 a	1.26 a
	50	28.7 b	10.2 ab	48.4 b	30.7 b	1.22 b
	100	28.2 b	9.74 bc	48.3 b	29.5 b	1.20 bc
	200	27.6 bc	9.62 c	47.8 b	28.7 b	1.19 c
	400	26.2 c	9.55 c	46.6 b	28.7 b	1.19 c
Dose	F	22.5**	4.91*	55.7**	11.9**	12.1**

**$p \leq 0.01$ statistically significant within error bounds
*$p \leq 0.05$ statistically significant within error bounds

Ahmad et al. (2011), investigated the phytotoxic effect of macro and micronutrients on the yield and macro and microelement concentration of *Helianthus annuus* L. plants with Ni applications (0, 10, 20, 30 and 40 mg L^{-1}). They observed a decrease in concentrations of N, P, K, Ca, Fe, Zn, Cu and Mn elements by the increase of Ni doses, on the other hand Ahmad et al. (2009), reported that increased Ni (10, 20, 30, 40, 50 and 60 mg L^{-1}) applications have been associated with the effect of 5 different sunflowers (*Helianthus annuus* L.) seeds, indicating a decrease in Mg and K contents due to increased Ni dose.

It was observed that the effects of Ni applications on microelement concentrations of *Xanthium strumarium* L. plant were statistically significant ($p \leq 0.01$) (*Table 4*).

When the rise in Ni doses was compared with the control group on the microelements (Fe, Zn, and Mn) of the plants, it was found that the concentration of these elements was decreased, while the Cu element concentration was increased (*Table 4*). Previous studies have shown that high amount of Ni applications can reduce the

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DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

amount of Fe (Chen et al., 2009; Pandey and Sharme, 2002). Rahman et al. (2005) reported that microelements (Fe, Zn and Mn) reduced in other applications except for 1.0 μM Ni, whilst investigating the effect of Ni doses (0, 1.0, 10 and 100 μM) on the growth and composition of barley plants.

Table 4. *The effect of different doses of Ni on Fe, Zn, Cu, and Mn concentrations in Xanthium strumarium L. (n = 3)*

	Ni	**Fe**	**Zn**	**Cu**	**Mn**
	(mg kg^{-1})	**(mg kg^{-1})**			
Xantium strumarium L.	0	49.0 a	15.7 a	43.0 c	116 a
	50	48.3 a	15.3 ab	45.3 c	111 ab
	100	45.7 a	13.7 bc	45.7 c	107 b
	200	45.0 a	12.9 c	54.3 b	107 b
	400	35.0 b	10.9 d	63.3 a	106 b
Dose	F	11.1**	13.06**	15.7**	5.58**

**$p \leq 0.01$ statistically significant within error bounds

Conclusion

Increasing doses of Ni used in this study induced a decrease in chlorophyll content and dry weight of plants. The main causes of this decrease are thought to be the accumulation of the Ni element in plant tissues at toxic levels and the negative effects of metabolisms that allow the plant to grow and develop.

The effect of Ni applications on GSH concentration according to the control group was varied between 225-336 μg mL^{-1}, which was exhibited the highest at 200 mg Ni kg^{-1} application, while the lowest Ni content was at 33.2 μg plant^{-1} and the highest 530 μg plant^{-1} with 400 mg Ni kg^{-1}.

The content of Ni has increased with varying dose applications, and no chlorosis or necrosis has been observed in the plant. *Xanthium strumarium* L. plant is found to be an important step for investigating the biochemical and physiological responses of plants against Ni toxicity and suitable for increasing the phytoremediation efficiency.

In addition, *Xanthium strumarium* is a native and aggressive weed in many countries. Although the species can be used in phytoremediation, it absolutely should be grown on controlled conditions.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20052013

AROMATIC PLANTS IN WEED CONTROL: INFLUENCE ON THE BROMUS ANTIOXIDANT SYSTEM AND SOIL MICROORGANISM GROWTH

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(Received 26th Oct 2018; accepted 7th Jan 2019)

Abstract. Natural compounds produced in plant metabolism can offer an alternative method to the chemical control of weeds. The aim of this study was to evaluate the effects of *Salvia sclarea* L. and *Clinopodium menthifolium* Host on the bromus (*Bromush mollish* L.) antioxidant properties to explore the potential of these aromatic plants in weed control. The impact of bioherbicides on the growth of beneficial microorganism present in the soil is less known. Consequently, the second aim was to evaluate the effects of *S. sclarea* and *C. menthifolium* on the growth of beneficial microorganism so as to assess theirs possible side effects when applied as bioherbicides. Two different concentrations (0.1% and 0.2%) of the aqueous extract of *S. sclarea* and *C. menthifolium* were assayed. Both tested extracts caused a change in peroxidase (POD) activity in leaves and roots of the bromus plants. Furthermore, both extracts induced lipid peroxidation in bromus leaves. In addition, tested extracts showed positive effects on the growth of some beneficial bacteria. The results indicate that *S. sclarea* and *C. menthifolium* aqueous extracts have negative effects on the antioxidant system in bromus plants with no inhibitory effects on the growth of the beneficial mycorrhizal bacteria and fungi.
Keywords: *allelochemicals, beneficial bacteria, bioherbicides, Bromush mollish L., enzyme activity*

Introduction

Throughout the years, the separation, characterization and production of natural products have been used as pesticides against insects, weeds, plant pathogens, and nematodes in the field. In the context of green chemistry (Abd El-Gawad, 2016), there is an urgent need for development of natural products called biopesticides as an alternative for maintaining high production with low ecological impact (Hermosa et al., 2012). Weeds have been documented as serious plant pests which constantly compete with crops for light and nutrients which cause considerable losses in their productivity (Jabran et al., 2015). Therefore, nitrogen (N), phosphorus (P), and potassium (K) uptake is reduced (Gonzalez Ponce et al., 1996). One of the highly resistant weed species in our crops is bromus.

With the constant implementation of synthetic herbicides in crop protection systems weeds have developed resistance. Herbicide resistance demands a new solution to cope with economic losses generated by weeds (Abd El-Gawad, 2016). Allelopathy is an ecological phenomenon where plants produce a great variety of secondary metabolites, called allelochemicals (Abd El-Gawad, 2016; Céspedes et al., 2014). These compounds belong to numerous chemical groups including: phenolic acids, flavonoids, triketones, terpenes, benzoquinones, coumarins, terpenoids, tannins lignin, fatty acids and non-protein amino acids (Soltys et al., 2013). They are important in mediating interactions

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DOI: http://dx.doi.org/10.15666/aeer/1702_20152031

between plants and their biotic environment (Céspedes et al., 2014). Allelochemicals either have an inhibitory or stimulatory effect on plants, and they are considered to be a natural defense mechanism of plants (Abd El-Gawad, 2016).

Allelochemicals may be synthesised in all plant organs like leaves, stems, flowers, fruits, seeds and roots (Farooq et al., 2011). One of the main invisible effects of allelochemicals on the target plant is uncontrolled production of reactive oxygen species (ROS) (Bogatek et al., 2006). Under stress conditions, the generation of ROS is greatly increased (Soares et al., 2016). ROS are highly reactive and in the absence of any protective mechanism, they can seriously damage vital biomolecules such as lipids, proteins and nucleic acids (Meloni and Martinez, 2009). To mitigate the oxidative damage induced by ROS, plants have developed antioxidant defense systems, enzymatic and non-enzymatic (Azevedo Neto et al., 2006). During oxidative stress, plants produce hydrogen peroxide which is considered harmful to plant cells (Šimonovičová et al., 2004). Peroxidases are one of the major H_2O_2-scavenging enzymes. The cellular level of H_2O_2 could be toxic enough to inhibit the enzymes' activity, leaving the plant vulnerable to oxidative damage (Mandal et al., 2013). Allelopathic interactions between plants may become an alternative to pesticides for weed control (Khalid et al., 2002).

Salvia sclarea, commonly called clary sage, and *Clinopodium menthifolium* belong to Lamiaceae. *S. sclarea* is an important medicinal herb (Kumar and Sharma, 2012). The major phytochemicals of the sage plant are phenols and terpenoids. Different bioassays of plant extracts have shown biological activities such as antimicrobial, antioxidant, cytotoxic, antiprotozoal, antidiabetic (Mahmood et al., 2012). The aqueous extract of aromatic plants, which is rich in phenols, is easily used for foliar application due to their rich water solubility and their ability to create a uniform spray mixture.

The major objective of this study was to evaluate the allelopathic effects of the aqueous extract of two aromatic plants, *Salvia sclarea* L. and *Clinopodium menthifolium* Host, on bromus (*Bromush mollish* L.) antioxidant properties to explore the potential of this species in weed control. The effect of the two concentrations (0.1 and 0.2%) of *S. sclarea* and *C. menthifolium* aqueous extracts on the lipid peroxidation process (LP), as well as the activity of peroxidase (POD) antioxidant enzymes (pyrogallol and guaiacol peroxidases) in leaves and roots of bromus seedlings were examined 24, 72 and 120 h after the treatment. Due to strong antimicrobial activity of aromatic plants, the impact of tested aqueous extracts on the growth of beneficial microorganism present in the soil was additionally investigated.

Materials and methods

Plant materials and preparation of the aqueous extracts

Salvia sclarea L. was collected in the south of Serbia, around Vranje town (longitude: 21°53'09.23" E, latitude: 42°22'40.44" N, altitude: 494 m), in July of 2012. *Clinopodium menthifolium* (Host) was collected at localities near the Adriatic coast in Montenegro, around Sutomore town and Čanj town (longitude: 19°00'30.10" E, latitude: 42°09'52.19" N, altitude: 31 m), in May of 2012. Voucher specimens *Salvia sclarea* L. Nº 2-1545 and *Clinopodium menthifolium* (Host) Nº 2-1543 were confirmed and deposited at the Herbarium of The Department of Biology and Ecology, Faculty of Science, University of Novi Sad (Holmgren and Holmgren, 2003).

The plants were dried at 30 ºC for two weeks, and the dried plants were then ground into powder. The powdery material (10 g) was spilled with 100 mL of boiling distilled

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water (10%, w/v) and left for 24 h. After 24 h, the extracts were filtered through Whatman No. 4 filter paper and kept at 4°C in the fridge until application.

Seedling growth

The experiment was performed at the Laboratory of Biochemistry, Faculty of Agriculture, Novi Sad and conducted under controlled conditions (28 °C, 60% relative humidity, a photoperiod of 18 h, and a light intensity of 10.000 lx). The bromus (*Bromus mollish* L.) seeds were surface-sterilized with 3% H_2O_2 (v/v), washed with deionised water, placed in plastic pots containing sterile sand and maintained under dark conditions. Thirty-day-old seedlings were transplanted in plastic pots containing 700 mL of Hoagland's solution prepared according to Hoagland and Arnon (1950), and 7 or 14 mL of 10% *S. sclarea* and *C. menthifolium* aqueous extract, separately, while pots of control contained the same volume of nutrient solution. When 7 mL of the plant extract was added to the solution, the final concentration of the extract was 0.1%. When 14 mL of the plant extract was added to the solution, the final concentration of the extract was 0.2%. The bromus plants were harvested for determining the investigated biochemical parameters 24, 72 and 120 h after the treatments with the plant aqueous extracts.

Analysis of the POD antioxidant enzymes and MDA content

For the determination of the peroxidase (POD) activity and malondialdehyde (MDA) content, 2 g of fresh plant material (bromus leaves and roots from each growth condition: control, 0.1 and 0.2% plant aqueous extracts) was homogenized in 10 mL of phosphate buffer (0.1 M, pH 7.0) prepared in-house. After centrifugation, supernatants (bromus extracts) were used for protein quantification and POD activity assays. Biochemical analyses were carried out spectrophotometrically using an UV/VIS spectrophotometer (Thermo Scientific Evolution 220 (USA)). A measurement of protein levels in the supernatants was performed according to the method of Bradford (Sedmark and Grossberg, 1977; Spector, 1978). The activity of the POD (EC 1.11.1.7) was measured using guaiacol and pyrogallol as substrates according to Morkunas and Gmerek (2007). The absorbance was recorded at 436 nm. The activity of the POD was expressed in U/mg of proteins. The MDA content, an end product of lipid peroxidation process, was measured at 532 nm using the thiobarbituric acid (TBA) test (Mandal et al., 2008). The total amount of TBA-reactive substances was given in nmol of MDA equivalents/mg of proteins.

Bacteria culture

The test microorganism used in this study were as follows: Azotobacter–isolates 1 and 2; Pseudomonas–isolates 1, 2, and Marker; Bacillus–Bacillus subtilis marker 44, Bacillus subtilis and Bacillus megaterium; Rhizobium–isolate D_1, Bradyrhizobium japonicum isolate S511, Rhizobium trifolii 1; fungi–Penicillium sp., Alternarium sp. and Trichoderma asperellum. The collection of microbial soil isolates is from the Laboratory of Microbiology, Faculty of Agriculture, University of Novi Sad, Serbia).

Microorganism cultivation

Azotobacter isolates were grown on mannitol selective nutrient medium (mannitol 20.0 g, $K_2 HPO_4$ 0.3 g, $CaHPO_4$ 0.2 g, $MgSO_4$ 0.3 g, NaCl 0.5 g, $FeCl_3$ 0.1 g, $CaCO_3$

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2.5 g, microelements solution 1.0 mL, distilled H_2O 1000.0 mL, pH 8.2) for 48 h on 150 rpm at 28 °C in shaker incubator (Aquilanti et al., 2004). After OD determination on 600 nm all inocula were adjusted at 10^8 cell mL^{-1}. *Pseudomonas* isolates were grown on King – B nutritet medium (pepton 10.0 g, trypton 10.0 g, K_2HPO_4 1.5 g, $MgSO_4$ 1.5 g, glycerol 10.0 mL, distilled H_2O 1000.0 mL, pH 7.2) for 48 h on 150 rpm at 28 °C in shaker incubator (Valls et al., 1999). After OD determination on 600 nm all inocula were adjusted at 10^8 cell mL^{-1}. *Bacillus subtils* and *Bacillus megaterium* strains were grown on the L – agar, selective liqud nutrient medium (distilled H_2O 1000 mL, tripton 10.0 g, yeast extract 5.0 g, NaCl 5.0 g,) for 48 h on 150 rpm at 28 °C in shaker incubator (Valls et al., 1999). After OD determination on 600 nm all inocula were adjusted at 10^8 cell mL^{-1}. *Bradyrhizobium japonicum* isolate S511 and *Rhizobium* isolates were grown on YMB selective liqude medium (mannitol 10.0 g, yeast extract 0.5 g, K_2HPO_4 0.5 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.2 g, NaCl 0.1 g distilled H_2O 1000.0 mL, pH 7.2), *B japonicum* for 5 days and *Rhizobium* sp. for 3 days on 150 rpm at 28 °C in shaker incubator. After OD determination on 600 nm all inocula were adjusted at 10^8 cell mL^{-1}.

Pure cultures of fungi - *Trichoderma asperellum*, *Penicillium* sp., and *Alternarium* sp. were isolated from serial dilutions and grown on potato dextrose agar (PDA, Difco®) at 25 ± 1 °C for at least 7 days. Single colonies were purified by re-isolation on PDA and a single hyphal tip was isolated and grown on PDA. The sporulated colony arising from this hyphal tip was used to make inocula with 10^6 spore mL^{-1} for each fungi.

Disc diffusion method

The evaluation of the *S. sclarea* and *C. menthifolium* aqueous extracts on the growth of microorganism was carried out by the disc diffusion method described by Prabuseenivasan et al. (2006). Sterilized Petri dishes with agar were inoculated with microorganism cultures. The paper discs impregnated with plant aqueous extracts were placed on the agar surface. The plates were incubated at 28°C. After incubation (72 h and 120 h), the plates were examined for the stimulation/inhibition zone (*Fig. 1*). The test was repeated three times to ensure reliability.

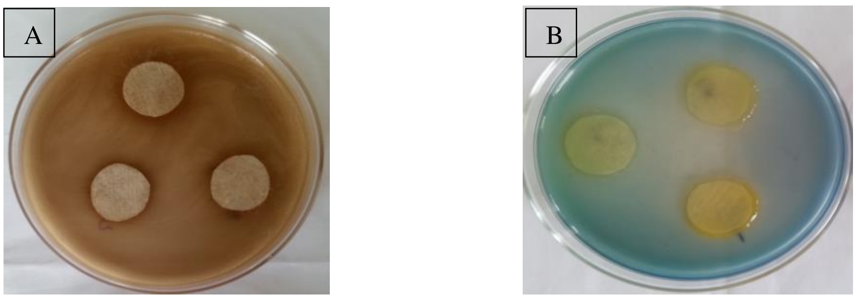

Figure 1. *The bacterial strain growth stimulator zone Azotobacter isolate 1 (A) and Bradyrhizobium japonicum isolate S511 (B) under the influence of the S. sclarea aqueous extract*

Statistical analysis

All measurements were performed in triplicates. Values of the biochemical parameters were expressed as mean ± standard error of mean and tested by ANOVA followed by comparison of the means by Duncan's multiple range test ($P < 0.05$). Data were analyzed

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using STATISTICA for Windows version 11.0. Comparable percentage was done by *Equation 1*.

$$\Delta(\%) = (100 \text{ x sample / control}) - 100 \quad \text{(Eq.1)}$$

Results

POD activity and MDA content in bromus leaves and roots

In leaves of the bromus plants, a significant decrease in activity of POD was detected 72 h after the treatment with 0.1% *S. sclarea* aqueous extract (*Fig 2*). The activity of pyrogallol peroxidase showed a 30% decrease, while the activity of guaiacol peroxidase showed a 36% decrease (*Table 1*). In the treatment with a higher concentration of the *S. sclarea* aqueous extract (0.2%), there were no significant differences in the activity of POD in the leaves of treated bromus plants compared to plants from the control group. On the other hand, in the roots of bromus, a lower tested concentration of *S. sclarea* aqueous extract (0.1%) significantly increased the activity of POD 120 h after the treatment. The activity of pyrogallol and guaiacol peroxidases was increased by 117% and 248%, respectively. In the treatment with a higher concentration (0.2%) a significant increase in the activity of pyrogallol peroxidase was detected 72 h after the treatment (98%).

Table 1. *The effect of the two concentrations (0.1 and 0.2%) of the S. sclarea aqueous extract on the activities of the antioxidant enzymes (U/mg protein) and on MDA content (nmol/mg protein) in leaves and roots of the bromus seedlings compared to the control group*

Time		**24 h**	**72 h**	**120 h**
Leaves				
Guaiacol peroxidase	Control	$(4.01 \pm 0.09) \cdot 10^{2\ a}$	$(3.56 \pm 0.32) \cdot 10^{2\ a}$	$(5.17 \pm 0.18) \cdot 10^{2\ b}$
	0.1%	$(4.77 \pm 0.27) \cdot 10^{2\ b}$	$(2.29 \pm 0.17) \cdot 10^{2\ c}$	$(3.74 \pm 0.13) \cdot 10^{2\ a}$
	0.2%	$(3.44 \pm 0.04) \cdot 10^{2\ a}$	$(5.46 \pm 0.25) \cdot 10^{2\ b}$	$(5.05 \pm 0.41) \cdot 10^{2\ b}$
Pyrogallol peroxidase	Control	$(3.87 \pm 0.18) \cdot 10^{2\ a}$	$(3.51 \pm 0.10) \cdot 10^{2\ a}$	$(3.41 \pm 0.37) \cdot 10^{2\ a}$
	0.1%	$(3.35 \pm 0.39) \cdot 10^{2\ a,b}$	$(2.48 \pm 0.14) \cdot 10^{2\ b}$	$(3.35 \pm 0.98) \cdot 10^{2\ a,b}$
	0.2%	$(3.63 \pm 0.31) \cdot 10^{2\ a}$	$(3.21 \pm 0.33) \cdot 10^{2\ a,b}$	$(3.40 \pm 0.34) \cdot 10^{2\ a}$
MDA content	Control	$3.83 \pm 0.02^{a,b}$	3.26 ± 0.15^{c}	5.48 ± 0.07^{f}
	0.1%	4.87 ± 0.05^{e}	4.44 ± 0.03^{d}	3.60 ± 0.05^{a}
	0.2%	3.70 ± 0.10^{a}	4.00 ± 0.03^{b}	7.04 ± 0.09^{g}
Roots				
Guaiacol peroxidase	Control	$(2.09 \pm 0.21) \cdot 10^{3\ a,b}$	$(1.56 \pm 0.05) \cdot 10^{3\ a}$	$(1.03 \pm 0.00) \cdot 10^{3\ c}$
	0.1%	$(1.71 \pm 0.12) \cdot 10^{3\ a}$	$(0.59 \pm 0.02) \cdot 10^{3\ c}$	$(3.59 \pm 0.17) \cdot 10^{3\ d}$
	0.2%	$(2.53 \pm 0.26) \cdot 10^{3\ b}$	$(2.04 \pm 0.16) \cdot 10^{3\ a}$	$(1.68 \pm 0.08) \cdot 10^{3\ a}$
Pyrogallol peroxidase	Control	$(1.75 \pm 0.02) \cdot 10^{3\ a}$	$(0.53 \pm 0.02) \cdot 10^{3\ c}$	$(0.94 \pm 0.16) \cdot 10^{3\ b,c}$
	0.1%	$(1.27 \pm 0.04) \cdot 10^{3\ b}$	$(0.47 \pm 0.01) \cdot 10^{3\ c}$	$(2.04 \pm 0.10) \cdot 10^{3\ a}$
	0.2%	$(1.99 \pm 0.11) \cdot 10^{3\ a}$	$(1.05 \pm 0.32) \cdot 10^{3\ b}$	$(0.97 \pm 0.23) \cdot 10^{3\ b,c}$
MDA content	Control	$1.66 \pm 0.16^{a,b}$	1.52 ± 0.13^{a}	$2.85 \pm 1.36^{a,b}$
	0.1%	$2.64 \pm 0.10^{a,b}$	$1.93 \pm 0.08^{a,b}$	$2.46 \pm 0.01^{a,b}$
	0.2%	3.22 ± 0.08^{b}	$2.36 \pm 0.10^{a,b}$	$2.16 \pm 0.04^{a,b}$

The data are mean values ± standard error
$^{a\text{-}f}$Values without the same superscripts within each column differ significantly (*P* < 0.05)

In the treatment with 0.1% *C. menthifolium* aqueous extract, there were no significant differences in the activity of pyrogallol peroxidase in the leaves of bromus between the plants from the control group and the treatments (*Table 2*). In the treatment with a higher concentration of the *C. menthifolium* aqueous extract (0.2%), a significant increase in the activity of pyrogallol peroxidase was detected 120 h after the treatment (26%). A significant increase in the activity of guaiacol peroxidase was detected in the leaves of bromus 72 h after the treatment. The activity of guaiacol peroxidase showed an increase of 88% in the treatment with 0.1% *C. menthifolium* aqueous extract and an increase of 85% in the treatment with 0.2% *C. menthifolium* aqueous extract. In the roots of bromus plants, both tested concentrations of *C. menthifolium* aqueous extract decreased the activity of pyrogallol and guaiacol peroxidases. In the treatment with a higher concentration (0.2%), a significant decrease in the activity of guaiacol peroxidase was detected (71% 24 h after the treatment, 75% 72 h after the treatment, and 55% 120 h after the treatment). In the treatment with 0.1% *C. menthifolium* aqueous extract, a significant decrease in the activity of guaiacol peroxidase was detected as well (52% 24 h after the treatment, 67% 72 h after the treatment, and 51% 120 h after the treatment). The activity of pyrogallol peroxidase showed a decrease of 48% in the treatment with 0.1% and a decrease of 68.5% in the treatment with 0.2% *C. menthifolium* aqueous extract 120 h after the treatment.

Table 2. *The effect of the two concentrations (0.1 and 0.2%) of the C. menthifolium aqueous extract on the activities of the antioxidant enzymes (U/mg protein) and on MDA content (nmol/mg protein) in leaves and roots of the bromus seedlings compared to the control group*

Time		24 h	72 h	120 h
Leaves				
Guaiacol peroxidase	Control	$(4.01 \pm 0.09)\cdot10^{2\ a,c}$	$(3.56 \pm 0.32)\cdot10^{2\ c}$	$(5.17 \pm 0.18)\cdot10^{2\ a,b,c}$
	0.1%	$(5.60 \pm 0.33)\cdot10^{2\ a,b}$	$(6.69 \pm 0.51)\cdot10^{2\ b}$	$(5.56 \pm 1.17)\cdot10^{2\ a,b}$
	0.2%	$(4.06 \pm 0.17)\cdot10^{2\ a,c}$	$(6.59 \pm 0.43)\cdot10^{2\ b}$	$(4.14 \pm 0.42)\cdot10^{2\ a}$
Pyrogallol peroxidase	Control	$(3.87 \pm 0.18)\cdot10^{2\ a,b}$	$(3.51 \pm 0.10)\cdot10^{2\ a,b,c}$	$(3.41 \pm 0.37)\cdot10^{2\ a,c}$
	0.1%	$(4.14 \pm 0.16)\cdot10^{2\ a,b}$	$(4.37 \pm 0.36)\cdot10^{2\ b}$	$(3.38 \pm 0.18)\cdot10^{2\ a}$
	0.2%	$(2.92 \pm 0.21)\cdot10^{2\ c}$	$(4.01 \pm 0.26)\cdot10^{2\ a,b}$	$(4.30 \pm 0.24)\cdot10^{2\ b}$
MDA content	Control	3.83 ± 0.02^{a}	3.26 ± 0.15^{c}	5.48 ± 0.07^{e}
	0.1%	$3.98 \pm 0.07^{a,b}$	4.76 ± 0.04^{d}	4.24 ± 0.07^{b}
	0.2%	4.22 ± 0.13^{b}	4.83 ± 0.15^{d}	5.45 ± 0.09^{e}
Roots				
Guaiacol peroxidase	Control	$(20.96 \pm 2.15)\cdot10^{2\ a}$	$(15.69 \pm 0.51)\cdot10^{2\ b}$	$(10.31 \pm 0.40)\cdot10^{2\ b}$
	0.1%	$(10.11 \pm 0.27)\cdot10^{2\ b}$	$(5.18 \pm 0.49)\cdot10^{2\ c}$	$(5.31 \pm 0.08)\cdot10^{2\ c}$
	0.2%	$(6.20 \pm 0.82)\cdot10^{2\ c}$	$(3.95 \pm 0.23)\cdot10^{2\ c}$	$(4.63 \pm 0.71)\cdot10^{2\ c}$
Pyrogallol peroxidase	Control	$(17.51 \pm 0.28)\cdot10^{2\ a}$	$(5.36 \pm 0.24)\cdot10^{2\ c,d}$	$(9.47 \pm 1.60)\cdot10^{2\ b}$
	0.1%	$(10.70 \pm 0.42)\cdot10^{2\ b}$	$(7.17 \pm 0.48)\cdot10^{2\ c}$	$(4.91 \pm 0.43)\cdot10^{2\ e}$
	0.2%	$(10.28 \pm 0.57)\cdot10^{2\ b}$	$(5.03 \pm 0.15)\cdot10^{2\ d}$	$(2.98 \pm 0.27)\cdot10^{2\ b,c}$
MDA content	Control	1.66 ± 0.16^{a}	1.52 ± 0.13^{a}	2.85 ± 1.36^{a}
	0.1%	1.72 ± 0.02^{a}	1.73 ± 0.01^{a}	1.79 ± 0.04^{a}
	0.2%	1.72 ± 0.22^{a}	1.56 ± 0.01^{a}	1.55 ± 0.04^{a}

The data are mean values ± standard error
$^{a\text{-}e}$Values without the same superscripts within each column differ significantly ($P < 0.05$)

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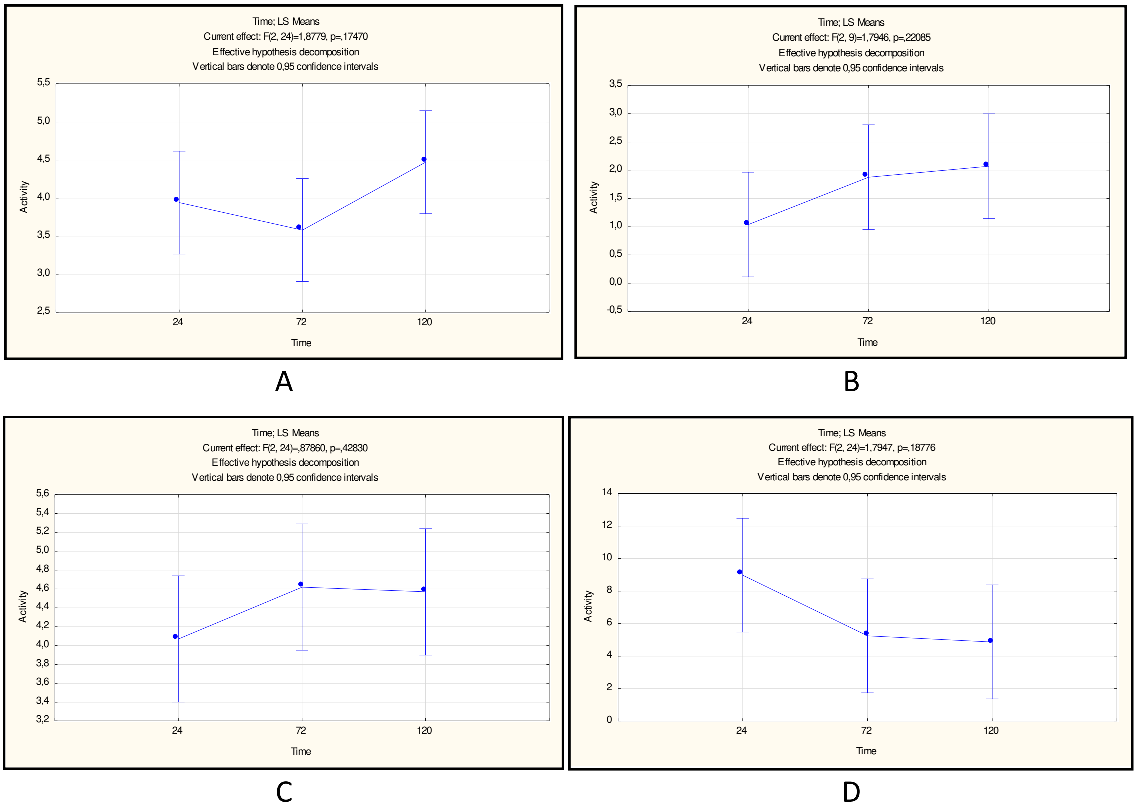

Figure 2. *The relationship between the period of time and the activities of the antioxidant enzymes in leaves of the bromus treated with S. sclarea aqueous extract (A), roots of the bromus treated with S. sclarea aqueous extract (B), leaves of the bromus treated with C. menthifolium aqueous extract (C), roots of the bromus treated with C. menthifolium aqueous extract (D)*

The accumulation of malondialdehyde (MDA), an end product of the lipid peroxidation process, was significantly higher in leaves of bromus plants 120 h after the treatment with *S. sclarea* extract and 72 h after the treatment with *C. menthifolium* extract (*Fig. 3*). In the treatment with 0.2% *S. sclarea* aqueous extract, the accumulation of MDA was 29% (*Table 1*). In the treatment with 0.1% and 0.2% *C. menthifolium* aqueous extract the accumulation of MDA was 46% and 48%, respectively (*Table 2*). Furthermore, the accumulation of MDA was higher in leaves of bromus plants after the treatment with *C. menthifolium* aqueous extract compared to *S. sclarea* aqueous extract. This observation could indicate that *C. menthifolium* possesses a higher phytotoxic effect than *S. sclarea*. On the other hand, in the roots of bromus plants, there was no significant increase in the lipid peroxidation intensity.

Disc diffusion method

In the treatment with both concentrations (0.1% and 0.2%) of *S. sclarea* and *C. menthofolium* aqueous extracts, separately, there were no stimulatory or inhibitory effects on the growth of the bacteria *Pseudomonas* – isolates 1, 2, and Marker; *Bacillus* – *Bacillus subtilis* and *Bacillus megaterium*; *Rhizobium trifolii 1*; and fungi – *Penicillium* sp., *Alternarium* sp. and *Trichoderma asperellum.*

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The tested extracts showed a stimulatory effect on the growth of the *Azotobacter* – isolates 1 and 2; *Bacillus subtilis marker 44*, *Rhizobium* isolate D_1, *Bradyrhizobium japonicum* isolate S511 120 h after the treatment with the zone of stimulation ranging from 2 to 8 mm (*Figs. 4* and *5*).

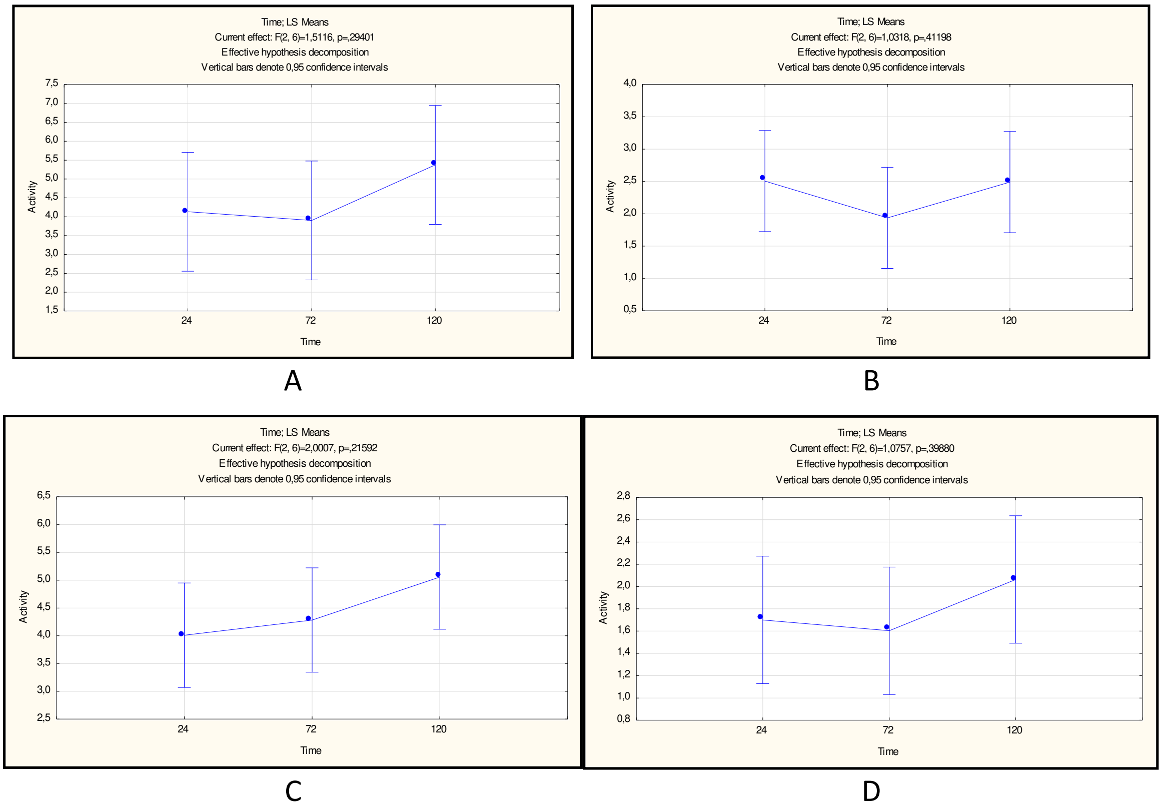

***Figure 3.** The relationship between the period of time and MDA content in leaves of the bromus treated with S. sclarea aqueous extract (A), roots of the bromus treated with S. sclarea aqueous extract (B), leaves of the bromus treated with C. menthifolium aqueous extract (C), roots of the bromus treated with C. menthifolium aqueous extract (D)*

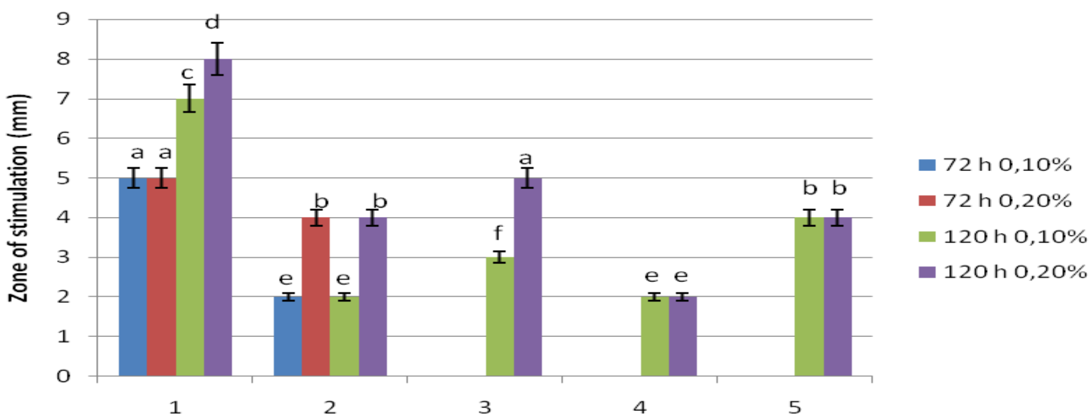

***Figure 4.** The stimulatory effect of the two concentrations (0.1 and 0.2%) of the S. sclarea aqueous extracts on the growth of test microorganism (1–Azotobacter isolate 1; 2–Azotobacter isolate 2; 3– Bacillus subtilis marker 44; 4–Rhizobium isolate D_1, 5–Bradyrhizobium japonicum isolate S511)*

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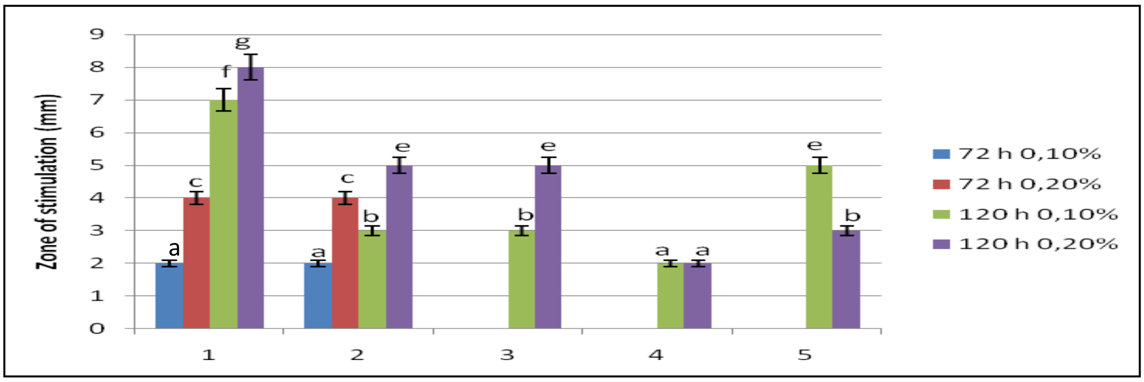

***Figure 5.** The stimulatory effect of the two concentrations (0.1 and 0.2%) of the C. menthifolium aqueous extracts on the growth of test microorganism (1–Azotobacter isolate 1; 2–Azotobacter isolate 2; 3– Bacillus subtilis marker 44; 4–Rhizobium isolate D1, 5–Bradyrhizobium japonicum isolate S511)*

Discussion

The activity of antioxidant enzymes is frequently used as an indicator of oxidative stress in plants caused by pro-oxidants (Li et al., 2013). For various plant species under oxidative stress, oxidative damage of cell membranes is observed. In this research, the phytotoxic effect of extracts was different between two examined plant tissues. Even though plant aqueous extracts affected the activity of the antioxidant enzymes in leaves and roots of the bromus seedlings, a significantly higher accumulation of MDA was detected only in leaves of bromus plants. The accumulation of MDA indicates that allelochemicals presented in plant extracts caused oxidative damage of membranes. No differences in MDA content in roots of bromus plants treated with extracts compared to untreated roots confirmed that leaves of bromus were more affected than roots. This is in accordance with the results reported in a study by Mahdavikia and Saharkhiz (2016) who reported that peppermint allelochemicals caused oxidative stress in the aerial parts of tomato seedlings. On the other hand, Chon et al. (2002) reported that even though phenolic compounds are involved in the inhibition of shoot growth, root length is a better indicator of phytotoxic effects of allelochemicals than shoot length. It is very important to know the mode of action of toxic compounds of plants. Non-protein amino acids produced by plants, such as *meta*-tyrosine (*m*-Tyr), modify the activity of non-enzymatic antioxidants while cell membranes are not primary cellular targets (Andrzejczak et al., 2018). Contrarily, plant phenolic compounds, such as p-cymene and cinnamic acid increase lipid peroxidation in tested plants and stimulate total SOD activity (Zhang et al., 2012; Ding et al., 2007).

In this study, changes in enzymatic activity were the highest 72 h after the treatment. This was accompanied by the accumulation of MDA in bromus leaves. This observation could indicate that plant extracts exhibit a toxic effect in the first 72 h. In the treatment with *C. menthifolium* aqueous extract both tested concentrations decreased the activity of antioxidant enzymes in roots of bromus plants. In spite of the increased activity of the enzymes, there were no significant changes in the lipid peroxidation intensity in roots of bromus between the plants from the control group and the treatments at the end of the experiment (120 h after the treatment). This could indicate that the allelopathy-provoked stress was not strong enough and scavenging effects of antioxidant enzymes could still prevent an oxidative burst and the induction of lipid peroxidation. In the leaves and roots of black nightshade, due to the exposure to *C. menthifolium* aqueous extract, an increase in the lipid peroxidation process was

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DOI: http://dx.doi.org/10.15666/aeer/1702_20152031

observed (Šućur et al., 2017), which points to the different responses of species when facing allelochemicals. Chemical compounds produced by plants could be allelopathic agents with an inhibitory effect on plant growth. Phenolic compounds are identified as the most common allelochemicals produced by plants. Some phenolic compounds can either promote or inhibit plant growth according to their concentration (Li et al., 2010). Nandakumar and Rangaswamy (1985) reported that some flavonoids had promotive effects on plant growth while in contrast to the flavonoids some phenolic acids suppressed plant growth and inhibited seed germinations. The allelopathic effects of plant extracts were investigated in a number of studies. For example, Islam et al. (2013) found that aqueous methanol extracts of *Leucas aspera* L. and *Hyptis suaveolens* L. possess strong allelopathic potential against barnyard grass. Franco et al. (2016) observed that *Copaifera langsdorffii* leaf extract had an inhibitory action on seed germination and root growth in sorghum. Furthermore, *Thymus kotschyanus* (Lamiaceae) exhibited dose-dependent allelopathic effects on *Bromus tomentellus* seed germination and seedling growth (Safari et al., 2010). *Salvia officinalis* (Lamiaceae) aqueous extract showed a strong inhibitory effect on *Amaranthus retroflexus* seed germination (Bajalan et al., 2013).

It is very important that herbicides or bioherbicides used for weed control have no inhibitory effect on the growth of beneficial mycorrhizal bacteria and fungi. *Bacillus*, *Pseudomonas*, *Azotobacter* and *Rhizobium* species are well known as plant growth-promoting rhizobacteria (PGPR). They play an important role in increasing soil fertility, promoting plant growth, and suppressing phytopathogens for the development of ecofriendly sustainable agriculture (Gupta et al., 2015). Plant growth-promoting fungi (PGPFs), such as species of the genera *Trichoderma* and *Penicillium*, also have the ability to stimulate the plant immune response upon enemy attack and growth promotion in crop plants (Jogaiah et al., 2013). It is a useful finding that the tested plant extracts showed a stimulatory effect on the growth of some beneficial bacteria.

Conclusions

Based on our current results, it can be concluded that *S. sclarea* and *C. menthifolium* aqueous extracts possess a negative effect against bromus, inducing oxidative stress accompanied by the induction of the lipid peroxidation process. *C. menthifolium* possesses a higher phytotoxic effect than *S. sclarea* whereas the accumulation of MDA was higher in leaves of bromus plants after the treatment with *C. menthifolium* aqueous extract compared to *S. sclarea* aqueous extract. In addition, negative effects are dependent on the plant tissues and the sensitivity of the plant is dependent on the concentration of applied extracts. Since the present investigation suggests that *S. sclarea* and *C. menthifolium* aqueous extracts possess a negative effect against the antioxidant system in weeds, and a stimulatory effect on the growth of some beneficial bacteria, it would be good to explore the aromatic plants in the development of natural pesticides.

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APPENDIX

ANOVA tables

Table A1. *The effect of the C. menthifolium aqueous extract on MDA content (nmol/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .03180, df = 18.000								
	{1} (3.8391)	{2} (3.9842)	{3} (4.2202)	{4} (3.2673)	{5} (4.7662)	{6} (4.8370)	{7} (5.4892)	{8} (4.2402)	{9} (5.4597)
1		0.332331	0.022278	0.001135	0.000044	0.000035	0.000023	0.019607	0.000027
2	0.332331		0.122672	0.000214	0.000129	0.000063	0.000027	0.112208	0.000031
3	0.022278	0.122672		0.000067	0.002049	0.000892	0.000032	0.892058	0.000036
4	0.001135	0.000214	0.000067		0.000031	0.000027	0.000022	0.000040	0.000023
5	0.000044	0.000129	0.002049	0.000031		0.632518	0.000226	0.002145	0.000276
6	0.000035	0.000063	0.000892	0.000027	0.632518		0.000458	0.000983	0.000594
7	0.000023	0.000027	0.000032	0.000022	0.000226	0.000458		0.000036	0.841999
8	0.019607	0.112208	0.892058	0.000040	0.002145	0.000983	0.000036		0.000060
9	0.000027	0.000031	0.000036	0.000023	0.000276	0.000594	0.841999	0.000060	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A2. *The effect of C. menthifolium aqueous extract on MDA content (nmol/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .65187, df = 18.000								
	{1} (1.6616)	{2} (1.7245)	{3} (1.7215)	{4} (1.5287)	{5} (1.7311)	{6} (1.5624)	{7} (2.8559)	{8} (1.7963)	{9} (1.5560)
1		0.929903	0.928749	0.856564	0.926590	0.882135	0.123705	0.858727	0.881857
2	0.929903		0.996542	0.796331	0.992170	0.825184	0.131357	0.919834	0.823156
3	0.928749	0.996542		0.797064	0.989849	0.822650	0.137598	0.921050	0.821561
4	0.856564	0.796331	0.797064		0.789782	0.963194	0.095978	0.724838	0.967538
5	0.926590	0.992170	0.989849	0.789782		0.822904	0.122637	0.922497	0.817360
6	0.882135	0.825184	0.822650	0.963194	0.822904		0.100134	0.758023	0.992459
7	0.123705	0.131357	0.137598	0.095978	0.122637	0.100134		0.125536	0.100811
8	0.858727	0.919834	0.921050	0.724838	0.922497	0.758023	0.125536		0.751855
9	0.881857	0.823156	0.821561	0.967538	0.817360	0.992459	0.100811	0.751855	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

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Table A3. *The effect of the S. sclarea aqueous extract on MDA content (nmol/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .01914, df = 18.000									
	Var1	{1} (3.8391)	{2} (4.8718)	{3} (3.7015)	{4} (3.2673)	{5} (4.4442)	{6} (4.0087)	{7} (5.4892)	{8} (3.6074)	{9} (7.0484)
1	1		0.000060	0.238798	0.000194	0.000126	0.150785	0.000036	0.066617	0.000031
2	2	0.000060		0.000036	0.000027	0.001512	0.000075	0.000189	0.000031	0.000075
3	3	0.238798	0.000036		0.001674	0.000066	0.018073	0.000031	0.416198	0.000027
4	4	0.000194	0.000027	0.001674		0.000031	0.000041	0.000023	0.007667	0.000022
5	5	0.000126	0.001512	0.000066	0.000031		0.001302	0.000075	0.000036	0.000060
6	6	0.150785	0.000075	0.018073	0.000041	0.001302		0.000060	0.003754	0.000036
7	7	0.000036	0.000189	0.000031	0.000023	0.000075	0.000060		0.000027	0.000161
8	8	0.066617	0.000031	0.416198	0.007667	0.000036	0.003754	0.000027		0.000023
9	9	0.000031	0.000075	0.000027	0.000022	0.000060	0.000036	0.000161	0.000023	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A4. *The effect of the S. sclarea aqueous extract on MDA content (nmol/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .64586, df = 18.000									
	Var1	{1} (1.6616)	{2} (2.6440)	{3} (3.2268)	{4} (1.5287)	{5} (1.9357)	{6} (2.3611)	{7} (2.8559)	{8} (2.4610)	{9} (2.1663)
1	1		0.199035	0.050623	0.841857	0.681258	0.341123	0.125384	0.286864	0.476813
2	2	0.199035		0.412212	0.150707	0.343830	0.688864	0.750672	0.783560	0.513248
3	3	0.050623	0.412212		0.036451	0.099244	0.249821	0.578961	0.298233	0.167079
4	4	0.841857	0.150707	0.036451		0.565382	0.267974	0.092955	0.221944	0.384671
5	5	0.681258	0.343830	0.099244	0.565382		0.548120	0.227765	0.472679	0.729488
6	6	0.341123	0.688864	0.249821	0.267974	0.548120		0.498447	0.880803	0.770136
7	7	0.125384	0.750672	0.578961	0.092955	0.227765	0.498447		0.576915	0.356484
8	8	0.286864	0.783560	0.298233	0.221944	0.472679	0.880803	0.576915		0.676742
9	9	0.476813	0.513248	0.167079	0.384671	0.729488	0.770136	0.356484	0.676742	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A5. *The effect of the C. menthifolium aqueous extract on the activity of the guaiacol peroxidase (U/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00763, df = 18.000								
	{1} (.40147)	{2} (.56029)	{3} (.40683)	{4} (.35696)	{5} (.66957)	{6} (.65905)	{7} (.51747)	{8} (.55683)	{9} (.41484)
1		0.061888	0.941056	0.540447	0.003605	0.004561	0.151624	0.063844	0.861890
2	0.061888		0.066902	0.020727	0.163236	0.183090	0.577717	0.961933	0.075823
3	0.941056	0.066902		0.517307	0.003927	0.004905	0.158298	0.067697	0.911897

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4	0.540447	0.020727	0.517307		0.001064	0.001350	0.056118	0.021426	0.466550
5	0.003605	0.163236	0.003927	0.001064		0.884374	0.069174	0.162901	0.004559
6	0.004561	0.183090	0.004905	0.001350	0.884374		0.083409	0.190978	0.005603
7	0.151624	0.577717	0.158298	0.056118	0.069174	0.083409		0.587873	0.167332
8	0.063844	0.961933	0.067697	0.021426	0.162901	0.190978	0.587873		0.074311
9	0.861890	0.075823	0.911897	0.466550	0.004559	0.005603	0.167332	0.074311	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A6. *The effect of the C. menthifolium aqueous extract on the activity of the guaiacol peroxidase (U/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .02164, df = 18.000								
	{1} (2.0964)	{2} (1.0112)	{3} (.62067)	{4} (.56909)	{5} (.51857)	{6} (.39514)	{7} (1.0317)	{8} (.53162)	{9} (.46335)
1		0.000075	0.000060	0.000036	0.000027	0.000022	0.000161	0.000031	0.000023
2	0.000075		0.004590	0.002373	0.001352	0.000210	0.866393	0.001482	0.000583
3	0.000060	0.004590		0.672836	0.446068	0.111363	0.004122	0.492737	0.253202
4	0.000036	0.002373	0.672836		0.696174	0.208052	0.001995	0.758857	0.430245
5	0.000027	0.001352	0.446068	0.696174		0.344026	0.001060	0.914771	0.651363
6	0.000022	0.000210	0.111363	0.208052	0.344026		0.000162	0.310922	0.577319
7	0.000161	0.866393	0.004122	0.001995	0.001060	0.000162		0.001187	0.000454
8	0.000031	0.001482	0.492737	0.758857	0.914771	0.310922	0.001187		0.598110
9	0.000023	0.000583	0.253202	0.430245	0.651363	0.577319	0.000454	0.598110	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A7. *The effect of the S. sclarea aqueous extract on the activity of the guaiacol peroxidase (U/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00169, df = 18.000								
	{1} (.40147)	{2} (.47785)	{3} (.34468)	{4} (.35696)	{5} (.22902)	{6} (.54691)	{7} (.51747)	{8} (.37484)	{9} (.50578)
1		0.035627	0.136981	0.225625	0.000177	0.000842	0.004638	0.438493	0.008104
2	0.035627		0.001810	0.003411	0.000032	0.073814	0.279081	0.008792	0.416899
3	0.136981	0.001810		0.718996	0.003070	0.000053	0.000204	0.407277	0.000364
4	0.225625	0.003411	0.718996		0.001806	0.000085	0.000377	0.601344	0.000685
5	0.000177	0.000032	0.003070	0.001806		0.000022	0.000023	0.000721	0.000028
6	0.000842	0.073814	0.000053	0.000085	0.000022		0.392736	0.000196	0.261713
7	0.004638	0.279081	0.000204	0.000377	0.000023	0.392736		0.001000	0.732088
8	0.438493	0.008792	0.407277	0.601344	0.000721	0.000196	0.001000		0.001811
9	0.008104	0.416899	0.000364	0.000685	0.000028	0.261713	0.732088	0.001811	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

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Table A8. *The effect of the S. sclarea aqueous extract on the activity of the guaiacol peroxidase (U/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .06693, df = 18.000								
	{1} (2.0964)	{2} (1.7118)	{3} (2.5328)	{4} (.56909)	{5} (.59976)	{6} (2.0422)	{7} (1.0317)	{8} (3.5923)	{9} (1.6836)
1		0.100717	0.053662	0.000029	0.000033	0.800615	0.000207	0.000076	0.088101
2	0.100717		0.001851	0.000113	0.000147	0.135309	0.006351	0.000036	0.895507
3	0.053662	0.001851		0.000023	0.000027	0.039904	0.000033	0.000240	0.001602
4	0.000029	0.000113	0.000023		0.886289	0.000034	0.051416	0.000022	0.000145
5	0.000033	0.000147	0.000027	0.886289		0.000038	0.055913	0.000023	0.000161
6	0.800615	0.135309	0.039904	0.000034	0.000038		0.000308	0.000060	0.124405
7	0.000207	0.006351	0.000033	0.051416	0.055913	0.000308		0.000027	0.006543
8	0.000076	0.000036	0.000240	0.000022	0.000023	0.000060	0.000027		0.000031
9	0.088101	0.895507	0.001602	0.000145	0.000161	0.124405	0.006543	0.000031	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A9. *The effect of the C. menthifolium aqueous extract on the activity of the pyrogallol peroxidase (U/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00186, df = 18.000								
	{1} (.38770)	{2} (.41418)	{3} (.29249)	{4} (.35137)	{5} (.43739)	{6} (.40171)	{7} (.34141)	{8} (.33818)	{9} (.43094)
1		0.486523	0.024042	0.315864	0.219410	0.695532	0.228930	0.212466	0.274280
2	0.486523		0.006272	0.117495	0.541449	0.727435	0.077614	0.069720	0.639831
3	0.024042	0.006272		0.141013	0.001849	0.011904	0.204510	0.210874	0.002532
4	0.315864	0.117495	0.141013		0.042101	0.192091	0.780603	0.727966	0.055206
5	0.219410	0.541449	0.001849	0.042101		0.364965	0.026290	0.023165	0.856957
6	0.695532	0.727435	0.011904	0.192091	0.364965		0.132082	0.120389	0.442851
7	0.228930	0.077614	0.204510	0.780603	0.026290	0.132082		0.928127	0.035046
8	0.212466	0.069720	0.210874	0.727966	0.023165	0.120389	0.928127		0.031161
9	0.274280	0.639831	0.002532	0.055206	0.856957	0.442851	0.035046	0.031161	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A10. *The effect of the C. menthifolium aqueous extract on the activity of the pyrogallol peroxidase (U/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .01249, df = 18.000								
	{1} (1.7518)	{2} (1.0703)	{3} (1.0281)	{4} (.53660)	{5} (.71705)	{6} (.50395)	{7} (.94740)	{8} (.49103)	{9} (.29886)
1		0.000162	0.000075	0.000031	0.000036	0.000027	0.000060	0.000023	0.000022
2	0.000162		0.649203	0.000063	0.001910	0.000046	0.218177	0.000039	0.000023

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3	0.000075	0.649203		0.000127	0.004236	0.000072	0.388464	0.000061	0.000028
4	0.000031	0.000063	0.000127		0.063602	0.724697	0.000438	0.642921	0.026336
5	0.000036	0.001910	0.004236	0.063602		0.038900	0.021330	0.033797	0.000500
6	0.000027	0.000046	0.000072	0.724697	0.038900		0.000269	0.889048	0.046060
7	0.000060	0.218177	0.388464	0.000438	0.021330	0.000269		0.000222	0.000033
8	0.000023	0.000039	0.000061	0.642921	0.033797	0.889048	0.000222		0.049609
9	0.000022	0.000023	0.000028	0.026336	0.000500	0.046060	0.000033	0.049609	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A11. *The effect of the S. sclarea aqueous extract on the activity of the pyrogallol peroxidase (U/mg protein) in leaves of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .00234, df = 18.000								
	{1} (.38770)	{2} (.33563)	{3} (.36302)	{4} (.35137)	{5} (.24814)	{6} (.32123)	{7} (.34141)	{8} (.33509)	{9} (.34087)
1		0.255383	0.539710	0.395622	0.005999	0.156661	0.295902	0.254290	0.299232
2	0.255383		0.540064	0.719503	0.055140	0.734634	0.892068	0.989366	0.895871
3	0.539710	0.540064		0.771357	0.019433	0.361510	0.611535	0.536521	0.613689
4	0.395622	0.719503	0.771357		0.032279	0.505083	0.803740	0.715576	0.804920
5	0.005999	0.055140	0.019433	0.032279		0.080667	0.048649	0.050154	0.046933
6	0.156661	0.734634	0.361510	0.505083	0.080667		0.651061	0.729571	0.654013
7	0.295902	0.892068	0.611535	0.803740	0.048649	0.651061		0.886979	0.989458
8	0.254290	0.989366	0.536521	0.715576	0.050154	0.729571	0.886979		0.891982
9	0.299232	0.895871	0.613689	0.804920	0.046933	0.654013	0.989458	0.891982	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120 h

Table A12. *The effect of the S. sclarea aqueous extract on the activity of the pyrogallol peroxidase (U/mg protein) in roots of the bromus seedlings compared to control group*

Cell No.	Duncan test; variable Var2 (Spreadsheet1) Approximate Probabilities for Post Hoc Tests Error: Between MS = .07228, df = 18.000								
	{1} (1.7518)	{2} (1.2701)	{3} (1.9958)	{4} (.53660)	{5} (.47918)	{6} (1.0592)	{7} (.94740)	{8} (2.0475)	{9} (.97299)
1		0.041693	0.281264	0.000097	0.000069	0.007276	0.003396	0.218168	0.003789
2	0.041693		0.005292	0.006664	0.004281	0.349384	0.193207	0.003843	0.215977
3	0.281264	0.005292		0.000033	0.000029	0.000837	0.000378	0.816350	0.000428
4	0.000097	0.006664	0.000033		0.796712	0.040651	0.077774	0.000029	0.074785
5	0.000069	0.004281	0.000029	0.796712		0.027111	0.057242	0.000025	0.051922
6	0.007276	0.349384	0.000837	0.040651	0.027111		0.636617	0.000590	0.699390
7	0.003396	0.193207	0.000378	0.077774	0.057242	0.636617		0.000261	0.908605
8	0.218168	0.003843	0.816350	0.000029	0.000025	0.000590	0.000261		0.000299
9	0.003789	0.215977	0.000428	0.074785	0.051922	0.699390	0.908605	0.000299	

*1-Control after 24 h, 2-Treatment with 0.1% after 24 h, 3-Treatment with 0.2% after 24 h, 4-Control after 72 h, 5-Treatment with 0.1% after 72 h, 6-Treatment with 0.2% after 72 h,7-Control after 120 h, 8-Treatment with 0.1% after 120 h, 9-Treatment with 0.2% after 120

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DOI: http://dx.doi.org/10.15666/aeer/1702_20152031

THE USE OF PREDATORY MITE *PHYTOSEIULUS PERSIMILIS* (ACARI: PHYTOSEIIDAE) IN THE CONTROL OF TWO-SPOTTED SPIDER MITE (*TETRANYCHUS URTICAE* KOCH, ACARI: TETRANYCHIDAE) AT GREENHOUSE CUCUMBER PRODUCTION IN TOKAT PROVINCE, TURKEY

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(Received 27th Oct 2018; accepted 11th Jan 2019)

Abstract. The effectiveness of the predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), as a suppressive agent of the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), was evaluated on greenhouse cucumber at predator:prey release ratios of 1:5, 1:15, and 1:30. Releases at each predator:prey ratio were made at 30 *T. urticae* densities per leaf. Evaluation was initiated 4 days after the release. On the under side of the leaves which were selected randomly from the lower, middle, and upper parts of the plants, the eggs and active forms of *T. urticae* and *P. persimilis* were counted using 10X hand magnifier for each treatment. In control treatments without predatory mite and acaricide application, population of *T. urticae* was constantly increased and reached 140 active forms/leaf in August. Subsequently the population decreased when the plants died. At ratios of 1:5, *P. persimilis* reached 8 active forms/leaf while *T. urticae* populations reached 11 active forms/leaf. At ratios of 1:15 *P. persimilis* population increased (3.4 active form per leaf) and *T. urticae* population decreased (1.6 active forms per leaf) in September and the latter one was kept by *P. persimilis* at low levels thereafter. Plant damage also was significantly reduced at these densities. *P. persimilis* population decreased when prey (*T. urticae*) population decreased. Our work demonstrates the potential of *P. persimilis* to provide effective control of *T. urticae* on a greenhouse-grown cucumber at a moderately low predator:prey ratio (1:15) in Tokat Province, Turkey.
Keywords: *predatory mites, biocontrol, cucumber pests, spider mites*

Introduction

Spider mites of the Tetranychidae are major pests of greenhouse cucumber and other agricultural crops worldwide. Foliar damage caused by spider mite feeding causes significant yield loss. To protect these plants from spider mite damage, farmers have traditionally relied on synthetic pesticides to suppress population outbreaks. However, due to resistance of spider mites to pesticides, negative effects of pesticides on natural enemies, and environmental concerns, other control tactics besides pesticides are needed.

An alternative to pesticides for suppression of spider mites is the inoculative release of predatory mites from the family Phytoseiidae. Predatory mites are important biological control agents of spider mites in many agricultural systems. Phytoseiids (*Typhlodromus* spp., *Amblyseius* spp., *Phytoseiulus* spp.) are effective at suppressing two-spotted spider mites in vegetables grown in greenhouse. Predatory mites have a shorter development

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20332041

periods and active feeding in each period, therefore they are very important biocontrol agents. In recent years, especially greenhouse vegetables and floriculture, as well as the control of the red spider species in the open field, primarily *Phytoseiulus persimilis* and other phytoseiid species have been used (Loginova et al., 1987; Jarasik, 1990; Jarasik and Pliva, 1990; Easterbrook, 1992; Zang and Sanderson, 1995; Boom et al., 2002; Lanzoni et al., 2017). Kim and Park (2006) reported that *Tetranychus urticae* was effectively controlled by the release of predatory mites at a rate of 3 predator/m^2. Limited studies were conducted in mass production of *P. persimilis* and it use in control of *Tetranychus cinnabarinus* on greenhouse growing strawberries (Kılınçer et al., 1992; Kazak et al., 1992; Öncüer et al., 1994; Kısmalı et al., 1999; Kazak et al., 2002; Çakmak et al., 2005; Çakmak et al., 2007) and cucumber (Kazak et al., 2000) in Turkey. In the study, conducted by Çakmak et al. (2005) reported that when the *T. cinnabarinus* population level reached 2-3 individuals per leaflet in strawberry plants, *P. persimilis* was released once at the rate of 1 predator to 20 prey, the predatory mites provided an effective control 15-20 days after release, and no additional release was needed during the rest of the growing season. At present, mass rearing of predator mite *P. persimilis* is not continued in Turkey. Commercial companies are exporting predator mites from abroad.

Aims of the present study is assess the effectiveness of the predatory mite *Phytoseiulus persimilis* at controlling two-spotted spider mite (*Tetranychus urticae*) when released into greenhouse growing cucumber plants in Tokat-Turkey.

Materials and methods

Rearing predatory mites

The stock cultures of *Phytoseiulus persimilis* were purchased from private company (Koppert Biological Systems, Netherland) and reared on the bean plants grown at 25°C and 60% ± 5% relative humidity at 16h:8h (L:D).

Screenhouse trial

The experiment was conducted in Plant Protection Department screenhouse at the Tokat Gaziosmanpasa University, Tokat. The screenhouse received natural light during the course of the treatment with no artificial light source or climate controls. Coco peat is used for growing cucumber plants (*Figure 1*). Necessary plant essentials were given by water dripplet system. The average temperature and relative humidity were 26±2°C and 60±5% during the course of the experiment in the screenhouse. Five treatments were evaluated in an randomized complete block design with three blocks and nine plants per replicates. A predator:prey ratio of 1:5, 1:15; 1:30 was calculated for each release. In addition to this, there is a control and acaricide treatment with classical acaricide (Spiromesifen 240 g/l (Oberon SC 240) 50 ml/da) application. Thirty *Tetranychus urticae* young female were placed on individual plants with the aid of a fine-tipped saber brush. Two days after *T. urticae* release, at 1:5, 1:15, and 1:30 ratios 6, 2, and 1 mated young *P. persimilis* female individuals were released (Kazakoğlu et al., 2000). Acaricide was applied at recommended concentration.

Evaluation was performed 4 days after the predatory mite release. Plants were evaluated every four days. In each plot lower, middle and upper parts of randomly selected plants 25 leaves were visually assessed and the number of *T. urticae* and *P.*

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DOI: http://dx.doi.org/10.15666/aeer/1702_20332041

persimilis motiles and eggs counted with hand-held 10X magnifier to quantify the effect of *P. persimilis* releases on *T. urticae* populations.

Figure 1. *A view from experiment unit*

Statistical analysis

The data were analyzed by the SPSS 17 statistical program (SPSS Inc., 2008) using general linear model to compare the effects of relase rates of predatory mites and time on controlling *Tetranychus urticae* at screenhouse conditions. ANOVA was used to compare means and Tukey's test was used to separate means.

Results

When *Phytoseiulus persimilis* was not released and without acaricide application, the population of *Tetranychus urticae* was constantly increased, and reached a peak of 141.11 motiles per leaf at week 5 and then decreased due to death of the infested cucumber plants ($F = 3.42$; $df = 4, 64$; $P < 0.05$) (*Figures 2, 3b*).

Two weeks after the predatory mite release, no *P. persimilis* motiles were recorded in the 1/5 predator:prey ratio treatment. However, at week 3 there were an average of 0.96 motiles per leaf ($F = 29.16$; $df = 4$; $P < 0.001$).

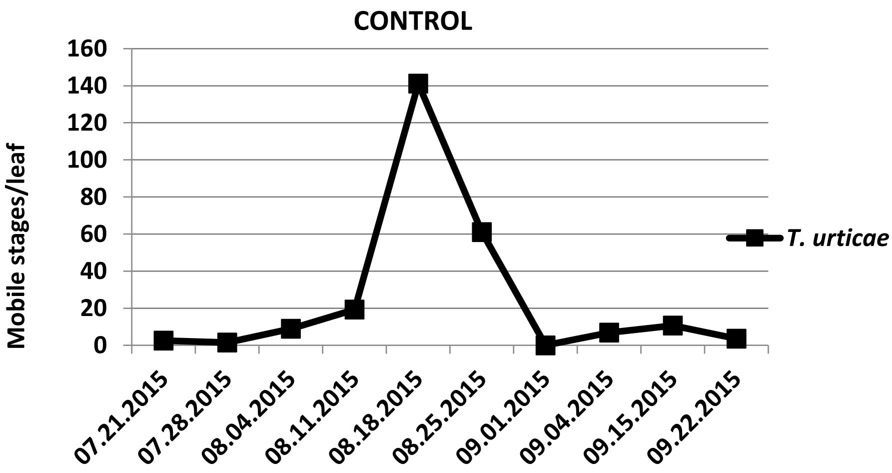

Figure 2. *Tetranychus urticae two-spotted spider mite density in untreated control*

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DOI: http://dx.doi.org/10.15666/aeer/1702_20332041

This increased to 8.07 motiles per leaf at week 4 (F = 10.33; df = 4; P < 0.001) and declined to 4 and 2 motiles per leaf at week 5 and 6 respectively and then remained at this level for the remaining 2 weeks (*Figure 4*). The parasitic mite *T. urticae* population was followed the same trend with the predator mite. It's population reached at peak of 10.66 motile per leaf at week 4 (F = 22.25; df = 4; P < 0.001) and declined to 3.33 and 2.04 motile per leaf at week 7 and 8, respectively (F = 9.92; df = 4; P < 0.001) (*Figure 4*).

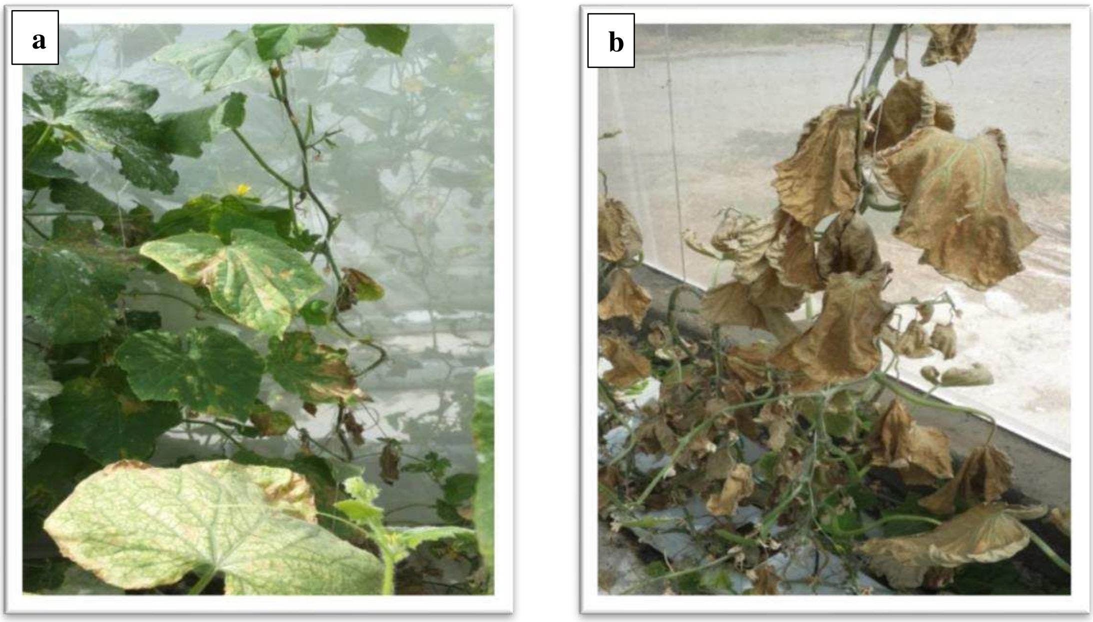

***Figure 3**. **a**. Cucumber plants in Phytoseiulus persimilis released plots **b.** Damages caused by Tetranychus urticae in untreated control plants*

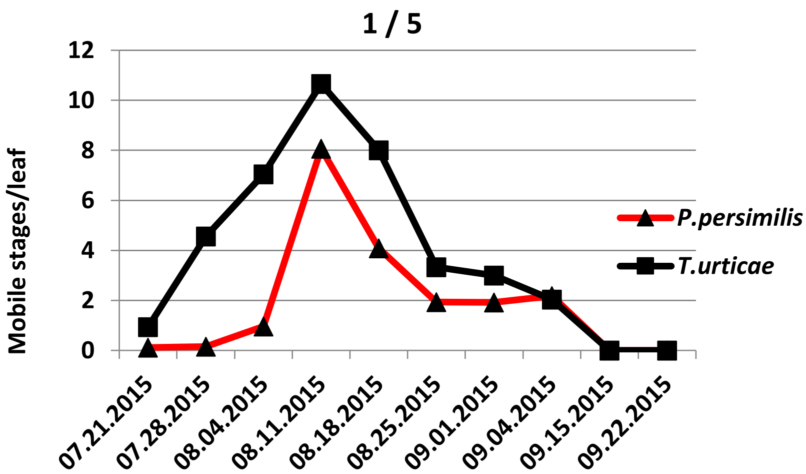

***Figure 4.** Tetranychus urticae two-spotted spider mite and Phytoseiulus persimilis density at 1/5 predator:prey ratio*

P. persimilis population density increased with increase in *T. urticae* population and decreased with decrease in prey density. The data demonstrated that releases of *P.*

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DOI: http://dx.doi.org/10.15666/aeer/1702_20332041

persimilis at a 1:15 predator:prey ratio maintained low *T. urticae* populations (*Figure 4*). Two weeks after the predatory mite release, no *P. persimilis* motiles were recorded in the 1/15 predator:prey ratio treatment. However, at week 3 there were an average of 1.0 motiles per leaf (F = 29.16; df = 4; P < 0.001). This increased to about 3.5 motiles per leaf at week 7 and declined to 3 and 0.5 motiles per leaf at week 8 and 9, respectively (F = 4.88; df = 4; P < 0.01) (*Figure 5*).

The parasitic mite *T. urticae* population was followed the parallel trend with the predator mite It's population reached at peak of 4.25 motile per leaf at week 2 (F = 22.11; df = 4; P < 0.001) and declined to 3.25 (F = 6.52; df = 4; P < 0.01), 1.75 (F = 22.11; df = 4; P < 0.001), 1.0 (F = 3.41; df = 4; P < 0.05) and 0.75 (F = 8.59; df = 4; P < 0.001) motile per leaf at week 3, 4, 5 and 6, respectively. *T. urticae* population reached average of 1.75 motile per leaf at week 7 and declined to 1.41 and 0.0 motile per leaf at week 8 (F = 9.91; df = 4; P < 0.002) and 9, respectively (*Figure 5*).

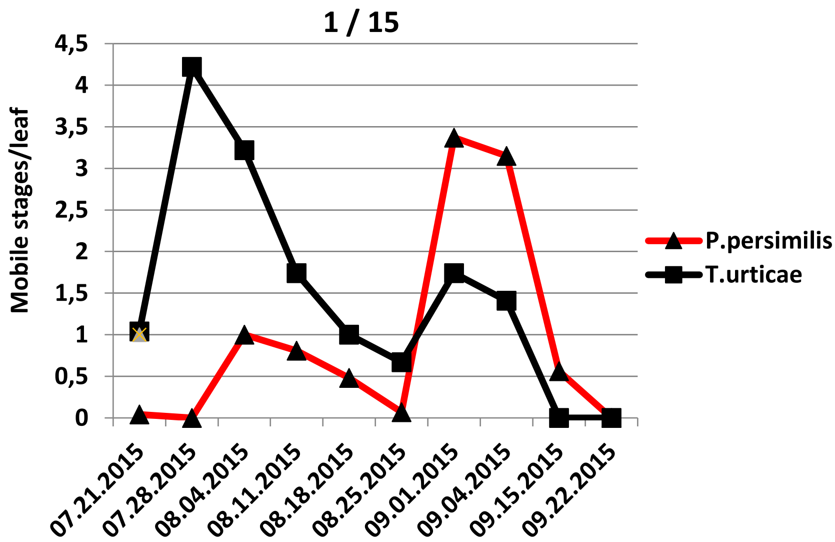

Figure 5. *Tetranychus urticae two-spotted spider mite and Phytoseiulus persimilis density at 1/15 predator:prey ratio*

At 1/30 predator:pery ratio treatment, despite the predator population reached peak of 22.0 motiles per leaf at week 4 (F = 10.30; df = 4; P < 0.001) it did not suppress the population of *T. urticae* which reached average of 40 motiles per leaf at week 4 (F = 22.25; df = 4; P < 0.001) (*Figure 6*). One week after predatory mite release, no *P. persimilis* motiles were recorded in the *P. persimilis* treatment. However, constant increase of *P. persimilis* observed after first week of release *P. persimilis* did not suppress *T. urticae* population effectively until week 5 (12.37 motiles/leaf) (F = 3.42; df = 4; P < 0.05). *Tetranychus urticae* population declined to average of 7.55 motile per leaf at week 6 (F = 8.59; df = 4; P < 0.003) then continued to decline constantly to 6.9 (F = 9.92; df = 4; P < 0.002), 2.2 (F = 6.28; df = 4; P < 0.01) and 0 motiles per leaf at week 7.8 and 9 respectively (*Figure 6*).

In the acaricide treatment, *T. urticae* population reached the peak of 2.8 motile per leaf at week 3 (F = 6.53; df = 4; P < 0.01) and declined to average of 0.8 motile per leaf at week 4 (F = 22.25; df = 4; P < 0.001) than increased up to average of 1.95 motile per leaf

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at week 5 (F = 3.42; df = 4; P < 0.05). Again the population of *T. urticae* started to decline at week 6 (F = 8.59; df = 4; P < 0.003) (*Figure 7*).

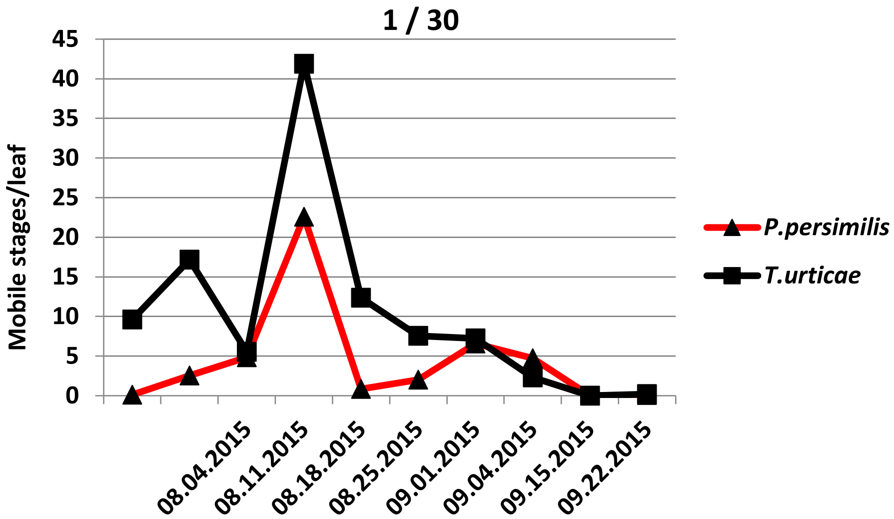

Figure 6. *Tetranychus urticae two-spotted spider mite and Phytoseiulus persimilis density at 1/30 predator:prey ratio*

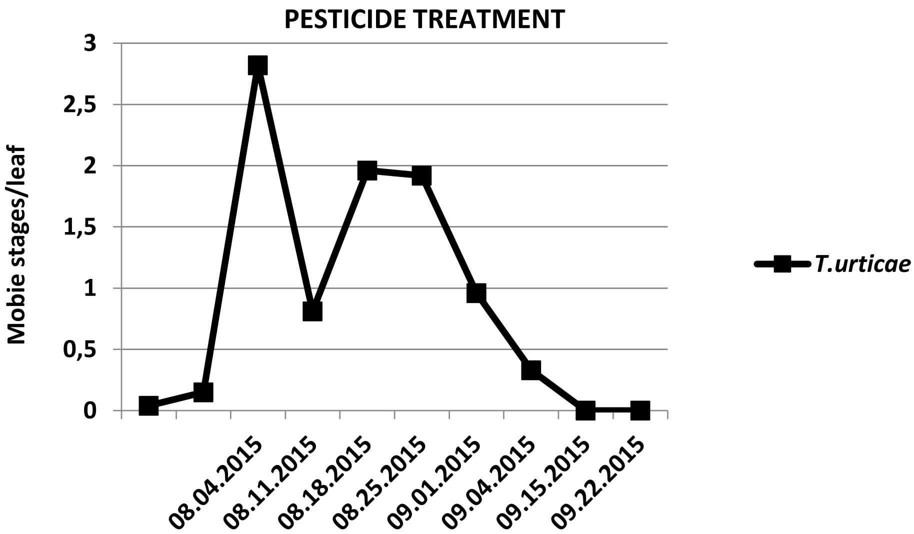

Figure 7. *Tetranychus urticae two-spotted spider density in acaricide treatment*

Discussion

The natural predator of two-spotted spider mites *Phytoseiulus persimilis* is the most widely used commercial predators for controlling mites. Having shorter development period from one generation to the next provides advantage to *P. persimilis* over the preys. However, the faster development potential of *P. persimilis* is an important factor in its success as a predator, it was reported that the initial ratio of pest to predator determines the outcome of a control program (Griffits, 2000; Lanzoni et al., 2017). Based on the

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results of the present study, population of *Tetranychus urticae* increased rapidly in control treatment after week 4. The rapid population growth of *T. urticae* in the control treatment was expected, given the high biotic potential of *T. urticae*. Indeed prevailing conditions in protected cultivation such as greenhouse often favor the development of *T. urticae* (Kim and Park, 2006). The present study indicated that the potential of the predator to reduce the population of *T. urticae* determined by the predator:prey ratios at the time of predator release. At the 1/5 and 1/30 predator:prey ratios, population of *T. urticae* increased, and reached the peak of 10.5 and 40.0 motiles per leaf respectively at week 4. At these two ratios tested *P. persimilis* population increased constantly up to 4th week of release and then began to suppress *T. urticae* population significantly ($p \leq 0.05$). On the other hand, population of *T. urticae* reached at peak of 4.25 motile per leaf at week 2 and declined to 3.25, 1.75, 1.0 and 0.75 motile per leaf at week 3, 4, 5 and 6, respectively. *Phytoseiulus persimilis* took to reach control over *T. urticae* was 14 days when the pest:predator ratio is 1/15. The finding of the present study demonstrated that predator:prey ratio was a determinative factor for the effective control of *T. urticae*. This finding is supported by another greenhouse study showed that Predator mite *Anthoseius recki* (Wainstein) (Mesostigmata: Phytoseiidae) suppressed the population of *Tetranychus cinnabarinus* at 1/30 and 1/15 predator:prey ratios effectively. However, at 1/60 predator:prey ratio the predator failed to provide effective control of the prey on strawberry plants (Şekeroğlu, 1984). Scopes (1985) reported that *P. persimilis* took to reach control over *T. urticae* was 16 days when the pest:predator ratio was 1:200, 13 days when it was 1:100, and 11 days when it is 1:50. This clearly demonstrates that early application is more efficient. *P. persimilis* gave effective control of *T. urticae* when it was released onto strawberry with low levels of *T. urticae* infestation in southeast Queensland, Australia (Waite, 1998). Bonomo et al. (1991) also reported that releases of *P. persimilis* gave effective control of *T. urticae* at lower density of it. Similarly Kim (2001) evaluated the effectiveness of *P. persimilis* against *T. urticae* on strawberry in five commercial greenhouses and got an excellent result by releasing the predator at a rate of 3/m^2 in Korea. Spicciarelli et al. (1992) and Boom et al. (2002) recommended that phytoseiid mites give good control of *T. urticae*, if one mite is released per plant when the infestation of *T. urticae* has reached two individuals per leaf, and about 30% of the leaves are infested.

Conclusion

This study was conducted to see controlling two-spotted spider mites using *P. persimilis* at cucumber plants in screenhouse conditions in Tokat. Our results indicate that when released at 1:15 predator:prey and when *T. urticae* population are low per leaf, *P. persimilis* is able to maintain populations of *T. urticae* below damaging levels throughout a growing season (*Figure 3a*). It would appear that *P. persimilis* can give good control of two-spotted spider mite on cucumber plants, particularly if the predator is introduced when numbers of *T. urticae* are fairly low. What we do for growers, we arrange field days in the light of experiences and the results are shared with our producers and the stakeholders.

Acknowledgement. This work was funded by Tokat Provincial Administration.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20332041

WATER USE EFFICIENCY OF DIFFERENT SUNFLOWER GENOTYPES UNDER DEFICIT IRRIGATION IN A SEMI-ARID REGION

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(Received 30th Oct 2018; accepted 11th Jan 2019)

Abstract. The factorial experiment was set up in a randomized complete block design as a split-plot arrangement with three replications. Irrigation as main-plot consisted of four levels: stopping of irrigation after 60, 75, and 90 days from planting, and full irrigation. The sub-plot encompassed three sunflower genotypes (Barolo RO, Velko, and Local) used under a semiarid condition in Sulaimani, Kurdistan Region of Iraq. Velko genotype under full irrigation produced the highest seed yield of 5716.685, and 5190.545 kg ha^{-1} at Kanipanka and Qlyasan locations respectively. Overall, 60 days of irrigation, and full irrigation showed the highest and lowest WUE or IWUE, respectively. All the genotypes offered a crop response factor of less than one at both locations indicating that the grown crop is more tolerant to water deficit, and recovers partially from stress. The result of this study concluded that the Barolo RO genotype has the highest performance under deficit irrigation. Therefore, this genotype is the most proper one for the study area as a part of drought-prone environments. Furthermore, the results obtained from this study indicated that the early stage of growth was more effective to increase the seed yield of sunflowers than irrigation in the middle and later stages.
Keywords: *sunflower water productivity, seed yield, deficit irrigation, and yield response factor*

Introduction

An estimated 70% of the world fresh water is used for agricultural productions, and this percentage could reach above 90% in some developing countries (WWP, 2017). The application of efficient water management strategies is a key element to increase water productivity (Mancosu et al., 2015). Further, water availability is one of the primary factors in sunflower seed production by irrigation depths (Gomes et al., 2012). The applied irrigation depths must be adequately quantified; otherwise, they may negatively affect the crop through water deficit or excess. Water deficit compromises photosynthesis, stomatal conductance, and transpiration, reducing plant biomass (Duarte et al., 2012). Water is decreasing day by day due to unavailability of natural rainfall, excessive withdrawal of groundwater, population growth, and increased use of irrigation water (Vörösmarty et al., 2000; Rosegrant et al., 2002; Rockström et al., 2009; Sarkar and Ali, 2009; Asraf et al., 2015). To deal with this situation, the lowest water usage with the highest yield has to be utilized in order to achieve maximum water productivity instead of highest yield, this method called, deficit irrigation and efficient use of water. Also, this technique can save irrigation cost with a negligible yield reduction consequently net farm income increase (Ali et al., 2007). This method shows a quantitative relationship between relative evaporation deficit and relative yield

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DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

decrease. Therefore, to accept this theory, higher crop production and water productivity can be achieved with better water management planning (Ali, 2009). The sunflower plant is categorized as one of the low to medium drought-sensitive crop. Many researchers found that both quantity and distribution of water have a significant impact on achene and oil yield in sunflower (Fereres et al., 1986; Andrich et al., 1996; Krizmanic et al., 2003; Reddy et al., 2003; Iqbal et al., 2005). Deficit irrigation causes a yield reduction to some extent which depends on both the severity and timing of the water deficits (Orgaz et al., 1992). In water deficit condition (when it occurs during the life cycle) plant can achieve maximum water productivity (Ali, 2009). Ayas and Korukcu (2010) reported the k_y factor of 0.909 in potato during growth period in Yenisehir, Bursa. Demir et al. (2006) estimated yield response factor k_y of sunflower to deficit irrigation in a sub-humid climate (Bursa, Turkey). They found k_y of 0.8382 for the total growth period of sunflower. However, by using furrow irrigation method (Karaata, 1991) found a k_y value of 0.91 for the whole growing season and 0.83 for the vegetative + yielding stage. Hence, it is understandable that response factor differs from location to location depending on weather conditions, soil types, variety, crop, season and also for individual growth stage to the total growing season (Ali, 2009). Therefore, it is utmost essential, to consider location-specific response factor for efficient management of water. A hybrid yield was conditioned by its capacity to use the environmental variables efficiently in different phenophases (Gonzáles et al., 2013). Thus, the genetic potential of the sunflower hybrid was reduced by the action of the growth factors, either environmental or technological ones. Agronomic practices in addition to high yielding varieties are the two most important items for higher productivity of the sunflower crop (Beg et al., 2007). Productivity per unit area of sunflower was determined by many factors, including plant population and variety (Ibrahim, 2012). The objective of this study was to determine the effect of different deficit irrigation levels on the yield, irrigation water use efficiency (IWUE), water use efficiency (WUE), and yield response factor k_y of some sunflower genotypes (*Helianthus annuus L.*) in the semi-arid conditions of Sulaimani region.

Material and methods

Study area and soil sampling

This experiment was conducted during the summer season of 2016 between July and October at two locations surrounding Sulaimani city. The first location was the Kanipanka Agricultural Research Station (latitude: 35° 22’ 22” N, Longitude: 045° 43’ 22” E, altitude: 548 masL) in Sharazoor valley. While the second location was Qlyasan, the farm of Crop Science Department, College of Agricultural Sciences, the University of Sulaimani, located at (latitude: 35° 34’ 17” N, Longitude: 045° 22’ 00” E, altitude: 757 masL) “Garmin, GPSmap60 Cx.” (*Fig. 1*). The total available water at these two locations is 151 and 147 mm m^{-1}.

At each location of the experiment, a composite soil sample of about 5 kg was obtained by mixing subsamples from 6 sites using a shovel. Each composite soil sample was freed from plant roots and other debris. Collected soil samples were placed in the open to be air dried at room temperature for about a week, then gently crushed and sieved at 2 mm with a stainless-steel sieve to avoid any contamination and then stored for subsequent analyses.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

Each experiment was set up in a randomized complete block design (RCBD) as a split-plot factorial arrangement with three replications with the deficit and full irrigation treatments as the main plot. Four levels were used: I_1 irrigation treatment (stopping of irrigation after 60 days from planting), I_2 irrigation treatment (stopping of irrigation after 75 days from planting), I_3 irrigation treatment (stopping of irrigation after 90 days from planting), and I_4 nonstop irrigation treatment (full irrigation), while the sub-plot factors encompassed three sunflower genotypes (Barolo RO, Velko, and Local). These genotypes were selected because of different responses to water stress.

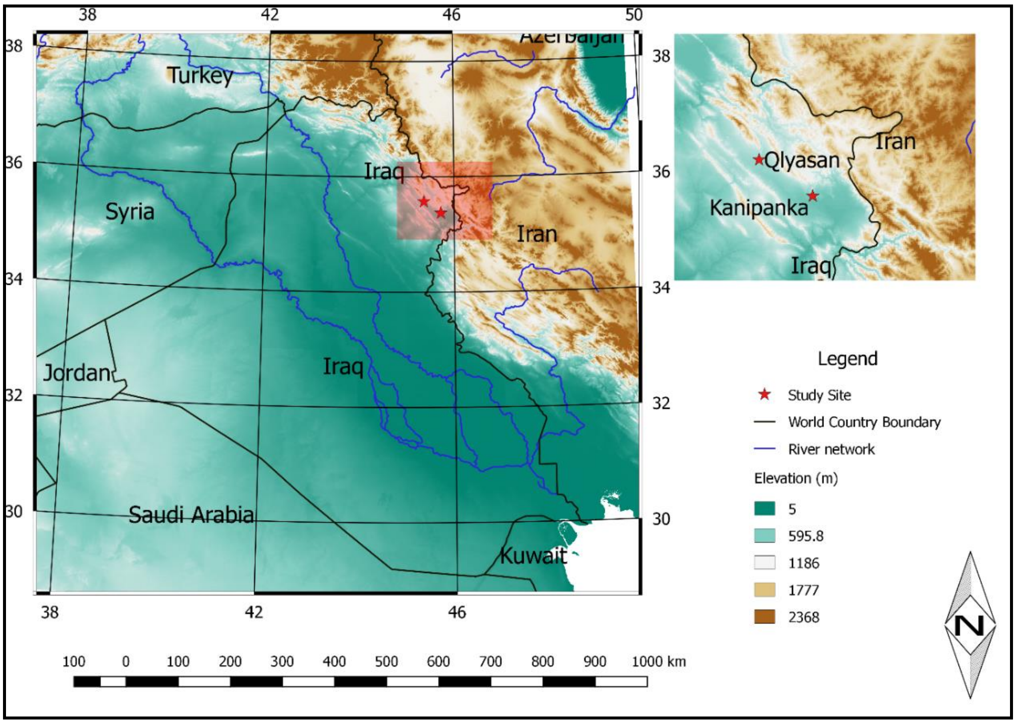

Figure 1. *Map of the study site in Sulaimani-Kurdistan Region, Iraq*

The sub-plots were 3 m by 1.8 m in size, and each consisted of three rows, spaced at 0.60 m with a plant distance of 0.30 m. The seeds were planted during July 11th, and July 15th at Kanipanka and Qlyasan location respectively. Three seeds hole^{-1} were placed at a depth of 2-4 cm. Two weeks after planting, the seedlings were thinned out to one plant hole^{-1}. Urea, as a source of nitrogen fertilizer, was applied to all plots in two equal split doses before the second irrigation and prior to flowering at a rate of 43 kg ha^{-1} as recommended. Hand weeding practiced as needed.

Climate conditions of Sulaimani Governorate

The climate of Sulaimani governorate is semi-arid environment: hot and dry in summer; cold and wet in winter. During July and August, the average temperature is between 39-43 °C, and often reaching nearly 50 °C. Autumn means high temperatures are 24-29 °C in October, cooling slightly in November. Precipitation is limited to winter and spring months, and the overall average annual rainfall of 666.8 mm was at Sulaimani city in 2016 (Kurdistan Regional Government, 2018). An overview of experimental conditions is given in *Table 1*.

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Table 1. *Some agrometeorological parameters at Kanipanka and Qlyasan locations*

Locations	Month	Air temperature (°C)		Average humidity (%)	Average wind speed (m s^{-1})	Precipitation (mm)
		Minimum	Maximum			
Kanipanka	July	27.05	44.33	20.30	1.65	0
	August	29.34	45.88	19.56	1.5	0
	September	21.42	39.55	22.68	1.61	0
	October	15.66	34.69	28.56	1.38	0
Qlyasan	July	25.84	43.46	22.9	1.77	0
	August	27.49	45.36	20.35	1.6	0
	September	19.95	38.26	26.17	1.65	0
	October	14.95	32.01	29.42	1.44	0

Watering schedule and restrictions

Irrigation timing method was based on an allowable root zone available water depletion (45%) during full and no deficit irrigation (Allen et al., 1998). The amount of irrigation water was measured with water flow meter devices (SOTERA digital display meter) (*Fig. 2*). The soil water content was monitored gravimetrically (Lorenz and Maynard, 1980), using a small auger 5 cm in diameter. The net water requirement (crop consumptive use) was calculated from soil moisture. When the available soil moisture was depleted by 45%, the soil moisture was brought to filed capacity. The sum of the crop consumptive use during the growing season was comparable well with that computed by Penman-Monteith equation

At each location, the matured plants from the central rows in each plot were harvested manually and then yield and yield components for each treatment at each replicate were determined. The yield data was taken at about 10% seed moisture level. The harvesting dates were on October 19th, 2016 for the deficit irrigation treatments, and October 24th for the full irrigation treatment at Kanipanka location, while October 25th were the harvesting dates for all the treatments at Qlyasan location respectively.

Figure 2. *SOTERA digital display meter*

The below-stated equation was used to estimate water use efficiency (WUE, *Eq. 1*), and irrigation water use efficiency (IWUE, *Eq. 2*), as the ratio of crop yield per unit of water applied (Kang et al., 2000):

$$WUE = \frac{Y}{ETc} \qquad \text{(Eq.1)}$$

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$$IWUE = \frac{Y}{I} \quad \text{(Eq.2)}$$

where:
Y = The total sunflower seed yield (kg ha^{-1})
ETc = The seasonal evapotranspiration (m^3 ha^{-1})
I = The total volume of applied irrigation water (m^3 ha^{-1})

While the irrigation application efficiencies for the locations under study based on average land slop and basic infiltration rate was calculated according to Karim and Karim (2001) and the results are revealed in *Table 2*.

Table 2. *Irrigation application efficiencies for the locations under study based on average land slope and basic infiltration rate*

Locations	Average land slope (%)	Basic infiltration rate, Ib (mmhr^{-1})	Irrigation application efficiency, Ea
Kanipanka	Nearly level	44	0.70
Qlyasan	1.16	80	0.65

The crop response factor *Ky* is the relationship between relative yield decrease and relative evapotranspiration deficit which was determined by the procedure given by Doorenbos and Kassam (1979) (*Eq. 3*).

$$\left(1 - \frac{Ya}{Ym}\right) = Ky\left(1 - \frac{ETa}{ETmax}\right) \quad \text{(Eq.3)}$$

where:
Ya = Actual crop yield (kg ha^{-1})
Ym = Maximum crop yield (kg ha^{-1})
Eta = Actual evapotranspiration (mm)
ETm = Maximum evapotranspiration (mm)
Ky = Yield response factor (dimensionless)

The k_y factor for the entire season determined by linear regression, adjusted through the origin, between the reduction in relative yield and deficit of relative evapotranspiration.

The uniformity coefficient (UC, *Eq. 4*) for some selected parameters was determined according to Devitt et al. (1992):

$$UC = 1 - (standard\ deviation/mean) \quad \text{(Eq.4)}$$

Analytical methods and laboratory analysis

The results of the studied soil parameters are shown in *Table 3*. Particle size fractionation and distribution were conducted by international pipette method as recommended by Black et al. (1965). EC and acidity (pH) of soil sample were measured in 1:10, soil to H_2O ratio suspension according to Thomas (1996) by using these models of instruments; pH-meter (model WTW 330i/ Germany); EC-meter (model WTW 330i/Germany). The percent of organic carbon (o.m%) in soil samples were determined by the Walkley-Black method (wet oxidation by potassium dichromate $K_2Cr_2O_7$ and concentrated H_2SO_4) as described by Black et al. (1965). Then the content of organic

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DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

matter (OM) was calculated as follows: % Organic matter = % organic carbon × 1.724 (factor). The percent of the total ($CaCO_3$%) was determined by the acid-neutralization method according to the method 23c of U.S. Salinity Laboratory Staff, 1954 (Black et al., 1965).

Table 3. *Some physicochemical properties of the soil samples for locations of the experiment*

Physicochemical properties		Locations	
		Kanipanka	**Qlyasan**
Particles size distribution (kg^{-1})	Sand	36	87
	Silt	529	435
	Clay	435	458
	Texture	SiC	SiC
PH		7.70	7.59
ECe (micro siemens cm^{-1}) or ($\mu S\ cm^{-1}$)		218	490
O.M. ($g\ kg^{-1}$)		14.8	22.4
CaCO3 ($g\ kg^{-1}$)		208.3	304.3

Statistical analysis

Statistical analysis for all measured variable was performed using the XLSTAT software (XLSTAT, 2017). For direct comparison of treatments, least significant difference tests (LSD) at levels of 0.05 and 0.01 levels were used. For testing the main effects of deficit irrigation on sunflower genotypes in a semi-arid region, the data were subjected to analysis of variance (ANOVA).

Results

Table 4 shows the seed yields, for irrigation water use efficiency (IWUE) and water use efficiency (WUE) for all three genotypes of sunflower at Kanipanka and Qlyasan locations under different deficit irrigation treatments during the summer season of 2016.

As seen in *Table 4,* highly significant differences among genotypes were recorded (see *Appendix*). The highest value of seed yield recorded with the Velko genotype under I_4 (full irrigation) at first location was 5716.685 kg ha^{-1} which predominated all combinations significantly while the lowest value was 2915.600 kg ha^{-1} recorded with the Local genotype under I_1 (stopping of irrigation after 60 days) at the same locations. The irrigation water use efficiency varied from as low as 4.511 kg ha^{-1} mm^{-1} for the Local genotype under I_3 (stopping of irrigation after 90 days) at the second location to as high as 9.382 kg ha^{-1} mm^{-1} for the Velko genotype under I_1 (stopping of irrigation after 60 days) at the same location, in which exceeded other combinations significantly. On the other hand, it was reported that the water use efficiency varied from a minimum of 6.754 kg ha^{-1} mm^{-1} for the Barolo RO genotype under I_4 (full irrigation) at the first location to a maximum of 14.437 kg ha^{-1} mm^{-1} for the Velko genotype under I_1 (stopping of irrigation after 60 days) at the second location.

Based on the average values of IWUE, and WUE for the two locations, the order of performance of the genotypes is as follows: Velko > Barolo RO > Local. Our study showed that the Velko and Local genotypes offered the highest and lowest performance respectively.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

Table 4. *Seed yield, irrigation water use efficiency, and water use efficiency of three sunflower genotypes as influenced by different irrigation treatments at two locations within Sulaimani City*

Sunflower genotypes and irrigation treatments		**Total x (mm)**	**Total x (m^3 ha^{-1})**	**Total x ET_a**	**Seed yield (kg ha^{-1})**	**Irrigation water use efficiency (kg ha^{-1} mm^{-1})**	**Water use efficiency (kg ha^{-1} mm^{-1})**
Kanipanka location							
Barolo RO	I_1 stopping of irrigation after 60 days	455.37	4553.7	3187.59	2977.020 e	6.538 cd	9.339 cd
	I_2 stopping of irrigation after 75 days	632.77	6327.77	4429.439	3269.690 de	5.167 e	7.382 e
	I_3 Stopping of irrigation after 90 days	713.7	7137.03	4995.921	3541.455 d	4.962 e	7.089 e
	I_4 Full irrigation	761.3	7612.96	5329.072	3599.175 d	4.728 e	6.754 e
Velko	I_1 Stopping of Irrigation after 60 Days	455.37	4553.7	3187.59	4168.790 c	9.155 a	13.078 a
	I_2 Stopping of irrigation after 75 days	632.77	6327.77	4429.439	4178.780 c	6.604 cd	9.434 cd
	I_3 Stopping of irrigation after 90 days	713.7	7137.03	4995.921	5149.475 b	7.215 bc	10.307 bc
	I_4 Full irrigation	761.3	7612.96	5329.072	5716.685 a	7.509 b	10.727 b
Local	I_1 Stopping of irrigation after 60 days	455.37	4553.7	3187.59	2915.600 e	6.403 d	9.147 d
	I_2 Stopping of irrigation after 75 days	632.77	6327.77	4429.439	3389.94 d	5.357 e	7.653 e
	I_3 Stopping of irrigation after 90 days	713.7	7137.03	4995.921	3491.690 d	4.892 e	6.989 e
	I_4 Full irrigation	761.3	7612.96	5329.072	3670.215 d	4.821 e	6.887 e
$LSD_{.05}$					404.034	0.689	0.984
Qlyasan location							
Barolo RO	I_1 Stopping of irrigation after 60 days	452.04	4520.37	2938.241	3644.685 d	8.063 b	12.404 b
	I_2 Stopping of irrigation after 75 days	583.33	5833.33	3791.665	3836.160 d	6.576 e	10.117 e
	I_3 Stopping of irrigation after 90 days	706.3	7062.96	4590.924	4116.620 c	5.828 f	8.967 f
	I_4 Full irrigation	754.26	7542.59	4902.684	4192.285 c	5.558 f	8.551 f

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

Velko	I_1 Stopping of irrigation after 60 days	452.04	4520.37	2938.241	4242.05 c	9.382 a	14.437 a
	I_2 Stopping of irrigation after 75 days	583.33	5833.33	3791.665	4817.030 b	8.258 b	12.704 b
	I_3 Stopping of irrigation after 90 days	706.3	7062.96	4590.924	4910.825 b	6.953 cd	10.697 cd
	I_4 Full Irrigation	754.26	7542.59	4902.684	5190.545 a	6.882 de	10.587 de
Local	I_1 Stopping of irrigation after 60 days	452.04	4520.37	2938.241	3283.01 e	7.263 c	11.173 c
	I_2 Stopping of irrigation after 75 days	583.33	5833.33	3791.665	3214.19 e	5.510 f	8.477 f
	I_3 Stopping of irrigation after 90 days	706.3	7062.96	4590.924	3186.440 e	4.511 h	6.941 h
	I_4 Full irrigation	754.26	7542.59	4902.684	3738.295 d	4.956 g	7.625 g
$LSD_{.05}$					253.587	0.375	0.577

As expected the percent yield reduction decreased progressively with an increase in the amount of applied water. Further, the Velko genotype offered the maximum percent of the reduction in yield (27.08%) under I_1 (stopping of irrigation after 60 days) at the first location. *Table 5* revealed that water stress imposed at the later stage of growth influence yield the least, and offered water saving of about 40% compared with the full irrigation treatment. The percent of the reduction under limited irrigation was less than 20% in most cases (*Table 5*).

Table 5. *Percent of yield reduction and water saving under limited irrigation*

Sunflower genotypes and irrigation treatments		**Total applied water (mm)**	**Seed yield (Kg ha^{-1})**	**Yield reduction (%)**	**Water saving (%)**
Kanipanka location					
Barolo RO	I_1 Stopping of irrigation after 60 days	455.370	2977.020 e	17.29	40.19
	I_2 Stopping of irrigation after 75 days	632.770	3269.690 de	9.15	16.88
	I_3 Stopping of irrigation after 90 days	713.700	3541.455 d	1.60	6.25
	I_4 Full irrigation	761.300	3599.175 d	0.00	0.00
Velko	I_1 Stopping of irrigation after 60 days	455.370	4168.790 c	27.08	40.19
	I_2 Stopping of irrigation after 75 days	632.770	4178.780 c	26.90	16.88
	I_3 Stopping of irrigation after 90 days	713.700	5149.475 b	9.92	6.25
	I_4 Full irrigation	761.300	5716.685 a	0.00	0.00

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DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

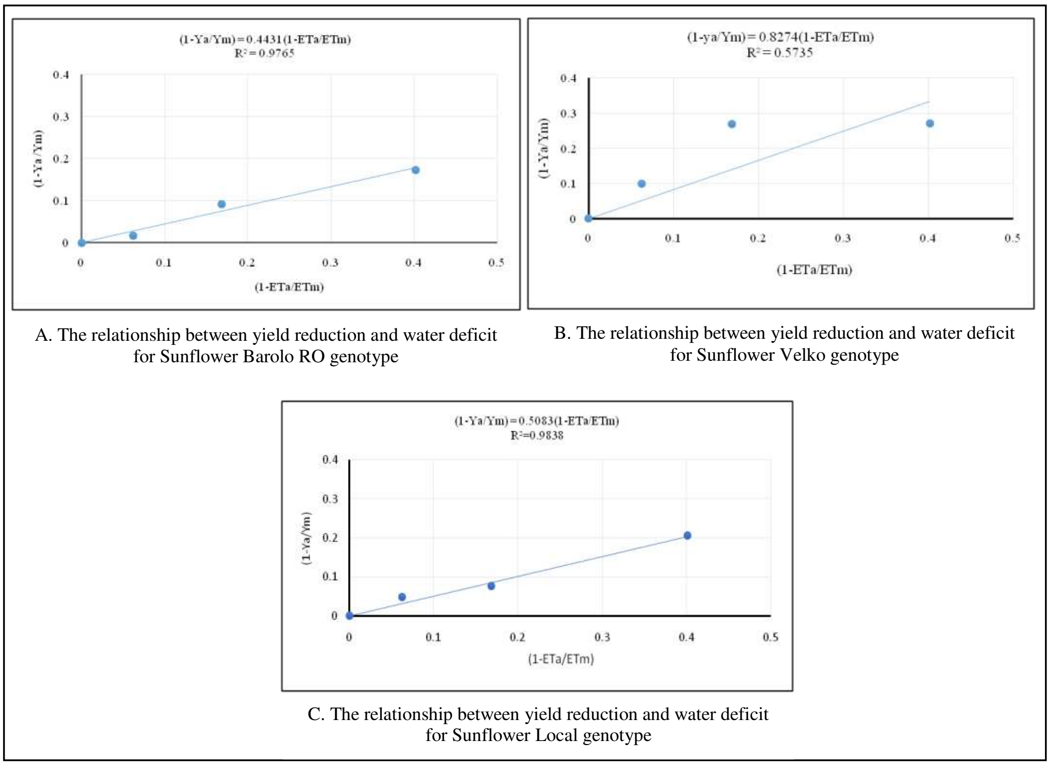
Local	I_1 Stopping of irrigation after 60 days	455.370	2915.600 e	20.56	40.19
	I_2 Stopping of irrigation after 75 days	632.770	3389.940 d	7.64	16.88
	I_3 Stopping of irrigation after 90 days	713.700	3491.690 d	4.86	6.25
	I_4 Full irrigation	761.300	3670.215 d	0.00	0.00
			$LSD_{.05}$ 404.034	SE = 2.943	
Qlyasan location					
Barolo RO	I_1 Stopping of irrigation after 60 days	452.040	3644.685 d	13.06	40.07
	I_2 Stopping of irrigation after 75 days	583.330	3836.160 d	8.50	22.66
	I_3 Stopping of irrigation after 90 days	706.300	4116.620 c	1.81	6.36
	I_4 Full irrigation	754.260	4192.285 c	0.00	0.00
Velko	I_1 Stopping of irrigation after 60 days	452.040	4242.050 c	18.27	40.07
	I_2 Stopping of irrigation after 75 days	583.330	4817.030 b	7.20	22.66
	I_3 Stopping of irrigation after 90 days	706.300	4910.825 b	5.39	6.36
	I_4 Full Irrigation	754.260	5190.545 a	0.00	0.00
Local	I_1 Stopping of irrigation after 60 days	452.040	3283.010 e	10.55	40.07
	I_2 Stopping of irrigation after 75 days	583.330	3214.190 e	12.43	22.66
	I_3 Stopping of irrigation after 90 days	706.300	3186.440 e	13.18	6.36
	I_4 Full irrigation	754.260	3738.295 d	0.00	0.00
			$LSD_{.05}$ 253.587	SE = 1.783	

As shown in *Table 6* and *Figures 3* and *4,* the crop yield response factor was estimated for sunflower at the study locations were calculated according to Doorenbos and Kassam (1979). The k_y values ranged from the minimum of 0.337 for the Barolo RO genotype at the second location to a maximum of 0.827 for the Velko genotype at the first location.

Table 6. *Crop response factor for different sunflower genotypes to limited irrigation*

Locations	**Genotypes**	**Seed yield (kg ha^{-1})**	**ETa (m^3 ha^{-1})**	**Ky**
Kanipanka	Barolo RO	3346.835 b	4485.506	0.443
	Velko	4803.433 a	4485.506	0.827
	Local	3366.861 b	4485.506	0.508
		$LSD_{.05}$ 202.017		$SE = 0.119$
Qlyasan	Barolo RO	3947.438 b	4055.878	0.337
	Velko	4790.113 a	4055.878	0.430
	Local	3355.484 c	4055.878	0.417
		$LSD_{.05}$ 126.794		$SE = 0.029$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

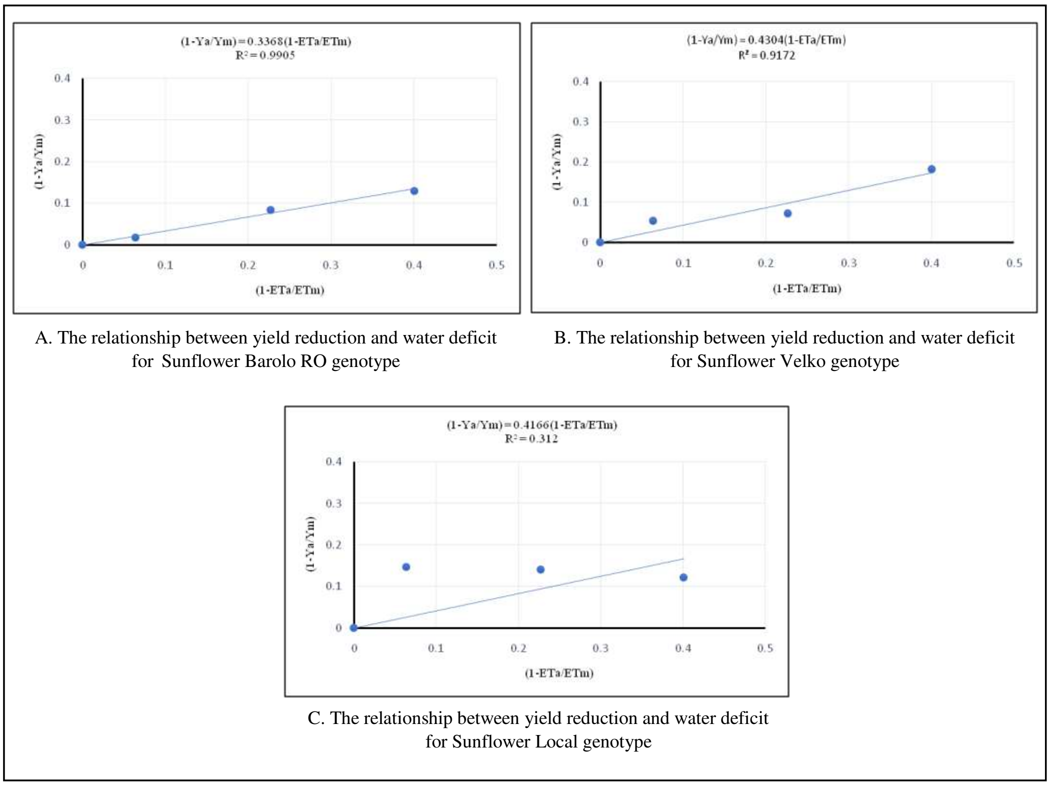


A. The relationship between yield reduction and water deficit for Sunflower Barolo RO genotype

B. The relationship between yield reduction and water deficit for Sunflower Velko genotype

C. The relationship between yield reduction and water deficit for Sunflower Local genotype

***Figure 3.** The relationship between yield reduction and water deficit for sunflower genotypes at Kanipanka location*



A. The relationship between yield reduction and water deficit for Sunflower Barolo RO genotype

B. The relationship between yield reduction and water deficit for Sunflower Velko genotype

C. The relationship between yield reduction and water deficit for Sunflower Local genotype

***Figure 4.** The relationship between yield reduction and water deficit for sunflower genotypes at Qlyasan location*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The result displayed in *Table 6* revealed that among the genotypes, Barolo RO exhibited the least value of k_y at both locations. The lower result indicates that this genotype is the most proper one for the study area as a part of drought-prone environments. Further examination of *Table 6,* revealed that the k_y values for both locations exhibited similar trends. The Barolo RO and Velko offered the minimum and maximum values for k_y respectively at both locations.

Based on the k_y values listed in *Table 7,* deficit irrigation may be needed at different stages for water limiting areas. Also, it was noticed that the uniformity coefficient values were below 0.86. The Velko genotype showed a uniformity coefficient of 0.55. Therefore, statistical differences between genotype and locations can exist.

Table 7. *Yield response factor (Ky) of sunflower genotypes under different irrigation treatments*

Genotypes	ETa (m^3 ha^{-1})		Ky value		Mean	Standard deviation (SD)	Uniformity coefficient (UC)	Coefficient of variance (CV) %
	Kanipanka location	Qlyasan location	Kanipanka location	Qlyasan location				
Barolo RO	4485.506	4055.878	0.443	0.337	0.390	0.0752	0.807	19.28
Velko	4485.506	4055.878	0.827	0.430	0.629	0.281	0.554	44.64
Local	4485.506	4055.878	0.508	0.417	0.462	0.065	0.860	14.02

Discussion

The results of the study indicated that the highest seed yield was obtained from the control treatment (full irrigation) and drought stress statistically decreased seed yield compared to no stress. These results collaborate (Tabatabaei et al., 2012; Dehkhoda et al., 2013; and Hussain et al., 2013). Unger (1982) found that limited irrigation water resulted in higher water use efficiency than full irrigation.

The higher IWUE and WUE values were due to limited irrigation treatments. Therefore, the increase of seed yields depends on genotypes and irrigation interval. Further, it can be reported, that with one exception, there is a steady decrease in both IWUE and WUE with an increase in the amount of applied water or plant evaporation. These changes may be due to a minimum water use efficiency of 6.754 kg ha^{-1} mm^{-1} under I_4 (full irrigation) for Barolo RO genotype, and maximum water use efficiency 14.437 kg ha^{-1} mm^{-1} under I_1 (stopping of irrigation after 60 days) for Velko genotype. However, Langeroodi et al. (2014) found that the highest water use efficiency achieved under several limited irrigation treatments for sunflower in the Islamic Republic of Iran was 7.1 kg ha^{-1} mm^{-1}. Also, Demir et al. (2006) found that the highest value was 10.19 kg ha^{-1} mm^{-1} under (full irrigation) at Bursa, Turkey. These findings support the results of Mahender et al. (2000) and Kakar and Soomro (2001) who pointed out that the increase in seed yield of sunflower depended on genotypes and irrigation intervals. Therefore, further studies are needed to investigate the important of water use efficiency on sunflower seed yields.

In regard to the genotypes, at the second and third stages, i.e., stopping of irrigation after 75 and 90 days was the critical stages for deficit irrigation. It was proved that stopping of irrigation after these periods can minimize crop yield to a great extent. Stopping of irrigation after 60 days should be preferred due to higher IWUE and WUE

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

if water resources are limited and irrigation water cost is high. Sunflower genotypes showed different responses to irrigation treatments under the conditions of the experiments.

It is interesting to note that the obtained values of the crop response factor were within the range of values documented for sunflower found in the literatures. There are numerous research reports exist on yield response of sunflower to water, a host of researchers found that the k_y values were in the range of 0.80–0.95 for sunflower (Doorenbos and Kassam, 1979; Moutonnet, 2002; Demir et al., 2006). Apart from this, Mila and Ali (2016) found that the k_y values were in the range of 0.25 to 0.64 for the entire growing season of sunflower. The factor k_y captures the essence of the complex linkages between production and water use by sunflower in this study. With no exception, all the genotypes yielded crop response factors were of less than 1.0. The decrease k_y value indicates that the grown crops are more tolerant to water deficit, and recovers partially from stress, exhibiting less than proportional reductions in yield with reduced water use. Based on the k_y values deficit irrigation may be needed at different stages for water limiting area. Our result collaborates with the FAO standard as reported by Steduto et al. (2012).

The Velko genotype showed a uniformity coefficient of 0.55. Therefore, statistical differences between genotype and locations can exist. These values vary depending on season, location and intensity of water deficit (Mila and Ali, 2016).

It is apparent from obtained results that the growth stage is most responsive to irrigation was early stage (the first 60 days), compared with other stages. Therefore irrigation during this period would ensure the least yield reduction of sunflower. These results implied that irrigation at an earlier stage was much useful to increase seed yield of sunflower genotypes rather than the middle and later irrigation. Enough root penetration without water deficit during the early stage may be responsible for the crop tolerance to drought at later stages of growth. Additionally, plant stress due to the increased production of antioxidant enzymes may also contribute to plant resistance to drought (Langeroodi et al., 2014). Therefore, tolerance of sunflower plants to drought makes sunflower more valuable under the prevailing climatic condition of the study area where the climate is characterized as semiarid due to irregular and insufficient rainfall and hot weather during vegetation period for sunflower production (Flagella et al., 2002; Reddy et al., 2003).

Conclusion

The results of this study indicated that Barolo RO genotype is the most proper one for the study area as a part of drought-prone environments. Additionally, the results indicated that full irrigation at the early stage of growth was more effective to increase the seed yield of sunflower genotypes rather than the middle and later stages of its growth. Thus some water must be ensured at this stage. Also, higher response factor indicates greater water stress. Therefore, the water supply must be applied at the vegetative and pre-flowering stage. However, this will vary with location, the intensity of water deficit, and growth stages. In view of the obtained values of water efficiency, it is recommended to give high priority to Velko genotype coupled with stopping irrigation after 60 days.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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APPENDIX

Appendix 1. *Mean squares of variance analysis for seed yield and some irrigation treatments at both locations*

S.O.V	d.f	Seed yield (kg ha^{-1})	Irrigation water use efficiency (kg ha^{-1} mm^{-1})	Water use efficiency (kg ha^{-1} mm^{-1})
Kanipanka location				
Block	2	112716.4	0.381	0.778
Irrigation	3	1726819**	6.275**	12.807**
E(a)	6	49336.73	0.189	0.386
Genotype	2	8371629**	20.472**	41.780**
Irrigation × genotype	6	287980**	0.433*	o.884*
E(b)	16	54482.28	0.158	0.323
Qlyasan location				
Block	2	90154.22	0.217	0.513
Irrigation	3	658319.5**	12.131**	28.713**
E(a)	6	42885.49	0.102	0.24
Genotype	2	6237340**	16.168**	38.269**
Irrigation × genotype	6	105323.3**	0.141*	0.334*
E(b)	16	21462.21	0.047	0.111

*Significant at 0.05
**Significant at 0.01

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20432057

GENETIC ANALYSIS FOR SOME PHENOLOGICAL AND MORPHOLOGICAL TRAITS IN WHEAT (*TRITICUM AESTIVUM* L.) UNDER TWO DIFFERENT SOWING WINDOWS

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(Received 31st Oct 2018; accepted 21st Dec 2018)

Abstract. Major effect of climate change can be seen in developing countries like Pakistan that make them food insecure. Grain yield of wheat (*Triticum aestivum* L.) is low as it is affected by erratic weather conditions. Latest development of wheat genotypes applicable for late planting is one of the prime objectives of wheat breeders. Keeping in view the climatic conditions of Pakistan, current investigations were conducted to figure out the inheritance pattern and extent of variations using generation mean analysis. For the aforementioned study the two genotypes Miraj 2008 and Punjab 2011 were evaluated along with following generations i.e. F_1, F_2, BC_1 and BC_2 in normal and late sowing trial in Faisalabad, Pakistan. The highest reduction percentage was observed in grain weight per spike (-33.6%). The inheritance of all traits revealed complex inheritance due to the involvement of non-allelic interactions. The traits like days to heading and days to maturity had both additive, additive × additive i.e. fixable and additive × dominant non fixable genetic effects. So, selection in later generation would be fruitful for these traits when combinations are fixed. Heritability estimates for narrow sense was also determined. Low to moderate heritability was depicted under late sowing conditions.
Keywords: *late planting, generation mean, reduction percentage, grain weight, heritability*

Introduction

Crop yield is reducing due to adverse weather conditions whereas, supplies are becoming scarce, and the price is rising ultimately leads to food insecurity. Global mean temperature has risen at a rate of 0.3°C per decade during the twentieth-century (IFPRI, 2009) and is expected to reach 1 and 3°C above the current value by 2025 and 2100 respectively, resulting in more severe global climatic changes in near future. Heat stress (late sowing) at grain filling stage of wheat causes physiological and biochemical processes abort (Gupta et al., 2013). Therefore, selection of genotypes tolerant to high temperature with proper sowing time is a priority to manage the adverse effects of environments (Alghabari et al., 2014). Time of planting is one of the most important non-monetary input for optimizing the growth according to prevailing agro-climatic conditions and genotypes (Jena et al., 2017). It is an ultimate truth that crop yield is the inter-play of crop genetics, environment and management practices (Mirshekarnezhad et al., 2018). It is impossible to control the environment, but we can develop genotypes that can cope with changing environment. Among many other factors, late sowing is one of the major causes of lower wheat yield in Pakistan. Many cropping patterns prevailing in Pakistan have rotation like wheat; cotton, rice, maize, vegetables, fodders and sugarcane. This inertia in crop rotation cannot allow wheat to have an optimum

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20592071

time of plantation resulting in a large spectrum of sowing dates. About 80% of the wheat crop is grown after cotton, sugarcane and rice which exposes the crop to high temperature during the gain filling time that is the terminal heat stress which leads to drastic loss in grain yield and ultimately shortage of food (Prasad et al., 2011). High temperature is found to be accompanied with drought stress because of the rise in evapotranspiration. Terminal heat stress has drastic impacts on reproductive development such as flower initiation, ovary and pollen development, fertilization leading to reduction in sink potential which further results in yield loss (Barnabas et al., 2008). Selection of appropriate variety with respect to date of sowing is important to cope with the expected temperature rise at reproductive stage without compromising yield. Thus improvement in the crop genetic is the only inexpensive, effective and targeted approach available for wheat breeders in developing countries like Pakistan (Saxena and Toole, 2002). It is really important to know about genetic components as well as their interactions to achieve a workable breeding program. Generation mean analysis is a unique biometrical technique based on phenotypic performance of traits under study (Mather and Jinks, 1982). Generation mean analysis is most trusted biometric technique to investigate the genetic and epistatic effects. The present study was designed to achieve the information regarding the extent of variation and inheritance patterns in wheat genotypes under two different sowing windows.

Materials and Methods

Plant material

The current study was carried out in the research area of Department of Pant Breeding and Genetics University of Agriculture Faisalabad, Pakistan. The investigational material comprised of two bread wheat cultivars Miraj-2008 and Punjab-2011 (*Table 1*) as parents and their generation's i.e. F_1, F_2, BC_1, and BC_2 were established from two contrasting cultivars selected based on their planting time and commercial importance.

Table 1. *Name and pedigree of parental bread wheat cultivars (spring type)*

Parent	Name	Pedigree
1	Miraj-2008	SPARROW/INIA//V7394/WL711/13/BAUS
2	Punjab-2011	AMSEL/ATTILA//INQ-91/PEW'S'

All six generations of cross were sown on November 15, 2015 under normal (optimum) sowing conditions. The research trial was conducted in randomized complete block design (RCBD) with three replicates (P×P=15 cm; R×R= 30 cm). Dibbler method was used for sowing. To attain a single vigorous seedling per hole thinning was done after germination. To abate the border effect lines of non-experimental material was sown at the border of the experiment. Total four irrigations were applied. First irrigation was applied at tillering stage, second at booting stage, third at anthesis stage, fourth at milking stage. Fertilizer were applied at the rate of 46 kg nitrogen, 34 kg phosphorus and 25 kg potash per acre. Twenty well-guarded plants from P_1, P_2 and F_1 was tagged. Fifty plants from each back cross (BC_1 and BC_2) and two hundred plants from F_2 generation per replication were tagged prior to flowering. Similar activities were

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performed for the late sowing experiment on December 20, 2015. Harvesting was done 8-15 April, 2016. Data were recorded for the following traits: Plant height, tillers number per plant, Days taken to heading, days taken to maturity, grain filling duration (*Eq. 1*), grain filling rate (*Eq. 2*), harvest index (*Eq. 3*) and grain weight per spike.

$$\text{Grain filling duration} = \text{Days to maturity} - \text{days to heading} \quad \text{(Eq.1)}$$

Grain filling rate was estimated using following formulae (*Eq. 2*):

$$\text{Grain filling rate} = \frac{\text{Grain yield}}{\text{Grain filling period}} \times 100 \quad \text{(Eq.2)}$$

$$\text{Harvest index} = \frac{\text{Grain yield}}{\text{Biological yield}} \times 100 \quad \text{(Eq.3)}$$

Statistical analysis

Analysis of variance was done following Steel et al. (1997) and generation mean analysis (*Eq. 4*) was performed according to the method given by Mather and Jinks (1982). A computer program provided by Dr. H.S. Pooni, School of Biological Sciences, University of Birmingham UK was used for generation mean analysis by the method of Mather and Jinks (1982). The narrow sense heritability (h^2ns) was estimated by the method shown by Mather and Jinks (1982) and Saleem et al. (2016).

$$Y=m+\alpha[d]+\beta[h]+\alpha^2[i]+2\alpha\beta[j]+\beta^2[l] \quad \text{(Eq.4)}$$

where Y = mean of one generation, m = mean of all the generations, d = sum of additive effects, h = sum of dominance effects, i = sum of additive × additive gene interaction i.e. complementary, j = sum of additive × dominance α^2, $2\alpha\beta$ and β^2 were the coefficient of genetic parameters, l = sum of dominance × dominance interaction i.e. duplicate.

Results and discussion

Understanding the genetic makeup of any crop plants is dire to batten the crop performance in the current scenario of climate change. Analysis of variance revealed significant genotypic difference for each generation (P_1, P_2, F_1, F_2, BC_1 and BC_2) for all traits under study. Under both sowing conditions parents and generations exhibited significant differences (*Table 2*). The presence of genetic diversity in breeding material is basic criterion for success in any breeding program. Under different sowing conditions changed behavior of breeding material is an indication of significant environment into genotypes interaction (Akram, 2011; Saleem et al., 2016).

Comparison of two treatment means of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions

Expression of genes in any stress become restricted. Under late sowing trial the overall performance of all the traits decreased. The magnitude of sensitivity was different for the different traits. Most sensitive traits grains weight per spike with a reduction of 33.6% succeeded by tillers number per plant 28.3% and harvest index

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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27.1%. In contrast the lowest reduction was shown by days to heading 12.3% followed by grain filing rate 13.4% and grain filling duration 17.0% (*Table 3*). Scientists have also exhibited that under stressed environment, traits do not fully express themselves (Tahir et al., 2009; Sohail et al., 2014).

Table 2. *Analysis of variance of different traits under studied of cross Miraj-2008 × Punjab-2011 under normal and late sowing conditions*

Traits	Normal sowing	Late sowing
Plant height	61.1819**	57.4181**
Tillers numbers per plant	0.72056**	1.29956*
Days to heading	25.5520*	12.6195**
Days to maturity	20.1437**	15.3067**
Grain filling duration	16.2646**	18.8524**
Harvest Index	13.1339*	8.12861**
Grain filling Rate	2.15689**	8.01126*
Grain weight per spike	0.03896**	0.12190*

*, ** Significant at 5% and 1%, probability levels, respectively

Table 3. *Growth and yield parameters of six generations means of Cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions with % reduction under late sowing conditions*

Traits	Normal sowing	Late sowing	% change
Plant height	101.81	76.747	-24.4
Tillers numbers per plant	12.372	8.8756	-28.3
Days to heading	96.200	84.393	-12.3
Days to maturity	133.92	114.55	-14.5
Grain filling duration	37.772	31.336	-17.0
Harvest Index	42.377	30.878	-27.1
Grain Filling Rate	94.535	81.857	-13.4
Grain weight per plant	2.2550	1.4978	-33.6

Phenological traits

Days to heading

Heading time is phenological stage as it determines the pattern of yield potential and stability. When overall reduction percentage was exhibited from pooled means (*Table 3*) of both parents (P_1 and P_2) and their generations (F_1, F_2, BC_1 and BC_2) the results depicted that growth span reduction percentage maximum in BC_2 (15.81%) and minimum in F_2 (10.28%), and when individual performance of parents and generation was investigated for mean values the response was different in both normal and late sowing conditions (*Figure 1*). In late sowing condition F_2 depicted maximum mean value for days to heading (117.35) which revealed more variation in the segregating generation. Those observation were in line with the finding of Kilic and Yagbasanlar (2010). The study of controlling days to flowering is really pivotal as per genetic needs because genetic potentials would show negative indicators with the onset of erratic weather conditions. The genotypes with more days to heading in delayed planting

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conditions produce more grain yield because these genotypes assimilate nutrient from stem to kernels very precisely and grains enjoyed large size ultimately increased grain weight.

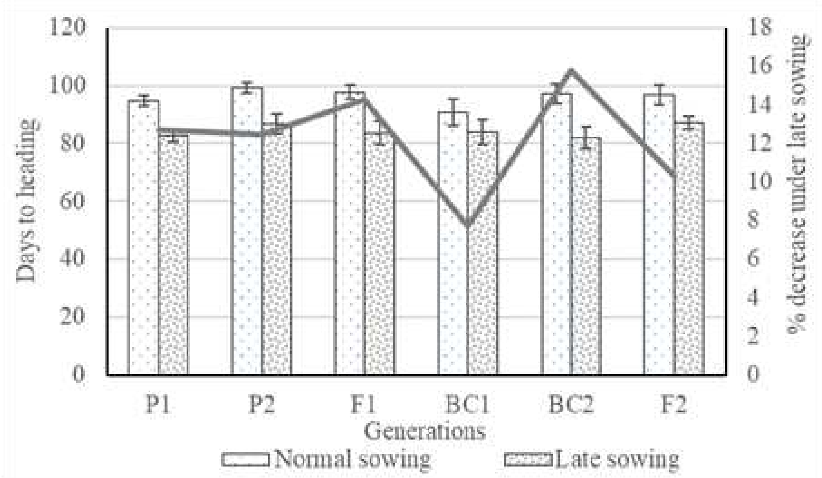

Figure 1. *Days to heading of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

Days to maturity

The stress environment forced early maturity and abridged the life cycle of the genotypes and their generation. P_1 exhibited uppermost reduction percentage (16.5%) for maturity days and lowermost (11.52%) was revealed by F_2 (*Table 3*). It means P_1 was more sensitive to environmental stress. While in late sowing date F_2 depicted highest days to maturity (117.3) and lowest magnitude (112.2) were documented for BC_1 (*Figure 2*). Those statement were also analogous with the finding of Kilic and Yagbasanlar (2010).

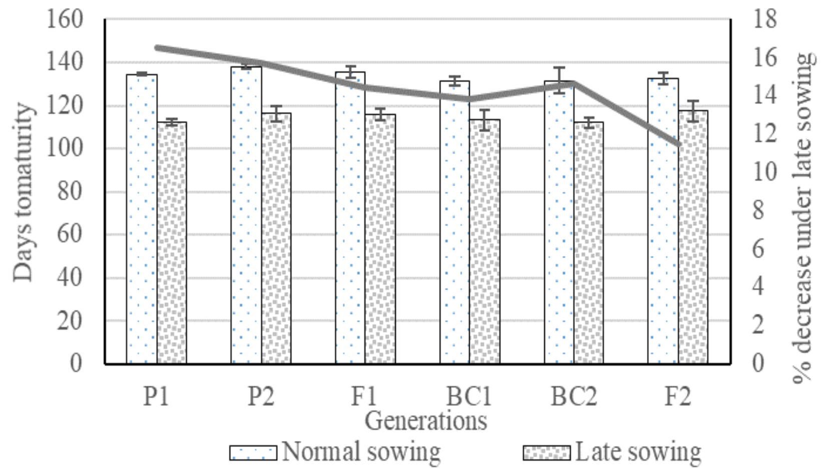

Figure 2. *Days to maturity of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

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Grain filling duration

When genotypes were sown in normal dates more grain filling days were produced comparative to late sowing (*Table 3*). For grain filling duration in pooled mean values of both parents and generations back cross 1 attained highest reduction (27.9%) percentage while lowest value (13.59%) was noted in F_2 depicted in *Figure 3*. The losses percentage was out of parents range. In late planting conditions P_2 (33.86) illustrated maximum grain filling days and P_1 (28.60) showed lowest value when separate performance of parents and generations was observed. Delayed sowing shortens the duration of each development phase which ultimately reduces grain filling period resulting in lower grain weight (Kaur, 2017). It additionally shows that the optimum planted genotype relished a prolonged development stage and favorable pre-heading environments, which have had a persist impact on grain weight via stem reserves mobilization (Baloch et al., 2012).

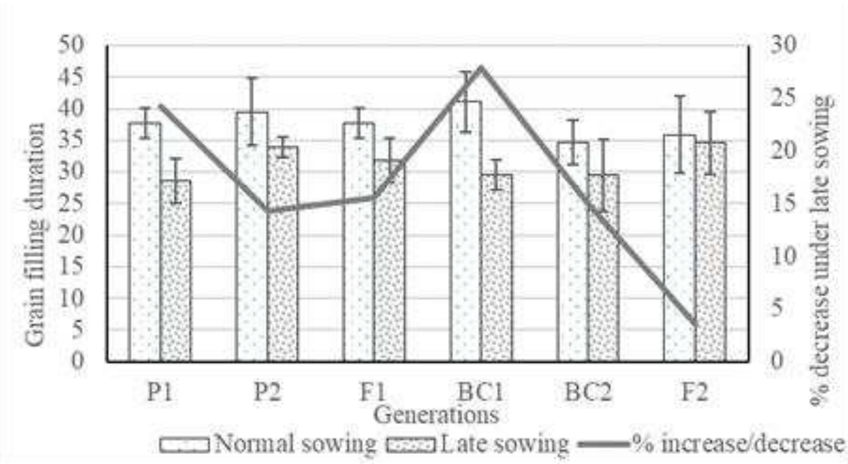

Figure 3. *Grain filling duration of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

Grain filling rate

It is also an important penolohgiacal trait. In pooled mean values for both parents and their generations P_1 showed maximum reduction percentage magnitude (14.96%) and minimum value showed by F_2 that was (11.52%). For observing individual performance in all generation and parents under both normal and late sowing trial the uppermost mean magnitude revealed by F_2, while the lowermost value was perceived by BC_1 (93.41) in normal and P_1 (80.05) scored minimum in late planting experiment (*Figure 4*). Similar results were presented by Tahir et al. (2009), Laghari et al. (2012) and Golparvar (2012). Grain filling rate is critical growth phase where hostile environmental circumstances leads to harmful effect on grain yield and quality. Delay planting face high temperature at the time of maturity and reduced grain filling duration. It has been noted in the recent studies high temperature stress increase rate of stem reservoir mobilization towards grain filling but not reimburse for short grain filling days (Farooq et al., 2011). Due to this reason grain could not reach maximum size ultimately decline in grain yield occured.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20592071

Harvest index

It may be explained as translocation of nutrients from vegetative part to reproductive part. In pooled mean value of both parents and generations, highest reduction percentage was noted in F_2 segregating generation (32.89%) while the lowest percentage was revealed by F_1 (23.62%) of cross Miraj 2008 × Punjab 2011. while in detecting individual performance of parents and generations under normal planting trial F_2 represented (44.10%) highest mean value and BC_2 showed (38.59%) the lowest value, whereas in late planting trial P_2 obtained (32.23%) highest mean value (*Figure 5*). Harvest index deliver fruitful information regarding selection of genotypes in late sowing conditions. It has been noted in literature the genotypes with more harvest index attained more biomass and performed well in delay sowing conditions (Akram, 2011; Mushtaq et al., 2011).

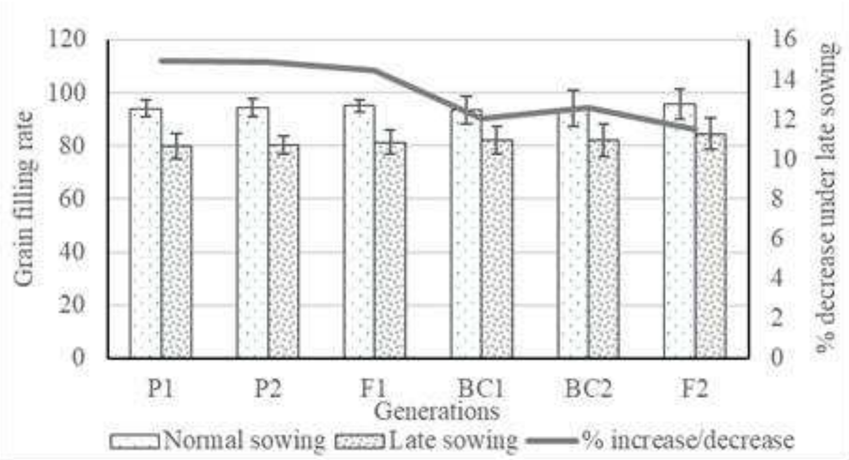

Figure 4. *Grain filling rate of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

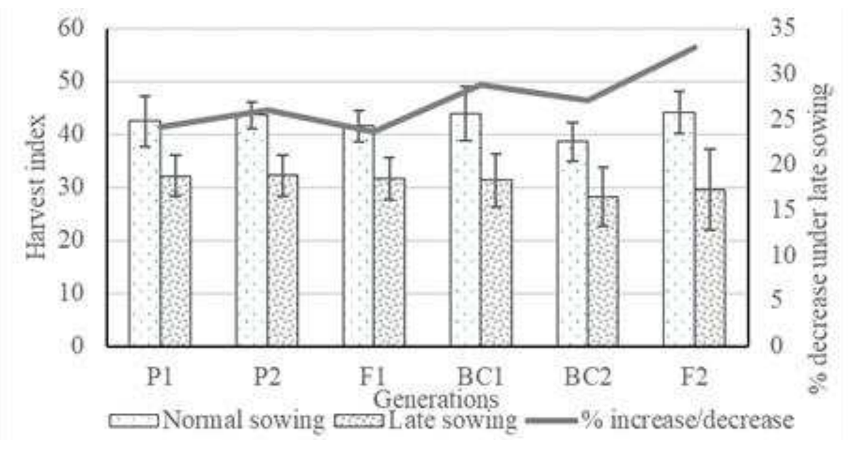

Figure 5. *Harvest index of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

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DOI: http://dx.doi.org/10.15666/aeer/1702_20592071

Morphological and yield related traits

The yield associated traits like Plant height (*Figure 6*), tillers number per plant (*Figure 7*) and grain weight per spike (*Figure 8*) were advisedly affected under late sowing conditions. Grain weight per spike revealed maximum decline, as all other yield related traits add up to decrease grain yield in delay planting condition. To select genotypes with optimum plant height with vigorous vegetative growth is painstaking criteria. Tillering in wheat crop help to compensate the yield even though plant population is not met adequately. Number of tillers per plant is reportedly decreased with the onset of late planting due to less growing degree days. Grain weight per spike is more sensitive trait under late sowing conditions.

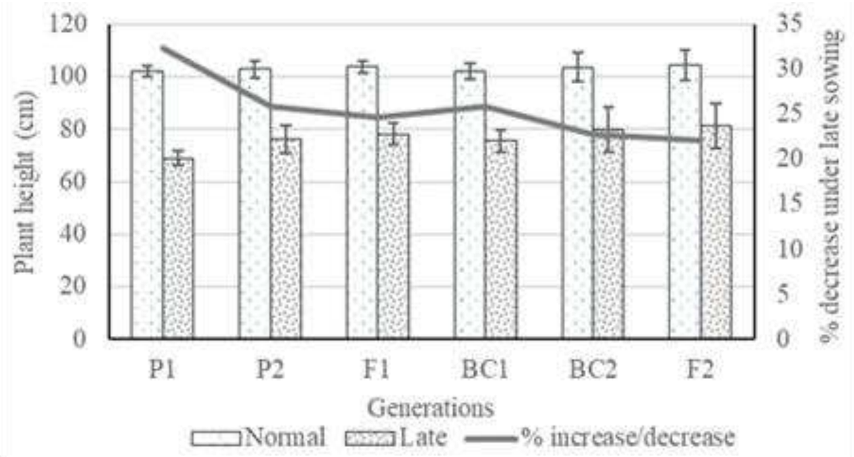

Figure 6. *Plant height of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

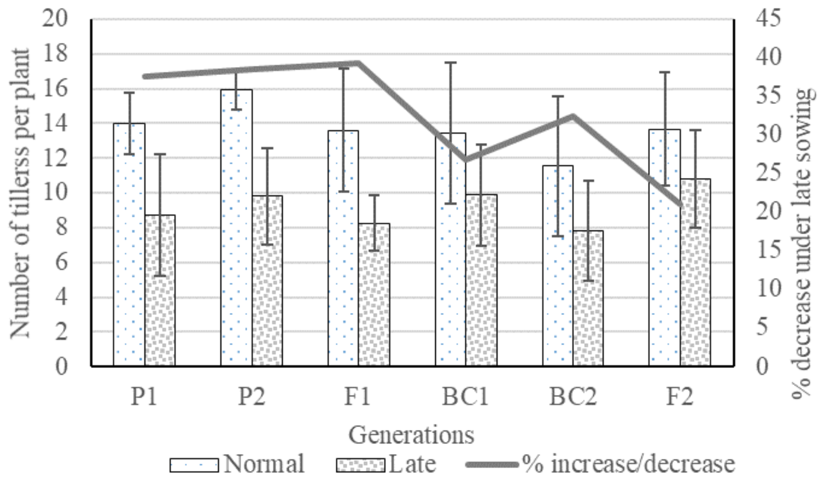

Figure 7. *Tillers number per plant of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

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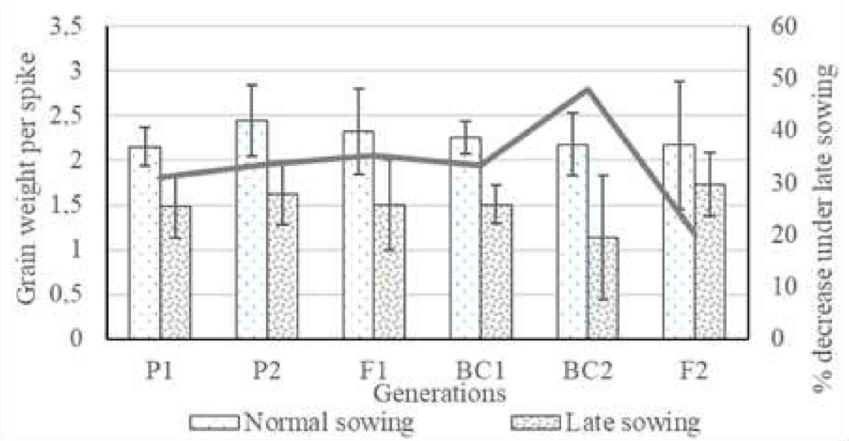

Figure 8. *Grain weight per spike of six generations of cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions along with % increase or decrease. Error bar represents standard errors and overlapping bars are statistically at par with each other*

Gene action

Generation mean analysis was employed to assess the mean [m], additive [d], dominance [h], additive × additive [i], additive × dominance [j] and dominance × dominance [l] gene actions and magnitudes. The best efficacy of model was selected using two things i.e. lowest standard error and non-significant value of chi square χ^2 test for all traits under observation. For the detection of inheritance array in normal conditions for plant height five parameter model was best and vastly presence of additive [d] genetic effect than dominance [h], while the adequacy of three parameter model in late sowing conditions observed best fitted. Additive × additive [i] gene effects were significant. This suggests that selection should be carried out in later generations and the interaction should be fixed by selection under selfing conditions. These results are agreed with the observation of Farshadfar et al. (2001). Ojaghi and Akhundova (2010) noted additive, while conflicting results were depicted by Saleem et al. (2016). For tillers number per plant significant additive genetic effects with epistatic effects were also recorded. Additive × additive [i] interaction is more valuable for genetic improvement of characters through selection. In normal planting condition five [mdhij] and four [mdhi] parameter models in late planting conditions were observed best tailored to explain the genetic variation existed in breeding material for number of tiller per plant. Such type of findings were documented via Badran and Moustafa (2014) and conflicting conclusion were publicized by Ullah et al. (2010) and Rashid et al. (2012). Generation mean analysis revealed that days to heading was governed by polygenes i.e., complex genetic inheritance. The negative magnitude of [i] additive × additive epistatic effect showed in late sowing conditions gene expression not fully expressed in stressed condition and nature of epistasis was duplicate as main additive effect [d] were also reported. Five parameters [mdhij] in late and four [mdhi] parameter models obtained best fit to examine the variation in inheritance pattern (*Table 4*). Zara-Kohan and Heidari (2012) witnessed similar results. They revealed both fixable and non-fixable gene action. According to the nature of treatments in cross for days taken to maturity five parameter model [mdhij] was investigated fit for detection of gene action under both planting conditions. Hybrid breeding is fruitful for selection of trait because

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prominent dominant [h] effect was paving. In normal planting non fixable gene action somewhat more percentage than fixable gene action.

Additive type of gene action was prominent in grain filling period in normal planting conditions with [i] and [j] epistatic effects but under late planting conditions change in gene action was witnessed (*Table 4*). It viewed under the effect of environment and duplicate type of epistasis was found because of main dominance effect [h] and epistatic dominance × dominant [l] effect had opposite sign under late planting time. The similar situation was examined by Nazeer et al. (2004). Zara-Kohan and Heidari (2012) reported selection in stable generation would be fruitful due to governing of non-fixable type of gene action for this parameter. For the inheritance of grain filing rate under late planting conditions four parameter model [mdhl] was found best fit (*Table 4*).

Table 4. *Estimates of the best fit model for generation means parameters (±, standard error) by weighted least squares analysis in cross Miraj 2008 × Punjab 2011 under normal and late sowing conditions*

Traits	Cross	Genetic Effects						χ2 (df)
		m ± S.E	[d] ± S.E	[h] ± S.E	[i] ± S.E	[j] ± S.E	[l] ± S.E	
Plant height	N	124.21±3.06	4.48±0.231	49.54±8.07	25.61±3.05	-	31.84±5.13	0.2864(1)
	L	84.40±1.05	3.61±0.23		11.79±1.08	-		0.8651(3)
Tiller number per plant	N	11.53±0.53	0.19±0.20	1.05±0.79	1.22±0.57	7.10±1.45	-	1.7860(1)
	L	7.18±0.42	0.41±0.14	1.04±0.40	1.97±0.45	-		4.455(2)
Days to heading	N	97.03±0.16	2.47±0.15	5.45±0.88	6.21±0.90	-	-	1.7548(2)
	L	88.96±0.65	1.95±0.22	-5.42±0.80	4.35±0.70	-7.55±1.58	-	1.1269(1)
Days to maturity	N	136.87±0.43	1.73±0.12	1.99±0.62	0.80±0.45	1.42±0.32	-	1.9142(1)
	L	133.24±1.90	1.77±0.20	46.30±4.99	-18.92±1.89	28.97±3.20	-	1.5337(1)
Grain filling duration	N	34.27±0.52	0.88±0.22	-3.60±0.72	4.44±0.57	-14.58±1.30	-	1.9844(1)
	L	31.23±0.24	2.63±0.24	6.19±1.02	-	-	-5.47±1.34	1.2870(2)
Harvest index	N	38.79±0.51	0.567±0.21	2.76±0.68	4.236±0.56	11.81±1.17	-	0.0158(1)
	L	26.84±0.74	1.01±0.27	4.43±1.23	-5.24±0.81		6.58±1.37	2.2183(1)
Grain filling rate	N	94.48±0.27	0.44±0.17	3.79±1.48	4.78±2.19	-	2.99±1.22	0.1496(1)
	L	80.70±1.28	5.19±1.12	8.50±5.04	-	-	7.72±2.22	1.1150(2)
Grain weight per spike	N	2.01±0.06	0.14±0.02	-	-	-0.44±0.20		4.5642(3)
	L	1.55±0.01	0.06±0.01	0.80±0.10	-	-0.88±0.12	0.74±0.10	1.2521(1)

N = normal sowing, L= late sowing conditions

Due to presence of non-allelic interaction this attribute was governed by multiple genes. Hybrid breeding is done for the improvement of grain filling rate due to presence of dominance component [*h*]. To obtain maximum grain filing rate selection could be done in succeeded generations. These results are lined with Nazeer et al. (2010) and clashing conclusions obtained by Ullah et al. (2010). For harvest index five parameters models i.e. [mdhij] and [mdhil] were found best fit under both normal and late planting environments. In late sowing condition [i] epistatic gene action showed negative sign which exhibited that gene interacting with one another (*Table 4*). Due to presence of main dominance effect [h] and dominant × dominant [l] selection can also performed in later generation and also hybrid breeding for harvest index would be fruitful. Farshadfar

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DOI: http://dx.doi.org/10.15666/aeer/1702_20592071

et al. (2001) displayed same type of conclusion while. Grain weight per spike five parameter model [mdhjl] was adequate to elucidate the gene inheritance in late planting environment (*Table 4*). In order to attain higher grain weight per spike selection in succeeded generation could be helpful. Parallel results are confirmation with Amin (2006) and El-Rahman (2013).

Heritability studies

Estimation of heritability is another important estimate in order to understand the potential of characters to be improved through selection and also tells about the possibility of accumulation of favorable gene in the successive generation after selection. It also indicates the amount of genetic variability relative to environmental affects and is considered a good index of transmission of characters from parents to their offspring. Under normal sowing conditions range of narrow sense heritability was from 0.35 to 0.90 (*Table 5*). Most of the examined traits revealed moderate to high heritability except grain filling rate and grain weight per spike. The range (0.23 to 0.71) was observed under late sowing conditions (*Table 5*). Low to medium narrow-sense heritability can be attributed to the reduction of genetic variability in the late sowing environment. The higher heritability value will make the selection process effective, which will result in higher response. Selection of traits with high heritability in early generation is more fruitful. Abinasa et al. (2011) and Saleem et al. (2016) found high heritability for plant height, days to heading and days to maturity. While moderate type of heritability was observed by Nazeer et al. (2010). Zara-Kohan and Heidari (2012) and Khan and Naqvi (2011) have also noted high heritability for grain filling period. The reduction in heritability value under late sowing conditions indicated that gene responsible for the heritability of trait do not expressed themselves strongly in late sowing. Usually late sowing produce early senescence hence decrease in grain filling period causing reduction in yield. Therefore the genotypes which have longer grain filling period under stress conditions would be better yielder.

Table 5. *Estimation of narrow sense heritability of different traits under studied*

Traits	Normal sowing	Late sowing
Plant height	0.90	0.67
Tiller number per plant	0.76	0.23
Days to heading	0.89	0.71
Days to maturity	0.78	0.55
Grain filling duration	0.64	0.26
Harvest index	0.62	0.65
Grain filling rate	0.35	0.33
Grain weight per spike	0.47	0.41

Conclusion

Optimum sowing date for any cultivar is very crucial to accumulate ample dry matter and grain weight per spike yet to elude potential yield losses form high temperature stress during critical time mainly flowering initiation to grain filling stage. Genetic analysis showed that all traits under study were under the control of complex inheritance due to presence of epistasis. So, selection for these traits would be done in

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later generations when combinations fixed. The phenological traits grain filling rate, grain filling duration and harvest index considered pivotal for the development of genotypes in late sowing conditions. So, for efficient breeding utilization of these phenological traits like grain filling rate, grain filling duration and harvest index are necessary to improve crop yield for late sowing wheat areas.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20592071

DIVERSITY AND COMMUNITY STRUCTURE OF FUNGI IN THE ROOTS OF *MACHILUS PAUHOI* IN DIFFERENT AGE GROUPS

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(Received 7th Nov 2018; accepted 11th Jan 2019)

Abstract. In this study, in order to investigate the diversity of fungi in the roots of *Machilus pauhoi* in different age, we investigated the fungi diversity and community structure in the roots of *Machilus pauhoi* in the age of 2-year-old, 5-year-old, 8-year-old and 20-year-old by Illumina Hiseq sequencing platform. 424 fungi operational taxonomic units (OTU) were obtained. There was no significant difference in fungi abundance and diversity index between the age of 2-year-old, 5-year-old and 20-year-old, but they were higher than those in the age of 8-year-old. On phylum level, *Ascomycota*, *Basidiomycota* and *Glomeromycota* were the dominant species with a relative abundance of more than 1%. Besides, with the increase of age, the fungi community in the roots of *Machilus pauhoi* showed a certain degree of change while then decreased to the equivalent level in the young age. On genus level, *Clitocybula*, *Chaetosphaeria*, *Phylchara* and *Ochroconis* were the dominant species, accounting for more than 10% of the investigated species. The specific species of *Phyllachora* appeared in the highest proportion in 8-year-old roots. Abundance heat map analysis showed that the fungi communities in the 2-year-old and 20-year-old roots were clustered into one category and those in the 5-year-old and 8-year-old were classified as another group. These results indicated that the fungi community in the roots of *Machilus pauhoi* was greatly affected by the growth age.
Keywords: *endophytic fungi, high throughput sequencing, relative abundance, Venn diagram, phylogenetic tree analysis*

Introduction

Soil microbes are the basis for the recycling and utilization of materials in ecosystems and are also a guarantee for the stability and normal functions of ecosystems (Chapin et al., 1997). Soil, as a site for soil microbial activity, has an important impact on microbial community and activity (Jeanbille et al., 2016; Kuramae et al., 2011). Fungi in soils play a huge role in improving the plant stress resistance and maintaining normal plant growth (Mukerji et al., 1996; Smith et al., 1999). Endophytic fungi in roots mainly results from soil microorganisms infection when contacting with soil. Soil fungi are closely related to plant growth and can affect the health and productivity of plants. As an important part of the whole plant, the endophytic fungi play a very important role in plant growth, development, accumulation of secondary metabolites and adaptation to the environment (Vandenkoornhuyse et al., 2015). Some species can enhance the resistance and daptability of the host (Kaushik et al., 2013; Iranshahi et al., 2016; Zhao et al., 2013; Kumar et al., 2013) and promote the uptake of nitrogen, phosphorus and other elements (Rodrigue et al., 2004; Azevedo et al., 2000). Some have no significant effect on plants, neither attacking plants nor promoting plant growth (Rout, 2014). And some are latent in plant tissues and remain dormant. When plants suffer from physiological and biochemical dysfunction caused by changes in the external environment, they become active to attack plants (Petrini, 1991). Endophytic fungi are

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ubiquitous in a variety of plants. However, in over ten thousand kinds of plants on the earth, only hundreds of endophytic fungi have been studied (Stone et al., 2000). In addition, the distribution of endophytic fungi in the host is often affected by various factors such as the forest age, season, altitude, and sampling number (Schmit et al., 2004).

Machilus pauhoi belongs to Lauraceae, Machilus Nees, which is a large evergreen tree with rapid growth and adaptability. It is a national second-class protected plant with high economic value in both wood and seeds (Guo et al., 2017). Because of its important ecological and economical value, *Machilus pauhoi* has been listed as a key tree species by Guangxi Forestry Department. It is worthy of vigorous promotion in depth (Wei et al., 2011). At present, there are few studies on *Machilus pauhoi* at home and abroad, and most of articles only briefly describe the cultivation techniques, ecological characteristics and basic measures for afforestation (Zhong et al., 2009). However, the effects of growth age on the diversity and community of fungi in the roots of *Machilus pauhoi* have not been reported. In addition, through field investigations, after 5 to 6 years of growth, yellow, brown to black irregular shape lesions appear in large areas of leaves, stems and other parts above ground, and sometimes the whole leaves are even covered by black spots, which form a black-colored convex stroma, seriously inhibiting the photosynthesis of the plant. As a result, a significant part of the plant would die, greatly reducing the survival rate of the *Machilus pauhoi*. Whether this phenomenon is related to some pathogens in the roots of the *Machilus pauhoi* is worth exploring.

At present, many researchers at home and abroad are committed to the restoration and reconstruction of fragile or degraded ecosystems, and plantations have become a better means of repair (Rappaport et al., 2014). It is necessary to restore the plants on the ground and the functional microorganisms underground that are symbiotic with plants. Given to the universal distribution of endophytic fungi in terrestrial ecosystems, the diversity of ecological functions and their important roles in the restoration and reconstruction of degraded ecosystems, and taking into account the development potential and economic value of *Machilus pauhoi*, this research studied the cultivated *Machilus pauhoi* in different age groups in Yangshan County, Qingyuan City, Guangdong Province, China for the first time. The high-throughput sequencing technology was used to reveal the diversity of fungi in the roots of *Machilus pauhoi* in different developmental stages. This study helped to screen the mycorrhizal fungi which could promote the growth of *Machilus pauhoi.* Besides, it can effectively guide the introduction and cultivation application of *Machilus pauhoi*. At the same time, it is necessary to explore whether the scabs formed in *Machilus pauhoi* are related to certain pathogens. The results of this study not only help to understand the role and status of soil microbes in the different growth years of *Machilus pauhoi*, but also provide a theoretical basis for the sustainable management of *Machilus pauhoi*.

Materials and methods

Overview of the study area

The plantation forest of *Machilus pauhoi* is located in Yangshan County, Qingyuan City, Guangdong Province, China (N24°26’, E112°36’), which belongs to the monsoon climate zone with transition from south subtropical to mid-subtropical. The annual

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average temperature is 20.3 °C, and the average annual rainfall is about 1850 mm. The altitude is in the range of 10 to 270 m, and the soil type is mainly red soil.

The original study area is mainly mountain rain forest. However, it has been damaged by human activities for a long time, resulting in secondary forests and extremely irregular physiognomy. Since 1995, the artificial test plantation of *Machilus pauhoi* has been constructed with similar site conditions. At present, there are four different developmental stages of *Machilus pauhoi* plantation with forest ages of 2 years, 5 years, 8 years and 20 years respectively. The site conditions of the plots of the test plantation were basically the same, and the row spacing was 3×3 m. A map indicating the location of sampling site was given (see *Fig. 1,* which was generated by Google earth). Some photos about the habitat of experimental sites are also given in *Figure 2*.

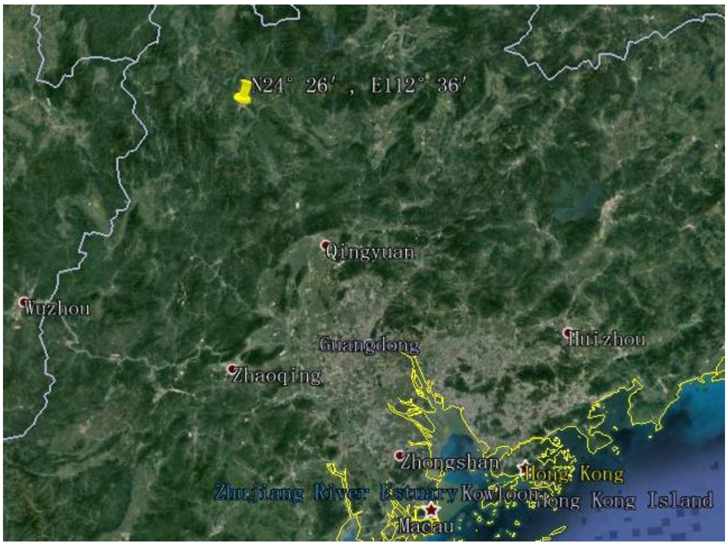

Figure 1. *GPS coordinates of the sampling site*

Figure 2. *Habitat of the experimental sites*

Sampling method

In October 2017, root samples at 4 different stages of development, i.e., 2a, 5a, 8a and 20a, were selected as the research objects from the test plantation. In order to

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DOI: http://dx.doi.org/10.15666/aeer/1702_20732083

reduce the error, each sample was selected in random and three parallel samples were selected. The samples of the 2a *Machilus pauhoi* were numbered as 2YR.1, 2YR.2, 2YR.3, and the 5a *Machilus pauhoi* were numbered as 5YR.1, 5YR.2, 5YR.3, 8a *Machilus pauhoi* were numbered as 8YR.1, 8YR.2, 8YR.3, and the 20a *Machilus pauhoi* were numbered as 20YR.1, 20YR.2, and 20YR.3, respectively. When selecting samples, the end part of taproots with low lignification degree were collected. Root samples were cut into 2-3 cm long segments first, then rinsed with tap water, then rinsed with distilled water, then soaked in 0.1% mercury bichloride for 5-10 min, then rinsed with distilled water, then soaked in 75% alcohol for about 20 s, then rinsed with distilled water, then dried with silica gel in aseptic self-sealing bags and marked, and then stored in a refrigerator at 4 °C. A total of 12 *Machilus pauhoi* samples were collected.

High-throughput sequencing methods

In this study, high-throughput sequencing technology was used. DNA samples were extracted from root samples with the kit (Plant Genomic DNA Kit, Tiangen, China) using the modified Cetyl Trimethyl Ammonium Ammonium Bromide (CTAB) method (Guo et al., 2000). The concentration and purity of the extracted DNA were measured by spectrophotometer (Thermoelectric Corporation, USA). The integrity of the DNA sample was measured by 0.8% agarose gel electrophoresis at a voltage of 120 V and an electrophoresis time of about 20 mins.

30 μL PCR reaction system: 15 μL of Phusion Master Mix (2×), 3 μL of Primer (2 μmol/L), 10 μL of gDNA (1 ng/μL), and 2 μL of H_2O. The amplification procedure: pre-denaturation at 98 °C for 1 min; 30 cycles including denaturation at 98 °C for 10 s; annealing at 50 °C for 30 s; extension at 72 °C for 30 s; and extension at 72 °C for 5 min. Library preparation, homogenization, detection, computer sequencing and data quality control were all completed by Guangzhou Meg Biotechnology Co., Ltd.

The phylum and genus level was identified based on the alignments of majorizing sequence and reference sequence in the database. The uclust del method (Caron et al., 2009) was used to cluster OTUs with high-quality sequences with a similarity ≥ 97%. According to the results of cluster analysis, alpha diversity analysis was carried out. The Observed species index and the Chao1 index reflect the richness of the community in the sample, that is, simply refer to the number of species in the community (the number of OTUs); the Shannon index and the Simpson index reflect the diversity of the community, and are affected by species richness and species uniformity in the sample community. The results were processed and bioinformatics analysis was carried out using Microsoft Excel 2013 and R 3.4.2 software (Knezevic et al., 2007).

Results and analysis

Sequencing depth evaluation

12 fungi samples were sequenced and a total of 557,906 sequences were obtained after sequence optimization. The sequences were randomly selected. Curves of the number of selected sequences versus the number of OTUs they represent were drawn. The OTU was clustered at 97% similarity level and the rarefaction curves of each sample was prepared (*Fig. 3*). The curves gradually flatten indicating a reasonable number of sequencing.

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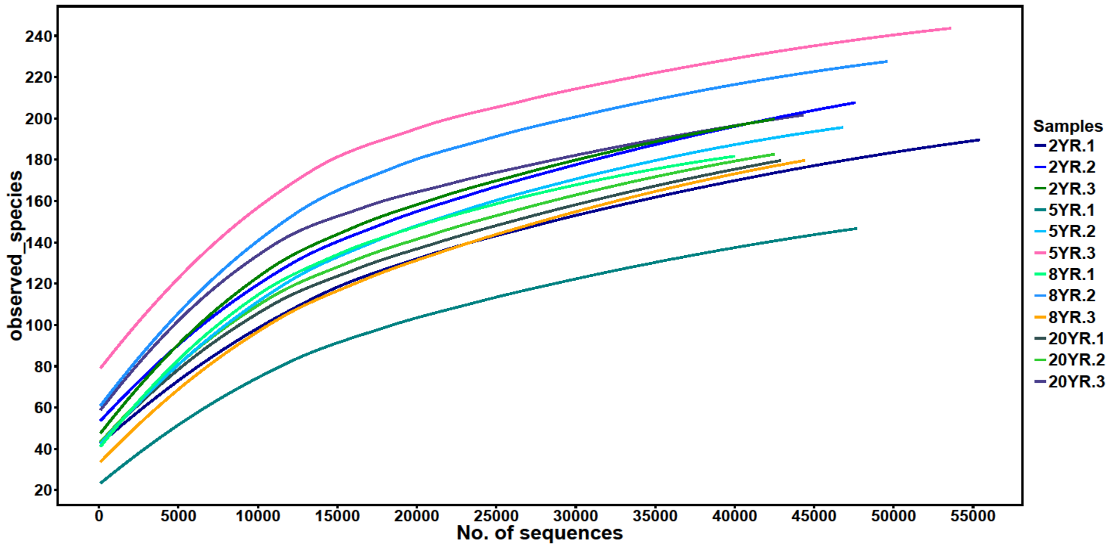

Figure 3. *Rarefaction curves of roots of Machilus pauhoi*

Fungi diversity and OTUs in roots of Machilus pauhoi in different age groups

It can be seen from *Table 1* that the fungi richness and diversity index in the roots of 8a *Machilus pauhoi* are significantly lower than the other three ages. However, there is no significant difference in the abundance and diversity index of fungi communities in the roots of the 2a, 5a and 20a *Machilus pauhoi*.

Table 1. *Diversity indexes of fungi in roots of Machilus pauhoi in different age groups*

Treatment	Observed species index	Chao1 index	Shannon index	Simpson index
2a	183.67±11.72a	270.9±14.11a	3.16±0.04a	0.78±0.04a
5a	187.00±15.59a	269.02±31.96a	3.17±0.3a	0.77±0.09a
8a	165.33±16.06b	223.32±19.3b	2.11±1.91b	0.40±0.38b
20a	190.33±22.68a	251.65±24.47a	3.31±0.58a	0.82±0.05a

According to the Venn diagram (*Fig. 4*), which represents the relationship between the number of OTUs in the roots of *Machilus pauhoi* in different age, 424 fungi OTUs were obtained from all samples in 4 age groups, including 223 in the second year, 233 in fifth year, 216 in eighth year and 220 in twentieth year. The unique OTUs in the roots of 2a, 5a, 8a and 20a *Machilus pauhoi* are: 53, 47, 50 and 51, accounting for 12.50%, 11.08%, 11.79% and 12.03% of the total OTU, respectively. There are 106 OTUs shared by samples in different age groups, accounting for 25.0% of the total number of OTUs. The common OTU in the roots of *Machilus pauhoi* in fifth year and eighth year is the most, up to 151. And the common OTUs in the roots of *Machilus pauhoi* in second year and twentieth year is the least as 135. The common OTUs shared by other age groups are: 141 OTUs for the second and fifth year, 143 OTUs for the second and eighth year, 147 OTUs for the twentieth and fifth year, and 135 OTUs for the twentieth and eighth year.

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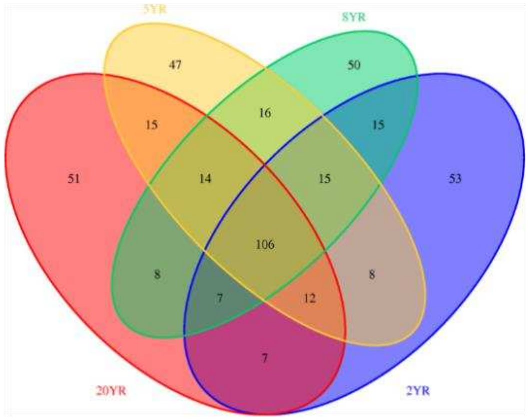

***Figure 4.** OTU Venn diagram of root samples in various age groups of stand*

Analysis of fungi community in roots of Machilus pauhoi in different age groups

At the phylum level, there are 3 species with relative abundance more than 1%. *Ascomycota* fungi accounts for the highest proportion (60.98%), followed by *Basidiomycota* fungi (33.62%), and *Glomeromycota* fungi accounts for the smallest proportion (2.74%). According to the analysis of the proportion of all kinds of fungi, the fungi community in roots of *Machilus pauhoi* in different age groups presents a significant change (*Fig. 5*). From the interannual variation, in the roots of 5a *Machilus pauhoi*, the relative abundance of *Basidiomycota* is the highest while that of *Ascomycota* is the lowest. However, in the case of the roots of 8a *Machilus pauhoi* is the exact opposite. The relative abundance of *Glomeromycota* increases first and then decreases with the growth age. Compared with the 20a *Machilus pauhoi*, the fungi community in the roots of the 2a *Machilus pauhoi* has little change. On the whole, with the increase of growth age, the fungi community in the roots of *Machilus pauhoi* shows a certain degree of change and then returns to the similar structure in the young age (2a) at the phylum level.

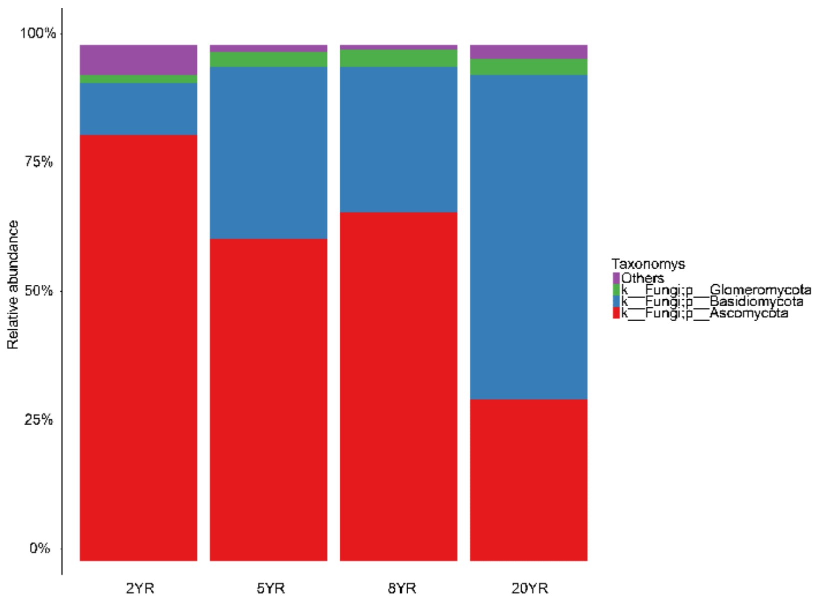

***Figure 5.** Composition of fungi community at a phylum level*

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At the genus level, in each community, there were 8 genera with the relative abundance more than 1% in the roots of *Machilus pauhoi* (*Fig. 6*): *Archaehorhizomyces*, *Chaetosphaeria*, *Clitocybula*, *Capronia*, *Muyocopron*, *Ochroconis*, *Parascutellinia* and *Phylchara*. Among them, *Clitocybula*, *Chaetosphaeria*, *Phylchara* and *Ochroconis* were dominant species, accounting for 36.16%, 21.54%, 11.31% and 19.11%, respectively. From the perspective of interannual variation, the composition of fungi communities in roots of *Machilus pauhoi* in different age groups also changes significantly (*Fig. 4*). The relative abundance of Clitocybula first increases and then decreases and further increases to the peak level at 20a. The relative abundance of *Ochroconis* decreases first, then increases and then decreases. The relative abundance of *Chaetosphaeria* increases with the extension of the growth age. The relative abundance of *Parascutellinia* does not change much with the increase of growth age. The relative abundance of *Capronia* is small, which appears to decrease first and then increase with the increase of growth age. *Archaeorhizomyces* has a relatively low abundance at young age, and reaches the maximum value after 20 years. It is worth noting that *Phylchara* only appears in the roots of 8a *Machilus pauhoi*, and ranks the largest proportion of each species.

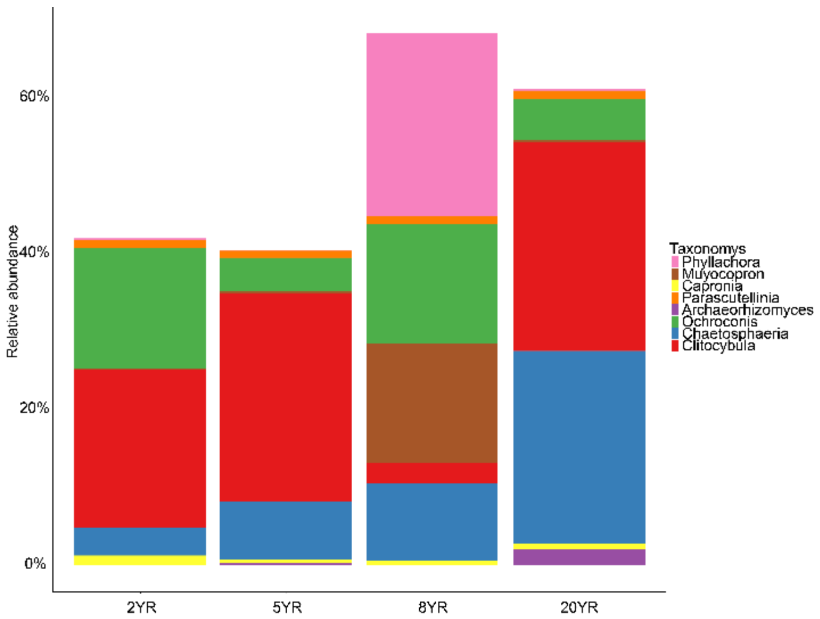

Figure 6. *Composition of fungi community at a genus level*

The relative abundance distribution of fungi in roots of Machilus pauhoi in different age groups

In terms of their abundance, the top 30 species are selected (all species are selected if there are less than 30 species). The relative abundance of fungi in the roots of *Machilus pauhoi* of different age groups are clustered based on their average abundance of different growth age at both the phylum and genus level, and the heatmap is obtained (*Fig. 7*). At phylum level, *Blastocladiomycota* and *Annelida* are most abundant in the roots of 20a *Machilus pauhoi*; *Basidiomycota* and *Chytridiomycota* are most abundant in the roots of 5a *Machilus pauhoi*; *Arthropoda* is most abundant in the roots of 2a *Machilus pauhoi* and *Kickxellomycotina* and *Zoopagomycotina* are most abundant in the

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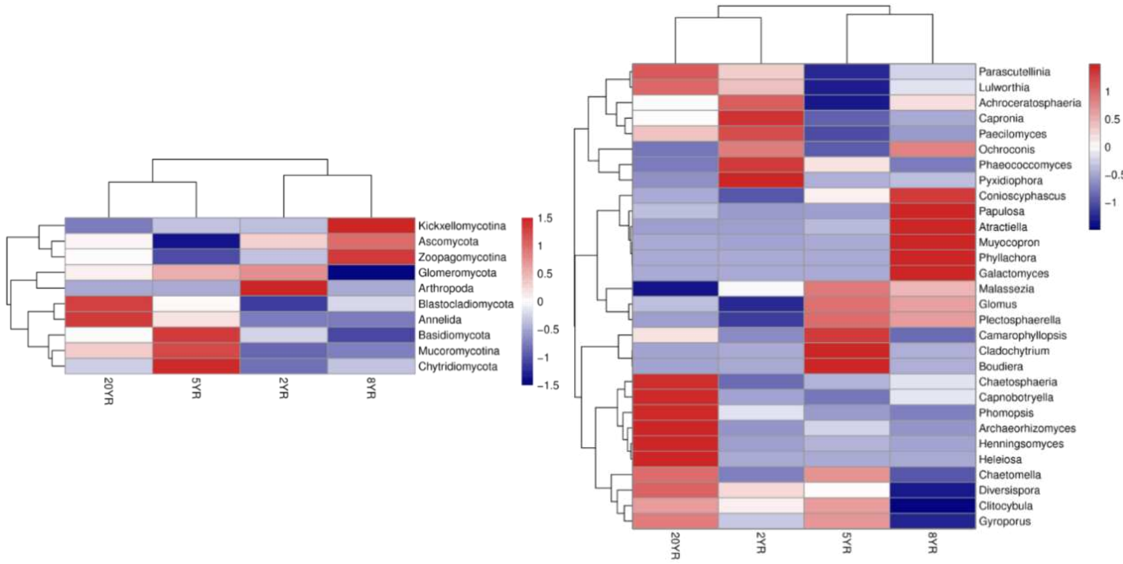
DOI: http://dx.doi.org/10.15666/aeer/1702_20732083

roots of 20a *Machilus pauhoi*. The genera with high relatively abundances in each sample are also different: *Chaetosphaeria*, *Capnobotryella*, *Phomopsis*, *Archaehorhizomyces*, *Henningsomyces* and *Heleiosa* are most abundant in the roots of 20a *Machilus pauhoi*; *Capronia*, *Phaeococcomyces* and *Pyxidiophora* are most abundant in the roots of 2a *Machilus pauhoi Camarophyllopsis*, *Cladochytrium* and *Boudiera* are most abundant in the roots of 5a *Machilus pauhoi* and *Conioscyphascus*, *Papulosa*, *Atractiella*, *Muyocopron*, *Phylchara* and *Galactomyces* are most abundant in the roots of 8a *Machilus pauhoi*, which indicates a difference in the abundance of fungi in the roots of *Machilus pauhoi* in different age groups. Due to the high similarity between the 2a and the 20 samples, they are grouped together. For the same reason, 5a and 8a samples are clustered as one group.



a b

Figure 7. *Abundance heatmap of species at different level of root samples in various age groups of stand (a: phylum; b: genus)*

Discussion

High-throughput sequencing accurately reflects the composition and proportion of the fungi with different abundance in the roots of *Machilus pauhoi*, especially the species with low abundance, and objectively and comprehensively reveals the fungi structure in the roots in different age groups, making contributions to enriching plant endophytic resources and giving full play to high-throughput sequencing.

In this study, based on the Illumina Miseq sequencing platform, the diversity of fungi in the roots of *Machilus pauhoi* has been successfully detected, and a large amount of comprehensive and in-depth information on the fungi communities has been obtained. A total of 557,906 optimized sequences and 424 OTUs were obtained. A total of 10 fungi phyla and 32 genera were separated from the samples in different age groups. The rarefaction curves analyzed the sampling depth of root samples of different age groups. The number of OTUs in each sample presented the same or similar variation trend, that was, with the increase of sequencing depth, the increase of OTU number tended to slow down and eventually, reached basic saturation.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20732083

The *Machilus pauhoi* in this study were in the same plantation but in different age groups, so the environmental difference was very small. The air humidity, climate, temperature, latitude and longitude, altitude, soil pH and concentrations of N, P, K and other soils in the same area were basically the same. During the experiment, a unified experimental method and data analysis method were employed. Therefore, the growth age became the dominant factor in the diversity of fungi in the roots of *Machilus pauhoi*.

A total of 424 fungi OTUs were obtained from *Machilus pauhoi* in 4 different age groups. According to the Venn diagram (*Fig. 4*), in the second and eighth year, the number of OTU is large, but it is small in fifth and twentieth year. With the increase of growth age, the fungi abundance and diversity index decreases first and then increases, reaching minimum value in the eighth year. In the analysis of community structure, with the increase of the age, the community structure of endophytic fungi in the roots of *Machilus pauhoi* changes dramatically. In the cluster analysis of relative abundance in different age groups, the fungi in 2a and 20a samples are clustered as one group and fungi in 5a and 8a samples are grouped together, indicating that the composition of endophytic fungi in second and twentieth year is more similar but is different from that in the fifth and eighth year. The results indicate that, with the increase of age, the fungi community in the roots of *Machilus pauhoi* changes to a certain degree and then goes back to the young age.

Through the above results, it can be clearly seen that there are a large number of endophytic fungi colonization with great diversity in the roots of *Machilus pauhoi* in the plantation. However, with the growth and development of *Machilus pauhoi*, endophytic fungi colonized in its roots have undergone certain changes, which might be related to the regulation of plants themselves in the process of adapting to the environment. When the plant was initially planted in the plantation field, there were lots of endophytic fungi in the roots. However, as the growth and development of plant and adaption to the environment, the number of competitive relationships among endophytic fungi colonized in the roots decreases. Then, after the plants slowly adapt to the surrounding environment, some fungi have established stable parasitic relationships with plants. Therefore, the number of fungi colonized in the roots of the plants increases.

It is worth noting that the characteristic genus, *Phylchara*, appears in the roots of 8a *Machilus pauhoi*, and its relative abundance is the highest among all species. This might be related to the decline in quality and productivity in forest land soil. *Ascomycotina*, *Ascomycetes*, *Phyllachorales*, and *Phyllachoraceae* are a class of obligate parasitic vegetative fungi (Mccoy et al., 2018; Denman et al., 1999). The *Phyllachora* fungi are widely distributed in plants in tropical and subtropical regions. *Phyllachora graminis* is a main kind of plant disease for precious tropical native species in Hainan Province, such as *Dalbergia odorifera*, *Pterocarpus macrocarpus*, and *Pterocarpus santalinus* (Liu et al., 2016). Combined with the symptoms of *Phyllachora graminis* (Levente et al., 2018; Reddell et al., 1999; Egham et al., 1992), it can speculate that the scabs formed on *Machilus pauhoi* might be related to these species, but further experimental verification is needed. Moreover, the reduction of fungal community diversity in eighth year might be related to the dominant position of phytopathogenic fungi.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20732083

Conclusions

This study, for the first time, used high-throughput sequencing method to study the fungi community structure in the roots of *Machilus pauhoi*, a secondary protected plant, in the experimental forest in Yangshan County, Qingyuan City, Guangdong Province. The results show that endophytic fungi in the roots have a great diversity and great research value. The dominant species are *Ascomycota* and *Basidiomycota* at phylum level and *Clitocybula*. *Ochroconis* and *Chaetosphaeria* at genus level. With the increase of the age, the fungi abundance and diversity index decreases first and then increases. *Phylchara*, might be related to scabs formed on *Machilus pauhoi*, appears in the roots of 8a *Machilus pauhoi.* These conclusions can provide a theoretical basis for the protection of the community of *Machilus pauhoi*. Personally, the experiment only focused on the change of fungal community structure, ignoring the effect of other factors such as bacteria. Future researches should carry out multiple factors analysis and comparative experiments to explore the regulatory relationship between other factors such as microorganisms, insects and human activities destruction and the structure and function of the fungal.

Acknowledgements. This work was financially supported by China West Normal University Doctoral Startup Research Project (412666).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20732083

RESPONSES OF TROPHIC STRUCTURE VARIATION IN FISH ASSEMBLAGE TO HYDROLOGIC REGULATION IN A TRIBUTARY OF THE THREE GORGES RESERVOIR, CHINA

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(Received 8th Nov 2018; accepted 11th Jan 2019)

Abstract. Responses of trophic structure to reservoir regulation are important to manage and conserve the river ecosystem influenced by dams. We used stable isotope-derived community-wide metrics to describe spatiotemporal variation in trophic niches and trophic structures of fish assemblages in a tributary of the Three Gorges Reservoir (TGR) in China where water level regulation is put into operation annually. A longitudinally increasing trophic space was discovered in this tributary especially when the water level was low. Trophic diversity declined and trophic redundancy increased from the non-flood period to flood season at the upper backwater region. However, trophic space was more stable in the estuarine site. The site differences were associated with availability of food resource and pathway of consumers, as specifically, disturbance derived from flow pulse at the end of backwater site altered diet composition of the fish consumers, while immense water body in the lower confluence area limited the flood influences as well as promoted exploitation of diverse available sources. Moreover, the declined water level control facilitated the contraction of the trophic niche size at the tail backwater site because of remarkable riverine morphology alternation leading to decreasing water body whereas the rising water level regulation homogenized trophic structure along the downstream river. Our study revealed that there was different response of fish assemblages along longitudinal fluvial gradient to the water level regulation in the reservoir and targeted management and conservation measures are needed for the different sections of the tributary.

Keywords: *reservoir regulation, stable isotope, community metrics, pathway, food resource*

Introduction

For purposes of flood control, power generation and/or navigation, dams have been constructed on the majority of large rivers globally since the last century (Avakyan and Iakovleva, 1998) and these river systems have been affected spatiotemporally to varying

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

extent (Nilsson et al., 2005). By altering thermal and flow regimes as well as sediment transport (Kondolf, 1997; Graf, 2006), the presence of dams leads to subsequent changes in biotic assemblages (Cumming, 2004; Freedman et al., 2014) and contributions of allochthonous and autochthonous nutrients (Doi et al., 2008; Sabo et al., 2018). As a result, a variety of trophic shifts may occur resulting in alteration for trophic diversity and food web structure (Mercado-Silva et al., 2009; Growns et al., 2014; Kaymak et al., 2018), which ultimately affect river ecosystems function. Although numerous studies have been devoted to understanding how dams influence biodiversity and food-web interactions (Holmquist et al., 1998; Bunn and Arthington, 2002; Cross et al., 2011), responses of rivers and river ecosystems to dams are intricately varied and usually show contrasting results (Growns et al., 2014), as they depend on local geomorphic, hydrological and biologic attributes (Power et al., 1996).

Tributaries could promote the function of large river ecosystems, such as enhancing the overall biodiversity of large rivers (Fernandes et al., 2004), resetting the ecological continuum of the mainstem (Kiffney et al., 2006), and maintaining fish reproductive success with retained natural flow regime (Pracheil et al., 2009). Certain tributaries can sometimes serve as a sanctuary for many indigenous species after the mainstem has been altered by dams (Park et al., 2003; Pracheil et al., 2013). Hence, tributaries, by virtue of lower degree of alteration could provide underappreciated opportunities for conserving biodiversity (Pracheil et al., 2013) especially in the impounded reaches where lotic species are more susceptible to environmental changes. However, much research focuses on downstream effects (Cross et al., 2011; Sabo et al., 2018) or on longitudinal patterns of the river continuum (Mercado-Silva et al., 2009; Cross et al., 2013; Growns et al., 2014) from the dam. There is a paucity of information pertaining to variation in trophic diversity in the upper tributaries which are also influenced by the regulated reservoir.

The Three Gorges Dam (TGD) is built in the middle of the Yangtze River, the largest river in China, and has created a reservoir 600 km long with a total storage capacity approaching 40 billion m^3. The dam has been put into operation in 2003 and filled initially up to 175 m, the normal pool level, in 2010. Since then, the reservoir would be drawn down to 145 m water level during the flood season (May-September) for flood control, and impounded to 175 m water level after the flood season and maintained until the next year for power generation and navigation. In the Three Gorges Reservoir (TGR) region, six tributaries with the watershed area larger than 3000 km^2 (ten tributaries more than 1000 km^2) flow into the Yangtze River. These tributaries have supported the mainstream biodiversity and possess the potential to be suitable refuges for the affected biota (Park et al., 2003). However, the same water fluctuation cycle as the mainstream occur at the lower reaches influencing the fish assemblages (Yang et al., 2013; Zhao et al., 2015) and thus would likely impact the energy flow and trophic structure as well. The Daning River is one of these important tributaries with less damaged natural landscape in particular in the pristine upper reaches. Previous study has analyzed whether the impoundment affected assimilation of carbon sources by fish consumers in this tributary (Deng et al., 2018). Isotope mixing model results indicated that riparian C_3

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

plants are the consistently major energy source for fish consumers irrespective of reaches or feeding strategies because of abundant standing stock of riparian vegetation and hydrologic regimes in this river (Deng et al., 2018). This tributary suffers the same hydrological regulation in the backwater accompanied by seasonal flood flows from the uppers as other tributaries in TGR. However, trophic structure shifts on account of spatiotemporal variation in environmental conditions are still unknown. The present study extends previous research on the Daning River to explore the effects of water level regulation on food web structure of local fish assemblages.

Stable isotopes, providing a concrete quantitative measure of niche width and trophic diversity (Bearhop et al., 2004; Layman et al., 2007a; Schmidt et al., 2007; Jackson et al., 2011), are widely used to examine structural features and variations of food webs influenced by species invasion (Jackson et al., 2012), overexploitation recovery (Hamilton et al., 2014), land use (de Carvalho et al., 2017), river fragmentation (Freedman et al., 2014; Kaymak et al., 2018). In this study, we employed stable isotope-derived community metrics to describe and compare trophic diversity of fish assemblages among sites and between periods. The purpose of this study was to examine the spatiotemporal variation in trophic niches and trophic structures of fish assemblages and further how local food webs responded to changes in the environmental conditions enhanced by hydrological regulation. We hypothesized that trophic niches size of fish assemblage increased along longitudinal gradient within the tributary due to the enlarged water body and the effects of flood flow on structure diversity would mitigate by water level conditions. The study on spatiotemporal variation of food web structure in association with flood flows and water level fluctuation in this important tributary could help provide the necessary references for understanding how reservoir regulation could affect overall river systems in the region. Further, this information could be benefit to ongoing management and conservation efforts in the tributaries alike.

Materials and Methods

Study sites and sampling design

This study was conducted in the Daning River, a tributary of the Yangtze River, located in the central part of the Three Gorges Reservoir (*Fig. S1*) in China. The tributary watershed covers an area of 4426 km^2, and is 200 km long with 47-60 km backwater channel due to the impoundment of TGR. The backwater area vary in length and depth according to the hydrologic regulation of the TGR. The river is canyon bound scattering several open sections with less steep slopes where both C_3 and C_4 vegetation dominate the riparian areas. The valley has subtropical humid monsoon climate with mean average temperature of 16.6°C and an annual mean precipitation of 1124.5 mm, about 80% of which falls in May-October. As a result, the maximum flow in the river is generally observed from June to September (*Fig. 1*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

Three sites representing the estuary area (Wushan), the end of the backwater water region (Dachang) and the natural fluvial site (Wuxi) were selected for sampling (*Fig. S1*, *Table 1*). The Wushan site is located in the confluence to the reservoir, 123 km upstream of the Three Gorges Dam. This site is characterized by steep littoral zones with little vegetation cover because of the populated areas. The estuary water area is nearly 1.2 km wide and 55 m deep at the lowest water level and 1.3 km wide, 68 deep when the water level rises to 175 m (*Table 1*). The Dachang site is located 45 km above the estuary, near the end of the backwater water. In this zone, the channel is wider with less steep slopes, which forms 9 km^2 lacustrine environment with the depth of 33 m at the highest water level (*Table 1*).

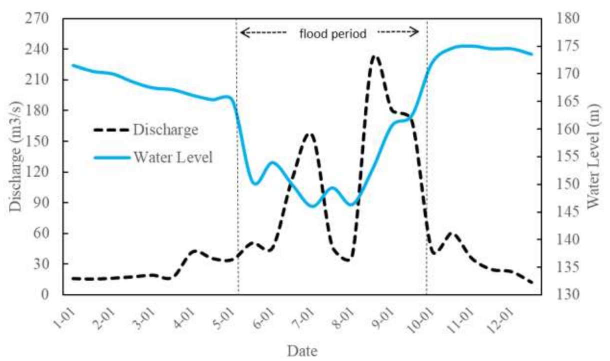

Figure 1. *Water discharge (dash line) from Wuxi hydrologic gauge station (in the upper stream) and water level (solid line) at the Wushan site in Daning River*

Table 1. *Environmental characteristics of the sampling sites along the Daning River (NF = non-flood period or high water level; F = flood period or low water level)*

	Wushan		Dachang		Wuxi	
	NF	F	NF	F	NF	F
Water depth (m)	68	55	33	19	1.5	3
Width (m)	1300	1200	1000	700	25	60
Discharge (m^3/s)	—	—	—	—	34	122
Velocity (m/s)	0.010	0.015	0.008	0.035	0.82	1.38
Transparency(m)	1.85±0.82	0.46±0.18	1.70±0.47	0.68±0.50	—	—
Water temperature (ºC)	19.1±2.9	21.5±0.2	17.8±2.0	21.3±0.1	13.6±2.1	18.3±0.2
Dissolved oxygen (mg/L)	7.9±2.8	7.4	8.2±2.1	8.6	9.6±2.7	9.8
Total phosphorus (mg/L)	0.11±0.07	0.14±0.05	0.07±0.03	0.03±0.02	0.03±0.02	0.08±0.05
Density of phytoplankton (million cells/L)	5.55±8.46	21.11±12.77	1.55±1.37	1.22±0.31	0.18±0.21	1.37±0.34

During the lower water levels, however, large lateral habitats characterized by muddy substrates expose out of the water and dense macrophyte mats would appear along the littoral zone. The Wuxi site is an upstream site where riverine system has not yet affected by the TGR. The section is characterized by lotic and shallow habitats with the substrate dominated by rock and cobble. Due to pronounced seasonality in rainfall, highest annual river discharges typically occur from June to September with an average

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

of 122 m^3/s and the average discharge drops to 34 m^3/s between October to May (*Fig. 1*). According to the water-level regulation in the TGR as well as river monthly discharges from Wuxi hydrologic gauge station, two sampling periods that reflected strong seasonal patterns in precipitation, water-level and thus, the potential for observing changes in trophic structure of fish assemblages were chosen (*Fig. 1*). In order to detect temporal differences in trophic structure based on consumer stable isotope composition, enough time need for muscle tissue to reflect any new diet (Abrantes et al., 2014). At each period, consumers were living for more than two or three months under that condition before sampling (Boecklen et al., 2011; Abrantes et al., 2014). Therefore, in the Daning River, fishes collected in September-October were referred as samples from the flood period and in December-May were referred as from the non-flood period (*Fig. 1*). All the data we used were collected from 2011 to 2013 and were classified into two periods.

Sample collection and analysis

Fish were collected from the river using 2 m by 50 m gillnets with different mesh sizes (30, 60, 80 and 120 mm). After captured, the number and weight of fish species collected were recorded. Fish were classified into five trophic groups based on available literature in Daning river (The Fish Laboratory, 1976; Zeng, 1990; Ding, 1994), which included planktivores, herbivores, benthivores, omnivores and piscivores (*Table S1*). A total of 68 fish species in this tributary were captured in our several surveys. However, the most dominant species from the different trophic groups were selected for isotopic analysis based on the abundance and frequency of capture in the surveys (Yang et al., 2013) so that the species analysed at each site during each period represented well their trophic groups. For each species, only large adult individuals were selected in case of the influence of ontogenetic diet shifts on isotopic value. For selected individuals, fish were weighed (g), measured (mm), and dissected to extract approximately 5–10 g of dorsal muscle tissue for isotopic analysis. Samples of basal carbon sources were also collected and detailed information are described in Deng et al. (2018).

All fish samples were dried at 60 °C for at least 48 h and ground into a fine powder and stored in glass vials. Carbon and nitrogen stable isotopic analysis was done at the Stable Isotope Laboratory, Chinese Academy of Forestry, Beijing, China, using a Flash EA1112 HT Elemental Analyzer coupled to a DELTA V Advantage Isotope Ratio Mass Spectrometer. Stable isotope ratios are expressed as delta (δ) and defined as parts per thousand (‰) relative to the laboratory standard material. The ratios is calculated by *Equation 1*:

$$\delta X = (R_{\mathrm{sample}} - R_{standard})/R_{standard} \times 10^3‰ \quad \text{(Eq.1)}$$

where X is ^{13}C and ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. R is the molar ratio of the heavy to light isotope of the sample and standard (‰). The standard

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material used for carbon and nitrogen are Pee Dee Belemnite (PDB) limestone and atmospheric nitrogen. Precision of the isotopic analysis was 0.1‰ for carbon and 0.2‰ for nitrogen.

Data analysis

Five quantitative community-wide metrics derived from stable isotope data (Layman et al., 2007a) were used to describe variation in trophic structure of fish assemblages at each site during each period. Because not all species occurred at all sites, analyses were based on fish trophic guilds. Three of these metrics are community-wide measures of trophic diversity, including $\delta^{13}C$ range (CR), which depicts basal source diversity, $\delta^{15}N$ range (NR), which gives information on trophic length, and mean distance to centroid (CD), which is calculated as the mean Euclidean distance of each assemblage component to the centroid and a measure of the average degree of trophic diversity within the assemblage (Layman et al., 2007a). Two additional metrics reflect the extent of trophic redundancy, namely mean nearest neighbour distance (NND) and standard deviation of the nearest neighbor distance (SDNND). MNND is an estimate of density and clustering of species within the community and SDNND measures the evenness of the groups' spacing in the bi-plot space (Masese et al., 2018). Smaller NND and SDNND indicate greater trophic redundancy, which means, groups have more similar trophic niches (Abrantes et al., 2014; Masese et al., 2018). Because riparian plants are the major carbon source to fishes and widespread along the river channel (Deng et al., 2018), the range in potential source $\delta^{13}C$ was similar for all sites, meaning that the community-wide metrics are comparable among sites and periods (Layman et al., 2007a). In order to provide more robust metrics that are unbiased with respect to variations in the number of groups and allow for statistical comparisons among sites and periods, Bayesian methodology was implemented to generate a posterior distribution of estimates of these metrics, thus providing a measure of uncertainty of these metrics for the fish communities at each site and period (Jackson et al., 2011). Then the comparison was conducted graphically based on the visual analysis of the credibility intervals and the degree of overlap between the Bayesian distributions was used as an indication of similarities or dissimilarities between sites and periods (Abrantes et al., 2014; Masese et al., 2018).

The area of $\delta^{13}C$- $\delta^{15}N$ space occupied by all consumers generally represents a measure of trophic niche size and thus a proxy for the total extent of trophic diversity (Layman et al., 2007a). Instead of total area, which is highly sensitive to sample size, standard ellipse area (SEA) was used to quantify the trophic niche space of fish assemblages (Jackson et al., 2011). SEA is equivalent to bivariate standard deviations and robust to variation in sample size or the number of groups (Jackson et al., 2011). Two versions of SEA, Bayesian standard ellipse area (SEA_b), which estimate via Bayesian approach taking account of sampling error and returning probability distributions, and size-corrected standard ellipse area (SEA_c), which correct bias towards underestimation of the SEA when sample sizes are small, were calculated for

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niche space comparisons of different fish assemblages. All metrics were calculated using the R statistical computing package, "Stable Isotope Analysis in R"(SIAR) and "Stable Isotope Bayesian Ellipses in R" (SIBER) (R Development Core Team, 2007; Parnell et al., 2008, 2010; Jackson et al., 2011).

T-test and ANOVA were used to compare $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures data from different periods or sites. Pairwise differences were tested using Dunnett T3's post hoc test. Statistical analyses were conducted using IBM SPSS Statistics (16.0). Statistical significance was determined at $p = 0.05$.

Results

Spatiotemporal differences in $\delta^{13}C$ and $\delta^{15}N$ values of fish assemblages

At Wushan, a total of 42 fish species were found. Each 22 dominant fish species were sampled during the non-flood and flood period (*Table S1*). The $\delta^{13}C$ and $\delta^{15}N$ values of fish assemblages ranged from -29.74‰ to -17.60‰ and 4.54‰ to 17.51‰, with an average of -23.52‰ and 9.37‰ in the non-flood period and an average of -22.02‰ and 10.94‰ in the flood period, respectively. There are also 42 fish species captured at Dachang site. The 27 and 23 dominant species were sampled in the non-flood and flood period (*Table S1*). Fish $\delta^{13}C$ values varied from -28.57‰ to -16.61‰, with an average of -23.70‰ in the non-flood period and -23.51‰ in the flood period. Fish $\delta^{15}N$ values varied from 4.91‰ to 14.15‰ and the average $\delta^{15}N$ values of fish assemblages were 9.10‰ and 9.78‰ in the non-flood and flood period, respectively. At Wuxi, 35 fish species were captured during the surveys. The 13 and 15 dominant fish species were sampled in the non-flood and flood period (*Table S1*). The range of $\delta^{13}C$ and $\delta^{15}N$ signatures was from -25.31‰ to -21.56‰ and 2.25‰ to 10.81‰.

There were significant differences in fish $\delta^{13}C$ between the non-flood and flood period at Wushan site (t-test, $t = 2.855$, $p < 0.01$) and no significant differences at Dachang (t-test, $t = 0.382$, $p = 0.703$) and Wuxi site (t-test, $t = 1.297$, $p = 0.201$). During the non-flood period, there are no significant differences in $\delta^{13}C$ among sites (ANOVA, $F = 0.096$, $p = 0.908$). However, the average fish $\delta^{13}C$ value displayed significant differences among sites (ANOVA, $F = 4.577$, $p < 0.05$) during the flood period. Fish assemblages at Wushan were more enriched in $\delta^{13}C$ than that at Dachang (Dunnett T3's method, $p < 0.05$) and Wuxi (Dunnett T3's method, $p < 0.05$) in the flood period. Similarly, differences in fish $\delta^{15}N$ signature between the non-flood and flood period were significant at Wushan (t-test, $t = 2.497$, $p < 0.05$) but not significant at Dachang (t-test, $t = 1.916$, $p = 0.058$) and Wuxi site (t-test, $t = 0.947$, $p = 0.349$). There are significant differences in $\delta^{15}N$ values among sites within each period (non-flood: ANOVA, $F = 14.129$, $p < 0.01$; flood: ANOVA, $F = 18.158$, $p < 0.01$) and only $\delta^{15}N$ values at Wushan and Dachang did not differ significantly (Dunnett T3's method, $p = 0.07$) during the non-flood period. Overall, average $\delta^{15}N$ value of fish assemblages increased from upstream site to the downstream site.

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Spatial differences in trophic diversity and structure

SEAs of fish communities differed in size, shape and position in the bi-plots (*Fig. 2*). Generally, standard ellipse areas sizes declined with sites upstream from the confluence. In the flood period, the SEA of the Wushan site was larger than that of Dachang site, of which was sequentially greater than the SEA of the Wuxi site. SIBER indicated that the SEA_b at the downstream sites had more than 97% probability of being larger than the SEA_b at the upstream sites for the flood period in each two-sites comparison (*Table 2*). In the non-flood period, however, the SEA sizes were more similar between at the Wushan and Dachang sites, which were both larger than at the Wuxi (*Table 2*). The probability that the SEA_b of the Wushan was larger than that of the Dachang in the non-flood period was only 56% (*Table 2*) and this lack of differences was also demonstrated by the high overlaps in the SEA_b 50% CIs between these two sites (*Fig. 3*). Besides, there was more overlap between the different SEA in the non-flood period than in the flood period (*Fig. 2*, *Table 2*). SEAs of the Wushan site showed larger overlap with the Dachang site during non-flood period than in the flood period (14.41‰^{2} in the non-flood period and 4.02‰^{2} in the flood periods) (*Table 2*) and the distributions of Bayesian results (SEA_b) showed that the probability was 100%.

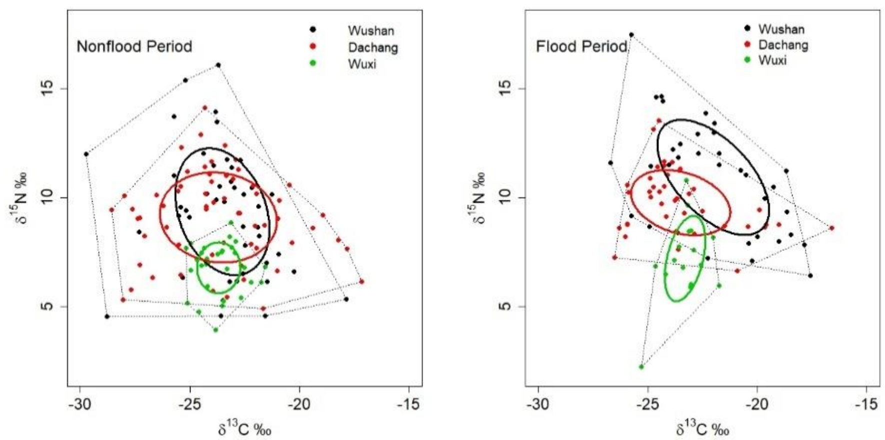

Figure 2. *Mean stable isotope composition of the different fish species from the three study sites for the non-flood and flood period. Solid lines represent the standard ellipses area, and dotted lines are the convex hull areas of fish assemblages for each site*

Five Layman's community metrics also varied among sites in each period (*Figs. 4*, *5*). In the flood period, all the metrics at the Wushan were the largest than the other two sites except SDNND, whereas the metrics at the Wuxi showed the widest range of values (*Figs. 4*, *5*). In the non-flood period, the five metrics were more similar between Wushan and Dachang, with a large overlap of the 50% CIs for each metrics in these two sites (*Figs. 4*, *5*). However, the Wuxi site showed the smallest mode value for all the metrics compared to the two downstream sites in the non-flood period.

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Table 2. *Small size-corrected standard ellipse areas (SEAc), probability that the SEA in the flood period is larger than the SEA at the non-flood period, overlap in SEA between periods for the same site and overlap in SEA between pairs of sites*

	SEA_c (‰^{2})	p Value SEA flood > non-flood	Overlap in SEA_c (SEA_b) between periods (‰^{2})	SEA overlap between pairs of sites (‰^{2})		
				Wushan	Dachang	Wuxi
flood period						
Wushan	17.42	0.720	**5.80** (3.69-10.64)		**4.02** (1.98-6.65)	**0.00** (-0.06-0.89)
Dachang	10.18	0.001	**9.89** (5.88-10.99)	1.00		**0.83** (-0.18-1.96)
Wuxi	5.15	0.861	**2.87** (1.15-3.77)	0.97	1.00	
non-flood period						
Wushan	18.87				**14.41** (9.76-15.67)	**1.92** (0.54-3.43)
Dachang	17.30			0.56		**1.24** (0.04-2.20)
Wuxi	3.62			1.00	1.00	

Note: The top halves of the matrices show the SEA overlap between sites, in ‰^{2}. The bottom halves of the matrices with grey shading show the Bayesian probability (in %) that the SEA of group 1 (sites from the first column) is smaller than that of group two (sites from the first row). Values in brackets are 95% Bayesian credibility intervals estimates by models. Values in bold are calculated by SEA_c

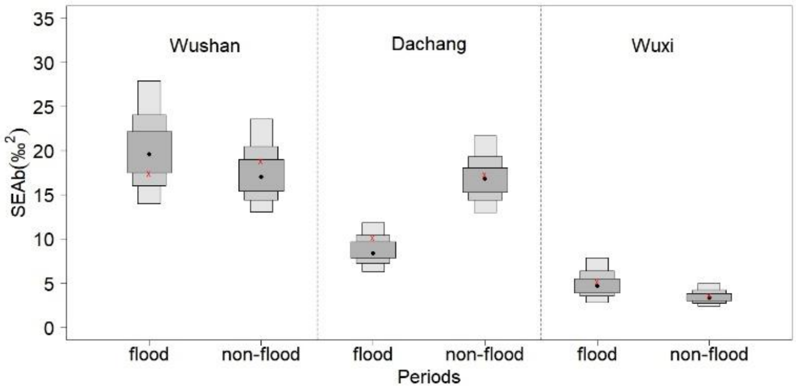

Figure 3. *Density plots of the credibility intervals of the standard ellipse areas (SEA). Black dots are the mode SEA, and boxes indicate the 50%, 75% and 95% credible intervals. Red crosses are the small sample size-corrected SEA (SEA_c)*

Temporal variation in trophic diversity and structure

For Wushan site, there was a temporal change in SEA position with higher $\delta^{13}C$ and $\delta^{15}N$ values in the flood period (*Fig. 2*). However, SEA did not differ in size between periods at this site (*Table 2*, *Fig. 3*). The overlap in SEA between periods was relatively low (5.85‰^{2}, corresponding to 33.32% and 30.77% of the total SEA for the flood and non-flood period, respectively; *Table 2*). CR, NR, CD and NND were wider in flood period than in non-flood period with the slight overlap in the 75% or 95% CIs of the distributions of Bayesian results (*Figs. 4*, *5*), suggesting that the fluctuation of water-levels and the discharge during the flood period increases isotopic trophic diversity and decreases trophic redundancy at the Wushan site.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

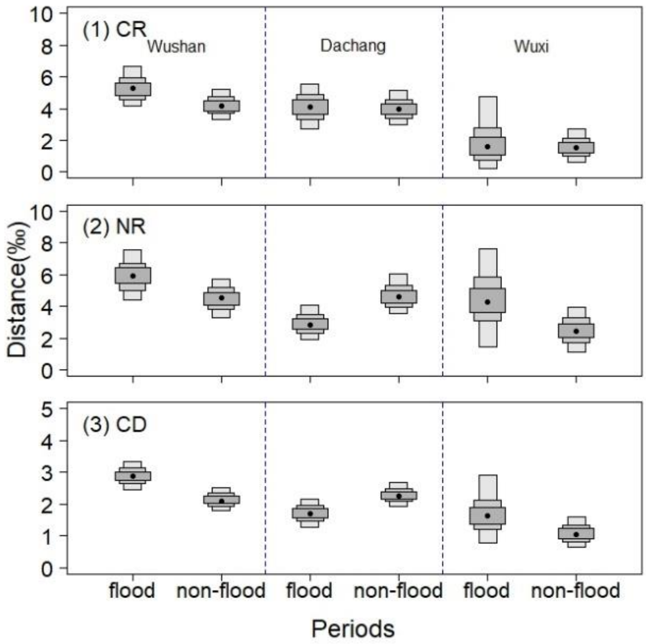

Figure 4. *Bayesian results for the three Layman stable isotope-based community-wide metrics that provide information on trophic diversity at each site. CR: Stable isotope of carbon ($\delta^{13}C$) range; NR: stable nitrogen isotope ($\delta^{15}N$) range; CD: mean distance to centroid. Black dots are the mode value (‰) and boxes indicate the 50%, 75% and 95% credibility intervals*

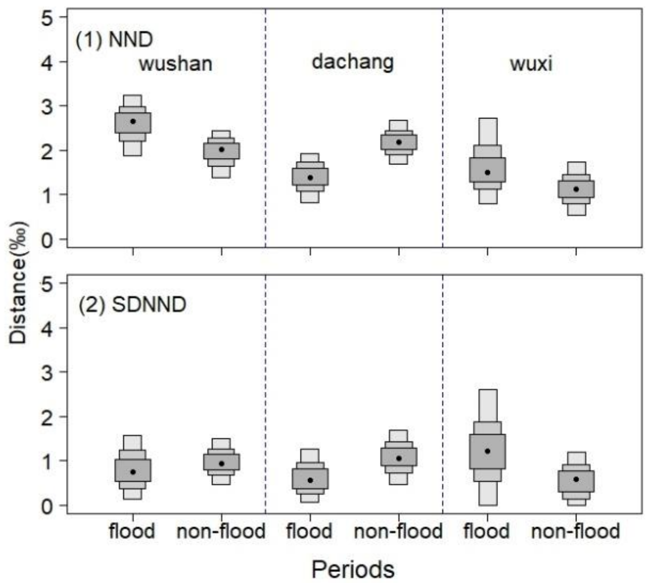

Figure 5. *Bayesian results for the two Layman stable isotope-based community-wide metrics that provide information on trophic redundancy at each site. NND: mean nearest neighbor distance; SDNND: standard deviation of mean distance to centroid. Black dots are the mode value (‰) and boxes indicate the 50%, 75% and 95% credibility intervals*

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Conversely, fish groups from the Dachang site showed no evidence of temporal changes in SEA position but increased in size in the non-flood period when 99.9% of the SEA_b was larger than that in the flood period (*Table 2, Figs. 2, 3*). The area overlap between the two periods' standard ellipses was 9.89‰^{2}, corresponding to 97.21% and 57.21% of the total SEA of the flood and non-flood period, respectively (*Table 2*). NR, CD and NND were significantly narrower in flood period (*Figs. 4, 5*) which confirmed by Bayesian results that 98.40%, 97.15% and 97.93% of the metrics in flood period were smaller than in the flood period, respectively. However, CR was similar for both periods and there was high overlap in the 95% CIs for SDNND (*Fig. 5*).

As for Wuxi site, there was slight seasonal differences in SEA size (*Fig. 3*). The SEA_b in the non-flood period had 89.8% probabilities of being larger than the SEA_b in the flood period and the SEA overlap between periods was 2.87 ‰^{2} (*Table 2*), which accounted for 55.81% and 79.36% of the whole SEA in the flood and non-flood period, accordingly. As well, for the five Layman's metrics, there was little evidence of temporal differences, which were mostly a result of the large overlap in the 95% CIs between periods, due to the wide range of every value in the flood period (*Figs. 4, 5*).

Discussion

Longitudinal patterns of trophic diversity in fish assemblages

The lowest site, Wushan, displayed the largest SEA while the upper site showed the lower values (*Fig. 3*) suggesting the greater niche space in the downstream, which was also supported by higher CR and CD values implying higher trophic diversity in Wushan (*Fig. 4*). This longitudinally increasing tendency, which is also discovered in other impounded river (Kaymak et al., 2018), is much more evident in the flood season (*Fig. 3*) when the water level is relatively low and the discharge varies. It is reported that there are 42 and 35 fish species recorded at downstream and upstream respectively after the impoundment of TGR (Yang et al., 2013). Increased fish species richness in the downstream gives the opportunities for consumers to exploit more food resources and occupy new niches (Roach et al., 2009). Nevertheless, the opposite was the case in the upstream, Wuxi site, where fish community revealed a lack of planktivorous group and preponderance of omnivorous group (*Table S1*) (Ding, 1994). Besides, several fish species, such as *Coreius heterodon*, distributed mainly in the mainstream, was only found in the estuary in this tributary. These fish species could integrate the food carbon source from the mainstream. On the other hand, although the former study has verified that riparian C_3 plants are the main basal food source irrespective of reaches or feeding groups (Deng et al., 2018), the CRs, which are indicative of food source diversity in the backwater area are significantly larger than those in the upper lotic reaches (*Fig. 4*). This is likely explained by the massive backwater area as well as large periodic inundated zone in the downstream that boost diverse fish species assemblage and alternative food resource presence (Deng et al., 2018).

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DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

Temporal variation in trophic structure of fish assemblages

Seasonal differences in food web structure were observed at the downstream sites. At Wushan, the slight overlap of the SEA (*Table 2*) suggested that the position of the fish community had change substantially between two periods although the area occupied was similar. This position alternation is attributed to the change in both the base and the vertical structure of the food web influenced during the flood period (*Figs. 2, 4*). A widening of food source (larger CR) in flood season when the water level is low and fluctuant probably results from the carbon subsidies in the estuary from the mainstream where terrestrial C_4 plants is important to fish species (Wang et al., 2014). Therefore, the fish community had significantly higher $\delta^{13}C$ values in flood season (*Fig. 2*). The increase in trophic length (NR) is also likely attributed to enlarged optional food resource. There is strong evidence that food web length increases with productivity or resource availability (Pimm and Kitching, 1987; Sabo et al., 2009; Takimoto and Post, 2013). Previous reports have confirmed that planktonic algae, of which biomass in the estuary peaked in June and August during the flood season (*Table 1*) (also see Zhu et al., 2013), is an important secondary carbon source in the downstream (Deng et al., 2018). Exploitation of diverse available sources, both abundant from terrestrial and aquatic, as well as tributary and mainstream, leads to a higher trophic diversity (larger CD) and lower trophic redundancy (higher NND) at the lower water level (*Figs. 4, 5*).

In the tail of backwater region (Dachang site), there was a decrease in niche area and trophic diversity during the flood season. In general, an increased range of available food sources during the flood season, e.g. increased input of terrestrial organic matter by surface run-off, leads to a higher consumer trophic diversity and expanded niche sizes (Junk et al., 1989). However, the considerably overlapping in CR between two periods suggested that fishes exploited similar food sources, which could be consolidated by the importance of C_3 plants as the major and consistent carbon source supporting fish species because of the predominance of nutritious riparian C_3 plants in vast periodic inundated area at Dachang (Deng et al., 2018). Consequently, the decrease of niche sizes was associated with the shorter trophic length in the flood season (*Figs. 2, 4*), perhaps as a function of fluctuant water level and discharge resulting in limiting intermediate predators as prey for fish (McHugh et al., 2010). Environmental disturbance such as hydrologic variability can influence food web length in many ecosystems (Post, 2002; Sabo et al., 2009) and some argue that flood disturbance with more unpredictable flow regimes generally reduce food web length in temperate stream systems (Marty et al., 2009; Sabo et al., 2010; McHugh et al., 2010). In flood season, an effect of drastic perturbation on the backwater ecosystem in Daning River derives from dramatically descending water level and stochastic discharges from the upstream (*Fig. 1*). As a result, trophic length at Dachang would be shorten through disturbance-induced change in diet strategies according to relative accessibility of a prey (McHugh et al., 2010; Ruhí et al., 2016). In addition, the flood disturbance could further enhance fish consumers to prefer abundant C_3 plants than diverse food sources resulting in increased trophic redundancy (lower NNR) during the flood season.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The effect of hydrological regulation on the trophic structure

The larger overlap of SEA with the similar trophic metrics between Wushan and Dachang sites during non-flood period (*Table 2*) inferred that trophic structure were becoming more homogenous after the impoundment. This could be explained by expanding backward mixing processes of the whole backwater zone increasing longitudinal connectivity during the higher-water-level period (Ran et al., 2010; Zhao et al., 2015). Due to extended water retention time, it is reported that uniformity of environmental conditions (*Table 1*) (Zhu et al., 2013) and fish community (Yang et al., 2013) among different sections occur in the backwater area when the water level rises.

The hydrological regulation also could adjust response of fish assemblages from different sites to flood. After the water level declines, the significant alternation of fluvial morphology and riverside zone appears in the tail of the backwater zone but seems much less in the estuary area. The depth of water drops remarkably from an average of 33 m to 19 m at Dachang section in the flood period, which would produce a large area of inundated riparian rocky and muddy substrates outcrops and thereby compress overall ecosystem size. However, the estuary area characterized by an incised channel with steep slopes has a mean depth of more than 50 m even at the lowest water level. Accordingly, fish assemblages at Dachang site is likely more susceptible to the unpredictable flow pulse in the flood period considering comparatively smaller water body in the tail of the backwater zone than the estuary area. On the contrary, the vast water storage in the estuarine confluence zone that dampens the effect of unregulated flow pulse but likely subjects to the mixing processes with the mainstream (Ran et al., 2010). Consequently, owing to the constant contribution from C_3 source within this tributary (Deng et al., 2018), the shrinkage of ecosystem size interacting with drastic flow pulse from the upper contribute directly to contraction of the niche size and trophic diversity in Dachang region by reductions in prey diversity (Layman et al., 2007b), that is, preference of more easily accessible resources which are at lower trophic levels (Roach et al., 2009; Ruhí et al., 2016). Nevertheless, the subsidies from the mainstream and autochthonous production due to relatively stable ambient at Wushan site facilitate trophic diversity increase and maintain ecological stability.

Conclusion

Overall, river impoundment have altered the hydrology at locations in the downstream and hence the food web structure throughout the Daning River. The lower sites with larger water storage had broader trophic niches and higher trophic diversity for fish assemblages reside. The temporal variation in trophic structure changed differently according to their distinct habitats. The trophic diversity shift in the estuarine area is caused mainly by availability of basal food sources either from the mainstream or aquatic algae. However, the variation of niche diversity in the end of backwater site results from disturbance on the vertical structure of the fish food web, specifically, disturbance-induced change in diet composition based on changeless food resource

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choice because nutritious vegetation contribution prevail within this tributary. The water level control promote disparate response for trophic structure of fish assemblages at different sites to the flood. The descending water level concomitant with unpredictable flow would shrunken the trophic niche size for fish assemblages in the end of backwater where considerable riverine morphology alternation resulted in water body decrease, whereas the stability of trophic size in the estuarine area were due to less perturbation mitigated by immense water body. In addition, the rising water level regulation could homogenize trophic structure along the river. The findings supplement the information of trophic ecology in the impound rivers and have important implications for development plans in this tributary.

Acknowledgements. This research was funded by the Hubei Key Laboratory of the Three Gorges Project for Conservation of Fishes (Grant NO. SXSN14381), Key Research and Development Project of Chongqing Science and Technology Commission (cstc2017shms-zdyfX0040), and the National Key Technology R&D Program (2015BAD13B02). We are grateful to Ke Yuan for the assistance with laboratory analysis of carbon and nitrogen isotope ratios.

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DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

APPENDIX

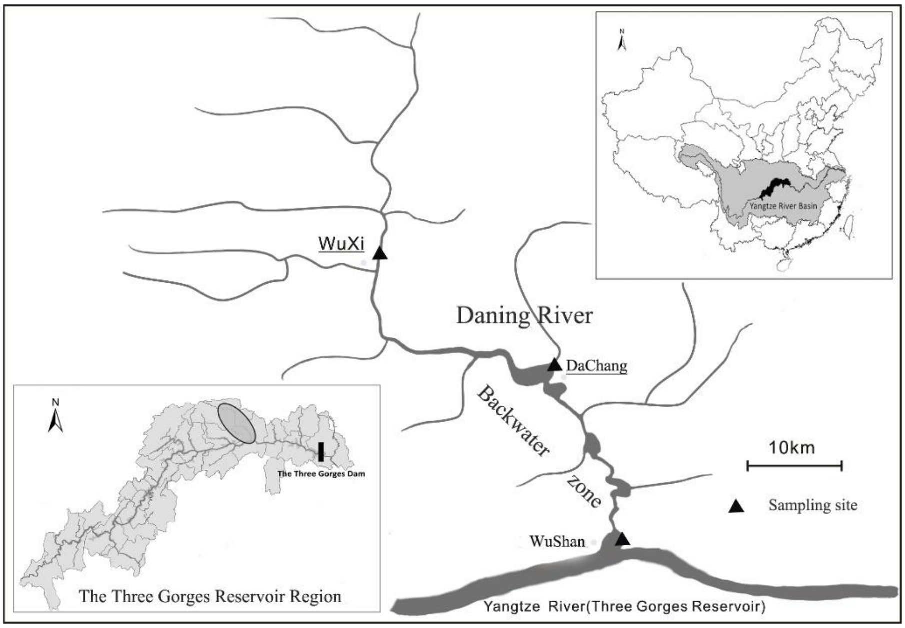

Figure S1. *The location of the Daning River and the sampling sites*

Table S1. *Trophic group, size range and stable isotope composition (mean ± SD) of fishes collected at each site and period (n = Number of samples analysed)*

	Trophic group	Fish species	($\delta^{13}C \pm SD$)‰	($\delta^{15}N \pm SD$)‰	Size (mm)	*n*
Non-flood period	**Wushan**					
	Benthivores	*Pelteobagrus nitidus*	-23.80 ± 0.06	13.72 ± 0.33	96 - 108	2
		Coreius heterodon	-25.10 ± 0.68	12.83 ± 2.30	122 - 220	3
		Pelteobagrus vachelli	-22.87 ± 1.94	8.61 ± 2.25	130 - 185	2
	Herbivorous	*Ctenopharyngodon idella*	-22.57 ± 1.44	4.59 ± 0.01	175 - 200	2
		Squaliobarbus curriculus	-22.19 ± 0.51	9.20 ± 1.36	220 - 260	2
		Megalobrama amblycephala	-23.5	5.29	220	1
		Parabramis pekinensis	-23.45 ± 2.21	9.36 ± 0.34	149 - 165	2
	Omnivores	*Hemiculter leucisculus*	-22.84 ± 0.50	6.99 ± 1.18	115 - 143	2
		Cyprinus carpio	-20.73 ± 2.58	5.91 ± 0.47	97 - 220	3
		Opsariichthys bidens	-22.7	11.72	140	1
		Saurogobio dabryi	-23.82 ± 0.15	13.80 ± 3.29	115 - 117	2
		Pseudobrama simoni	-22.57	8.69	97	1
		Squalidus argentatus	-23.88 ± 1.84	7.97 ± 2.04	98 - 106	2
		Carassius auratus	-20.60 ± 0.49	7.03 ± 0.57	162 - 210	2
	Piscivores	*Leiocassis longirostris*	-21.89	8.65	112	1
		Culter dabryi dabryi	-22.57 ± 0.56	10.47 ± 0.01	247 - 302	2
		Siniperca kneri	-22.19 ± 1.29	10.77 ± 0.86	165 - 262	2
		Culter alburnus	-23.32	11.78	330	1
		Elopichthys bambusa	-23.41 ± 0.25	10.38 ± 0.48	106 - 242	3
	Planktivores	*Neosalanx taihuensis*	-27.73 ± 2.84	12.87 ± 1.24		2
		Hypophthalmichthys molitrix	-26.76 ± 1.61	7.93 ± 2.30	148 - 250	4
		Aristichthys nobilis	-24.56 ± 1.08	8.12 ± 2.55	210 - 243	2
	Dachang					
	Benthivores	*Pelteobagrus nitidus*	-24.33	14.15	106	1
		Pelteobagrus vachelli	-25.21 ± 3.43	9.38 ± 0.13	92 - 220	2
		Silurus asotus	-25.12	11.21	220	1
	Herbivorous	*Ctenopharyngodon idella*	-21.21 ± 3.58	6.05 ± 0.29	131 - 282	3
		Megalobrama amblycephala	-23.31	5.46	181	1
		Spinibarbus sinensis	-21	6.66	195	1
		Parabramis pekinensis	-24.75 ± 1.10	7.62 ± 0.33	170 - 202	2
	Omnivores	*Hemiculter bleekeri*	-27.35 ± 0.06	9.06 ± 0.03	95 - 104	2

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	Trophic group	Fish species	($\delta^{13}C \pm SD$)‰	($\delta^{15}N \pm SD$)‰	Size (mm)	*n*
		Hemiculter leucisculus	-22.66 ± 1.29	8.61 ± 1.23	100 - 182	5
		Cyprinus carpio	-20.07 ± 0.58	9.05 ± 1.39	162 - 182	3
		Opsariichthys bidens	-22.52 ± 0.57	11.17 ± 0.85	110 - 135	2
		Saurogobio dabryi	-27.07 ± 0.80	8.86 ± 2.17	99 - 135	3
		Pseudobrama simoni	-22.47 ± 0.37	8.60 ± 1.00	125 - 150	2
		Acanthorhodeus chankaensis	-18.93	9.21	82	1
		Squalidus argentatus	-23.32 ± 1.04	10.56 ± 0.93	117 - 130	4
		Carassius auratus	-19.34 ± 2.28	7.80 ± 0.22	152 - 205	3
	Piscivores	*Leiocassis longirostris*	-21.29 ± 0.42	8.90 ± 0.23	195 - 210	2
		Pseudobagrus crassilabris	-24.3	10.18	190	1
		Culter dabryi dabryi	-23.78 ± 0.40	10.63 ± 0.72	230 - 255	3
		Siniperca kneri	-23.91 ± 0.47	11.53 ± 0.82	150 - 210	3
		Culter mongolicus	-23.97 ± 0.75	11.45 ± 2.07	190 - 195	2
		Culter alburnus	-24.59 ± 1.55	10.72 ± 0.52	250 - 320	3
		Elopichthys bambusa	-25.38	12.32	350	1
	Planktivores	*Neosalanx taihuensis*	-28.57	9.46		1
		Hypophthalmichthys molitrix	-26.86 ± 1.33	6.69 ± 1.48	220 - 263	3
		Aristichthys nobilis	-25.67 ± 2.76	6.56 ± 1.61	165 - 255	4
		Hyporhamphus intermedius	-26.2	9.64	187	1
	Wuxi					
	Benthivores	*Rhinogobius cliffordpopei*	-23.15 ± 0.03	8.55 ± 0.46	75 - 83	2
		Hemibarbus labeo	-23.78 ± 0.69	6.84 ± 0.14	134 - 202	2
		Paracobitis variegatus	-24.49 ± 0.66	7.25 ± 0.57	92 - 115	4
	Herbivorous	*Discogobio yunnanensis*	-23.98 ± 1.09	5.10 ± 0.27	98 - 125	4
	Omnivores	*Trilophysa bleekeri*	-23.51	7.57	80	1
		Onychostoma macrolepis	-23.22 ± 2.06	6.65 ± 0.74	107 - 142	2
		Acrossocheilus monticolus	-21.89 ± 0.47	6.47 ± 0.49	77 - 145	2
		Cyprinus carpio	-23.47	5.26	210	1
		Schizothorax prenanti	-22.51	6.81	150	1
		Saurogobio dabryi	-22.97 ± 0.09	7.73 ± 0.25	145 - 180	3
		Carassius auratus	-23.84	3.93	176	1
	Piscivores	*Glyptothorax fukiensis*	-24.75 ± 0.58	7.69 ± 0.01	77 - 92	2
		Zacco platypus	-24.16 ± 0.26	7.02 ± 0.63	80 - 135	5
Flood period	**Wushan**					
	Benthivores	*Pelteobagrus nitidus*	-24.32	14.45	125	1
		Coreius heterodon	-25.77	17.51	231	1
		Pelteobagrus vachelli	-24.86	11.44	135	1
		Silurus asotus	-24.33 ± 0.42	13.07 ± 2.20	191 - 205	2
	Herbivorous	*Ctenopharyngodon idella*	-18.65	9.37	251	1
		Squaliobarbus curriculus	-20.25	7.1	244	1
		Spinibarbus sinensis	-20.42	7.92	318	1
		Parabramis pekinensis	-20.36 ± 2.69	7.77 ± 0.75	245 - 250	2
	Omnivores	*Hemiculter bleekeri*	-19.68	9.97	100	1
		Hemiculter leucisculus	-17.85	7.86	105	1
		Cyprinus carpio	-17.6	6.43	256	1
		Saurogobio dabryi	-23.74 ± 0.18	12.05 ± 0.28	113 - 121	2
		Pseudobrama simoni	-19.52 ± 0.70	8.11 ± 0.14	95 - 100	2
		Squalidus argentatus	-20.66 ± 0.18	11.17 ± 0.16	84 - 103	2
	Piscivores	*Culter dabryi dabryi*	-18.97 ± 0.41	10.88 ± 0.52	101 - 109	2
		Siniperca kneri	-22.16 ± 0.28	13.66 ± 0.32	225 - 232	2
		Culter mongolicus	-21.87 ± 0.16	12.52 ± 0.68	222 - 251	2
		Culter alburnus	-22.72 ± 0.03	12.49 ± 0.64	223 - 260	2
		Elopichthys bambusa	-22.63 ± 1.27	12.00 ± 0.67	174 - 203	2
	Planktivores	*Neosalanx taihuensis*	-24.38	14.65	113	1
		Hypophthalmichthys molitrix	-24.28 ± 0.91	8.54 ± 0.21	195 - 629	2
		Aristichthys nobilis	-26.23 ± 0.66	10.39 ± 1.71	227 - 772	2
	Dachang					
	Benthivores	*Pelteobagrus vachelli*	-23.57 ± 0.63	9.83 ± 0.64	168 - 210	3
		Silurus asotus	-24.75 ± 0.25	10.86 ± 0.79	170 - 181	2
	Herbivorous	*Ctenopharyngodon idella*	-18.76 ± 3.04	7.62 ± 1.40	190 - 245	2
		Spinibarbus sinensis	-23.52 ± 0.27	8.88 ± 0.57	220 - 269	2
		Parabramis pekinensis	-22.83 ± 0.42	9.81 ± 0.56	234 - 280	2
	Omnivores	*Hemiculter bleekeri*	-24.88	10.03	105	1
		Hemiculter leucisculus	-25.94	8.77		1
		Cyprinus carpio	-19.32 ± 0.40	8.78	226 - 230	2
		Saurogobio dabryi	-25.93	10.6	135	1
		Pseudobrama simoni	-23.63	7.62	115	1
		Procypris rabaudi	-22.88	8.33	130	1

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DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

	Trophic group	Fish species	$(\delta^{13}C \pm SD)$‰	$(\delta^{15}N \pm SD)$‰	Size (mm)	*n*
		Squalidus argentatus	-24.43 ± 0.06	10.00 ± 0.39	103 - 133	2
		Rhinogobius giurinus	-23.76	9.89	55	1
		Carassius auratus	-20.37 ± 0.81	8.88 ± 0.38	157 - 225	4
	Piscivores	*Culter dabryi dabryi*	-24.19 ± 0.12	11.27 ± 0.29	232 - 270	2
		Siniperca kneri	-23.96 ± 0.50	12.17 ± 1.20	178 - 227	3
		Culter mongolicus	-24.75	10.49	347	1
		Culter alburnus	-24.62 ± 0.34	11.53 ± 1.42	240 - 490	3
		Elopichthys bambusa	-25.87	10.25	270	1
	Planktivores	*Neosalanx taihuensis*	-24.27	11.68		1
		Hypophthalmichthys molitrix	-26.23 ± 0.41	8.04 ± 1.08	321 - 342	2
		Aristichthys nobilis	-26.32	8.62	312	1
		Hyporhamphus intermedius	-26.03	8.21	176	1
	Wuxi					
	Benthivores	*Rhinogobius cliffordpopei*	-23.17	9.67	68	1
		Hemibarbus labeo	-23.16 ± 0.34	7.20 ± 0.57	125 - 258	2
		Paracobitis variegatus	-23.12	8.47	100	1
	Herbivorous	*Discogobio yunnanensis*	-23.08	5.92	115	1
		Spinibarbus sinensis	-25.31	2.25	126	1
	Omnivores	*Trilophysa bleekeri*	-23.58	7.77	78	1
		Onychostoma macrolepis	-21.74	5.98	98	1
		Acrossocheilus monticolus	-23.04	8.50	123	1
		Cyprinus carpio	-22.58	6.93	234	1
		Schizothorax prenanti	-23.02	6.02	78	1
		Saurogobio dabryi	-22.04	8.20	140	1
		Carassius auratus	-24.64	6.88	87	1
	Piscivores	*Siniperca kneri*	-23.23	10.81	177	1
		Glyptothorax fukiensis	-23.81	7.15	91	1
		Zacco platypus	-24.19	6.48	116	1

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):2085-2104.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_20852104

IMPACT OF GROWTH BIOSTIMULATORS AND HERBICIDE ON THE CONTENT OF ASCORBIC ACID IN EDIBLE POTATO TUBERS (*SOLANUM TUBEROSUM* L.)

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(Received 12th Nov 2018; accepted 16th Jan 2019)

Abstract. The objective of the study was to define the impact of nursing methods with the application of growth biostimulators and herbicide on the content of ascorbic acid in edible potato tubers. A series of field experiments were carried out in the years 2015-2017 in the region of eastern Poland (52°02'N; 23°07'E). The examined factors included: factor I – three early cultivars of edible potato (Owacja, Bellarosa, Vineta), factor II – five methods of nursing with the application of growth biostimulators: GreenOK-Universal Pro and Asahi SL as well as their combination with the Avatar 293 ZC herbicide. The treatment methods applied in the experiment with the use of growth biostimulators and herbicide had a significant impact on the rise of the content of ascorbic acid in edible potato tubers in comparison with only mechanical treatment. The highest concentration of ascorbic acid (on average 193.88 mg kg^{-1}) was marked in the tubers gathered from object 4., in case of which mechanical treatment has been made. Then the Avatar 293 ZC herbicide and the GreenOK Universal-PRO biostimulator were applied. The content of ascorbic acid in potato tubers was also significantly impacted by genetic characteristics of individual cultivars and weather conditions during the conduct of tests.
Keywords: *nutrients, Solanum tuberosum L., GreenOK Universal-PRO, Asahi SL, Avatar 293 ZC*

Introduction

Experiment background

Conventional, intensive methods of plant production have a significant impact on the natural environment and such impact is most frequently a negative one. In particular, the „high-tech-agriculture" model may constitute a threat not only for the health of consumers but also for natural resources on which we are entirely dependent (Scherr and McNeely, 2008; Głodowska and Gałązka, 2018).

Presently, within plant production, including potato (*Solanum tuberosum* L.) cultivation an increasing level of attention is drawn not only to the size of crops but also to the health-related qualities, while agricultural practice continues to evolve towards ecological, balanced cultivation systems, which are at the same time environment friendly (Bulgari et al., 2015).

In modern agriculture various types of agroecosystem friendly preparations have become commonly applied which are classified as growth biostimulators. They limit the application of agro-chemicals and thus, contribute to the development of balanced methods of plant production (Radkowski and Radkowska, 2013; Calvo et al., 2014; Du Jardin, 2015).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21052118

Growth biostimulators

According to the definition of The European Biostimulant Industry Council (EBIC), growth biostimulators are preparations which are applied on a plant, seeds or a root zone in order to stimulate the natural processes, increasing the effectiveness of use of nutrients, tolerance to abiotic stress and/or quality of crops. Their activity does not depend on the content of nutrition elements. Biostimulators do not directly counteract pests, therefore they are not classified as pesticides (European Biostimulants Industry Council, 2012; Matyjaszczyk, 2015). These preparations do not also act as fertilisers, while their main role is to control and accelerate the life processes of plants and to increase their resistance to abiotic stress (Matyjaszczyk, 2015). In the opinion of Calvo et al. (2014), interest in the use of biostimulators in agricultural practice continues to increase and the global market of these preparations may in 2018 reach the level above 2 241 million dollars.

Growth biostimulators constitute preparations of natural or synthetic origin the chemical content of which is characterized by a number of bioactive organic and non-organic components such as: effective micro-organisms, humus substances, extracts from seaweed and fruits, enzymes, phytohormones, chitin, chitosan, poli-oligosaccharides and chemical elements, inorganic salts (including phosphonates), phenolic compounds, antitranspirants and other substances of stimulating properties (Hamza and Suggars, 2001; Kauffman et al., 2007; Khan et al., 2009; Du Jardin, 2012; Przybysz et al., 2014).

Biostimulators contribute to an increase and growth of plants for their entire life cycle: They improve some physicochemical properties of soil and support the growth of useful soil microorganisms, stimulating germination and plant growth (Vernieri et al., 2002; Yildirim et al., 2002). They impact bio-chemical processes, facilitate assimilation, translocation and the use of nutrients (Kauffman et al., 2007; Kunicki et al., 2010; Du Jardin, 2012; Calvo et al., 2014). They may increase tolerance of plants towards biotic and abiotic stress as well as accelerate their regeneration (Ziosi et al., 2013).

Growth biostimulators may also impact the nutritional value of arable crops, including also the content of ascorbic acid (Dudaš et al., 2016; Mikos-Bielak et al., 1999a, b).

Ascorbic acid – Albert Szent-Györgyi

In 1928, Albert Szent-Györgyi – Hungarian scientist, biochemist, laureate of the Nobel prize (1937) extracted and identified vitamin C from pepper plant (ascorbic acid ($C_6H_8O_6$) – Latin: *acidum ascorbicum*). This compound was named ascorbic acid since its shortage or lack in nourishment leads to the development of scurvy (Latin: *scorbutus*). Five years later the first synthetic ascorbic acid was obtained (Gábor, 1982; Szarka and Lőrincz, 2013; Szendrei and Háznagy-Radnai, 2013).

Ascorbic acid is an organic chemical compound from the group of unsaturated polyols; it is essential for the functioning of living organisms. Vitamin C belongs to the most commonly known vitamins with multidirectional functions. It is an activator of various enzymes, performing a key role in cellular respiration. It also plays a role in the course of many metabolic processes. It participates in production of collagen and the basic proteins in the whole organism (bones, cartilages, tendons, ligaments). It helps the wounds, breaks to heal, inhibits the healing of bruises, occurrence of haemorrhage and gum bleeding, activates our immunological system stimulating the growth and

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effectiveness of T and B type immunological cells as well as other white blood cells (Moszczyński and Pyć, 1999; Huang et al., 2002; Nowak, 2004; Janda et al., 2015; Carr and Maggini, 2017). It participates in the synthesis of hormones and transmitters (Moszczyński and Pyć, 1999). Ascorbic acid is one of the strongest water-soluble antioxidants which performs the protective function against cancer diseases (Asensi-Fabado and Munné-Bosch, 2010). It is necessary in prevention of circulatory system diseases – it regulates the production of cholesterol in liver and its transformation into bile acids (it decreases the general level of "bad" LDH cholesterol in blood, increasing the volume of "good" HDL cholesterol) (Nowak, 2004; Janda et al., 2015).

Human beings do not possess metabolic pathways leading to the synthesis of C vitamin, therefore it must be provided on an ongoing basis by means of nourishment (Tabatabaei-Malazy et al., 2014). Vitamin C appears in the greatest volumes in fresh vegetables and fruits, thus it is extremely important to enrich our diet in these products.

Within our everyday diet, the cheapest and most widespread source of vitamin C are the edible potato tubers. This stems from the fact that edible potato is cultivated in approx. 160 countries worldwide and it is consumed on a daily basis by over a billion of persons (Bishwoyog et Swarnima, 2016; Baranowska, 2018). Content of vitamin C in potato tubers amounts to from 11.5 to 27.8 mg 100 g^{-1} (Grzesińska and Kierebiński, 1990; Kris-Etherton et al., 2002; Love and Pavek, 2008).

One must underline that in the course of preparation of meals and potato preserves, losses in the content of ascorbic acid occur, caused mainly by the operations of high temperature, enzymes, oxygen and light. Already the peeling process of potatoes causes a 6% loss of ascorbic acid. Once we chop the peeled potatoes before boiling, losses of vitamin C will be even more substantial. The largest losses occur as a result of slicing or dicing the tubers (15-16%), whilst the smallest – as a result of cutting into poles (9%). Losses of vitamin C during technological processes may range between: 27 and 33% - blanching process, up to 50% boiling in water, up to 83% - frying of crisps. However, the greatest losses of ascorbic acid (up to 93%) occur in the course of production of dry potato cubes. In order to minimize the losses of vitamin C it is most beneficial not to peel or cut the tubers, but rather wash them thoroughly prior to boiling (boiling with an addition of a small amount of hot water and kitchen salt in accuthermal dishes, pressure cookers, microwaves or steaming is recommended). One ought to further limit to the minimum the time of operations of destructive factors impacting the content of vitamin C, such as high temperature (Rytel and Lisińska, 2007; Janda et al., 2015). Despite the losses of ascorbic acid in the course of preparation of meals or potato preserves, potato is one of the main sources of vitamin C in human diet due to the significant volumes in which it is consumed. Consumption of 200 g of potatoes covers daily demand for vitamin C in approx 50% (Leszczyński, 2000).

Purpose of the experiment

Sparse and ambiguous so far empirical results of studies on the impact of growth biostimulators on the chemical composition of edible potato tubers, including also the content of ascorbic acid incline to carrying out further studies.

The purpose of the experiment was to specify the impact of five methods of treatment with the application of growth biostimulators and herbicide on the content of ascorbic acid in tubers of three cultivars of edible potato. Research hypothesis assumes that plant-care procedures with the use of growth biostimulators and herbicide will impact the content of ascorbic acid in edible potato tubers.

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Materials and methods

Location of the experiment

The field experiment was conducted in the years 2015-2017 in the region of eastern Poland (*Fig. 1*), in the Biała Podlaska Commune (52°02'N; 23°07'E) in the Lublin Voivodship, on acidic light soil.

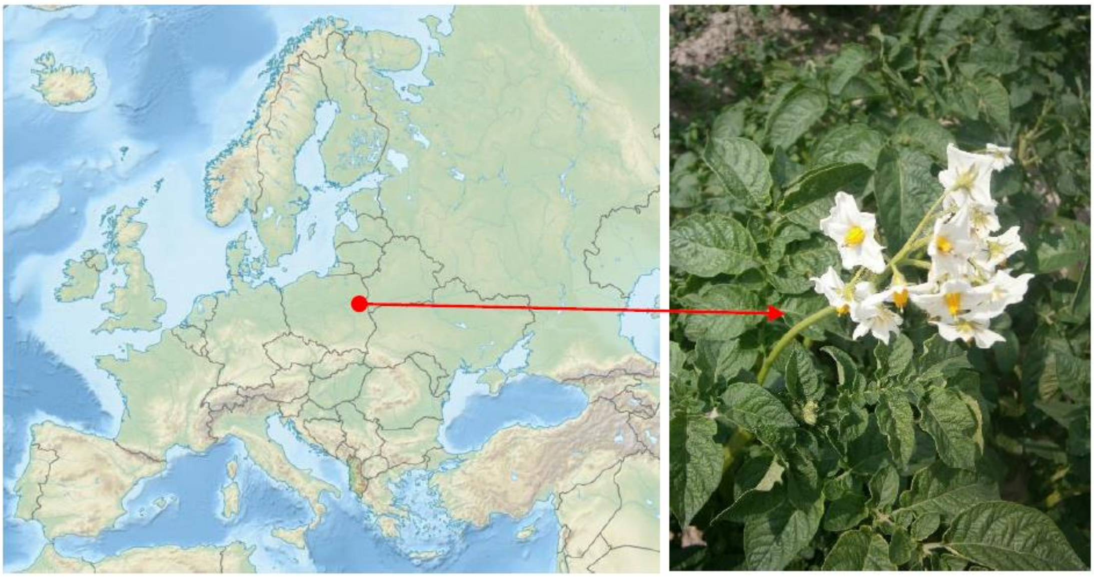

Figure 1. *Location of the experiment*

Experiment and plant material

The experiment was performed in the split-plot system, as two-factor in three replications. The influence of two factors was tested:

I. Factor – three early edible potato cultivars: Bellarosa, Owacja, Vineta,

II. Factor – five methods of treatment with the application of growth biostimulators and herbicide:

1. Standard object – mechanical treatment (without biostimulators and herbicide),
2. From sprouting of potato plants – mechanical treatment and after sprouting – GreenOK Universal–PRO bioactivator, three times to leaves: at a dose of 0.10 dm^3 ha^{-1} – peak–end of sprouting (phase BBCH 13-19) + 0.15 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55),
3. From sprouting of potato plants – mechanical treatment, and after sprouting – Asahi SL bioactivator, three times to leaves at a dose of 0.50 dm^3 ha^{-1} – peak–end of sprouting (phase BBCH 13-19) + 0.50 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.50 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55),
4. From sprouting – mechanical treatment, and after the final shaping of ridges and just before sprouting Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05). After sprouting – three applications of GreenOK Universal–PRO bioactivator at a dose of 0.10 dm^3 ha^{-1} – peak–end of sprouting

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(phase BBCH 13-19) + 0.15 dm^3 ha^{-1} – covering of interrows (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} – flower bud break (phase BBCH 51-55),

5. From sprouting – mechanical treatment, and after the final shaping of ridges before sprouting of potato plants – Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05).

The characteristics of the potato varieties and preparations used in the experiment are presented in *Tables 1* and *2*.

Table 1. *Characteristics of varieties of potatoes grown in the experiment. (Source: Plant Breeding and Acclimatization Institute – National Research Institute)*

Variety	Year of inscription to variety register	Breeder of variety	Yield t ha^{-1}	Colour of skin	Colour of pulp
Bellarosa	2006	Europlant, Pflz. GmbH Germany	38.3	Red	Yellow
Owacja	2006	PMHZ Strzekęcin Poland	41.4	Yellow	Light yellow
Vineta	1999	Europlant, Pflz. GmbH, Germany	39.1	Yellow	Yellow

Table 2. *Characteristics of biostimulators and herbicide applied in the experiment. (Source: information disclosed by agents' manufacturers)*

Name of preparation	Holder of the authorization	Content of active substances
GreenOK Universal-PRO – natural fertilizer with biostimmulating effect	Latvian Institute of humic substances Brivibas Street 144, Riga, Latvia, LV–1012	Humus substances ≤ 20 g dm^3 (NPK 0.13-0.09-0.7), dry mass 22 g dm^3, humidity 96%, organic substances 3%, pH 7-9
Asahi SL – growth regulator (known under the name of Atonik in Poland)	Asahi Chemical Europe s.r.o., Lužná 591/4 – Vokovice, 160 00 Praha 6, The Czech Republic	Sodium para-nitrophenolate (substance of nitrophenoles group) – 3 g dm^3 (0.3%) sodium orto-nitrophenolate (substance of nitrophenolates group) – 2 g dm^3 (0.2%) sodium 5-nitroguaiacolate (substance of nitrophenolates group derivatives) – 1 g dm^3 (0.1%)
Avatar 293 ZC – herbicide	FMC Chemical sprl, Boulevard de la Plaine 9/3, B–1050 Brussels, The Kingdom of Belgium	Clomazone (substance of isoxalodine group) – 60 g dm^3 (5.13%) metribuzin (substance of triazinones group) – 233 g dm^3 (20.64%)

In autumn, before the experiments, natural manure fertilization was used at a dose of 25 t ha^{-1}, as well as mineral fertilization with phosphorus 44.0 kg P ha^{-1} (triple superphosphate 46%) and potassium 124.5 kg K ha^{-1}(potassium salt 60%), and during spring – nitrogen fertilization (ammonium nitrate 34%) at a dose of 100 kg N per 1 ha. Potato tubers were planted in the second decade of April (in 2015 and 2016) and in the third decade of April (in 2017). Protection treatment against diseases and pests was used in accordance with the plant protection recommendations. In the period of vegetation, potato crops were protected by means of insecticides: Actara 25 WG (thiamethoxam 250 g kg^{-1}) at a dose of 0.08 kg ha^{-1}, Calypso 480 SC (tiachlopryd 480 g dm^{-3}) at a dose

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of 0.1 dm^{-3} ha^{-1} and fungicides: Copper Max New 50 WP (copper 500 g kg^{-1}) at a dose of 2.0 kg ha^{-1} Dithane Neo Tec 75 WG (mancozeb 750 kg ha^{-1}) at a dose of 2.0 kg ha^{-1}. Harvest was performed in the phase of full technological maturity of tubers.

Chemical analysis methods

The content of ascorbic acid was marked in fresh mass of tubers via Tillmans method modified by Pijanowski which consists of reducing the colour solution of 2.6-dichlorphenolindophenol to colourless leuco compound by acid solution of ascorbic acid (Pijanowski et al., 1964; Rutkowska, 1981).

Statistical analysis

The obtained results of research regarding the content of ascorbic acid of have been developed statistically by means of the analysis of variance method. Significance of variability sources was tested with the Fischler-Snedecor *F* test, and the assessment of significance of variance at the significance level $p = 0.05$ between the compared averages using Tukey's range test.

Weather conditions

Meteorological conditions in potato growing season have been presented in *Figures 2–5* and *Table 3*. The data come from the Meteorological Station of the Research Centre for Cultivar Testing (COBORU) in Słupia Wielka and they were compiled for the Cultivar Assessment Experimental Station situated near Biała Podlaska.

Meteorological conditions were characterized on the basis of monthly precipitation sums (*Figs. 2* and *3*), average air temperatures (*Figs. 4* and *5*), and hydrothermal coefficient of Sielianinov (*K*) (*Table 3*) (*Eq. 1*):

$$\mathbf{K} = \frac{\mathbf{P}}{\mathbf{0.1} \sum \mathbf{t}} \quad \text{(Eq.1)}$$

K – value of hydrothermal coefficient, *P* – sum of monthly rainfall, *t* – sum of daily air temperatures from a given month, where: *P* – signifies the monthly sum of rainfall, $\sum t$ – monthly sum of air temperatures >0 °C.

Extremely dry and extremely humid conditions were distinguished, performing a division of values of Sielianinov coefficient (*K*) into nine classes. The following divisions of values were applied: borderline dry $K \geq 0.4$, very dry $0.4 < K \geq 1.0$, dry $0.7 < K \geq 1.0$, rather dry $1.0 < K \geq 1.3$, optimal $1.3 < K \geq 1.6$, rather humid $1.6 < K \geq 2.0$, humid $2.0 < K \geq 2.5$, very humid $2.5 < K \geq 3.0$, borderline humid $K > 3.0$ (Chereszkowicz, 1979; Skowera and Puła, 2004; Skowera et al., 2014)

During the conduct of tests diverse meteorological conditions occurred (*Figs. 2–5* and *Table 3*). The lowest precipitation sum (288 mm) was recorded in 2015 with mean air temperature of 15.7 °C. In this season, precipitation shortage occurred from June to August, while August was extremely dry with precipitation sum of 7 mm and high atmospheric air temperatures. This season was defined as dry ($0.7 < K \geq 1.0$). Whilst, the highest precipitation sum was recorded in the growing season of 2017 (425 mm) in which the average air temperature was 15.4 °C; it was the coldest year in comparison with other years covered by the research. Within the growing season of 2016 the most humid months were: July with a sum of rainfall of 121 mm and high air temperatures

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(on average: 19.8 °C) and June with a sum of rainfall 84 mm and average air temperature of 18.4 °C. This season was specified as rather dry (1.0 < $K \geq 1.3$) (*Figs. 2–5* and *Table 3*).

Table 3. *Sielianinov's hydrothermic coefficient (K)*

*Sielianinov's hydrothermic coefficients *(K)*							
Month/Year	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**Mean IV–IX**
2015	1.43	2.28	0.59	0.80	0.11	1.66	0.99
2016	1.19	0.47	1.53	1.97	0.48	0.26	1.00
2017	2.68	0.93	1.97	1.24	0.66	2.37	1.50

*Sielianinov hydrothermal coefficient (K) – formula as in research methodology: Month's classification according to (Chereszkowicz, 1979; Skowera and Puła, 2004; Skowera et al., 2014): extremely dry K ≥ 0.4, very dry 0.4 < K ≥ 0.7, dry 0.7 < K ≥ 1.0, quite dry 1.0 < K ≥ 1.3, optimal 1.3 < K ≥ 1.6, quite humid 1.6 < K ≥ 2.0, humid 2.0 < K ≥ 2.5, very humid 2.5 < K ≥ 3.0, extremely humid K > 3.0

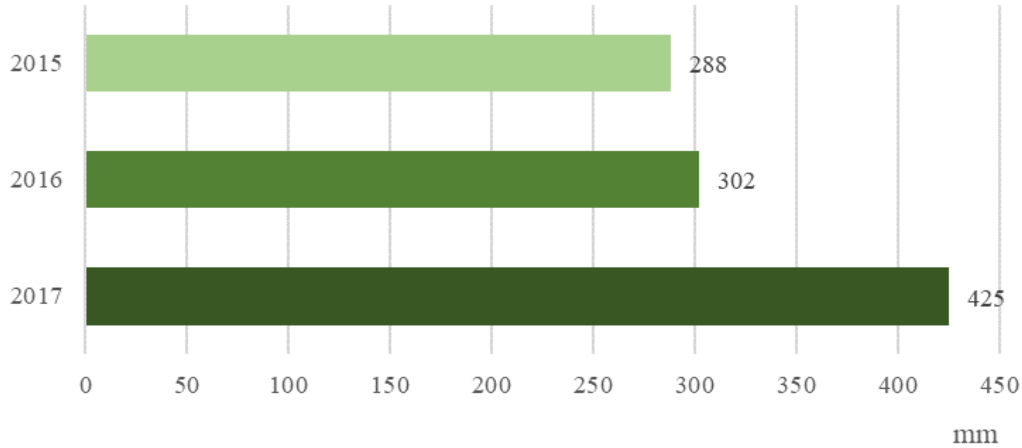

Figure 2. *The sum of precipitation (IV-IX) for the years 2015-2017 (mm)*

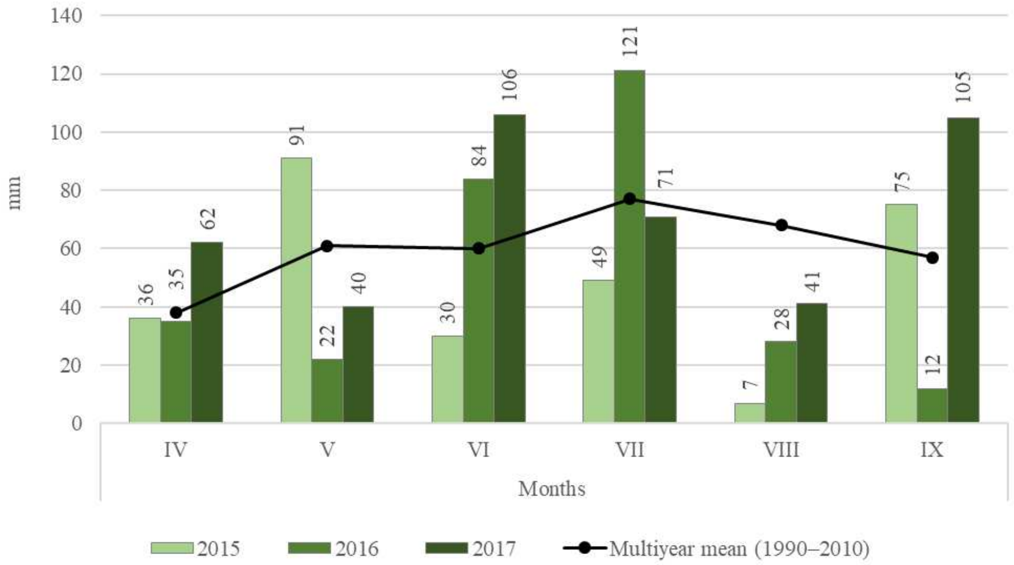

Figure 3. *The sum of precipitation in the vegetation period of potato in 2015-2017 (mm)*

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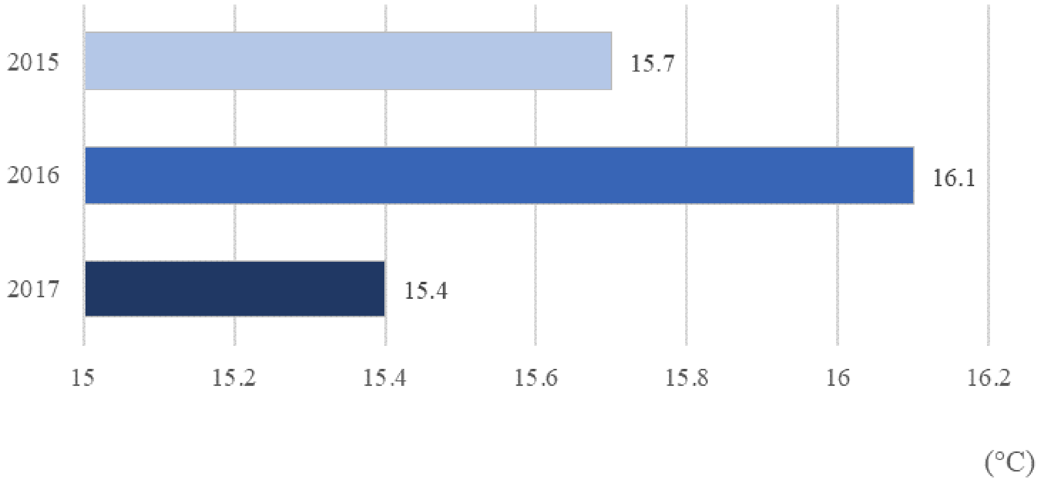

Figure 4. *The average air temperature (IV-IX) for the years 2015-2017 (°C)*

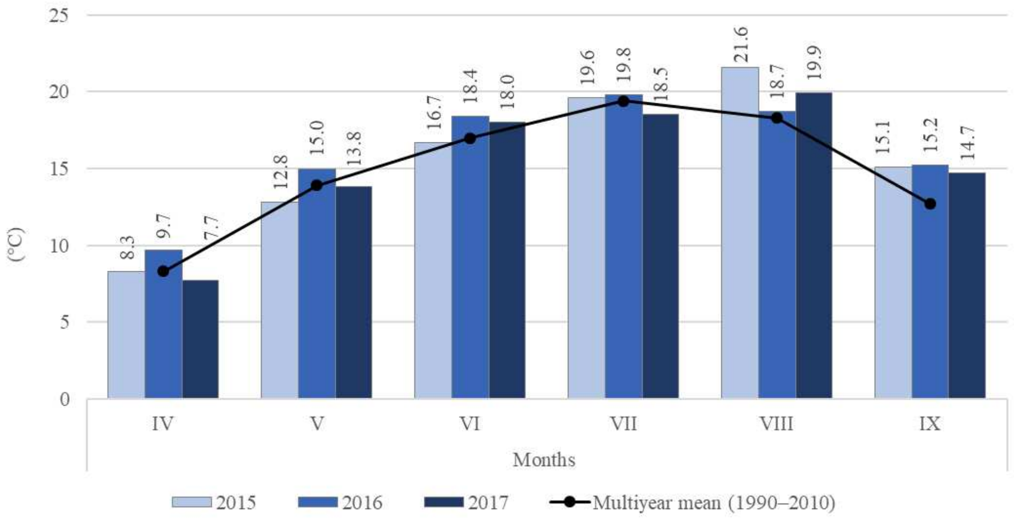

Figure 5. *Air temperature during the growing season of potato in 2015-2017 (°C)*

Results and discussion

As a result of the carried out research, it was proved that both applied cultivation methods ($F(4;120) = 4.23$; $p = 0.0031$), potato cultivars cultivated in the experiment ($F(2;79.06) = 12.93$; $p < 0.0001$), as well as meteorological conditions ($F(2;120) = 170.99$; $p < 0.0001$) that prevailed in particular research years had a significant impact on the content of ascorbic acid (*Tables 4* and *5*).

The cultivation methods applied in the experiment with the use of growth biostimulators and herbicide had a significant impact on the rise of content of ascorbic acid within potato tubers (object 2.-5.) in comparison with only mechanical treatment (object 1.) Statistically significant differences occurred also between object 3. and 4. ($p = 0.0164$) (*Table 4*). The largest concentration of vitamin C was reported in tubers gathered from object 4., (on which mechanical treatment was applied from sprouting, followed by application of Avatar 293 ZC at a dose of 1.5 $dm^3\ ha^{-1}$, and after sprouting, triple application of bioactivator GreenOK Universal-PRO at a dose of 0.10 $dm^3\ ha^{-1}$ +

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0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} (on average 190.06 mg kg^{-1}). Positive impact of herbicides on the content of ascorbic acid was observed also in the research carried out by Zarzecka and Gugała (2003), Rymuza et al. (2013). Barabaś and Sawicka (2015) who noted a greater tendency to accumulate vitamin C after application of a mixture of herbicides Sencor 70 WG and Titus 25 WG, applied after sprouting of potato plants.

Mikos-Bielak et al. (1999a, b) applying growth regulator Mival (1 chloromethylosilatran) and Potejtin (mixture of sodium N oxide 2, 6 lutidine and succinic acid) noted an increased content of vitamin C in potato tubers (24 out of 37 tested cultivars). Trawczyński and Prokop (2017) post applying biostimulators obtained from marine algae: Fertiliser Gold and Fertiliser Axis obtained significantly larger content of vitamin C in tubers in comparison to the standard object. Similar results were obtained also by Dudaš et al. (2016) who applied preparation Bio-algeen S-90.

Whilst, according to the research carried out by Rudzińska-Mękal (2000) application of Moddus 250 ME regulator (ethyl trinexapac) and Atonik (polish name Asahi SL) – decreases the content of ascorbic acid in tubers regardless of the term and method of application. According to Mikos-Bielak (2005) one may assume that Atonik biostimulator not so much hinders biosynthesis of vitamin C but rather its transport to floral organs and spare organs.

***Table 4.** Content of ascorbic acid in fresh mass of edible potato tubers depending on the treatment methods and cultivars (average from the years 2015-2017) (mg kg^{-1})*

Methods*	Cultivars			Mean
	Owacja	Bellarosa	Vineta	
1.	170.00	188.30	176.33	$178.20^{a,b,c,d}$
2.	176.33	195.60	183.33	185.08^{a}
3.	174.66	192.73	181.36	$182.92^{b,e}$
4.	183.66	199.83	186.70	$190.06^{c,e}$
5.	177.00	192.96	184.33	184.76^{d}
Mean	176.33^{f}	$193.88^{f,g}$	182.41^{g}	184.20
**$LSD_{0.05}$ for: cultivars – 4.620; methods – 2.634; methods x cultivars – n.s.				

*As in the experiment's methodology:
1. Standard object (without biostimulators and herbicide)
2. GreenOK Universal-PRO 0.10 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1}
3. Asahi SL 0.50 dm^3 ha^{-1} + 0.50 dm^3 ha^{-1} + 0.50 dm^3 ha^{-1}
4. Avatar 293 ZC 1.5 dm^3 ha^{-1} + GreenOK Universal-PRO 0.10 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1}
5. Avatar 293 ZC 1.5 dm^3 ha^{-1}

**$LSD_{0.05}$: Least significant difference, n.s. – not significant
[a, b, c,…]Values marked with the same letter differ significantly

It was noted that the content of vitamin C in potato tubers significantly depended on genetic conditions of the cultivars. Bellarosa cultivar gathered the greatest level of vitamin C (on average 193.88 mg kg^{-1}), while Owacja gathered the smallest amount of it (on average 176.33 mg kg^{-1}) (*Fig. 6*). Content of Vitamin C within Bellarosa tubers was substantially higher than in the tubers of the remaining cultivars (Bellarosa-Owacja $p < 0.0001$; Bellarosa-Vineta $p = 0.0054$). In case of the discussed feature no significant differences between Owacja and Vineta cultivars were observed ($p = 0.1363$) (*Table 4*).

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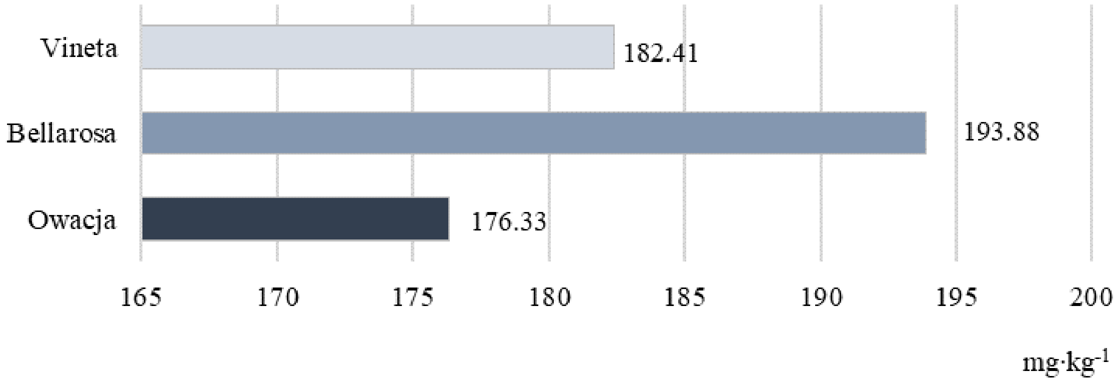

Figure 6. *Average content of ascorbic acid in the wet weight of edible potato tubers depending on the variety (averages from 2015-2017) (mg·kg^{-1})*

These results are compliant with the tests performed by Weber and Putz (1999), who carried out the tests with 26 German potato cultivars and noted that the content of vitamin Ca in tubers is determined by genetic factor. Also within the research by Wichrowska and Pobereżny (2008), Gugała and Zarzecka (2012) the content of vitamin C in tubers depended to a great degree on the genetic conditions of the analysed cultivars. In the opinion of Sowicka et al. (2014) differences in the content of vitamin C, in potato tubers are conditioned by phenotypic switching of cultivars which is a joint effect of genetic and environmental volatility.

A significant impact on the content of vitamin C in edible potato tubers was also assigned to atmospheric conditions in the years of research conduct (*Table 5* and *Fig. 7*).

Table 5. *Content of ascorbic acid in fresh mass of edible potato tubers depending on the treatment method and years of conduct of tests (average from years 2015-2017) (mg kg^{-1})*

Methods*	Year			Mean
	2015	2016	2017	
1.	195.56	184.03	155.03	178.20^{a,b,c,d}
2.	203.53	191.03	160.70	185.08^{a}
3.	200.33	189.73	158.70	182.92^{b,e}
4.	206.36	194.73	169.10	190.06^{c,e}
5.	202.36	191.26	160.66	184.76^{d}
Mean	201.62^{f,g}	190.15^{f,h}	160.83^{f,g,h}	178.20^{a,b,c,d}
**$LSD_{0.05}$ for: years – 4.620; methods – 2.634; methods x years – n.s.				

*As in the experiment's methodology:
1. Standard object (without biostimulators and herbicide)
2. GreenOK Universal-PRO 0.10 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1}
3. Asahi SL 0.50 dm^3 ha^{-1} + 0.50 dm^3 ha^{-1} + 0.50 dm^3 ha^{-1}
4. Avatar 293 ZC 1.5 dm^3 ha^{-1} + GreenOK Universal-PRO 0.10 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1} + 0.15 dm^3 ha^{-1}
5. Avatar 293 ZC 1.5 dm^3 ha^{-1}

**$LSD_{0.05}$: Least significant difference, n.s. – not significant
$^{a, b, c,…}$Values marked with the same letter differ significantly

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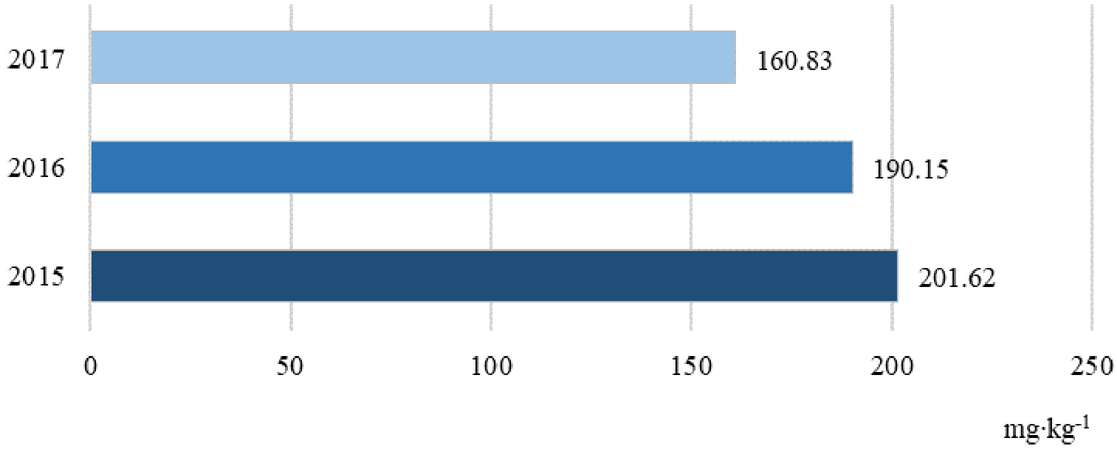

Figure 7. *Average content of ascorbic acid in the wet weight of edible potato tubers in the years 2015-2017 (mg·kg^{-1})*

The largest concentration of this component was noted in the growing season of 2015 (on average 201.62 mg kg^{-1}), which was warm and characterized by the smallest volume of rainfall (288 mm) in comparison to the other tests. Whilst, the smallest concentration of ascorbic acid was noted in humid and cool season of 2017 (on average 160.83 mg kg^{-1}). Statistically significant differences were observed in all medium pairs, that is between 2015 and 2016 ($p < 0.0001$), between 2016 and 2017 ($p < 0.0001$) and between 2015 and 2017 ($p < 0.0001$) (*Table 5*). Similar results of tests were obtained by Mazurczyk and Lis (2001), Love and Pavek (2008). According to Trawczyński and Prokop (2016) years with moderate volume of rainfall and close to approximate with multiple year air temperature positively impact the content of vitamin Ca in potato tubers. Sawicka et al. (2014) they proved that the content of vitamin C is decided by the content of chlorophyll in leaves and the intensity of the photosynthesis process.

Conclusions

Growth biostimulators continue to trigger an increasing interest both in science and agricultural practice. However, the impact of these preparations on the more crucial quality features of arable crops, including also on the content of ascorbic acid, has not yet been fully discovered and requires carrying out further scientific researches. Obtaining the results of own elaborations has allowed to note that treatment methods using growth biostimulators and herbicide significantly impacted the growth of content of ascorbic acid in potato tubers in comparison to the control object subjected to purely mechanical treatment. The content of ascorbic acid was substantially influenced also by genetic features of varieties as well as atmospheric conditions occurring in the subsequent years of conducting research. The greatest concentration of the discussed component was observed in Bellarosa variety of tubers whilst the smallest – in case of Owacja variety. Significantly higher content of ascorbic acid in the tubers of potato was noted in the growing season of 2015, which was characterized by the smallest sum of precipitation in comparison to the other years of research conduct.

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DOI: http://dx.doi.org/10.15666/aeer/1702_21052118

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21052118

Editorial Communication

RETRACTED

EVALUATION OF MORPHO-PHYSIOLOGICAL CHARACTRISTICS OF SUNFLOWER UNDER STRESS AND NON-STRESS DROUGHT CONDITION

This article has been retracted after OnlineFirst publication and DOI registration for the request of the authors.

THE EFFECT OF DIFFERENT IRRIGATION APPLICATIONS ON THE BLOSSOM-END ROT IN TREATED WASTEWATER-IRRIGATED TOMATOES (*LYCOPERSICON ESCULENTUM*)

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(Received 14th Nov 2018; accepted 16th Jan 2019)

Abstract. The study investigates the effect of water deficit conditions on blossom-end rot (BER) in tomato. Drip irrigation was used in irrigation. Therefore, experiment was conducted as a randomized complete block design (three replicates), in a 2 × 5 factorial arrangement, corresponding to two different water resources [treated wastewater (TWW) and freshwater (FW)] and five different irrigation practices. The irrigation strategies comprised full irrigation (100%), deficit irrigation, which involves the use of 75% and 50% of the water used in full irrigation, and partial root drying (PRD). According to the study results, in the full irrigation applications, blossom-end rot according to the fruit number values were 10.85% in fresh water irrigation while it was the lowest with 8.57% for treated wastewater. Also, in full irrigation applications, blossom-end rot values according to fruit weight were found to be lower compared to those in other applications. It was determined that there was a negative relation between blossom-end rot and Ca value. The lowest blossom-end rot incidence was observed in the full irrigation applications, while it was lower in the PRD applications than in the D applications. In the production of marketable tomato, the PRD applications can be more advantageous than the D applications under water shortage conditions.
Keywords: *BER, wastewater, tomato, PRD, deficit irrigation*

Introduction

The gap between water supply and demand is growing with the increasing global population and has reached life-threatening levels in certain regions of the world (Hussain et al., 2002). Moreover, although water of higher quality is suitable for household use, lower-quality waters are used in agricultural irrigation due to increasing demand for water in arid and semi-arid regions (Kızıloğlu et al., 2008). Thus, the need for the re-use of treated wastewaters is increasing. Today, according to recent estimates, 20 million hectares of agricultural land are irrigated with raw, processed or partially-diluted wastewaters worldwide (Drechsel et al., 2010). In agricultural irrigation, water saving-oriented applications are also used in addition to the use of wastewaters to protect surface and underground water resources. Deficit irrigation and partial root drying methods are examples of these applications. The studies have shown that deficit irrigation and partial root drying yielded positive results in the reduction of agricultural water use (Doğan Demir, 2016). Partial root drying and deficit irrigation are fundamentally similar to each other but in the partial root drying method, a portion of the root remains dry while the rest is wetted depending on alternating wetting (Stikic et al., 2003).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

Various plant species are irrigated using wastewater with partial root drying and deficit irrigation. High yields can be achieved with the irrigation of tomato plants with partial root drying and deficit irrigation either using clean water or wastewater and there are various studies investigating this subject (Al-Lahham et al., 2007; Li et al., 2007; Ekici, 2002; Alrajhi et al., 2015; Zegbe et al., 2007), but certain diseases may develop and cause yield losses due to applications. In tomatoes, blossom-end rot is the most common issue with these applications. Blossom-end rot in tomato has been described as a physical disorder for more than a century (Selby, 1896; Spurr, 1959; Saure, 2001; Ho and White, 2005; Taylor and Locascio, 2004). Although low calcium content has generally been reported to be the cause of blossom-end rot in tomato, various factors including acidic growing media, high N content, high soil salinity, low soil moisture and soil dryness have also been reported to affect the development of blossom-end rot (Selby, 1896; Shaykewich et al., 1971; Pill and Lambeth, 1980; Winsor and Adams, 1987; Ikeda and Osawa, 1988; Ho et al., 1999; Saure, 2001; Taylor and Locascio, 2004; Zhai et al., 2015).

There is limited information about the effects of water deficit and water stress on plant nutrition. In general, the nitrogen concentration in plant tissues increases with water stress. On the other hand, the phosphorus concentration in plants has been reported to decrease with decreasing water (Reichman and Grunes, 1966; Greenway et al., 1966; Thorup, 1969). The relationship between moisture deficit in soils and plant nutrition affects blossom-end rot development in tomatoes (Shaykewich et al., 1971; Bost, 2010). Tan and Dhanvantari (1985) reported that blossom-end rot development in tomatoes was reduced with irrigation. The water stress in plants increases under water deficit conditions, which results in blossom-end rot (Shaykewich et al., 1971; Ward, 1973).

Although partial root drying is a variation of deficit irrigation, deficit irrigation has been reported to aggravate blossom-end rot development in tomatoes (Adams and Ho, 1992; Obreza et al., 1996; Taylor et al., 2004; Sun, 2013). The irrigation method also affects the development of blossom-end rot (Carrijo et al., 1983).

For the detection of blossom-end rot (BER), total fruit number and rotten fruit number were determined. The number of the fruits with a rotten blossom-end were divided by the total fruit number and the blossom-end rot was given in percentages in the calculations. Furthermore, blossom-end rot value was also determined in percentages with respect to fruit weight. For this purpose, the weights of the fruits with a rotten blossom-end were measured and divided by the total weight of the fruits (Koral, 2006).

Studies have reported that the ratio of K/Ca was a more deterministic indicator of blossom-end rot in tomato than Ca content and the ratio between K and Ca and Ca content were related with each other (Gerard and Hipp, 1968; DeKock et al., 1982; Nukaya et al., 1994). Thus, this study also investigates the relationships between the Ca, K/Ca and K+Mg/Ca contents of the leaves and upper 30 cm of the soil and blossom-end rot. Moreover, the study examines the relationship between blossom-end rot and nitrogen and salinity values, which are thought to affect blossom-end rot.

The study investigates the effects of partial root drying (PRD) and deficit irrigation (D) on blossom-end rot (BER) in the tomato plants irrigated with treated wastewater.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Materials and methods

The mean elevation of the study area (*Fig. 1*) from sea level was 1030 m and the study area is located at latitude 38°53’01.91” - 38°53’01.52” N and longitude 40°32’57.82” - 40°32’56.73” E.

Figure 1. *Study area*

Table 1 shows the long-term average climatic data and climatic data for the study year in Bingöl, Turkey, as obtained from the meteorology station.

Table 1. *The long term average climatic data and climatic data for the study year in Bingöl, Turkey*

Year	Month	Temperature (°C)	Relative humidity (%)	Wind speed (m/s^{-1})	Daily sunshine (h)	Evaporation (mm)	Precipitation (mm)
Long term (1960-2013)	May	16.3	55.8	1.9	7.31	116.5	75.1
	June	22.1	43.5	2.1	9.40	179.1	20.6
	July	26.7	35.9	2.2	9.54	231.2	5.7
	August	26.4	35.1	2.1	9.24	221.7	3.3
	September	21.1	41.0	1.9	8.31	158.2	10.4
2013	May	18.7	47.8	0.87	7.20	12	7.0
	June	22.8	33.3	1.04	8.84	181	6.2
	July	27.2	26.4	1.28	9.39	278	-
	August	26.4	26.2	1.08	9.19	255	-
	September	20.3	34.5	0.83	7.95	111	10.9

[&]Includes the vegetation period in May and September of 2013
*The precipitation and evaporation values for 2013 were measured using a rain gauge and an A-class evaporation pan installed in the study area.

A profile was dug to determine the soil properties in the study area and disturbed and undisturbed soil samples were collected from the depths of 0-30, 30-60 and 60- 90 cm of the soil profile, followed by the analyses of the physical, chemical and hydraulic properties of the soil samples (*Table 2*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 2. *Basal properties of experimental field soil prior to trial*

Parameter	Soil layer (cm)		
	0-30	**30-60**	**60-90**
Texture	Clay	Clay	Clay
Bulk density (mg m^{-3})	1.30	1.31	1.36
Field capacity (% of weight)	28.5	30.3	30.8
Wilting point (% of weight)	17.2	18.1	18.4
pH	8.01	7.94	7.92
EC (dS m^{-})	0.528	0.509	0.450
Organic matter (%)	1.6	1.3	1.1
$CaCO_3$ (%)	4.60	3.40	2.10
Total N (%)	0.08	0.07	0.05
P_2O (kg da^{-})	8.30	5.7	1.5
K_2O (kg da^{-})	71.3	66.2	58.8
Ca (cmol kg^{-})	25.1	28.7	29.4
Mg (cmol kg^{-})	5.60	4.63	4.84
Na (cmol kg^{-})	0.50	0.40	0.40
B (mg kg^{-})	0.57	0.51	0.54
Fe (mg kg^{-})	14.5	15.7	15.0
Zn (mg kg^{-})	0.60	0.80	0.40
Cu (mg kg^{-})	0.60	0.80	0.80
Mn (mg kg^{-})	13.2	11.3	12.7
Cd (mg kg^{-})	0.20	0.30	0.30
Ni (mg kg^{-})	1.90	1.40	0.90
Pb (mg kg^{-})	0.09	0.09	0.05

The Joker-F_1 (*Lycopersicon esculentum*) variety was used in the study. In the trials, Joker F1 tomato variety was chosen due to the fact that it is a very strong variety that can cover the fruit, it is more resistant to diseases, it has can highly adapt to the region, it yields a hard fruit with a long shelf-life and it is suitable for temporary and long-term harvest. The seedlings were planted in the field on May 20. Prior to planting, plough cultivation was applied, large clods were crushed and field surface was smoothed to prepare the conditions for planting. The plants were planted in 5 rows with an inter-row spacing of 100 cm and intra-row spacing of 50 cm. Weeding was carried out manually through hoeing three times until the first harvest. Nitrogenous, phosphorous and potassium fertilizers were used in fertilization. Prior to planting, compound Diammonium Phosphate (DAP) (20:20:0) was applied in the dose of 50 kg per decare. Equal fertilization to each plant was carried out until reaching a cover level of 30% and irrigation conditions were met; after planting, in each irrigation, the NPK 15:15:15 and potassium nitrate 13-0-46 fertilizers were applied in the amount of 10 kg (50 kg in total) using a drip irrigation system. To control the more cricket population, the Korban-4 insecticide was used after planting. The experiments were carried out using waters of two different qualities in the applications, i.e. freshwater (FW) and treated wastewater (TWW). The quality result of each parameter in the *Table 3* shows the three sampling periods (June, July, and August). The clean water was obtained from the open irrigation channel at the entrance of the field and the wastewater was obtained from the exit of the wastewater treatment plant of Bingöl. *Table 3* shows the properties of the irrigation waters.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

Table 3. *The quality of the irrigation waters*

Parameter	June		July		August	
	TWW	FW	TWW	FW	TWW	FW
pH -	7.08	7.75	8.15	8.49	8.05	8.22
EC (dS m^{-})	0.480	0.172	0.529	0.159	0.533	0.158
TSS (mg l^{-})	20.3	21.2	16.0	17.8	28.0	21.3
Total N (mg l^{-})	14.9	-	13.0	-	13.41	-
Total P (mg l^{-})	1.95	-	2.00	-	1.86	-
CO_3 (me l^{-})	0	0	0	0	0.10	0.03
HCO_3 (me l^{-})	0.30	0.27	0.51	0.34	0.29	0.22
SO_4 (me l^{-})	3.90	1.20	2.85	1.43	2.89	1.41
Cl (me l^{-})	0.50	0.65	1.30	0.45	1.92	0.23
Ca (me l^{-})	1.59	1.16	2.58	1.23	2.11	0.91
Mg (me l^{-})	0.99	0.77	1.09	0.83	1.88	0.74
Na (me l^{-})	2.02	0.16	0.54	0.13	0.95	0.23
K (me l^{-})	0.14	0.07	0.41	0.04	0.49	0.05
B (mg l^{-})	0.19	0.03	0.54	0.34	0.57	0.39
Fe (mg l^{-})	0.46	0.23	0.09	0.06	0.15	0.10
Cu (mg l^{-})	0.13	0.09	0.05	0.03	0.09	0.04
Ni (mg l^{-})	0.03	0.04	0.05	0.05	0.05	0.04
Cd (mg l^{-})	0.07	0.05	0.11	0.07	0	0
Mn (mg l^{-})	0.11	0.06	0.09	0.03	0.02	0.01
Zn (mg l^{-})	0.05	0.04	0.04	0.04	0.08	0.04
Pb (mg l^{-})	0.02	0.02	0.09	0.07	0.08	0.05
Cr (mg l^{-})	0.35	0.25	0.46	0.42	0.41	0.37
Co (mg l^{-})	0.19	0.17	0.20	0.16	0.19	0.17
BOD_5 (mg l^{-})	24.0	-	25.0	-	36.0	-
COD (mg l^{-})	67.2	-	63.8	-	96.0	-
%Na (%)	42.62	20.22	11.69	5.30	17.49	9.50
SAR -	1.77	0.54	0.40	0.12	0.67	0.22
RSC (me l^{-})	-2.28	-1.87	-3.16	-1.94	-3.60	-1.89

TWW treated wastewater, *FW* freshwater, *EC* electrical conductivity, *TSS* total suspended solids, *BOD_5* 5-day biochemical oxygen demand, *COD* chemical oxygen demand, *SAR* sodium adsorption ratio, *RSC* residual sodium carbonate

Drip irrigation was used in irrigation. Therefore, experiment was conducted as a randomized complete block design (three replicates), in a 2 × 5 factorial arrangement, corresponding to two different water resources [treated wastewater (TWW) and freshwater (FW)] and five different irrigation practices. The irrigation strategies comprised full irrigation (100%), deficit irrigation, which involves the use of 75% and 50% of the water used in full irrigation, and partial root drying (PRD). In all applications, waters of two different qualities (clean water and wastewater) were used. In each parcel, 6 lateral pipes were installed in the middle of each plant row pair with a spacing of 100 cm; the irrigation water was applied from all lateral pipes in full and deficit irrigation applications, while the irrigation water was alternately applied in the partial root drying method [from no. 1-3-5 lateral pipes in odd-number irrigations; from

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DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

no. 2-4-6 lateral pipes in even-number irrigations]. The amount of irrigation water were equal in wastewater and clean water applications and adjusted in accordance with the control application in which 100% full irrigation was applied. The irrigations were commenced immediately after planting and finalized on September 29, when the growing season ended. *Table 4* shows the total and monthly irrigation water amounts in different applications. A total of 640.2 mm irrigation water was used in the 100% full irrigation application to tomato plants. The lowest irrigation water amount was 338.3 mm and applied in the 50% D and 50% PRD applications (*Table 4*).

Table 4. *The amounts of monthly and seasonal irrigation water applications to tomato plants in different irrigation applications (mm)*

Application	Months					
	May	June	July	August	September	Total
% 100	10.6	37.0	219	207.6	166.1	640.2
% 75 D	10.6	34.2	164.3	155.7	124.6	489.3
% 50 D	10.6	31.4	109.5	103.8	83.1	338.3
% 75 PRD	10.6	34.2	164.3	155.7	124.6	489.3
% 50 PRD	10.6	31.4	109.5	103.8	83.1	338.3

%100 full irrigation, *75D* and *50D* 75 and 50% deficit irrigation, *75PRD* and *50PRD* 75 and 50% deficit irrigation with PRD

Irrigation quantities in the scheduled irrigation period were corrected using a coefficient of 0.50 for the 50D and 50 PRD treatments and using a coefficient of 0.75 for the 75D and 75PRD treatments (*Table 4*).

BER were calculated using the below equations (*Eqs. 1–2;* Koral, 2006):

$$BER = \frac{Nf}{Ntf} x100 \quad \text{(Eq.1)}$$

where *BER* is the blossom-end rot (%), *Nf* number of rotten fruits, *Ntf* total number of fruits.

$$BER = \frac{Mf}{Mtf} x100 \quad \text{(Eq.2)}$$

where *BER* is the blossom-end rot (%), *Mf* rotten fruits weight (kg), *Mtf* total fruit weight (kg).

The changes in soil moisture were measured before the irrigations and showed that moisture did not drop to the wilting point in any of the applications throughout the growing season and the available water was reduced about 40% in the full irrigation application. A growing period of minumum 30% was provided and water was given equal to each plant until irrigation issues started. In general, the soil moisture values in the 75% PRD and 50% PRD applications were the closest values to the wilting point according to the soil moisture values. The changes in soil moisture during the growing season were close to each other in all applications (*Table 5*.)

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DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

Table 5. *The changes in soil moisture in different irrigation applications in the study year (%)*

FW	100%	%50 D	%50 PRD	%75 D	%75 PRD	TWW	100%	%50 D	%50 PRD	%75 D	%75 PRD
Irrigation1	24.79	24.79	24.79	24.79	24.79	**Irrigation1**	24.79	24.79	24.79	24.79	24.79
Irrigation2	25.29	25.29	25.29	25.29	25.29	**Irrigation2**	25.29	25.29	25.29	25.29	25.29
Irrigation3	25.25	25.25	25.25	25.25	25.25	**Irrigation3**	25.25	25.25	25.25	25.25	25.25
Irrigation4	23.89	23.89	23.89	23.89	23.89	**Irrigation4**	23.89	23.89	23.89	23.89	23.89
Irrigation5	25.37	25.37	25.37	25.37	25.37	**Irrigation5**	25.37	25.37	25.37	25.37	25.37
Irrigation5	23.57	23.57	23.57	23.57	23.57	**Irrigation5**	23.57	23.57	23.57	23.57	23.57
Irrigation6	24.97	24.97	24.97	24.97	24.97	**Irrigation6**	24.97	24.97	24.97	24.97	24.97
Irrigation7	24.02	24.02	24.02	24.02	24.02	**Irrigation7**	24.02	24.02	24.02	24.02	24.02
Irrigation8	24.79	22.98	21.88	23.54	22.42	**Irrigation8**	24.91	22.07	21.01	23.92	23.28
Irrigation9	24.37	22.10	21.08	23.83	21.91	**Irrigation9**	24.89	20.92	22.86	23.91	23.39
Irrigation10	24.86	23.26	20.85	23.59	22.08	**Irrigation10**	24.72	22.75	24.08	23.72	22.97
Irrigation11	24.80	21.22	20.64	23.94	21.70	**Irrigation11**	24.99	22.40	23.63	23.81	22.32
Irrigation12	24.64	22.38	20.51	23.77	21.45	**Irrigation12**	24.69	22.47	23.44	23.63	22.81
Irrigation13	24.96	22.78	20.94	23.17	21.70	**Irrigation13**	25.02	22.94	21.04	23.14	21.64
Irrigation14	24.37	21.66	21.21	23.39	21.59	**Irrigation14**	25.07	22.76	21.66	23.63	22.58
Irrigation15	24.92	22.82	21.68	23.23	22.28	**Irrigation15**	24.98	22.82	21.16	23.32	21.88
Irrigation16	25.01	22.07	20.91	23.54	22.11	**Irrigation16**	24.93	22.46	21.19	23.76	22.59
Irrgitaion17	25.06	21.88	21.16	23.73	21.60	**Irrgitaion17**	25.11	22.01	21.62	23.69	22.51
Irrigation18	24.87	23.25	21.58	23.85	21.62	**Irrigation18**	24.96	22.18	21.23	23.72	22.57
Irrigation19	25.04	22.51	21.49	23.45	22.00	**Irrigation19**	25.01	22.31	21.63	23.74	22.50
Irrigation20	24.69	23.04	21.39	23.91	21.66	**Irrigation20**	24.86	22.39	22.01	23.84	22.80
Irrigation21	25.08	24.01	23.66	24.19	23.97	**Irrigation21**	25.16	24.31	23.84	24.31	24.04
Irrigation22	24.97	23.19	21.61	24.09	21.03	**Irrigation22**	25.34	23.61	22.46	24.19	22.53
Irrigation23	18.79	13.97	12.80	15.75	14.12	**Irrigation23**	20.93	18.11	17.75	19.98	19.04

FW freshwater, *TWW* treated wastewater, *%100* full irrigation, *75D* and *50D* 75 and 50% deficit irrigation, *75PRD* and *50PRD* 75 and 50% deficit irrigation with PRD

The variance analyses were carried out using the SAS software (SAS, 2000) and the Duncan multiple comparison test of the Minitab software was used to compare the significant averages (Kesici and Kocabaş, 2007).

Results and discussion

Tables 6 and *7* show the effects of PRD and D applications on the blossom-end rot in tomato. According to *Table 6*, blossom-end rot incidences with respect to fruit number varied between 8.57% and 33.22%. The highest blossom-end rot incidence was obtained in the 50% D freshwater applications. The differences between the averages obtained for water resources were statistically not significant, while the differences between the applications were significant. The lowest blossom-end rot incidence was obtained in the 100% full irrigation applications, while the highest blossom-end rot incidence was obtained in the 50% D applications. The PRD applications had less effect

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

on blossom-end rot than the deficit irrigation applications. In their study, Zegbe et al. (2007) reported that the highest blossom-end rot was obtained in the D (37%) and PRD (22%) applications and determined that blossom-end rot was higher in the deficit irrigation application than in the partial root drying application. Obreza et al. (1996) found that blossom-end rot was five times higher in the 30% deficit irrigation application than in the full irrigation application. In the same vein, Dorji et al. (2005) determined that the highest blossom-end rot incidences were obtained in the deficit irrigation and partial root drying applications. The increased blossom-end rot incidences in deficit irrigation and partial root drying applications are attributable to the water deficit-induced decrease in the calcium content of the fruits (Adams and Ho, 1992).

Table 6. *Blossom-end rot incidences with respect to fruit number (%)*

Applications	% 100	% 75 D	% 50 D	% 75 PRD	% 50 PRD	*Means*
FW	10.85^{cd}	18.18^{bcd}	33.22^{a}	18.42^{bcd}	21.30^{bc}	18.71
TWW	8.57^{d}	21.59^{bc}	24.61^{ab}	19.39^{bcd}	19.42^{bcd}	20.39
Means	9.71**C**	19.88**B**	28.91**A**	18.90**B**	20.36**B**	

FW freshwater, *TWW* treated wastewater, *%100* full irrigation, *75D* and *50D* 75 and 50% deficit irrigation, *75PRD* and *50PRD* 75 and 50% deficit irrigation with PRD, $p<0.01$

Table 7. *Blossom-end rot incidences with respect to fruit weight (%)*

Applications	% 100	% 75 D	% 50 D	% 75 PRD	% 50 PRD	*Means*
FW	26.28	22.71	30.41	16.96	27.68	24.81
TWW	19.96	30.96	23.43	26.83	25.11	23.93
Means	19.79	26.83	26.92	21.90	26.39	

FW freshwater, *TWW* treated wastewater, *%100* full irrigation, *75D* and *50D* 75 and 50% deficit irrigation, *75PRD* and *50PRD* 75 and 50% deficit irrigation with PRD

According to *Table 7*, the blossom-end rot values with respect to fruit weight varied between 16.96 and 30.96%. The differences between the applications in the ratios of the weight of fruits with blossom-end rot to total fruit weight were not statistically significant.

The higher incidences of blossom-end rot in the deficit irrigation applications is attributable to the activation of the self-defense mechanisms of the plants in response to water stress. Moreover, irregular irrigation and saline water conditions have been reported to be the most common causes of blossom-end rot (Cuartero and Fernandez-Munoz, 1999; Franco et al., 1999; Zhai et al., 2015). The decrease in the soil moisture in the D and PRD applications can contribute to the increase in blossom-end rot incidences. According to *Table 6*, the highest incidence of blossom-end rot was observed in the deficit irrigation applications due to decreased soil moisture (*Tables 4* and *5*). In their study, Shaykewich et al. (1971) reported that blossom-end rot increased in soils with decreased moisture content. Pill and Mambeth (1980) observed that hardness and blossom-end rot increased in tomatoes under conditions of low NH_4 fertilization and soil water potential. It has also been reported that low Ca content in the fruit and deficit irrigation application affected the development of blossom-end rot (Adams and Ho, 1993; Taylor et al., 2004; Ho and White, 2005). Researchers have

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DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

noted that the PRD method is a variation of the deficit irrigation applications and one of its negative effects on the tomato plants is blossom-end rot development (Obreza et al., 1996; Zegbe et al., 2004). In their study in which the effects of PRD and D applications on tomato were investigated, Sun et al. (2013) reported that the PRD applications had a lesser effect on the development of blossom-end rot than the D applications and associated it with the increase in the abscisic acid concentration and the subsequent increase in stomatal conductivity leading to increased calcium uptake and, thus, decreased blossom-end rot development.

There was a statistically significant relationship between blossom-end rot and Ca contents of the fruit and leaves (*Table 8*). The first studies about the issue generally reported that blossom-end rot occurred under conditions of Ca deficiency (Hamner et al., 1942; Ward, 1973; Bradfield and Guttridge, 1984; Adams and Ho, 1992). However, blossom-end rot is not only affected by Ca deficiency but also affected by high Mg, Na, K and NH_4 concentrations, rapid growth, irrigation amount, low Ca content in soil and high or low transpiration (Geraldson, 1955; Kirby and Pilbeam, 1984; De Kreij, 1996; Paiva et al., 1998; Marcelis and Ho, 1999; Franco et al., 1999; Saure, 2001; Taylor and Locascio, 2004). The K and N contents of the fruits were also determined to have statistically significant effects on blossom-end rot while in soil, only the effects of K+Mg/Ca and EC on blossom-end rot were statistically significant. The results indicated that various factors affected blossom-end rot in addition to Ca deficiency.

Table 8. *The linear correlation between BER and Ca, K, N, K/Ca K+Mg/Ca and EC*

	Fruit	**Leaf**	**Soil**
Ca	-0.557**	-0.691**	-0.055
K	-0.407*	-0.280	-0.480
N	-0.399*	-0.300	-0.160
K/Ca	-0.264	0.380*	-0.040
K+Mg/Ca	-0.290	0.465**	0.396*
EC	-0.002	-	-0.535**

**p < 0.01, *p < 0.05

There was a significant negative relationship between blossom-end rot and the potassium content of the fruits, while the relationship between blossom-end rot and the potassium contents of leaves and soil was not statistically significant. Moreover, there was a significant negative relationship between the nitrogen content of the fruits and BER. There was a significant positive relationship between the K/Ca and K+Mg/Ca ratios in leaves and BER. BER had a significant positive relationship with the K+Mg/Ca ratio in soil, while it had a significant negative relationship with EC (*Table 8*). DeKock et al. (1982) reported that the ratio of K/Ca was a better determinant of blossom-end rot than the Ca content. However, Wada et al. (1996) and Bar-Tal and Pressman (1996) argued that the relationship between K/Ca and blossom-end rot did not have a predictive value. Albahou (1999) reported that high salinity contributed to the decrease in the blossom-end rot incidences. However, various researchers are of the opinion that blossom-end rot increases with increasing salinity and NH_4 content (Pill and Lambeth, 1980; Nonami et al., 1995; Saure, 2014).

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DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

Conclusion

In the study in which the effects of the D and PRD methods on blossom-end rot were investigated, the lowest blossom end-rot incidence was observed in the 100% full irrigation applications. The D application had the highest effect on blossom-end rot development. The PRD method had a lower effect than the deficit irrigation application. The lower incidence of blossom-end rot in the PRD applications compared with the D applications was associated with increased abscisic acid concentration in the xylem, decreased stomatal conductivity and the more effective use of water leading to increased Ca uptake and reduced blossom-end rot development. Therefore, the PRD can be a promising method for water saving under water deficit conditions and reduction of blossom-end rot development in tomatoes.

The results indicated that blossom-end rot was affected by various factors in addition to Ca deficiency. Furthermore, the results showed that in the production of marketable tomato, the PRD method is more advantageous than the D method under water shortage conditions where full irrigation is limited.

Acknowledgements. A. D. D. thanks the Ataturk University for the financial support of this research project (BAP-2012/414).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21352147

THE EFFECT OF MAIZE-SOYBEAN INTERCROPPING SYSTEMS ON A SET OF TECHNOLOGICAL AND PHYSIOLOGICAL PROPERTIES

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(Received 14th Nov 2018; accepted 16th Jan 2019)

Abstract. This study set out to determine which seeding rate produces the maximum grain yield through the monocropping and intercropping of maize and soybeans using different seeding rates on the research and land of the Faculty of Agriculture, Ordu University, in 2016. To this end, the study used a randomized block experimental design with three replications. The treatments included a sole crop of maize, a sole crop of soybean, one row of soybean intercropped with one row of maize, two rows of soybeans intercropped with one row of maize, three rows of soybeans intercropped with one row of maize, one row of soybean intercropped with two rows of maize, and one row of maize intercropped with three rows of soybean. According to the results on maize, the chlorophyll concentration index ranged from 45.50 to 50.93, leaf area from 360.51 to 510.19 cm^2, ash content from 5.76 to 9.30%, protein content from 9.26 to 10.77%, crude fat content from 3.35 to 4.85%, palmitic acid content from 12.17 to 13.12%, stearic acid content from 1.98 to 2.44%, oleic acid content from 23.86 to 24.95%, linoleic acid content from 58.97 to 60.90%, linolenic acid content from 0.91 to 1.13%, unsaturated fatty acid content from 84.62 to 85.84%, saturated fatty acid content from 14.15 to 15.37%, unsaturated to saturated fatty acid ratio from 5.50 to 6.08%, and grain yield from 32.01 to 89.512 kg ha^{-1}. According to the results on soybean, the chlorophyll concentration index ranged from 42.33 to 44.43, leaf area from 32.41 to 41.38 cm^2, ash content from 9.67 to 10.82%, protein content from 42.92 to 45.38%, crude fat content from 19.65 to 20.77%, palmitic acid content from 10.39 to 10.77%, stearic acid content from 3.81 to 4.28%, oleic acid content from 19.86 to 20.29%, linoleic acid content from 58.01 to 58.98%, linolenic acid content from 6.58 to 7.07%, unsaturated fatty acid content from 84.92 to 85.54%, saturated fatty acid content from 14.45 to 14.99%, unsaturated to saturated fatty acid ratio from 5.66 to 5.91%, grain yield from 10.36 to 61.11 kg ha^{-1}, and the Land Equivalent Ratio (LER) from 1.03 to 1.21. Considering all the properties investigated and LER together, three rows of soybean intercropped with one row of maize were found to be the most appropriate intercropping system.
Keywords: *crude fat content, fatty acids, leaf area, LER, protein content*

Introduction

Maize or corn occupies an important place both in Turkey and throughout the world because it has greater adaptability and the highest yield among cereals. 35% of the maize produced in Turkey is used in human nutrition, 30% in animal nutrition, 20% in the feed industry, and 15% in other areas (Gençtan et al., 1995). Maize is also a good source of energy because the majority of maize (corn grains) is composed of starch. Not only is corn starch obtained from maize, but also corn gluten meal and bran are manufactured from maize embryos for use in the oil and feed industry (Hallauer, 2001). The widespread use of oil and starch as bioenergy and the high yield of the maize plant have led to maize manufacture in every region where climatic conditions allow (Abbasi and Abbasi, 2010).

Soybean plants consist of 18 to 26% fat and 40% protein. The quality of soybean oil is increased by the high amount of oleic and linoleic fatty acids and the low amount of linolenic fatty acids. By-products obtained from soybean (flour, lecithin, protein, and fat) are used as raw materials in the industry (Cole and Erdahl, 2018). A wide range of products are manufactured from soybeans, including coffee cream, cooking oil, margarine, mayonnaise, medication, pharmaceuticals, salad dressing, anti-corrosion agents, anti-static agents, construction materials, ink printing materials, epoxies, fungicides, soap, detergents, bread, confectionery, biscuits, soya coffee, packaging foils, antibiotics, textiles, cat and dog foods, and fish feed (Endres, 2001).

In the world and in Turkey, the limited possibility of increasing the number of cultivable agricultural areas and the rapid increase in population have caused to agricultural workers to seek ways to exploit the maximum amount of agricultural land. Intercropping is one of these ways. Intercropping is recommended in order to produce greater and more balanced yield from unit area and to bolster defence against weeds (Acar et al., 2006).

Intercropping is an effective alternative for the prevention of loss within a complete growing season. The objective of intercropping is to create beneficial biological interactions between plants. Using legumes, it improves soil fertility through biological nitrogen fixation and ensures better soil conservation than monocropping. Ecological resources can be used better and far superior efficiency can be achieved in intercropping compared to monocropping. Having said that, there is a competition between intercropped inter- and intra-species for water, light, and nutrients. Therefore, it is of utmost importance to determine appropriate species and the ratio of crop mixtures in order to gain the expected benefit from intercropping (Lithourgidis et al., 2011).

Against this background, the purpose of the present study is to identify the effects of sole-cropped and intercropped maize and soybeans on a set of technological and physiological properties, which are manufactured to obtain material in primarily human nutrition and secondly animal nutrition.

Material and method

The trial was conducted on the research and land of the Faculty of Agriculture, Ordu University, in 2016. The land is located at 40°58′13.4″N latitude and 37°56′16.6″E longitude at an altitude of three meters (*Fig. 1*).

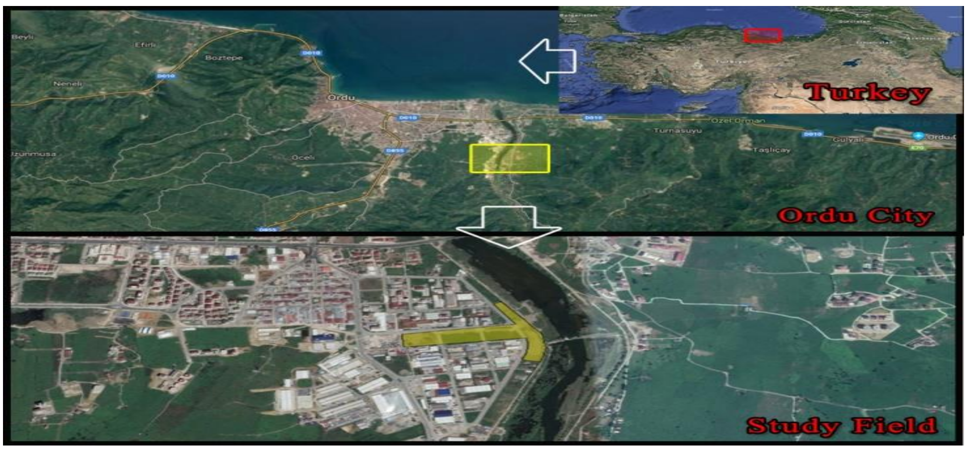

Figure 1. *Location of the study field*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

During the vegetation period of maize and soybean, the total precipitation at the trial site is 474.7 mm, the average temperature is 19.7ºC, and the average humidity is 74.3%. During the vegetation period of maize and soybean in 2016, the total precipitation at the trial site was 624 mm, the average temperature was 22.1ºC, and the average humidity was 68.3%. The total precipitation and average temperature recorded in the year of trial 2016 are above the average of long years (1965-2015) and the average humidity is below the average of long years (1965-2015). According to the results of soil analysis, the soil sampled from the trial site is clayey and neutral in terms of soil reaction and has a moderate amount of organic matter. Its less chalky soil is also less chalky and more saline.

The present study used Arısoy soybean seed variety supplied from the Black Sea Agricultural Research Institute, and SY Inove maize seed supplied from a private seed company. The trial was established in Ordu on June 13, 2016, under ecological conditions of the main crop. The study used a randomized block experimental design with three replications. The plots at the trial site were seeded with sole crops of maize and soybean, intercropped maize and soybean. *Table 1* shows the patterns of seeding. Prior to seeding, soybean seeds were inoculated using Rhizobium japonicum L. bacteria supplied from the Soil, Fertilizer and Water Resources Central Research Institute. Each plot was designed to have a length of 4 meters, a width of 7.7 meters, and 12 rows of seeding (30.8 m^2). An inter-row spacing of 70 cm was maintained for all row arrangements. An intra-row spacing of 20 cm was maintained for both the distinct row intercrops and the sole crop of maize. An intra-row spacing of 5 cm was maintained for both the distinct row intercrops and the sole crop of soybean.

Table 1. *Row Arrangements of Intercropping*

Crop Rows	Intercropping
Maize	Sole Crop
Soybean	Sole Crop
1 row of soybean + 1 row of maize	Distinct-row
2 rows of soybean + 1 row of maize	Distinct-row
3 rows of soybean + 1 row of maize	Distinct-row
1 row of soybean + 2 rows of maize	Distinct-row
1 row of soybean + 3 rows of maize	Distinct-row

Fertilization was carried out on the basis of the three periods of the growing cycle of maize and soybean. Diammonium phosphate (DAP, 18.46.0) was used as the bottom fertilizer and calcium ammonium nitrate (CAN, 46%). *Table 2* describes the time and amount of fertilizers applied to the plots.

Table 2. *Time and Amount of Fertilizers Applied on the Plots*

Time of Fertilizer Application	Fertilized Plot	Amount of Fertilizer
Pre-seeding	Sole Maize Soybean– Maize	Pure 0.7 kg ha^{-1} N/, 0.7 kg ha^{-1} P_2O_5
	Sole Soybean	Pure 0.3 kg ha^{-1} N 0.7 kg ha^{-1} P_2O_5
Bolting Time	Sole Maize Soybean – Maize	Pure 0.7 kg ha^{-1} N
Maize Dent Stage	Sole Maize Soybean – Maize	Pure 0.7 kg ha^{-1} N

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

Hoeing and weeding were done, when necessary, to control weeds and air the soil on all sole-cropping and intercropping plots. According to crop water needs, crops were irrigated four times using springer irrigation system: the time when crops attain a height of 20 cm, at maize tasseling and soybean flowering stages, at pollination stage, and at maize dent and soybean seed-filling stages.

Technological and physiological measurements made in the experiment are given in *Table 3*.

Table 3. *List of technological and physiological traits measured*

Trait	Maize	Soybean
Chlorophyll Concentration Index	Chlorophyll concentration index of 10 plants randomly selected from each plot chlorophyll concentration index was determined by measuring chlorophyll in sunny and cloudy days in the leaves of corn plant by using Apogee tool.	Chlorophyll concentration index of 10 plants randomly selected from each plot chlorophyll concentration index was determined by measuring chlorophyll in sunny and cloudy days in the leaves of soybean plant by using Apogee tool.
Leaf area	$LA=\left[\begin{array}{c}-5.87+(2.76xW)+(1.11xL)+(0.04X\ L^2) \\ +[0.05x(LxW^2)]\end{array}\right]$ LA: Leaf Area, L: Leaf length, W: Leaf width	Digimizer is determined using the package program.
Ash Content	The grains of 10 plants were randomly harvested from each parcel and ground to a 1 mm sieve. Calculation of ash ratio calculated using the formula given below $Ash\% = \frac{\text{Weight of burnt sample x 100}}{\text{Weight of unburned sample}}$	
Protein content, Crude fat content	The grains of 10 plants taken from each parcel were randomly blended and identified in non-grinded samples using the IC-1020WE maize calibration kit in the "Near Infrared Reflection" (NIRS) device.	The grains of 10 plants were randomly harvested from each plot and were determined in the non-grinded samples using the IC-0923FE soybean calibration set in the Near Infrared Reflecting" (NIRS) device.
Fatty acids	The grains of 10 plants randomly collected from each plot were blended and the amount of fatty acids was determined as % in Gas Chromatography.	
Grain Yield	In 10 plants randomly collected from each plot, the grains are blended and the grain moisture is arranged according to 14%. 10 plant yield per hectare multiplied by the number of plants and yield per hectare	
Land Equivalent Ratio (LER)	The land equivalent ratio (LER) was calculated as the sum of the relative yields of maize and soybean in the intercropping plots to the monocropping plots using the following equation: $LER=\left[\frac{Yint\text{-}soy}{Ymono\text{-}soy}\right]+\left[\frac{Yint\text{-}maize}{Ymono\text{-}maize)}\right]$ LER = LERsoy+LERmaize =Yint–soy +Yint–maize Ymono–soy Ymono–maize where Yint–soy, Ymono–soy, Yint–maize, and Ymono–maize are the grain yields (kg ha^{-1}) of intercropped soybean, monocropped soybean, intercropped maize, and monocropped maize, respectively.	

After crops attained full maturity, they were harvested October 25, 2016 (*Fig. 2*). After edges of 50 cm were cut to avoid edge effect during harvesting, technological properties were analyzed on 10 crops randomly selected among maize and soybeans. LER values were calculated for the seeding plots to determine the efficiency of the land use by dividing the intercrop yields of maize and soybean to the sole yields of maize and soybean and then adding two values. SAS-JMP 10.0 statistical software was used to run an analysis variance on the values based on a randomized block experimental design. The Least Significant Difference (LSD) test was used to measure significant differences between means.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

Figure 2. *Some views from experimental plots*

Results and discussion

Chlorophyll Concentration Index (CCI) of Maize

Considering the effect of intercropping on the chlorophyll concentration content of the maize plant, no significant difference was found. The CCI varied from 45.50 to 50.93. The lowest CCI was observed in the sole-cropped maize, while the highest CCI was observed in two rows of soybean intercropped with one row of maize. The mean CCI of the intercropped maize was found to be 47.91 (*Table 4*).

Our results on the maize chlorophyll concentration index are reported 35.17 - 47.03 in Choudrary et al. (2014), 17.89 - 29.53 in Sabancı (2015).

Table 4. *The Values of CCI, Leaf Area, Ash Content, Protein Content, and Crude Fat Content for the Intercropped Maize*

Intercropping	CCI	Leaf Area (cm^2)	Ash Content (%)	Protein Content (%)	Crude Fat Content (%)
SM	45.50	436.58	7.02 bc	9.26	3.90
1 S + 1 M	48.23	430.27	5.76 c	10.11	3.93
1 S + 2 M	46.56	360.51	8.05 ab	10.03	3.35
1 S + 3 M	46.23	384.56	8.86 a	10.54	4.85
2 S + 1 M	50.93	476.39	9.30 a	10.77	4.15
3 S + 1 M	50.06	510.19	8.78ab	10.51	3.53
Mean	47.91	433.08	7.96	10.20	3.95
Significance	-	-	-	-	-
LSD	-	-	1.88	-	-

CCI: Chlorophyll Content Index, SM: Sole Maize, S: Soybean, and M: Maize

CCI of Soybean

Considering the effect of intercropping on the chlorophyll concentration content of the soybean plant, no significant difference was found. The CCI varied from 42.33 to 44.43. The lowest CCI was observed in the sole-cropped soybean, while the highest CCI was observed in three rows of soybeans intercropped with one row of maize. The mean CCI of the intercropped soybean was 43.30 (*Table 5*).

Sole cropping of corn had the least chlorophyll content, while in all intercropping treatments especially when corn was in neighboring of soybean, chlorophyll content of corn significantly increased (Amini et al., 2013).

Table 5. *The Values of CCI, Leaf Area, Ash Content, Protein Content, and Crude Fat Content for the Intercropped Soybean*

Intercropping	CCI	Leaf Area (cm^2)	Ash Content (%)	Protein Content (%)	Crude Fat Content (%)
SS	42.33	32.41	9.82	43.56 bc	20.20 b
1 S + 1 M	43.20	39.50	10.82	45.38 a	19.65 b
1 S + 2 M	42.53	32.72	10.74	44.70 ab	20.06 b
1 S + 3 M	44.13	39.96	10.72	44.62 ab	20.00 b
2 S + 1 M	43.23	41.38	10.44	44.34 ab	19.93 b
3 S + 1 M	44.43	37.01	9.67	42.92 c	20.77 a
Mean	43.30	37.16	10.36	44.08	20.10
Significance	-	-	-	*	*
LSD	-	-	-	1.35	0.56

CCI: Chlorophyll Content Index, SM: Sole Soybean, S: Soybean, and M: Maize

Maize Leaf Area (cm^2)

Considering the effect of intercropping on the leaf area of the maize plant, no significant difference was found. The leaf area of maize varied from 360.51 cm^2 to 510.19 cm^2. The smallest leaf area was observed in one row of soybean intercropped with two rows of maize, while the largest leaf area was observed in three rows of soybean intercropped with one row of maize. The mean leaf area of the intercropped maize was 433.08 cm^2 (*Table 4*).

Our results on the maize leaf area are above those reported to be 305.96 - 325.62 cm^2 in Subedi and Ma (2005) but below those reported to be 877.3 - 626 cm^2 in He et al. (2011). A positive significant relationship has been reported between the number of leaves per plant and the leaf area of maize (Öner et al., 2012).

Soybean Leaf Area (cm^2)

Considering the effect of intercropping on the leaf area of the soybean plant, no significant difference was found. The leaf area of soybean varied from 32.41 cm^2 to 41.38 cm^2. The smallest leaf area was observed in the sole-cropped soybean, while the largest leaf area was observed in two rows of soybean intercropped with one row of maize. The mean leaf area of the intercropped soybean was 37.16 cm^2 (*Table 5*).

Our results on the soybean leaf area are below those reported to be 68.70 - 35.50 cm^2 in Ngalamu et al. (2012).

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DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

Maize Ash Content (%)

The effect of intercropping on the ash content of the maize plant was found to be statistically significant (P<0.05). The ash content of maize varied from 5.76 to 9.30%. The lowest ash content was observed in one row of soybean intercropped with one row of maize, while the highest ash content was observed in two rows of soybean intercropped with one row of maize. The mean ash content of the intercropped maize was 7.96% (*Table 4*).

Our results on the maize ash content are above those reported to be 1.46 - 1.81% in Lucchin et al. (2003), and those reported to be 1.08 - 2.22% in Moreno et al. (2015).

Soybean Ash Content (%)

The effect of intercropping on the ash content of the soybean plant was statistically insignificant. The ash content of soybean varied from 9.67 to 10.82%. The lowest ash content was observed in three rows of soybean intercropped with one row of maize, while the highest ash content was observed in one row of soybean intercropped with one row of maize. The mean ash content of the intercropped soybean was 10.36% (*Table 5*). Our results on the maize ash content are above those reported to be 1.99 - 18.74% in Ahmad et al. (2016).

Maize Protein Content (%)

The effect of intercropping on the protein content of the maize plant was statistically insignificant. The protein content of maize varied from 9.26 to 10.77%. The lowest protein content was observed in the sole-cropped maize, while the highest protein content was observed in two rows of soybean intercropped with one rows of maize. The mean protein content of the intercropped maize was 10.20% (*Table 4*).

Our results on the maize protein content are consistent with those reported to be 7.94 - 10.61% in Bekele and Rao (2014), while they are below those reported to be 11.02 - 12.73% in Öner et al. (2011) but above those reported to be 6.21 - 8.65% in Vartanlı and Emeklier (2007).

Soybean Protein Content (%)

The effect of intercropping on the protein content of the soybean plant was statistically significant (P<0.05). The protein content of soybean varied from 42.92 to 45.38%. The lowest protein content was observed in three rows of soybean intercropped with one row of maize, while the highest protein content was observed in one row of soybean intercropped with one row of maize. The mean protein content of the intercropped soybean was 44.08% (*Table 5*).

Our results on the soybean protein content are consistent with those reported to be 44.30 - 47.20% in Şenyiğit et al. (2015), while they are above those reported to be 32.27 - 33.87% in Aydemir and Kızılşimşek (2018).

Maize Crude Fat Content (%)

Considering the effect of intercropping on the crude fat content of the maize plant, no significant difference was found. The crude fat content of maize varied from 3.35 to 4.85%. The lowest crude fat content was observed in one row of soybean intercropped with two rows of maize, while the highest crude fat content was observed in one row of

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soybean intercropped with three rows of maize. The mean crude fat content of the intercropped maize was 3.95% (*Table 4*).

Our results on the maize crude fat content are consistent with those reported to be 3.50 - 8.30% in Hartings et al. (2008), while they are below those reported to be 11 - 6.28% in Akıncı et al. (2011) but above those reported to be 2.71 - 3.24% in Sabancı (2016).

Soybean Crude Fat Content (%)

The effect of intercropping on the crude fat content of the soybean plant was statistically significant (P<0.05). The crude fat content of soybean varied from 19.65 to 20.77%. The lowest crude fat content was observed in one row of soybean intercropped with one row of maize, while the highest crude fat content was observed in three rows of soybean intercropped with one row of maize. The mean crude fat content of the intercropped soybean was 20.10% (*Table 5*).

Our results on the soybean crude fat content are consistent with those reported to be 17.54 - 19.90% in Kan et al. (2011), while they are below those reported to be 22.06 - 24.67% in Dolapçı (2012) but above those reported to be 12.00 - 14.00% in Karagül et al. (2011).

Maize Palmitic Acid Content (%)

Palmitic acid is the second most common saturated fatty acids, following oleic acid. Palmitic acid composes 15 - 50% of fatty acids found in most fats (Kale et al., 2017). The effect of intercropping on the palmitic acid content of the maize plant was statistically insignificant. The palmitic acid content of maize varied 12.17 to 13.12%. The lowest palmitic acid content was observed in one row of soybean intercropped with three rows of maize, while the highest palmitic acid content was observed in the sole-cropped maize. The mean palmitic acid content of the intercropped maize was 12.45% (*Table 6*).

Our results on the maize palmitic acid content are consistent with those reported to be 12.79 - 15.55% in Akıncı et al. (2011), while they are above that reported to be 11.20% in Kan et al. (2011).

Table 6. *The Values of Palmitic Acid (%), Stearic Acid (%), Oleic Acid (%), Linoleic Acid (%) and Linolenic Acid (%) for the Intercropped*

Intercropping	Palmitic Acid (%)	Stearic Acid (%)	Oleic Acid (%)	Linoleic Acid (%)	Linolenic Acid (%)
SM	13.12	2.25	24.51	58.97	1.13
1 S + 1 M	12.44	2.44	24.54	59.63	0.91
1 S + 2 M	12.46	2.08	24.94	59.43	1.06
1 S + 3 M	12.17	1.98	23.86	60.90	1.06
2 S + 1 M	12.29	1.99	24.95	59.80	0.93
3 S + 1 M	12.25	2.19	24.91	59.63	1.00
Mean	12.45	2.15	24.61	59.72	1.01
Significance	-	-	-	-	-
LSD	-	-	-	-	-

SM: Sole Soybean, S: Soybean, and M: Maize

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

Maize Stearic Acid Content (%)

The effect of intercropping on the stearic acid content of the maize plant was also statistically insignificant. The stearic acid content of maize varied from 1.98 to 2.44%. The lowest stearic acid content was observed in one row of soybean intercropped with three rows of maize, while the highest stearic acid content was observed in one row of soybean intercropped with one row of maize. The mean stearic acid content of the intercropped maize was 2.15% (*Table 6*).

Our results on the maize stearic acid content are consistent with that reported to be 1.88 - 2.13% in Kaplan et al. (2017), while they are below those reported to be 2.87 - 3.50% in Akıncı et al. (2011).

Maize Oleic Acid Content (%)

Oleic acid is the most common fatty acid in nature. More than half of the fatty acids in most fats is oleic acid. It is quite rare that oils have less than 10% oleic acid. All natural oils and phospholipids known to date have oleic acid (Kaplan et al., 2017).

The effect of intercropping on the oleic acid content of the maize plant was also statistically insignificant. The oleic acid content of maize varied from 23.86 to 24.95%. The lowest oleic acid content was observed in one row of soybean intercropped with three rows of maize, while the highest oleic acid content was observed in two rows of soybean intercropped with one row of maize. The mean oleic acid content of the intercropped maize was 24.61% (*Table 6*).

Our results on the maize oleic acid content are consistent with that reported to be 24% in Lambert (2000), while they are below that reported to be 20 - 50% in Olmos et al. (2018).

Maize Linoleic Acid Content (%)

The effect of intercropping on the linoleic acid content of the maize plant was also statistically insignificant. The linoleic acid content of maize varied from 58.97 to 60.90%. The lowest linoleic acid content was observed in the sole-cropped maize, while the highest linoleic acid content was observed in one row of soybean intercropped with three rows of maize. The mean linoleic acid content of the intercropped maize was 59.72% (*Table 6*).

Our results on the maize linoleic acid content are above those reported to be 41.84 - 50.70% in Betancourt et al. (2017).

Maize Linolenic Acid Content (%)

The effect of intercropping on the linolenic acid content of the maize plant was also statistically insignificant. The linolenic acid content of maize varied from 0.91 to 1.13%. The lowest linolenic acid content was observed in one row of soybean intercropped with one row of maize, while the highest linolenic acid content was observed in the sole-cropped maize. The mean linolenic acid content of the intercropped maize was 1.01% (*Table 6*).

Our results on the maize linolenic acid content are consistent with that reported to be 0.65-0.95% in Betancourt et al. (2017), while they are below those reported to be 1.31 - 2.02% in Akıncı et al. (2011).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

Maize Unsaturated Fatty Acid Content (%)

Unsaturated fatty acids have double bonds (Ramos et al., 2009). The effect of intercropping on the unsaturated fatty acid content of the maize plant was also statistically insignificant. The unsaturated fatty acid content of maize varied from 84.62 to 85.84%. The lowest unsaturated fatty acid content was observed in the sole-cropped maize, while the highest unsaturated fatty acid content was observed in one row of soybean intercropped with three rows of maize. The mean unsaturated fatty acid content of the intercropped maize was 85.37% (*Table 7*).

Maize Saturated Fatty Acid Content (%)

Saturated fatty acids have no double (Ramos et al., 2009). The effect of intercropping on the saturated fatty acid content of the maize plant was also statistically insignificant. The saturated fatty acid content of maize varied from 14.15 to 15.37%. The lowest saturated fatty acid content was observed in one row of soybean intercropped with three rows of maize, while the highest saturated fatty acid content was observed in the sole-cropped soybean. The mean saturated fatty acid content of the intercropped maize was 14.61% (*Table 7*).

Table 7. *The Values of Unsaturated Fat Acid Content, Saturated Fat Acid Content, and Unsaturated to Saturated Fat Acid Ratio for the Intercropped Maize*

Intercropping	Unsaturated Fat Acid Content (%)*	Saturated Fat Acid Content (%) **	Unsaturated to Saturated Fat Acid Ratio (%)
SM	84.62	15.37	5.50
1 S + 1 M	85.10	14.89	5.72
1 S + 2 M	85.44	14.55	5.87
1 S + 3 M	85.84	14.15	6.08
2 S + 1 M	85.70	14.29	5.99
3 S + 1 M	85.55	14.44	5.92
Mean	85.37	14.61	5.84
Significance	-	-	-
LSD	-	-	-

* Unsaturated fat acid content (%) = oleic acid + linoleic acid + linolenic acid
* * Saturated fat acid content (%) = palmitic acid + stearic acid

Maize Unsaturated to Saturated Fatty Acid Ratio (%)

The most important property that determines the quality of vegetable fats is the ratio of the total amount of unsaturated fatty acids to the total amount of saturated fatty acids. The effect of intercropping on the unsaturated to saturated fatty acid ratio of the maize plant was also statistically insignificant. The unsaturated to saturated fatty acid ratio of maize varied from 5.50 to 6.08%. The lowest ratio was observed in the sole-cropped maize, while the highest ratio was observed in one row of soybean intercropped with three rows of maize. The mean unsaturated to saturated fatty acid ratio of the intercropped maize was 5.84% (*Table 7*).

Soybean Palmitic Acid Content (%)

The effect of intercropping on the palmitic acid content of the soybean plant was statistically insignificant. The palmitic acid content of soybean varied 10.39 to 10.77%. The lowest palmitic acid content was observed in one row of soybean intercropped with

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DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

two rows of maize, while the highest palmitic acid content was observed in the sole-cropped maize and in one row of soybean intercropped with one row of maize. The mean palmitic acid content of the intercropped soybean was 10.64% (*Table 8*).

Our results on the soybean palmitic acid content are consistent with those reported to be 2.7 - 11.2% in Bilyeu et al. (2018), while they are below those reported to be 10.98 - 12.88% in Arıoğlu et al. (2012) but above those reported to be 8.80 - 10.30% in Arıoğlu et al. (2012).

Soybean Stearic Acid Content (%)

The effect of intercropping on the stearic acid content of the soybean plant was also statistically insignificant. The stearic acid content of soybean varied from 3.81 to 4.28%. The lowest stearic acid content was observed in the sole-cropped soybean, while the highest stearic acid content was observed in three rows of soybean intercropped with one row of maize. The mean stearic acid content of the intercropped soybean was 3.99 % (*Table 8*).

Our results on the soybean stearic acid content are consistent with those reported to be 3.60 - 4.70% in Kan et al. (2011), while they are below those reported to be 4 - 12.1% in Carrero et al. (2014) but above those reported to be 1.15 - 2.02% in Sultan et al. (2015).

Table 8. *The Values of Palmitic Acid, Stearic Acid, Oleic Acid, Linoleic Acid, and Linolenic Acid for the Intercropped Soybean*

Intercropping	Stearic Acid (%)	Palmitic Acid (%)	Oleic Acid (%)	Linoleic Acid (%)	Linolenic Acid (%)
SS	3.81	10.77	20.06	58.26	7.07
1 S + 1 M	3.99	10.77	19.86	58.59	6.78
1 S + 2 M	4.06	10.39	19.88	58.59	7.05
1 S + 3 M	3.84	10.76	19.87	58.61	6.91
2 S + 1 M	3.97	10.48	19.97	58.98	6.58
3 S + 1 M	4.28	10.71	20.29	58.01	6.61
Mean	3.99	10.64	19.98	58.50	6.83
Significance	-	-	-	-	-
LSD	-	-	-	-	-

Soybean Oleic Acid Content (%)

The effect of intercropping on the oleic acid content of the soybean plant was also statistically insignificant. The oleic acid content of soybean varied from 19.86 to 20.29%. The lowest oleic acid content was observed in one row of soybean intercropped with one row of maize, while the highest oleic acid content was observed in three rows of soybean intercropped with one row of maize. The mean oleic acid content of the intercropped soybean was 19.98% (*Table 8*).

Our results on the maize oleic acid content are consistent with that reported to be 20.09 - 20.88% in Sultan et al. (2015), while they are below those reported to be 22.20 - 25.90% in Kan (2011).

Soybean Linoleic Acid Content (%)

The effect of intercropping on the linoleic acid content of the soybean plant was also statistically insignificant. The linoleic acid content of soybean varied from 58.01 to

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DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

58.98%. The lowest linoleic acid content was observed in three rows of soybean intercropped with one row of maize, while the highest linoleic acid content was observed in two rows of soybean intercropped with one row of maize. The mean linoleic acid content of the intercropped soybean was 58.50% (*Table 8*).

Our results on the maize linoleic acid content are below those reported to be 50.30 - 54.30% in Kan (2011).

Soybean Linolenic Acid Content (%)

The effect of intercropping on the linolenic acid content of the soybean plant was also statistically insignificant. The linolenic acid content of soybean varied from 6.58 to 7.07%. The lowest linolenic acid content was observed in two rows of soybean intercropped with one row of maize, while the highest linolenic acid content was observed in the sole-cropped soybean. The mean linolenic acid content of the intercropped soybean was 6.83% (*Table 8*).

Our results on the soybean linolenic acid content are consistent with those reported to be 6.30 - 7.62% in Arıoğlu et al. (2012), while they are below those reported to be 7 - 10 % in Kim et al. (2015).

Soybean Unsaturated Fatty Acid Content (%)

The effect of intercropping on the unsaturated fatty acid content of the soybean plant was also statistically insignificant. The unsaturated fatty acid content of soybean varied from 84.92 to 85.54%. The lowest unsaturated fatty acid content was observed three rows of soybean intercropped with one row of maize, while the highest unsaturated fatty acid content was observed in two rows of soybean intercropped with one rows of maize. The mean unsaturated fatty acid content of the intercropped soybean was 85.33% (*Table 9*).

Our results on the soybean unsaturated fatty acid content are consistent with those reported to be 82.02 - 84.93% in Arıoğlu et al. (2012), while they are below those reported to be 88.12 - 88.58% in Sultan et al. (2015).

Soybean Saturated Fatty Acid Content (%)

The effect of intercropping on the saturated fatty acid content of the soybean plant was also statistically insignificant. The saturated fatty acid content of soybean varied from 14.45 to 14.99%. The lowest saturated fatty acid content was observed in two rows of soybean intercropped with one row of maize, while the highest saturated fatty acid content was observed in three rows of soybean intercropped with one row of maize. The mean saturated fatty acid content of the intercropped soybean was 14.64% (*Table 9*).

Our results on the soybean saturated fatty acid content are consistent with those reported to be 13.59 - 16.88% in Arıoğlu et al. (2012), while they are below those reported to be 17.60 - 20.36% in Barış (2016) but above those reported to be 11.42 - 11.80% in Sultan et al. (2015).

Soybean Unsaturated to Saturated Fatty Acid Ratio (%)

The effect of intercropping on the unsaturated to saturated fatty acid ratio of the soybean plant was also statistically insignificant. The unsaturated to saturated fatty acid ratio of soybean varied from 5.66 to 5.91%. The lowest ratio was observed in three rows of soybean intercropped with one row of maize, while the highest ratio was observed in one row of soybean intercropped with two rows of maize. The mean unsaturated to saturated fatty acid ratio of the intercropped soybean was 5.83% (*Table 9*).

Our results on the soybean unsaturated fatty acid content are consistent with those reported to be 6.25 - 4.90% in Arıoğlu et al. (2012), while they are above those reported to be 3.92 - 4.69% in Barış (2016).

Table 9. *The Values of Unsaturated Fat Acid Content, Saturated Fat Acid Content, and Unsaturated to Saturated Fat Acid Ratio for the Intercropped Soybean*

Intercropping	Unsaturated Fat Acid Content (%)*	Saturated Fat Acid Content (%) **	Unsaturated to Saturated Fat Acid Ratio (%)
SS	85.40	14.59	5.86
1 S + 1 M	85.23	14.76	5.79
1 S + 2 M	85.53	14.46	5.91
1 S + 3 M	85.39	14.60	5.85
2 S + 1 M	85.54	14.45	5.93
3 S + 1 M	84.92	14.99	5.66
Mean	85.33	14.64	5.83
Significance	-	-	-
LSD	-	-	-

* Unsaturated fat acid content (%) = oleic acid + linoleic acid + linolenic acid
* * Saturated fat acid content (%) = palmitic acid + stearic acid

Maize Grain Yield (kg ha^{-1})

The effect of intercropping on the grain yield of the maize plant was found to be statistically very significant (P<0.01). The grain yield of maize varied from 32.01 kg ha^{-1} to 89.52 kg ha^{-1}. The lowest grain yield was observed in three rows of soybean intercropped with one row of maize, while the highest grain yield was observed in the sole-cropped maize. The mean grain yield of the intercropped maize was 63.20 kg ha^{-1} (*Table 10*).

Our results on the maize grain yield are consistent with those reported to be 37.00 - 51.00 kg ha^{-1} in Ijoyah and Fanen (2012) and 67.50 kg ha^{-1} in Alı et al. (2015), while they are above that reported to be 17.70 kg ha^{-1} in Rajı (2007).

Soybean Grain Yield (kg ha^{-1})

The effect of intercropping on the grain yield of the soybean plant was also found to be statistically very significant (P<0.01). The grain yield of soybean varied from 10.36 kg ha^{-1} to 61.11 kg ha^{-1}. The lowest grain yield was observed in one row of soybean intercropped with two rows of maize, while the highest grain yield was observed in the sole-cropped soybean. The mean grain yield of the intercropped soybean was 33.59 kg ha^{-1} (*Table 10*).

Our results on the soybean grain yield are consistent with those reported to be 10.20 kg ha^{-1} in Adeniyan and Ayoola (2007), 10.2 kg ha^{-1} in Raji (2007), 7.80 - 12.10 kg ha^{-1} in Ijoyah and Fanen (2012), and 10.88 kg ha^{-1} in Ali et al. (2015).

Land Equivalent Ratio (LER)

LER refers to the land amount needed by sole-cropping to produce the equivalent yield obtained from intercropped maize and soybean. The LER value varied from 1.03 to 1.21. The highest LER was observed in three rows of soybean intercropped with one

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DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

row of maize, while the lowest LER was observed in one row of soybean intercropped with two rows of maize (*Table 10*).

Our results on the LER value are consistent with those reported to be 1.27 in Wekesa et al. (2015) and 1.27 in Zhang et al. (2015), while they are below those reported to be 1.30 in Jamkhanh et al. (2012), 1.29 in Osang et al. (2014), and 1.44 in Alı et al. (2015).

Table 10. *The Values of Grain Yield and LER for the Intercropped Maize and Soybean*

Intercropping	Maize Grain Yield (kg ha^{-1})	Soybean Grain Yield (kg ha^{-1})	LER
YM / YS	89.52 a	61.11 a	-
1 S + 1 M	59.33 c	27.16 c	1.11
1 S + 2 M	76.53 b	10.36 d	1.03
1 S + 3 M	75.71 b	17.79 d	1.14
2 S + 1 M	46.07 d	33.35 c	1.06
3 S + 1 M	32.01 e	51.75 b	1.21
Mean	63.20	33.59	-
Significance	**	**	-
LSD	6.249	80.32	-

Conclusions and Suggestions

The study has found significant differences between the applied cropping systems in terms of the maize ash content, soybean protein content, and soybean crude fat content but not in terms of the grain yields of maize and soybean. Considering the analysis results and LER values together, three rows of soybean intercropped with one row of maize were found to be the most appropriate intercropping system. However, this study needs to be carried out for 1 to 2 years for a more precise recommendation. In line with the findings of the study, further work could assess different systems of cropping and focus on the fodder and silage capacity of intercropped maize and soybean rather than grain yield.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21492165

EVALUATION OF THREE CANOLA (*BRASSICA NAPUS* L.) CULTIVARS FOR YIELD AND SOME QUALITY PARAMETERS UNDER THE ENVIRONMENTAL CONDITION OF SOUTHEASTERN ANATOLIA, TURKEY

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(Received 20th Nov 2018; accepted 11th Jan 2019)

Abstract. Canola is a significant alternate oilseed crop in Southeastern Anatolia, Turkey. While, there is no evidence for good yield potential canola cultivars are available in this area. Therefore, the study was conducted with three canola cultivars in consecutive two seasons (2013-14 and 2014-15) to find out the appropriate canola cultivars which are suitable to grow under the environmental condition of South-eastern Anatolia, Turkey. The experiment was carried out in a randomized complete block design with three replications. After two years of observation, it was found that plant height and branches plant^{-1} of all cultivars were varied significantly in both years. Considering the growing season, the maximum seed yield (2593.7 kg ha-1) was recorded in the first season (2014-15), while the minimum was recorded in the second season (2015-16). Among the cultivars, seed yield varied significantly and the maximum seed yield (2617.1 kg ha-1) was achieved in the variety 'Licrown'. The variety, 'Licord' was produced the highest oil content (37.92%) and protein (22.48%), while, variety 'Express' achieved the lowest oil content (37.43%). The greatest palmitic acid (4.67%), stearic acid (2.06%) and oleic acid (65.5%) content were produced by the variety 'Express'. While the variety 'Licord' produced the smallest value of stearic acid (1.86%), linoleic acid (17.17%). The variety 'Express' produced the lowest saturated fatty acid content (12.63%), while, the variety 'Licrown' achieved the highest (12.97%), although, the variety 'Licrown' produced the lowest (85.14%), and variety 'Express' produced the highest (85.47%) unsaturated fatty acid content. It is concluded that all cultivars have a good potential for the winter season of Southeastern Anatolia of Turkey. Accordingly, in the future the cultivar 'Express' could be used as a cultivar with high oleic and linoleic acid content and high yield under investigation conditions.
Keywords: *canola, genotypes, oil composition, saturated fatty acid, unsaturated fatty acids, yield*

Introduction

Canola (*Brassica napus* L.) is an essential oil seed crop in the world. Its oil is used as biofuel, human consumption (edible oil), feeding animals, and used in chemical and pharmaceutical industries (Friedt and Snowdon, 2009). It belongs to the family of Brassicaceae, which is becoming one of the major sources of the vegetable oil in the world (Baghdadi et al., 2013). It is the second largest oil seed after soybean worldwide, producing high-protein meal used for animal feed during processing (FAO, 2018). Presently, over 50% of vegetable oil, consumed in Turkey, is imported from abroad (İkiel and Kaymaz, 2005). To reduce the gap between oil consumption and the production of oilseed in Turkey, it is essential to grow alternate oilseed crops such as canola in crop rotation system in this region. Thus, the supplementary production of oilseed can meet up vegetable oil, increase biodiesel production, and decrease the import of vegetable oil (Öztürk, 2010; Cosgun and Öztürk, 2014).

Canola is a specific type of rapeseed correlated with high quality oil and meal. It is a special type of rapeseed that have less than 2% erucic acid in the oil and less than 30 micromoles per gram of glucosinolates in the oil-free meal (Anonymous, 2002). Moreover, canola contains 40-45% oil and 39% protein, and oil contains a desirable

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21672177

profile of saturated fatty acids (~7%), high level of unsaturated fatty acids like oleic acids (61%) and medium level of linoleic acid (21%) and linolenic acid (11%) (Molazem et al., 2013; El Sabagh et al., 2017; El Sabagh et al., 2018; Joughi et al., 2018). Hence, it represents a healthy edible oil. Oil seed of canola provide over 13.2% of edible oil in the world (Eskandari and Kazemi, 2012; Joughi et al., 2018). Gheorghe et al. (2013) found that grain yields significantly differences among fifty rapeseed cultivars grown under Romanian conditions. The agronomic management factors influenced the yield of the crop (Anonymous, 1992) and the cultivars with high yield potential play an important role in the increase of the yield per unit area (Sana et al., 2003). However, winter canola is a relatively new crop for Turkey, but the suitable cultivars for winter canola have not yet been established for this region of study (Coşgun and Öztürk, 2014). Therefore, in this study we considered three cultivars to evaluate the varietal performance of these cultivars, considering agronomic characteristics, oil quantity and quality parameters in Southeastern Anatolia of Turkey.

Materials and methods

Location, duration, edaphic and climatic conditions

Two field experiments were conducted during 2013/14 and 2014/15 growing seasons at the research area of Dicle University to evaluate the yield and quality properties of some canola cultivars in the Southeastern Anatolia region of Turkey. Soil samples at sowing were analyzed and was observed the soil was classified as clay (71.6%), 1.25% organic matter, Available Phosphorus 1.63 kg/da, 13.02% alkaline, 0.01-0.02% salt and 7.73 pH. The average, and minimum temperatures, monthly rainfall and relative air humidity data for 2013/2014-2014/2015 during the canola vegetation period are presented in (*Fig. 1*).

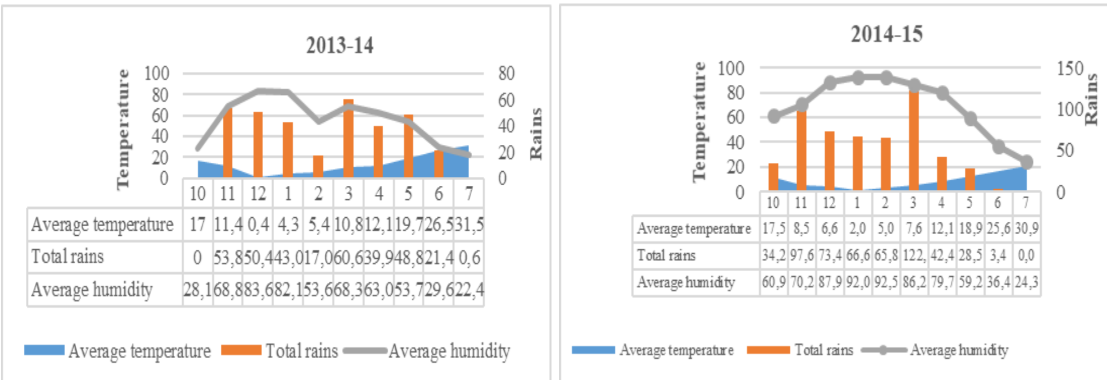

***Figure 1.** Monthly average air temperatures °C, totally rains (mm) and humidity (percentage), respectively during the growing seasons*

The monthly rainfall, humidity and average temperatures for 2013-14 and 2014-15 within the growing period of canola (October-May) are presented in *Figure 1*.

The average temperature was around 14.5 °C. The average rainfall was 42.07 mm. The highest rainfall was observed in March in 2013-14 as 60.6 mm and in March in 2014-15 as 122.2 mm. Air temperatures and humidity were close to the long term averages during the two growing seasons and but annual rainfall is different in the

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experimental years. 2013-14 growing season was a relatively dry year and monthly rainfall during the growing season (October–May) were lower than 2014-15 and long-term averages. Frost damage was not seen in 2014 and 2015 years.

Experimental design, plant materials, treatments and experimentation

The experimental area is located (37°532588 N, 40°162356 E) at 670 m above sea level. The field experiments were arranged in a randomized complete block design with three replications. Three canola (*Brassica napus* L.) cultivars i) Express, ii) Licord, and iii) Licrown were used as material in this study, which these cultivars are consider the most preferred are variants in Turkey. It has been used to determine the most suitable cultivars in southeastern of Anatolia. The area of each plot was including 6 rows with 0.7 m row distance, 5 m row length, plants in the row were 5 cm apart from each other and seed rate of 60 seed m^{-2}. All plots were received at 36 kg N ha^{-1} and 92 kg P_2O_5 ha^{-1} (18-16-00) before the sowing of crop and 80 kg N ha^{-1} was added as top dressing during stem elongation. The source of N was ammonium nitrate (33%N). The crop was sown on October 20 in both years (2013/14 and 2014/2015). The crop was harvest on June 20 in both years (2013/14 and 2014/15).

Data collection

Yield attributes: Randomize selected 10 plants each plot. Plant height, branch number, pod number and seed number. 1000-seed weight, and seed yield were examined. Plants were harvested 11.2 m^2. Seed yield (kg ha^{-1}) was calculated from the plants of the four ridges in each plot and the yield per hectare was measured.

Quality properties

Oil content, protein content and Fatty acid content: Protein content (%) was measured by Kjeldahl method (AOAC, 2000). Oil content (%) was measured by Soxhlet instrument with n-hexane (60 °C) as organic solvent. Fatty acid compositions of seeds were measured with a gas chromatography and the analyses were performed at the central laboratories of Tubitak–Mam (*Marmara Research Center*) according to the method described by Slover and Lanza (1979).

Statistical analysis

Data for the experiment was analysed by JMP 5.0 software program for comparison of data was analyzed by JMP package program Mean tested by Tukey at the 1% level.

Results and discussion

Agronomic traits

The results of ANOVA for agronomic traits are presented in *Table 1*. Statistical analysis for the yield attributes, the analysis of variance, year, and year x cultivar interactions were found significant on the plant height, and the number of branches per plant except for pod number, seed number, 1000-seed weight and seed yield. Moreover, the cultivars were insignificant for all parameters. According to the mean of two years were revealed that, the highest number of pod (122.5) was produced from Licrown and the lowest value (112.2) was produced from Express. These findings showed that, the

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highest value of seed number was achieved from Expres with 25.9, while the lowest value was produced from Licrown with 24.5 (*Table 2*).

***Table 1.** Analysis of variance and means of some yield traits of canola genotypes*

Source of variation	Df	Plant height (cm)	Pod number/plant	Seed number/pod^{-1}	1000-seed weight (g)	Branch number/plant	Seed yield (kg ha^{-1})
Block (B)	2	-	-	-	-	-	-
Years (Y)	1	**	ns	ns	ns	*	ns
Cultivars (C)	2	ns	ns	ns	ns	ns	ns
Y × C	2	*	ns	ns	ns	*	ns
Error	10						
CV		10.04	11.33	6.17	6.96	19.09	8.46

ns: not significant, *significant at P < 0.05, **significant at P < 0.01

***Table 2.** Response of canola cultivars on seed yield attributes and yield*

Cultivars	Plant height (cm)			Pod number/plant			Seed number/pod^{-1}		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	124.7 b	121.1	122.9	112.4	112.1	112.2	25.9	25.9	25.9
Licord	154.2 a	124.2	139.1	105.6	121.1	113.3	26.2	24.6	25.4
Licrown	159.2 a	120.4	139.8	127.2	117.9	122.5	25.3	23.7	24.5
Mean	146.0 a	121.9	133.9	115.1	117.0	116.0	25.8	24.7	25.3
LSD$_{caultivars}$	22.29	-	-	-	-	-			
LSD$_{year}$	13.81			-	-	-			

Cultivars	1000-seed weight (g)			Branch number/plant			Seed yield (kgha^{-1})		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	3.27	3.34	3.31	3.7	2.8	3.25	2317	2560	2439
Licord	3.38	3.57	3.47	4.0	2.9	3.45	2285	2652	2468
Licrown	3.27	3.58	3.42	3.6	3.3	3.45	2665	2568	2617
Mean	3.31	3.50	3.40	3.8 a	3.0 b	3.40	2422	2593	2508
LSD$_{cultivars}$	-	-	-	-	-	-	-	-	-
LSD$_{year}$	-		-	0.7		-	-	-	-

In each column, means followed by the same letter within columns are not significantly different (P < 0.01) according to Tukey test, ns: not significant

Considering the average two years yield, the greatest seed yield was achieved from second (2014-15) growing season with 2593.7 kg ha^{-1} and the minimum seed yield was achieved in first (2013-14) growing season. Regarding the averages, the difference between cultivars were statistically non-significant in terms of seed yield and the highest seed yields were produced from cultivar ‘Licrown’ (2617.1 kg ha^{-1}) in *Table 2*. Several factors affect the seed yield and yield attributes in crop, including cultivar, seasonal variation, location, planting date, soil nutrient, moisture availability, growing conditions in different crops (Abdelaal et al., 2017; Barutçular et al., 2017; Gormus et al., 2017a, b). Zhang et al. (2011) who pointed out the seed yield of different canola cultivars was significantly varied among them. Also, El Habbasha and El salam (2009) observed that there are significant differences among canola cultivars on the seed yield. It was assessed several genotypes of *Brassica napus* L. and found significant differences for some yield attributes. Likewise, various investigators studied for various mustard and canola cultivars, and observed the seed yield differences among the cultivars were significantly (Khehra and Singh, 1988; Stingram et al., 2002).

Quality traits

The statistical analysis (ANOVA) of data of quality traits are presented in (*Table 3*). It was observed that, the cultivars differed significantly and various cultivars non-significant in in oil quality traits. The means of some of quality properties of canola cultivars are given (*Table 4)*. As it can be seen from (*Table 4*), the oil and protein content among peanut varieties was significant, the cultivar Licord was recorded the highest oil content (37.92%) and protein (22.48%), while, the cultivar Express was produced the lowest oil content (37.43%). Oil content of canola depends on several factors such as variety, growth environment, agrotechnics and fertilization (Laaniste et al., 2004; Coşgun and Öztürk, 2014). It was found that the oil and protein percentage in various crops depending on genotype and growing conditions, and influenced by genotype, climatic conditions, geographical location, growing season and growing conditions (Yıldırım et al., 2018; Akdeniz et al., 2018a,b). Oil content is negatively related with protein content (Gül et al., 2005). Although, protein content may be affected by environments, they differ due to the genetic properties (Atakişi, 1977).

Generally, more work required to enhance oil quality among canola cultivars under investigation conditions. The main aims in canola breeding to reduction the long chain and saturated fatty acids in canola cultivars. Statistically significant differences among cultivars were found for fatty acids. The saturated fatty acids content in canola oil were affected by cultivars, the greatest value of palmitic acid (4.67%), stearic acid (2.06%) and oleic acid (65.5%) were produced from the variety Express. On the other hand, the cultivar Licord achieved the smallest value of stearic acid (1.86%) followed Licrown it statically group. According to Hassan et al. (2005) observed that significant differences for saturated fatty acids were due to the variances of the genotypes. However, the saturated fatty acids content in oil were affected by genotype (Isleib et al., 2008).

Table 3. *Analysis of variance and means of some quality properties of canola genotypes*

Source of variation	Df	Oil content	Protein content	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Alpha-linoleic acid	Gamma-linolenic acid	Arachidonic acid	Eicosadienoic acid	Erucic acid	Lignoceric acid	Nervonik acid	Saturated fatty acids (SFA)	Unsaturated fatty acids (UFA)	SFA/UFA	Oleic/linoleic
Block (B)	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Year (Y)	1	ns	*	ns	**	**	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
Cultivars (C)	2	ns	ns	*	ns	**	**	**	ns	*	**	*	ns	*	ns	**	ns	**	**
Y x C	2	*	*	ns	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Error	10																		
CV		2.31	5.03	4.05	2.77	4.57	0.64	1.27	5.88	9.04	4.63	9.92	11.06	8.87	10.11	0.83	0.14	0.96	1.80

ns: not significant, *significant at $P < 0.05$, **significant at $P < 0.01$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21672177

***Table 4**. Response of canola cultivars on protein and oil fatty acid contents*

Cultivars	Oil content (%)			Protein content (%)			Palmitic acid (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	36.27	38.60	37.43	20.62	23.91	22.26	4.68	4.66	4.67 b
Licord	38.33	37.50	37.92	21.57	23.40	22.48	4.19	4.42	4.30 b
Licrown	38.10	37.70	37.90	23.74	23.15	23.44	4.52	4.49	4.50 ab
Mean	37.57	37.93	37.75	21.98	23.48	22.73	4.46	4.52	4.92
LSD	-	0.87	-	-	-	-	-	-	0.23

Cultivars	Palmitoleic acid (%)			Stearic acid (%)			Oleic acid (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	0.173	0.177	0.175	1.99	2.12 a	2.06 a	65.2	65.8 a	65.5 a
Licord	0.160	0.180	0.170	1.74	1.98 b	1.86 b	64.4	64.9 b	64.6 b
Licrown	0.173	0.177	0.175	1.87	1.95 b	1.91 b	64.2	64.4 c	64.3 b
Mean	0.169	0.178	0.173	1.87	2.02	1.94	64.6	65	64.8
LSD_v	-	-	-	-	0.06	0.11	-	0.4	0.54

Cultivars	Linoleic acid (%)			Alpha-linoleic acid (%)			Gamma-linolenic acid (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	17.37 b	16.96 c	17.17 b	1.2	1.24	1.22	0.706	0.747	0.727 a
Licord	18.23 a	17.80 b	18.01 a	1.15	1.11	1.13	0.567	0.653	0.610 b
Licrown	17.93 b	18.24 a	18.09 a	1.22	1.13	1.17	0.653	0.653	0.653 a
Mean	17.84	17.67	17.75	1.19	1.16	1.17	0.642	0.684	0.663 a
LSD_v	0.48	0.16	0.29	-	-	-	-	-	0.077

In each column, means followed by the same letter within columns are not significantly different (P < 0.01) according to Tukey test, ns: not significant

Generally, in case of average values of both years, the variety Express achieved the smallest value (12.63%), while, the cultivar Licrown generated the greatest (12.97%) saturated fatty acid. In contrary, the maximum value (85.47%) and the minimum values (85.14%) of unsaturated fatty acid were observed in the variety Express and Licrown, respectively. Accordingly, the variety ‘Express’ achieved the highest rate for saturated fatty acids/unsaturated fatty acids (6.75%) and oleic/linoleic (3.82%). The potential impact of seed maturity is dependent on genotype, climatic conditions, and genotype/climate interactions was reported by (Onat et al., 2017). Lower temperature during the seed development normally is associated with more unsaturated oil due to the increased activity of oleatedesaturase, which promotes the synthesis of linoleic acid (Bovi, 1982). The increase in oleic acid with seed maturity is normally accompanied by a decrease in palmitic, linoleic, arachidic, eicosenic, behenic and lignoceric acid (Holaday and Pearson, 1974).

Concerning, the Erucic concentration, Erucic acid is one of the most essential fatty acids within Brassica genus. Low or zero erucic acid contents are desirable in edible oil. Data in *Table 5* revealed that the amount of Erucic percentage in all canola cultivars used ranged from the lowest (0.182) in Express to the highest (0.252 and 0.243) in Licord and Licrown. These results were supported by another investigations finding, who reported that, the safe limits for these compounds have been described as less than 2% of erucic acid in oil (Grombachr and Nelson, 1992; El sabah et al., 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 5. *Response of canola cultivars on oil fatty acid contents*

Cultivars	Arachidonic acid (%)			Eicosadienoic acid (%)			Erucic acid (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	5.85 b	5.60 b	5.73 b	0.380	0.387	0.383 a	0.203	0.160	0.182
Licord	6.71 a	6.26 a	6.48 a	0.313	0.333	0.323 b	0.243	0.260	0.252
Licrown	6.45 a	6.33 a	6.39 a	0.357	0.347	0.352ab	0.313	0.173	0.243
Mean	6.34	6.06	6.20	0.350	0.355	0.352	0.350	0.198	0.225
LSD_{year}	0.59	0.21	0.36	-	-	0.045	0.076	-	-

Cultivars	Lignoceric acid (%)			Nervonik acid (%)			Saturated fatty acids (SFA) (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	0.177	0.180	0.178 a	0.127	0.123	0.125	12.70	12.56 b	12.63 c
Licord	0.143	0.157	0.150 b	0.133	0.120	0.127	12.79	12.81 a	12.80 b
Licrown	0.173	0.163	0.168 ab	0.140	0.120	0.130	13.01	12.94 a	12.97 a
Mean	0.164	0.167	0.165	0.133	0.121	0.127	12.83	12.77	12.80
LSD_{year}	-	-	0.019	-	-	-	-	0.13	0.14

Cultivars	Unsaturated fatty acids (%)			UFA/SFA (%)			Oleic/linoleic (%)		
	2013/14	2014/15	Mean	2013/14	2014/15	Mean	2013/14	2014/15	Mean
Express	85.39	85.56 a	85.47 a	6.70	6.80 a	6.75 a	3.75 a	3.88 a	3.82 a
Licord	85.21	85.34 ab	85.27 ab	6.66	6.64 b	6.65 b	3.53 b	3.65 b	3.59 b
Licrown	85.03	85.25 b	85.14 b	6.63	6.67 c	6.65 c	3.58 b	3.53 c	3.55 b
Mean	85.21	85.38	85.29	6.63	6.67	6.65	3.62	3.68	3.65
LSD_{year}	-	0.21	0.21	-	0.06	0.08	0.13	0.05	0.08

In each column, means followed by the same letter within columns are not significantly different ($P < 0.01$) according to Tukey test, ns: not significant

The differences in the fatty acids among cultivars are due to differential growth habit, environmental factors and genetic variations. The statistically analysis of data indicated that cultivars differed significantly for oil content among cultivars, locations and their interactions in present study was confirmed the earlier by (Pritchard et al., 2000). Oil content of canola depends on several factors such as variety, growth conditions and fertilization (Laaniste et al., 2004). Bengtsson (1988) recorded 9% difference between two cultivars of winter canola, while, it was reported less difference of 2.3% between different *Brassica carinata* lines for seed oil content (Gentent et al., 1966). The protein contents of cultivar examined in the study varied between 21.98 to 23.48%. The protein content may be affected by environmental conditions (Barszczak et al., 1993), and Sargın (2012) noted that the variation largely depends on the genetic properties of variety.

The various cultivars of canola crop are differed to fatty acid under the current environments. It was observed that the most important fatty acid in rapeseed is oleic acid with 70-80% (Schierholt, 2000). Although the low level of palmitic acid and other saturated fatty acids (less than 5%) in canola oil is considered to be nutritionally desirable (Kay, 1988). In this concern McCartney et al. (2004) found that the major of the variation in the palmitic (C16:0) due to the impact of genotypic. The oil composition in peanut is affected by different factors involving environmental factors, genetic factors, and interaction between environmental and genetic factors (Mzimbiri et al., 2014; Gulluoglu et al., 2016). Fatty acid composition is the function of genotype, climate conditions, morphology and physiology as well as crop management (Arsalan, 2007).

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Conclusion

The results of the study suggested that, that all cultivars have a good potential for the winter season of Southeastern Anatolia of Turkey. Regarding the production, all cultivars were surpassed in yield as well as, others quality parameters such as high oleic, linoleic (Express) as well as high content in unsaturated Fatty Acids SFA/UFA. All cultivars produced low erucic acids, while the cultivar 'Licord' produced low linolenic acid content. The cultivar, 'Express' was produced a high oleic and linoleic acid content and these findings could be useful in maintaining the required yield and quality attributes of canola cultivars under investigation condition for future breeding programs.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21672177

HABITAT UTILIZATION AND WINTER TIME-ACTIVITY BUDGETS OF THE WHOOPER SWAN *CYGNUS CYGNUS* (LINNAEUS, 1758) POPULATION WINTERING AT THE VAN LAKE BASIN, TURKEY

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(Received 23rd Nov 2018; accepted 16th Jan 2019)

Abstract. In this study, daily behaviour patterns of whooper swans (*Cygnus cygnus*) were studied at the Van Lake Basin, which is one of the most important wintering areas of the species in Turkey. Winter-time activities of the swans were monitored from sunrise to sunset in 6 different categories: resting, feeding, preening, walking on land, swimming and flying. The most common daytime activity was resting (55%). Feeding (12.48%) and preening (10.98%) were the two other main diurnal activities of whooper swans during the present study. Resting peaked in midday period (08:30-13:30) while feeding was observed frequently in early morning hours (06:00-8:30) and late afternoon (13:30-17:00). At the beginning of severe winter period (December and January) whooper swans clearly reduced feeding time in all parts of the day, probably because they still met metabolic demands. A significant increase in feeding behaviour was observed in January and February when they were preparing to migrate. While open water surface was the most preferred area for resting, shallow coastal areas and reeds were the most utilized foraging habitats.
Keywords: *waterfowl, behaviour, diurnal period, movements, waterbirds, wetlands*

Introduction

Behaviour patterns of waterbirds vary with weather conditions, food availability, threat factors and the physiological status of the species (Weathers and Sullivan, 1993; Meissner and Markowska, 2009). These behaviours that birds show under different environmental conditions offer important tips in understanding their ecological requirements. Furthermore, changes in waterbird activity may provide an earlier indication of an impact than numbers of birds (Weathers and Sullivan, 1993; Hamilton et al., 2002).

Time-activity budget studies of wintering waterbirds give information about seasonal microhabitat selection and the influence of hunting pressure, habitat alteration and other threats on behaviour (Poulton et al., 2002; Michot et al., 2006; Crook et al., 2009). In this manner, such studies can be used to evaluate habitat quality and quantity, to compare activity patterns of populations in different geographic regions and contribute to wetland management or species conservation plans (Woodin and Michot, 2006).

Whooper swan (*Cygnus cygnus*) is mainly a winter visitor species to Europe and Turkey. Except reproduction period, they migrate in small flocks and congregating into flocks of up to 300-400 individuals in the winter (Johnsgard, 1978; Madge and Burn, 1988). The overall population trend is uncertain. Population size in Europe is estimated at between 25300-32800 pairs and the status is shown as “LC” in the IUCN category (Anonymous, 2015a). A limited number of studies about the Turkey population of the whooper swan indicate that the species has been wintering in the Van Lake Basin since the early 1990s. The species arrives to the basin from the beginning of the winter and stays at the basin until the beginning of spring and then leaves. Depending on the year,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21792185

the population size of the species wintering at Van Lake Basin ranges between 50-240 individuals (Adızel, 1995; Adızel et al., 2010; Azizoğlu et al., 2016, 2017; Çelik et al., 2016). In recent years, the number of individuals wintering in the Basin has increased while there is a lack of data about behaviour pattern of the species. The aim of this study was to determine the daily behaviour of a colony of whooper swan wintering in the Van Lake Basin.

Materials and methods

Study area

This research was carried out at Göründü Delta (38.328637° N, 42.927174° E) in the southern part of Van Lake. Göründü Delta that located on the 58th km of Van-Bitlis highway, is a wetland with 124 ha (*Fig. 1*). The delta, 1648 m in altitude and 5 km^2 in width, is an internationally important wetland that hosts large numbers of migratory and the resident waterbirds (Anonymous, 2015b; Aşur, 2017). In recent years, one of the unique habitats where the whooper swans were wintering in the eastern of Turkey is the Göründü Delta.

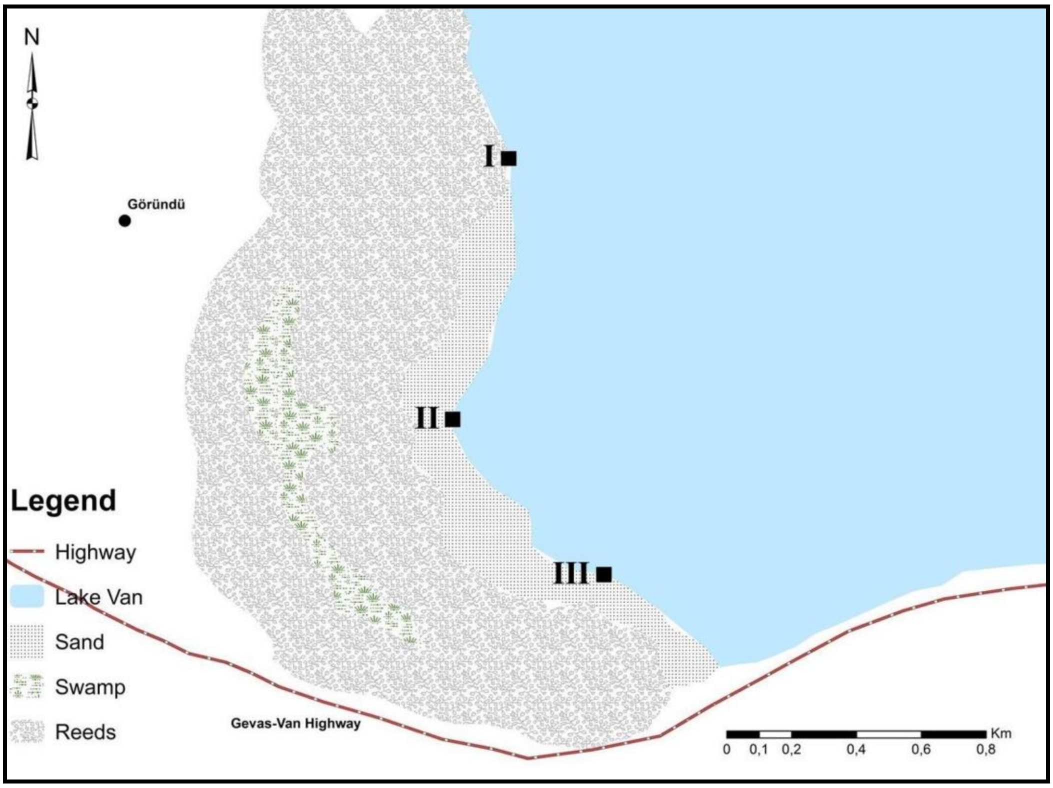

Figure 1. *Observation points at Göründü Delta*

Behavioural observations

In order to identify the wintering behaviour of whooper swan, observations were conducted between November 2015 and March 2016 on days without precipitation or strong wind. During this study period, a total of 70 days between November and March were used for diurnal observations.

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DOI: http://dx.doi.org/10.15666/aeer/1702_21792185

Scan sampling and focal sampling methods were used to evaluate different daily wintering behaviour of adults and juveniles (Altmann, 1974). The observations started in the morning before sunrise (06:00) and continued until sunset (17:00). This period was divided into 3 equal parts as morning (06:00-08:30), midday (08:30-13:30) and late day (13:30-17:00) with equal numbers of focal sample observations within them. Scan samplings were made at the beginning of each period to assess the total number of birds. When using this method, the behaviour of all individuals in a flock were recorded at predetermined time intervals during the daytime hours. In total 1540 observation sessions and 192.2 h of scanning were performed. Six main activities of whooper swan were evaluated in this study: resting, feeding, preening, swimming, flying and moving on land. In the determination of the behaviours, random focus observation method was used. The instantaneous behaviour of each individual in a flock with 90 individuals was recorded with a telescope in half-hour intervals. After scanning maximum 30 individuals, other individuals were evaluated as second and third groups. The scanning started from the first individual and continued towards left until the last individual. We quantified time-activity budgets by calculating the proportion of time spent in each behaviour for each focal sample.

Statistical analysis

The statistical software SPSS 20 was used to perform all statistical analyses. We identified diurnal period and winter period differences with ANOVA.

Results and discussion

During the five months of survey, daily activities of a whooper swan showed a major variation which occurred majorly due to the hydrobiological condition and ecological status of the coastal area. We detected that in winter season the whooper swans spent most of the time by resting (55%) and the rest by feeding (12.48%), preening (10.98%), walking on land (8.94%), swimming (7.73%) and flying (4.87%) (*Fig. 2*).

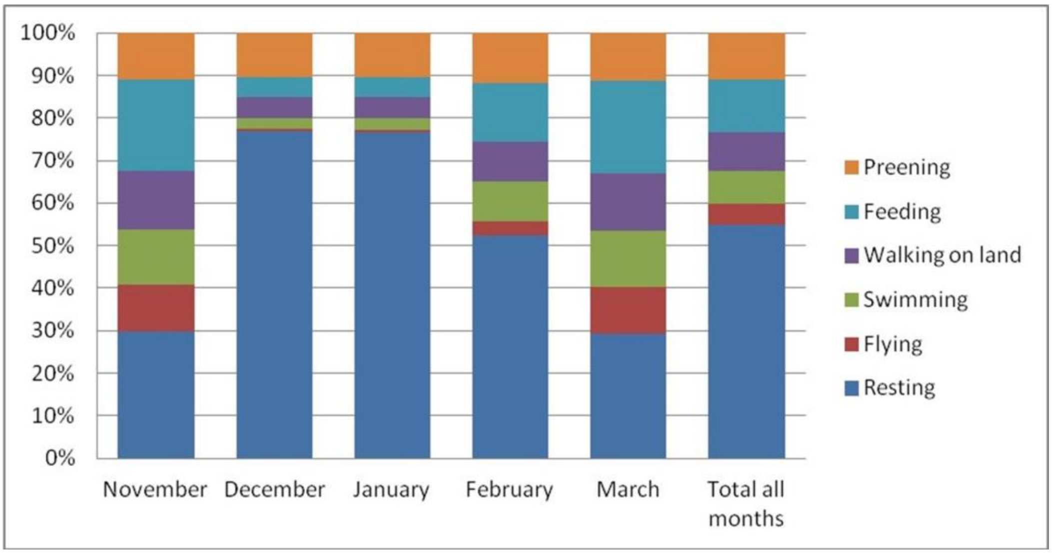

Figure 2. *The percentage of time spent to the activities by whooper swan at different parts of winter time (monthly and in total)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_21792185

Resting, feeding and preening were the main diurnal activities. Resting peaked in midday while feeding was observed frequently in morning and late afternoon (*Table 1*). The feeding pattern recorded was similar to patterns recorded earlier studies of swans (Tatu et al., 2007; Peihao et al., 2011).

Table 1. *Mean percentage of winter-time diurnal activities of the whooper swan in different time periods of the day*

Behaviour	**Morning (06:00-8:30) n: 43.45h**	**Midday (08:30-13:30) n: 87.5h**	**Late day (13:30-17:00) n: 61.25h**	**Overall**
Resting	41.8	62.9	60.3	55
Feeding	27.1	7.8	2.54	12.48
Preening	11.39	10.6	10.95	10.98
Walking on land	8.82	6.2	11.8	8.94
Swimming	7.25	6.74	9.2	7.73
Flying	3.64	5.76	5.21	4.87

n: observed hour

Temperature at a wintering site is an important determinant of the waterbirds' time budgets (Guillemain et al., 2002). The temperature averages according to the months were; November (4.4 °C ± 0.2), December (-2 °C ± 0.3), January (-3 °C ± 0.3), February (-4 °C ± 0.2) and March (-1 °C ± 0.4). The higher average temperature in November and March compared to the temperature average of December, January and February, affected the activities of the species. Resting activity in December, January and February was significantly higher whereas more time was spent on feeding activity in November and March ($P < 0.05$) (*Fig. 2*).

In this study, we detected that mean percentage of time spent to feeding activity was similar in November and March and the resting was similar in December and January. It was identified that all the activities in February shows temporal differences with the activities in the other months (*Table 2*).

Table 2. *Statistical analysis of the monthly activity budgets of whooper swan*

ANOVA		**Subset for alpha = 0.05**				
Behaviour	**n**	**November**	**December**	**January**	**February**	**March**
Resting	90	0.890667[c]	2.145259[a]	2.128333[a]	1.448571[b]	0.873056[c]
Feeding	90	1.178 [c]	0.246 [a]	0.26 [a]	0.7608 [b]	1.1853 [c]
Preening	90	0.6107[a,b]	0.5789 [b]	0.575 [b]	0.6405 [a]	0.6226 [a,b]
Moving on land	90	0.7457 [a]	0.2723 [c]	0.269 [c]	0.5233 [b]	0.7478 [a]
Swimming	90	0.7187 [a]	0.1409 [c]	0.149 [c]	0.5154 [b]	0.7238 [a]
Flying	90	0.6077 [a]	0.0375[c]	0.0303 [c]	0.1796 [b]	0.6124 [a]

*$P < 0.05$, n: individual number

When the activities of the species in November and March were considered, we revealed that diurnal activities as flying, swimming and moving on land were similar within themselves ([a]) ($P < 0.05$), feeding and resting were homogeneous within

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21792185

themselves ([c]) ($P < 0.05$). Preening was totally different from feeding and resting. However it was similar with the other activities ([a,b]) ($P < 0.05$). When daily activities in December and January were considered, feeding and resting were similar with themselves ([a]), while flying, swimming and on land movement were showing similarities within themselves ([c]). The preening activity was independent from both groups ([b]) ($P < 0.05$). In February all diurnal activities were similar with each other except preening (*Table 2*).

Results of our work confirm the findings by Squires and Anderson (1997) that swans spent more time resting as winter temperatures decreased. Percent of time spent on different daily activities differed significantly between day periods in mild and severe winter (*Fig. 2*). They spent the most of severe winter period inactive, apparently relaying on accumulated energetic reserves. Increasing energetic expenditure in the period of low temperatures is often compensated through more intensive and longer foraging (Guillemain et al., 2002; Meissner and Ciopcinska, 2007). However, during the harshest winter weather observation period in the Göründü Delta whooper swans reduced all daily activities primarily foraging and feeding. They spend less time on locomotion, but the most striking difference concerned feeding time. In December, January and February where the air temperature is the lowest -17 (average -4), the resting ratio is about 76%, and this ratio decreases 29% in November and March.

Despite lower air temperature in late winter period (in February), whooper swans had increased the total feeding time and they were more active. Because they had to store energy and increase fat reserves before the long migration period. At the same time they begin getting the nutrient reserves required for reproduction.

Black et al. (2010) indicated that 44.9% of the daily activities of the Tundra swan (*Cygnus columbianus*) in the Eel River Delta were related to the foraging activities. Rees and Bowler (1991) reported a similar proportion of 48.4% feeding in swan flocks in England in late winter/spring period. According to our results, daily foraging activities of whooper swans were decreased in the late winter period but lower than these rates. We think that the most important reason for this situation is low nutrient density, food availability and different climatic conditions in Göründü Delta.

Laubek et al. (1999) pointed out that the whooper swans use open water surface while performing their daily activities during the winter months. Similarly, we observed that the whooper swans were performed almost all their daily activity in water.

Conclusion

In the Göründü Delta where ducks winter in high density, whooper swans spent most of the brief severe winter period almost inactive, probably relying on accumulated energetic reserves. Diet of whooper swans mostly consist of aquatic plants such as *Algae* sp., *Chara* sp. and *Zostera* sp (Brazil, 1981; Del Hoyo, 1992; Kantrud, 1990; Squires and Anderson, 1995). Since the study area is associated with Van Lake and has soda-water, it is poor in terms of water plants. This factor reduces the feeding time of the species. The whooper swans were feeding in shallow water line and reeds. The caloric value in aquatic plants is lower than in terrestrial plants (Dourado et al., 2004). During the winter the species is known also to feed on agricultural land and takes agricultural grain (Brazil, 2003). Nevertheless, grazing on land was observed just a few times only in March. Intensive hunting pressure in the field is the most important reason for this situation. More comprehensive studies about whooper swans' winter food

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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supply and nutritional diversity would help to inform habitat management and protection for the swans.

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EFFECT OF FOLIAR APPLICATION OF SELENIUM ON NUTRIENT CONCENTRATION AND YIELD OF COLORED-GRAIN WHEAT IN CHINA

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(Received 8th Nov 2018; accepted 25th Jan 2019)

Abstract. A field experiment was conducted to evaluate the efficiency of foliar application of different concentrations of Se fertilization (37.50, 56.25, 75.00, 93.75, and 112.50 g ha^{-1} of Se) at the early grain filling stage on nutritional quality of colored-grain wheat. The results showed that Se plays a role in influencing the nutrients concentration of colored-grain wheat. When it was more than 75.00 g ha^{-1}, the Se concentration in the common wheat was excessive. However, when it was more than 37.50 g ha^{-1}, the Se concentration in the colored-grain wheat was excessive, indicating that colored-grain wheat was likely to be more sensitive to Se accumulation. Se concentration of colored-grain wheat was significantly increased under the optimal foliar application of Se concentration at 37.5 g ha^{-1}, decreased starch concentration, without affecting the yield. Particularly, the concentrations of gliadin and glutenin were significantly increased while both albumin and globulin were reduced with the increased Se concentration. Additionally, our research showed that foliar application Se increased the concentration of iron (Fe), zinc (Zn), but decreased the concentration of copper (Cu) and manganese (Mn). More importantly, the intake of Se promoted the production of amino acids and anthocyanins. This indicates the potential roles Se plays on regulating nutrition value in colored-grain wheat.
Keywords: *Se-enrich, total protein, minerals, amylase, amylopectin*

Introduction

Hidden hunger or micronutrient deficiency occurs when the food quality does not meet the requirements of essential nutrient need for the growth and development of humans. The main cause of hidden hunger is the deficiency of minerals (for examples, selenium, zinc, iron, and iodine), lack of vitamins in diet, and large consumption of staple food (wheat, rice, and maize) which lack these nutrients (Zhao et al., 2016). Hidden hunger is affecting approximately 2 billion people around the world including 300 million people in China (Toppe and Chomo, 2015; Kennedy et al., 2003; Maberly et al., 1994). Many chronic diseases, including diabetes, cardiovascular disease, cancer, and obesity were linked to the imbalance or deficiency of nutrient intake.

Selenium (Se) is an essential trace element for humans and animals. Numerous previous studies have proven it is beneficial to plants, however, it is considered non-essential for plant growth and development. Se plays an important role in the growth, development, and maintenance of the immune system of human and animals (Shenkin, 2006; Yadav et al., 2005; Speckmann and Grune, 2015). Se deficiency in the human body could cause about 40 kinds of diseases, such as Keshan and Kashin-Beck diseases (Tan et al., 2002; Zhang et al., 2014; Fernandes and Gandin, 2015; Chen et al., 2012; Kolesnikova et al., 2015). In 1982, it was reported that almost 72% of regions in China are facing the Se deficiency and about two-thirds of the population are experiencing

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21872202

deficiency of Se intake. Thus, the biofortification technology for increasing Se concentration in food crop has been paid more and more attentions by many researchers (Ma et al., 2008; Khanam and Platel, 2016).

Fertilization is short term and an effective method for improving micronutrient concentrations in food crop (Zhao and McGrath, 2009; Welch, 2008). Se fertilization is applied in soil and/or as a foliar application (Wang et al., 2017). Foliar fertilization is the most effective and safest way to increase the absorption of essential micronutrients in grain. Wheat is the most popular cereal crops worldwide and is a staple food for nearly half of the population in China where more than 85% of wheat is consumed as flour-derived products (Shewry, 2009; Li, 2006). Therefore, increasing Se nutrition in wheat grain is of the utmost importance when it comes to improving the nutritional status of people in China and other countries (Liu, 2007). In Hungary, colored-grain wheat cultivars are less known in contrast to East-Africa, New Zealand, Canada, and China, for example, where such forms occur in cultivated varieties (Varga et al., 2013). Wheat varieties with special colored-grain such as blue and purple grains had been proven to be rich in protein, amino acids, and trace elements or minerals (Zeven, 1991). It is well known that anthocyanins are functioning as antioxidants and, in addition, they also have anti-carcinogenic effects. Similarly, Se has anti-carcinogenic capabilities. Because of these health benefits, the combination of colored-grain wheat and Se is more beneficial to the development of food industry. Thus, colored-grain wheat is considered to be beneficial to the human body due to their high nutritional value and has become a special raw material for food industry (He et al., 2012). In addition, it was reported that colored-grain wheat was richer in Se than common wheat, suggesting a practical way to solve Se deficiency (Shi, 2006).

At present, there is little known about the enhancement of trace elements in colored-grain wheat and Se-enriched colored-grain wheat has not been fully utilized. The purpose of this study is to evaluate the effect of biofortification of Se in colored-grain wheat in Se deficient region of Northern China. In the present study, field experiment was conducted to (i) investigate the uptake of Se concentration in grains from three different varieties of colored-grain winter wheat in the middle area of Shanxi province in China, (ii) evaluate the effect of foliar applications of Se on yield, protein concentration, the concentration of Se and other minerals, starch and amino acid concentration of the grain, and (iii) examine the relationship between Se and anthocyanin.

Materials and methods

Field site description

The field experiment was carried out from September 2016 to June 2017 in an experimental site at Shanxi Agricultural University, Taigu (37°42’ N, 112°58’ E), Shanxi province, China (*Fig. 1*). The altitude of field experiment was 767-900 m, belong to the warm temperate zone continental climate, average annual precipitation was 450 mm, average temperature was 10 °C and average frost-free period is 160-190 d. The experimental field was plain and water land, planting one crop a year. There were four distinctive seasons. Soil samples were collected from the surface layer with a depth of 0-20 cm and the initial nutrient indices of the sampling soil were analyzed as follows: organic matter: 12.6 g kg^{-1}; total nitrogen: 1.8 g kg^{-1}; alkali-hydrolyzable nitrogen: 53.6 mg kg^{-1}; available phosphorus: 9.6 mg kg^{-1}; exchangeable potassium:

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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137.5 mg kg^{-1}; total Se: 0.171 mg kg^{-1} and available zinc: 4.375 mg kg^{-1}. Above mentioned indices were measured using the methods of potassium dichromate volumetric, Kjeldahl determination, alkali-hydrolyzable proliferation, sodium bicarbonate extraction, ammonium acetate flame photometric, hydride generation atomic fluorescence spectrometry, and concentrations of DTPA-extractable micronutrients respectively.

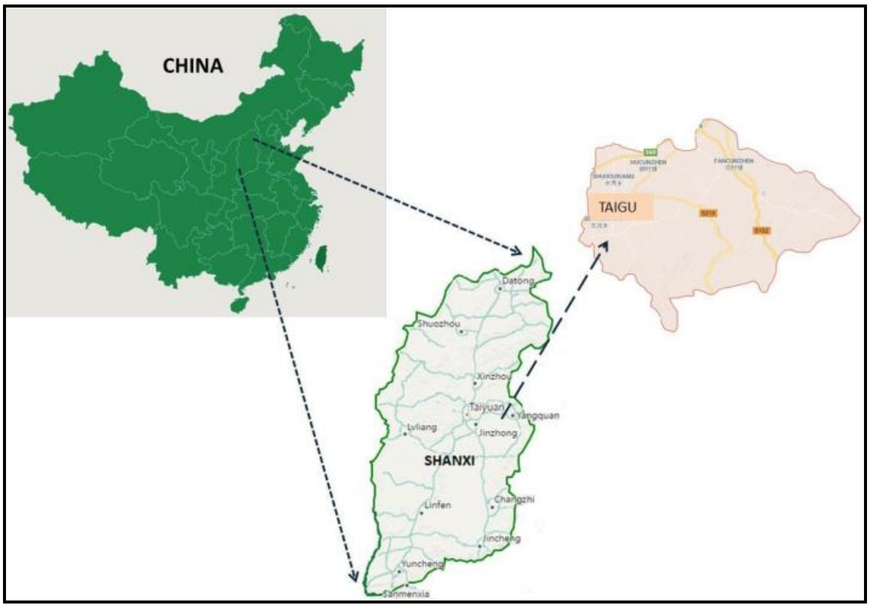

Figure 1. *Map of experimental region in China*

Experimental design

Two factors split plot design was used to optimize Se foliar fertilization content of colored-grain wheat by taking cultivars as a main factor and Se concentrations as a subplot factor. Three winter wheat cultivar Shannong 1 (purple), Shannong 031244 (blue), and Shannong 129 (white) were used in the experiment, among which Shannong 1 and Shannong 031244 were colored-grain wheat cultivar, while Shannong 129 was common wheat. Shannong 1 has a plant height of 60 ~ 70 cm, compact plant type, neat tiller, high heading rate, long awn, purple seed coat and rapid grouting. Shannong 031244 has a plant height of 80 ~ 90 cm, compact plant type, neat tiller, high heading rate, long awn, purple seed coat and rapid grouting. Shannong 129 has a plant height of 70 ~ 80 cm, compact plant type, neat tiller, high heading rate, long awn, white seed coat and rapid grouting. Se was added in the form of Na_2SeO_3 at rates of 0 (control), 37.50 g ha^{-1}, 56.25 g ha^{-1}, 75.00 g ha^{-1}, 93.75 g ha^{-1}, and 112.50 g ha^{-1} respectively. The six concentrations of Na_2SeO_3 were diluted with distilled water. A handheld compression sprayer was used to spray foliar fertilizer uniformly and about 750 L ha^{-1} solution was sprayed once. Wheat seeds were sown on September 30, 2016 at a sowing density of 600×10^4 plant ha^{-1} and row spacing of 20 cm. At the grain filling stage of winter wheat (May 20, 2017), each cultivar was sprayed with Se. No rainfall occurred after the spray. The plot sprayed with the same amount of distilled water was taken as the control. The harvest time was June 16, 2017. Each treatment was repeated three times. There were 54 plots in total. Area of each plot was 4 m^2 (2 m × 2 m) and total area was 216 m^2. Before sowing, 750 kg ha^{-1} of NPK compound fertilizer (N: P_2O_5: K_2O = 18%: 22%: 5%) was applied as a based fertilizer. Other field management measures (such as irrigation and weed maintenance) were carried out as common practices (*Fig. 2*).

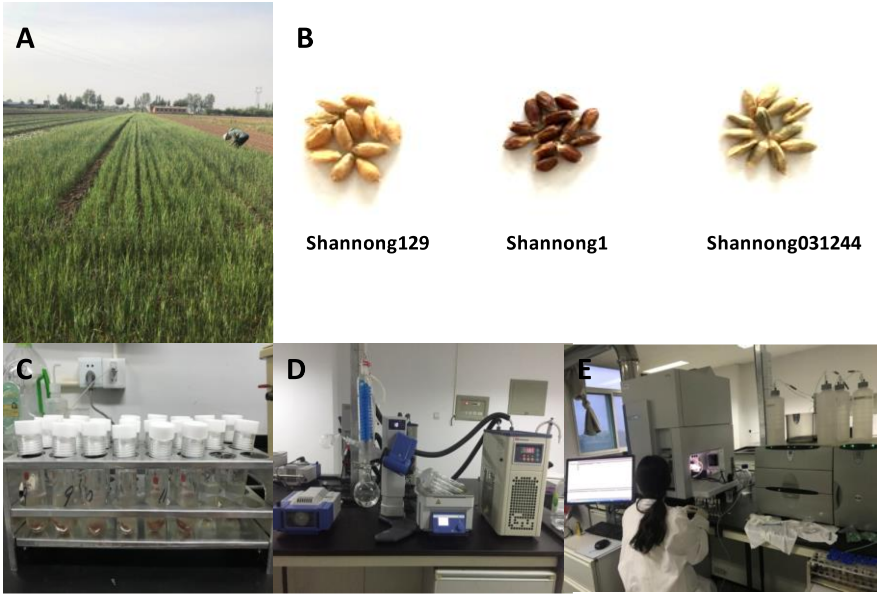

Figure 2. *Sampling sites for indices analysis at Shanxi province, China*

Se and minerals analysis

Wheat was harvested at full maturity stage to determine the yield as well as the contents of Se and minerals in grains. After determination of the yield, the obtained grain samples were washed carefully and rapidly with distilled water, and then dried at 65 °C for 10 h, grinded and sieved. The obtained flour samples were used to investigate the contents of total Se, minerals, protein, and the components. The flour samples were digested by using a HNO_3-H_2O_2 mixture in an alimentary furnace reaction system (LWY84B, China), and then the concentration of Se and minerals were measured by using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700x; Agilent Technologies, USA) (*Fig. 2*).

Protein and starch analysis

The flour protein and its components were measured using Kjeldahl nitrogen method (He, 1985). Amylasein and amylopectin were determined by dual wavelength method. The sum of the amylasein and amylopectin is the total starch.

Amino acids and anthocyanin analysis

The hydrolyzed samples were analyzed according to the national food safety standard of the China (GB5009.124-2016). The grain were dried, crushe and sieved (< 0.15 mm), which was weighed and put in the hydrolysis tube. First, 10 mL HCl and phenol (3-4 drops) were added. Second, the hydrolysis tube was put into the refrigerant (3-5 min) and attached to the suction pipe of the vacuum pump and fill nitrogen (fill

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DOI: http://dx.doi.org/10.15666/aeer/1702_21872202

nitrogen three times). After that the hydrolysis tube was sealed and put in thermostat (110 °C, 22 h). The hydrolysate was filtered into a 50 mL volumetric flask and constanted to the scale. The filtrate was put into the test tube. In a test tube enrichment apparatus decompression drying in 48 °C heating environment. The dried residue was dissolved by water. Then the solution was vacuumed and dried again and until the top up. Finally, the buffer solution of pH 2.2 sodium citrate was added to the test tube. The solution is to be measured passing through 0.22 nm membrane. Amino acid samples were separated by ion exchange chromatography and measured using the automatic amino acid analyzer (model Biochrom 30+, England). The anthocyanin analysis using pH differential method (Siriwoharn et al., 2004) (*Fig. 2*).

Statistical analysis

The experiment was laid out in a two factors split plot design with three parallel measurements. The data were subjected to a separate analysis of variance (ANOVA) for each cultivar, and the Tukey's multiple range test at $p < 0.05$ was used to determine differences between treatment means. The Pearson correlation procedure and general line regression model was used to evaluate the relationship between grain Se and anthocyanin. SAS software (SAS 8.0, USA) was used for all analyses.

Results

Grain yield and Se concentration

To evaluate the effects of foliar application of Se fertilizer to the grain yield and Se concentration in grains, we conducted the ANOVA analyses on the grain yield, concentration of the grain total Se and grain Se cumulant. The grain yield and Se analysis carried out on the experimental field with the colored-grain wheat showed foliar application of Se fertilizer at different concentrations did not significantly affect the yield of colored-grains ($p > 0.05$, *Table 1*). The grain yield of Shannong 1 and Shannong 031244 were higher than Shannong 129.

Nevertheless, there were significant effects of the total Se and Se cumulant of three wheat varieties ($p < 0.05$). The total Se and Se cumulant of three wheat varieties were increased significantly with the increase the concentration of foliar Se fertilizer. By the foliar application of the same concentrations of Se at grain filling stage of different colored-grain wheat, results of the total Se and Se cumulant showed colored-grain wheat (Shannong 031244 and Shannong 1) > common-grain wheat (Shannong 129). Compared to the grain Se concentration in the untreated control, the concentration of added Se increased by up to 18-fold at the maximum Se application of Shannong 129. However, it increased up to 10-fold and 8-fold in the colored-grain wheat (Shannong 031244 and Shannong 1) respectively. It indicated that the wheat grain had strong ability to concentrate exogenous Se under the condition of foliar application. Although there was a increase between the total Se concentration and the amount of Se applied in the grain, the utilization rate of Se decreased with the increase of the concentration of foliar Se fertilizer. When the concentration of foliar Se fertilizer was 37.50 g ha^{-1}, utilization rate of Se reached the maximum, with the pattern of (Shannong 031244 and Shannong 1) > common-grain wheat (Shannong 129) displayed in three wheat varieties. According to the National Food Safety Standard in China, Se concentration in Se-enriched wheat was prescribed in the range of 0.15-0.30 mg kg^{-1} (GB/T 22499-2008,

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China). When it was more than 0.30 mg kg^{-1}, the high concentration could be unhealthy for humans. The Se concentration in the grain of common wheat was over the above range when foliar application of Se concentration greater than 75.00 g ha^{-1}. However, when foliar application of Se concentration was more than 37.50 g ha^{-1}, the Se concentration in the colored-grain wheat was excessive. The results indicated that colored-grain wheat was more sensitive to Se accumulation.

Table 1. *Grain yield and Se concentration of colored-grain wheat affected by the different concentrations of Se fertilization*

Treatments		**Selenium (g·ha^{-1})**					
Indices	**Cultivar**	**0.00**	**37.50**	**56.25**	**75.00**	**93.75**	**112.50**
Yield (t ha^{-1})	Shannong 129	5.48aC	5.45aC	5.42aC	5.41aC	5.41aC	5.37aC
	Shannong 031244	5.76aB	5.77aB	5.76aB	5.70aB	5.65aB	5.62aB
	Shannong 1	6.34aA	6.42aA	6.35aA	6.39aA	6.34aA	6.33aA
Grain total Se concentration (mg kg^{-1})	Shannong 129	0.024eB	0.232dB	0.266cdC	0.283cC	0.350bB	0.443aB
	Shannong 031244	0.051eA	0.272dA	0.385cA	0.498bA	0.516bA	0.541aA
	Shannong 1	0.062fA	0.285eA	0.371dA	0.475cA	0.510bA	0.539aA
Grain Se cumulant (mg ha^{-1})	Shannong 129	130.45eB	1267.92dC	1441.15cdB	1527.06cB	1893.29bC	2379.72aC
	Shannong 031244	295.15eA	1569.04dB	2216.23cA	2835.68bA	2913.87bB	3040.98aB
	Shannong 1	391.99fA	1827.31eA	2351.35dA	3032.57cA	3231.26bA	3413.77aA
Se utilization rate (%)	Shannong 129	-	3.03aC	2.33abB	1.86bB	1.88bB	2.00bB
	Shannong 031244	-	3.40aB	3.42aA	3.39aA	2.79bA	2.44cA
	Shannong 1	-	3.83aA	3.37aA	3.18bA	3.03bcA	2.69cA

Values were means of three replicates. Different line lowercase letters (a, b, c, d, e, f) indicated significant differences among Se treatments, at p < 0.05 in Tukey test. Different column uppercase letters (A, B, C) indicated significant differences among cus, at p < 0.05 in Tukey test

Minerals concentration

To further understand the effects of different concentrations of Se on colored-wheat grains, the concentration of mineral elements in colored-grain wheat are analyzed and shown in *Figure 3*. The concentration of mineral elements in colored-wheat grains was higher than that in common wheat, indicating that the nutritional value of colored-wheat was higher than that of common wheat. After leaves were sprayed with Se concentrations of 37.50 g ha^{-1}, the concentration of Iron (Fe) in Shannong 1 and Shannong 031244 were significantly increased by 1.2-fold and 0.7-fold, respectively (p < 0. 05), the concentration of Zinc (Zn) in Shannong 1 and Shannong 031244 were significantly increased by 1.2-fold and 0.7-fold, respectively ($p < 0.05$). The concentration of Fe and Zn decreased but it was still hiher than the control value with the increase of Se concentration in foliar fertilizer. This indicates that the change of Fe and Zn in wheat grain presents a trend, which was low concentration promoted and high concentration inhibited by Se application. However, the supply of Se would inhibit the production of Copper (Cu) and Manganese (Mn). The decreased concentration range of Cu and Mn of all wheat were the same, indicating that the concentration variations of Cu and Mn of wheat grains were only affected by Se concentration other than wheat

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varieties. Overall, the results showed that the sensitivity of mineral concentration to Se in common wheat was lower than that of colored-grain wheat. The sensitivity of Se showed different in two colored-grain wheat, and purple wheat more sensitive than the blue one.

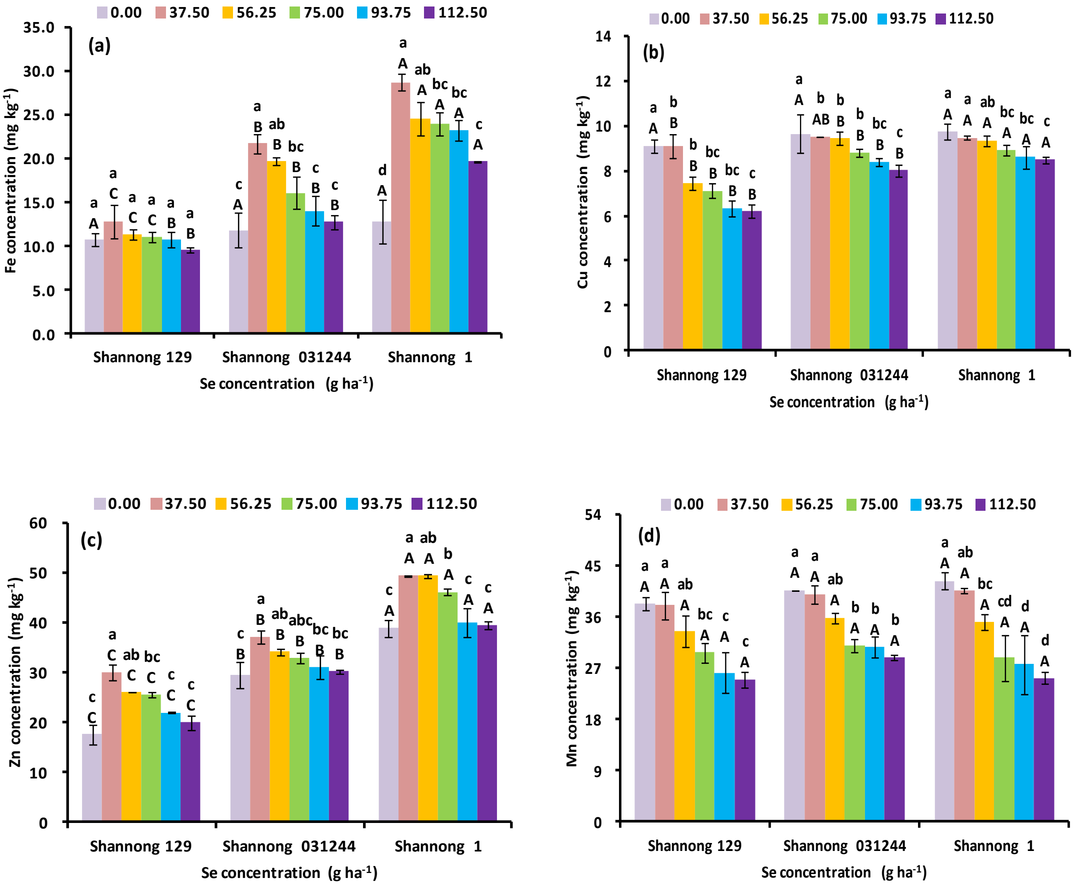

Figure 3. *Minerals in grains of colored-grain wheat cultivar under foliar application of Se. X axes show the concentrations of Se. Y axes show the concentrations of different minerals. Values were means of three replicates. The subfigures (a-d) show the concentrations of Fe, Cu, Zn and Mn, respectively. Different line lowercase letters (a, b, c, d) indicated significant differences among Se treatments, at p < 0.05 in Tukey test. Different column uppercase letters (A, B, C) indicated significant differences among cultivars, at p < 0.05 in Tukey test.*

Concentration of total protein, protein components

The concentrations of total protein and protein components showed significant changes within colored-grain wheat (*Fig. 4*, $p < 0.05$). Overall, total protein concentration was significantly lower in Shannong 129 than in Shannong 031244 without Se ($p < 0.05$). The concentration of total protein increased with the increase the concentration of foliar Se fertilizer. The concentration of albumin and globulin were increased, however, which concentration Se increase amplitude gradually decreased with the increase the concentration of foliar Se fertilizer with the foliar application of 37.5 g ha^{-1} which decreased at higher concentrations of Se, while gliadin and glutenin concentration s were increased in all wheat cultivar with increased concentrations of Se ($p < 0.05$). The highest gliadin and glutenin concentration was found at the highest

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concentration of Se. Glutenin concentration of colored-grain wheat (Shannong 1, Shannong 031244) was significantly higher than common wheat (Shannong 129); however, the concentration of albumin was highest in common wheat (Shannong 129). This indicates that the effect of foliar Se was significantly different for cultivar in terms of the protein components and total protein concentration.

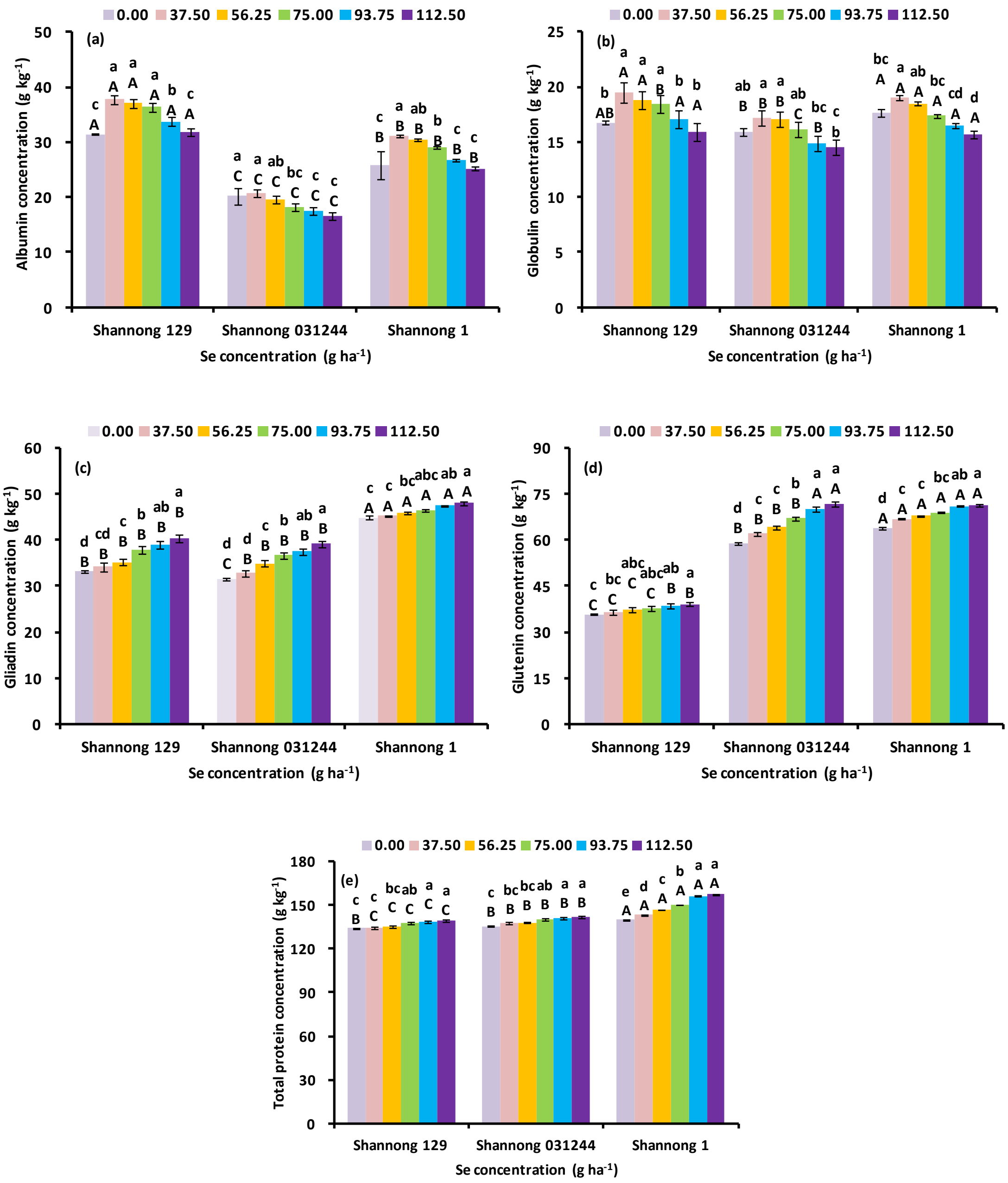

Figure 4. *Concentrations of total protein and protein components in grains of colored-grain wheat cultivar under foliar application of Se. X axes show the concentrations of Se. Y axes show the concentrations of different concentrations of total starch and different starch components. Values were means of three replicates. The subfigures (a-e) show the concentrations of albumin, globulin, gliadin, glutenin and total protein, respectively. Different line lowercase letters (a, b, c, d) indicated significant differences among Se treatments, at p < 0.05 in Tukey test. Different column uppercase letters (A, B, C) indicated significant differences among cultivars, at p < 0.05 in Tukey test.*

Concentration of total starch and its components

The concentration of total starch and its components showed significant changes within colored-grain wheat (*Fig. 5, p* < 0.05). Overall, the concentration of amylase was significantly lower in Shannong 129 and Shannong 031244 than in Shannong 1 without Se ($p < 0.05$). The highest concentration of total starch and its components was found at the Se concentration of 37.5 g ha^{-1} under foliar application. The concentration of total starch, amylase, and amylopectin first increased then decreased with the increase the concentration of foliar Se fertilizer. This indicates that the effect of foliar Se was significantly different for cultivar in terms of the starch components and total starch concentration.

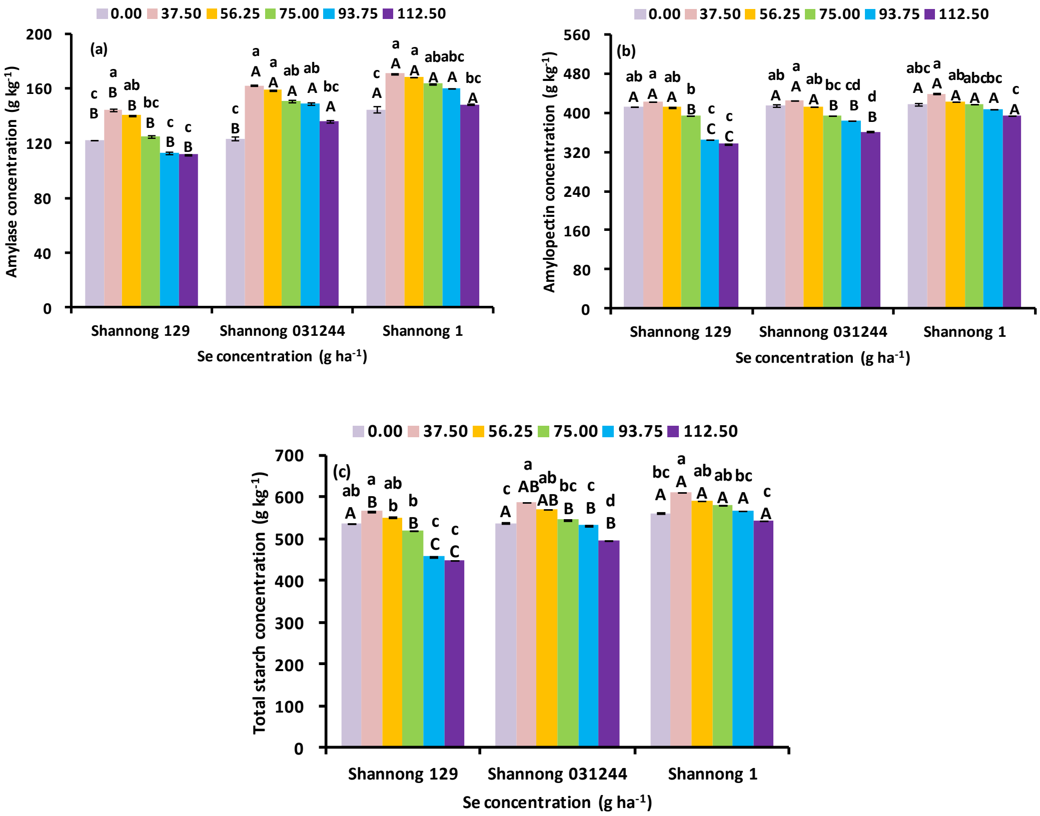

Figure 5. *Concentrations of total starch and different starch components in grains of colored-grain wheat cultivar under foliar application of Se. X axes show the concentrations of Se. Y axes show the concentrations of different concentrations of total starch and different starch components. Values were means of three replicates. The subfigures (a-c) show the concentrations of amylase, amylopectin and total starch, respectively. Different line lowercase letters (a, b, c) indicated significant differences among Se treatments, at p < 0.05 in Tukey test. Different column uppercase letters (A, B, C) indicated significant differences among cultivars, at p < 0.05 in Tukey test.*

Concentration of amino acids and anthocyanin

The concentration of amino acids was higher in colored-grain wheat than in common grain wheat without Se treatment (*Fig. 6*). In the present study, a small dose of Se had a positive effect on the concentration of the total amino acids.

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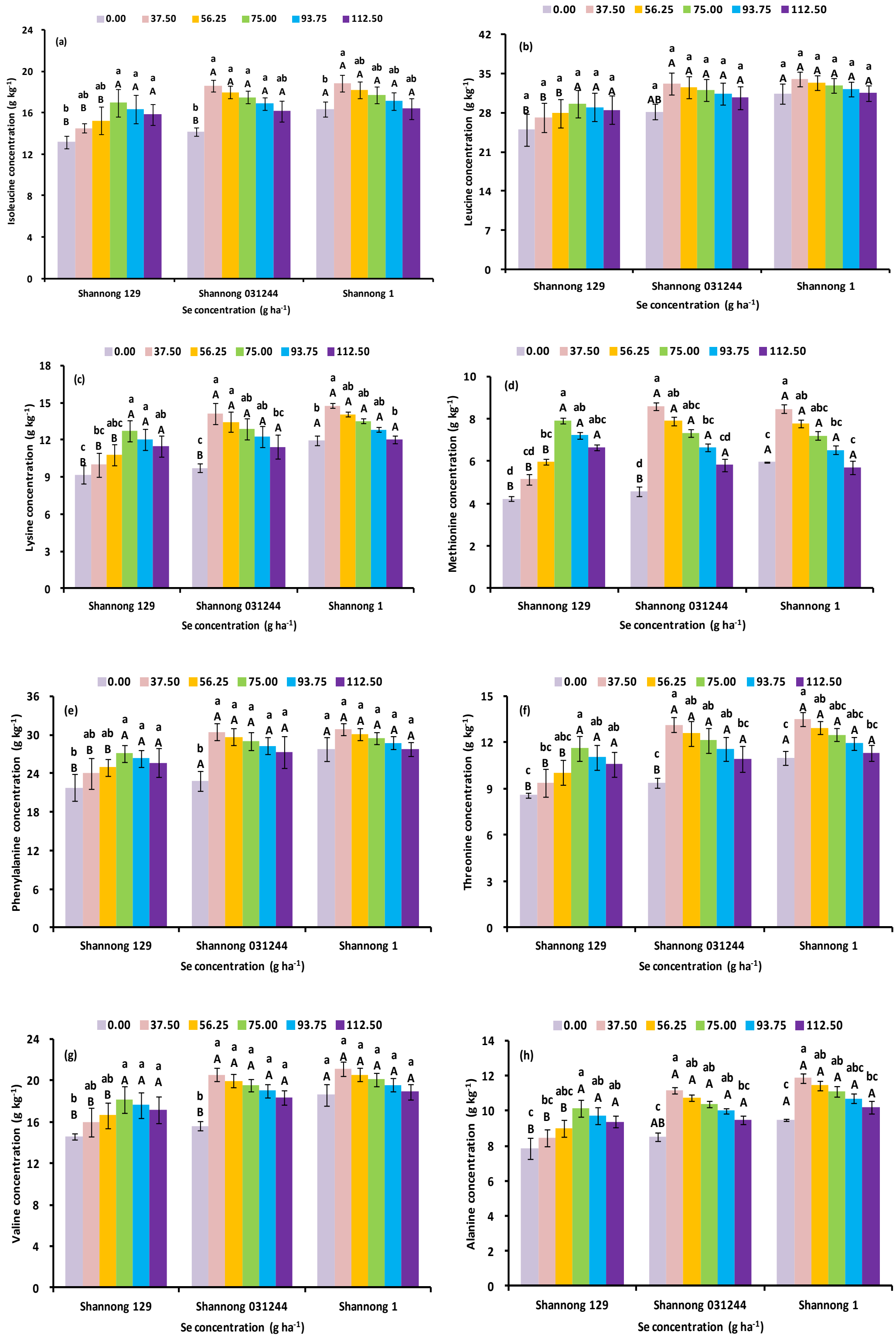

***Figure 6.** Concentrations of amino acids of colored-grain wheat affected by different concentrations of Se fertilization. X axes show the concentrations of Se. Y axes show the concentrations of different amino acids. Values were means of three replicates. The subfigures (a-p) show the concentrations of Ile, Leu, Lys, Met, Phe, Thr, Val, Ala, Arg, Asp, Glu, Pro, Gly, His, Ser and Tyr, respectively. Different line lowercase letters (a, b, c) indicated significant differences among Se treatments, at $p < 0.05$ in Tukey test. Different column uppercase letters (A, B, C) indicated significant differences among cultivars, at $p < 0.05$ in Tukey test.*

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However, higher levels of Se supplementation did not result in further increase of the concentration regarding the total amino acids. After applying a larger dose of Se, there was a small decrease in the total content of amino acids, but the overall concentration of amino acids was still larger than the control. There was a decreased trend after application Se concentration of 37.50 g ha^{-1} in colored-grain wheat, however, a decreased trend was found after Se concentration of 75.00 g ha^{-1} in common-grain wheat. The sixteen amino acids were detected (including seven essential amino acids and nine non-essential amino acids). All essential amino acids were significantly affected except leucine (Leu) ($p < 0.05$). The concentration of essential amino acids was significantly lower in Shannong 129 than in Shannong 1 and Shannong 031244 with Se. However, with the increase of Se concentration in foliar fertilizer, there were no significant effects of the essential amino acids of three wheat varieties ($p > 0.05$). The results for non-essential amino acids are also listed. The application of Se markedly influenced the concentration of non-essential amino acids except arginine (Arg) and tyrosine (Tyr) ($p < 0.05$). Meanwhile, with the increase of Se concentration in foliar fertilizer, there were aslo no significant effects of the non-essential amino acids of three wheat varieties ($p > 0.05$).

To examine the relationship between anthocyanins and Se, the concentration of anthocyanins was measured in colored-grain wheat (*Fig. 7*). We found the synergistic effect between anthocyanins and Se in colored-grain wheat, which indicated there was likely to be a great Se-enrichment potential in colored-grain wheat.

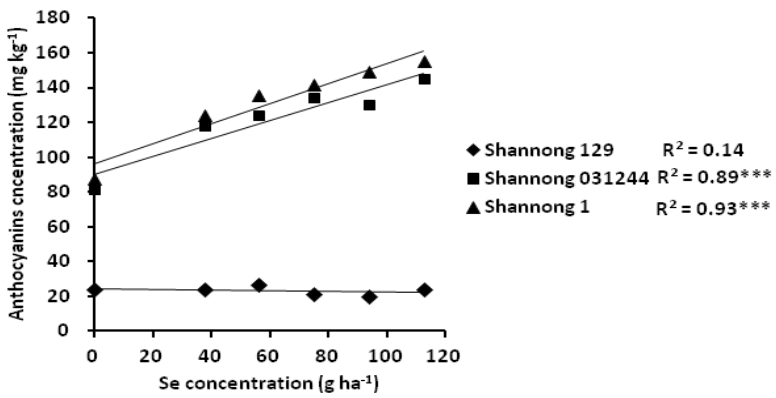

Figure 7. *The correlation between anthocyanin and foliar application of Se in grains of colored-grain wheat. X axes show the concentrations of Se. Y axes show the concentrations of different minerals*

Discussion

The customer's acceptance of innovative, new products is dependent on their feedback to the sensory and functional properties of flour, including color, flavor, nutritional value, and overall acceptability. In particular, for flour, it is necessary to maintain the typical sensory and nutritional characteristics for commercial success in the development of functional foods. The evidence that Se enrichment of colored-grain wheat improves the nutritional aspect of the flour without altering its sensory properties is critical for consumers to accept Se-enriched flour (Ficco et al., 2014). However, nutritional analyses of bio-fortified colored-grain wheat flour have not been studied extensively. Our study focused on the evaluation of the effect of Se on the nutritional

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contents of colored-grain wheat and provided a valuable practice on the development of new functional selenium-fortified colored-grain wheat.

The soil Se-supplement, dress seeds, soaking seeds, foliar application of Se and exogenous Se supplementation can increase the amount of Se in agricultural products (Ducsay et al., 2009). Among above foliar application of Se has the advantages of fast, efficient, low cost and low environmental risk. Our findings suggested the effectiveness of foliar Se fertilization as a suitable strategy to improve nutritional quality of colored-grain wheat, which is consistent with the previous studies which have shown the greater effectiveness of foliar Se treatment than other Se supply methods in wheat (Zhang et al., 2010; Galinha et al., 2015). Moreover, we found that all of the Se treatments were highly effective at increasing the Se concentration in the colored-grain wheat, for three wheat cultivars, and that the Se concentrations in the grain increased with increasing concentrations of Se application according to a positive correlation. This evidence is in agreement with several previous studies in which high correlation values were observed (Broadley et al., 2010; Rodrigo, Santamaría, López-Bellido and Poblaciones, 2013). The Se-enriched wheat required Se concentration to be 0.15-0.30 mg kg^{-1} (GB/T 22499-2008, China). Therefore, appropriate external Se will be necessary to make up the deficiency to develop the Se-enriched wheat flour. In our result, when the foliar application of Se at the optimal dose (37.5 g ha^{-1}), the concentration of Se in colored-grain wheat had reached the above standard. When the wheat flour above this standard will not be good for people to eat. The absence of Se poisoning observed in our study is an important consideration for farmers, because this foliar Se application could not cause any reductions in yield through a wide range of Se concentrations.

There was no significant difference in the yield of colored-grain wheat by foliar application of selenium fertilization, which was consistent with the previous studies, in which pre-harvest foliar application of mineral elements did not affect grain yield of crops (Chen et al., 2002; Rodrigo et al., 2013). There was much research about the effect of foliar application Se on common wheat, but little research has been done on colored-grain wheat. In the control group of our study, the Se concentration in the common wheat grain was a little lower than in the colored-grain wheat, which confirmed high nutritional value in the colored-grain wheat. The concentration of Se and the accumulation of Se increased with the increase of Se concentration; however, the increase varies depending on wheat varieties. The Se concentration in colored-grain wheat was significantly higher than common grain wheat which might be caused by the absorption, transformation, and transport capacity of the exogenous sodium Se solution of colored-grain wheat. The utilization efficiency of Se in wheat is similar to other elements, that is, under relatively stable technical conditions, the Se concentration of wheat increased with the increase of Se injection, but the utilization of Se goes down. This explains the high concentration of Se inhibited plant growth due to Se toxicity interfering with the plant metabolism. Clearly, our results showed the beneficial role of Se at low concentrations could promote colored-grain wheat growth, which is consistent with previous studies (Boldrin et al., 2016; Guerrero et al., 2014). For the first time, we compared the Se accumulation between common wheat and colored-grain wheat, and reported the significant increase of mineral concentrations in the colored-grain varieties comparing to the common variety, which indicated a great potential to increase the nutritional content of colored-grain wheat flour for the commercial uses by the strategy of Se foliar spray.

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DOI: http://dx.doi.org/10.15666/aeer/1702_21872202

It is generally considered that high dosages of Se may be toxic to plants by competing with some essential elements, such as Mn, Zn, Cu and Fe (Kabata-Pendias and Pendias, 2011; Fargasova et al., 2006), however, in some cases, the stimulating effects of Se on uptakes of Cu, Fe and Zn in plants were also observed (Feng et al., 2009). Intriguingly, foliar application of Se had a significant effect on mineral concentrations in wheat grains. The colored-grain wheat varieties had significantly higher mineral concentration than the common wheat. Our results showed foliar application of Se promoted the accumulation of Fe and Zn and inhibited Cu and Mn accumulation, similar to the previous suggestion by Feng et al. (2009).

As Se is incorporated into proteins as selenoaminoacids, the different aptitudes of these colored-grain varieties to accumulate Se might be linked to dissimilarities in their protein content (Barclay and MacPherson, 1992). If the protein content in wheat grain is high, Se is mainly associated with plant proteins. Nossier et al. (2011) found selenium was related to protein function. Se was further reported to be involved in protein metabolism as a component in an RNA chain in plants, with the main physiological function of transporting amino acids to synthesize proteins (Garousi, 2017; Schiavon et al., 2017). In our research, Se foliar fertilizer showed a significant impact on protein components in grains of colored-grain wheat varieties too. The content of albumin and globulin were significantly reduced but the content of gliadin and glutenin were significantly increased with the increase the concentration of foliar Se fertilizer. The significant effect of the high Se concentration on the protein components of colored-grain wheat was observed, with being more prominent in the deeper colored varieties. Furthermore, there was no effect of Se fertilization on gluten quality, similar to previous suggestions by Broadley et al. (2010). In addition to the protein components, the Se fertilization had no effect on either gluten or fat parameters, which indicated the foliar spray of Se will not affect other main organic nutrient than proteins in colored-grain wheat. Furthermore, the starch is the main source of wheat extract. In theory, amylose was converted into fermentable sugar and amylopectin was converted into fermentable sugar and low molecular dextrin under the action of enzyme. The possible reason for the decline of starch is that the high dose of Se supply made the grain filling speed decrease, thus preventing the transformation of starch component.

The significant influence of amino acid concentration in plant tissues can be caused by nutrition and fertilization, such as Se (Munshi, 1990). For exemple, methionine (Met) was the limiting essential amino acid, and application of Se in our study affected the Met content of colored-grain wheat. Moreover, the application of Se markedly influenced the content of aspartic acid (Asp) and Asparagine (Asn) which is resulted from amidation of Asp. Both Asp and Asn are important in the transport and storage of nitrogen in plant tissues (Lea et al., 2007). In addition, we found the synergistic effect between anthocyanins and Se in colored-grain wheat, which indicated there was likely to be a great Se-enrichment potential in colored-grain wheat.

In the future studies, the following aspects are expected to be focused: (i) The physiological regulation mechanism of Se in the process of migration, distribution, and accumulation in colored-grain wheat plants and the antagonistic mechanism of Se and heavy metals in colored-grain wheat plants. (ii) Further uses of advanced biotechnology on selecting and breeding new varieties of Se-enriched colored-grain wheat.

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DOI: http://dx.doi.org/10.15666/aeer/1702_21872202

Conclusion

It is the first time to report the Se-enrich colored-grain wheat with the nutritional analyses and evaluation upon different Se concentrations applied in foliar spray. The optimal fertilization of Se could enhance nutritional quality of colored-grain cultivar including the concentrations of protein, minerals and amino acid without affecting the grain yield, gluten, and fat content, however, the starch concentration was decreased. Additionally, wheat grain was reported to be enriched with Se through exogenous Se under the foliar application of Se. Thus, the foliar application of Se at the optimal dose (37.5 g ha^{-1}) is suggested to be used for Se bio-fortification of colored-grain wheat cultivar. Moreover, the purple grain wheat is more Se-enriched than blue one. Further studies are expected to understand the mechanisms of micro-nutrient uptake and transport to acquire more efficient accumulation of nutrients in colored-grain wheat. Our study provides a valuable practice in nutrition analyses of the colored-grain wheat and the development of nutrient-enriched wheat flour.

Acknowledgements. The authors wish to thank the mediums and sitters who volunteered their time for this study. This study was financially supported by the Shanxi Scholarship Council of China from the Ministry of Finance, PR of China (2015-Key4), the the Key project of Shanxi Key R&D Program of China (201703D211001-02) and the Special Plan of Scientific Research for Shanxi Agriculture Valley of China (SXNGJSKYZX 201702) and the China Agriculture Research System (CARS-03-01-24).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_21872202

THE IDENTIFICATION OF THE RESISTANCE LEVELS OF *FUSARIUM OXYSPORUM* F. SP. *RADICIS - LYCOPERSICI* AND *TOMATO YELLOW LEAF CURL* VIRUSES IN DIFFERENT TOMATO GENOTYPES WITH TRADITIONAL AND MOLECULAR METHODS

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(Received 14th Nov 2018; accepted 16th Jan 2019)

Abstract. *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) causes Fusarium crown and root-rot diseases and *Tomato yellow leaf curling virus* (TYLCV, Israel, Mild, Sardinian strains) infection leads to low yield and poor quality fruits, which causes important economic losses in tomato growing areas. In this study, previously developed molecular markers for FORL and TYLCV were used with 418 tomato genotypes which have characteristics of high yield, quality fruit and resistance to abiotic stresses. 62 tomato lines were obtained from Cukurova University (CU), 196 and 160 of tomato lines were generated from Western Mediterranean Agricultural Research Institute (BATEM) and private sector, respectively. All genotypes were tested with classical and molecular methods where species specific resistance RAPD and $SCAR_{Frl}$ primers for FORL, CAPS and Co-dominant SCAR primers for TYLCV resistance were used. Analyses revealed that 102 tomato genotype are resistant to FORL, 46 and 35 of plant genotypes contain *TY3* and *TY1* loci respectively. Three genotypes from BATEM and 4 genotypes from private sector contained the 3 target genes (FORL+TY1+TY3), however, none of the tested CU tomato genotype was resistant to the 3 target genes. Furthermore, yield and some fruit quality characteristics of 7 tomato lines which were resistant to both diseases were determined in molecular and classical tests.
Keywords: *Fusarium crown and root-rot, marker assisted selection, PCR, Tomato yellow leaf curl virus, tomato*

Introduction

It is extremely difficult to control the most significant form of Fusarium crown and root-rot disease (*Fusarium oxysporum* f. sp. *radicis-lycopersici* / FORL) that economically limits greenhouse tomato production since it is a soil borne disease (Colak and Bicici, 2011). FORL leads to recurrent infections during the season due to the spread of microconidia, especially in greenhouses, and results crop losses up to 90% in greenhouse tomato cultivation (Hibar, 2002). Commercially viable, agent-resistant indigenous varieties with adequate resistance to Fusarium crown and root-rot induced by FORL are not yet developed (Ozbay et al., 2004). In a study conducted in greenhouse tomato fields in Adana and Mersin provinces, *formae specialis* and strains of 87 *Fusarium oxysporum* isolates were determined in different locations and 60% of the isolates were identified as FORL while 40% isolate were identified as FOL. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22032218

results of this study demonstrated that FORL disease spreads rapidly in the region (Çolak and Biçici, 2013).

Tomato yellow leaf curl virus (TYLCV) is one of the most common diseases in greenhouse tomato fields for the last 20 years. Similar to other viral diseases, TYLCV is the most devastating virus disease active on tomatoes that can cause an 80-100% loss in tomato yields through epidemics in several countries and there are no chemical control agents against the disease which exhibits genetic differences similar to all virus diseases. It was first reported in 1964 on tomato plants (Moriones and Navas, 2000). TYLCV is persistently transported by silver leaf whitefly (*Bemisia tabaci*, Biotype B and Q). TYLCV has a quite broad host range. TYLCV is not mechanically transported and there are no records of transport via seeds (Czosnek, 2007; Mabvakure et al., 2016). Different TYLCV strains were reported at different parts of the world. The most common of these strains is the *Tomato yellow leaf curl virus*- Israel (TYLCV - IL), Israel strain. It is followed by *Tomato yellow leaf curl virus*- Sicily (TYLCSV - Sic), *Tomato yellow leaf curl virus*- Mild (TYLCV - Mld) and Sardinia (TYLCV - Sa) strains (Anfoka et al., 2005; Belabess et al., 2015). A study was conducted to identify the TYLCV strains present in Turkey in tomato cultivation fields in Antalya, Mersin and Adana provinces. The study determined that TYLCV-IL, TYLCV-Sa, TYLCV-Mld strains were present. It was observed that 93% of the whitefly samples collected on the infected tomato plants were *B tabaci* Biotype - B (*B. argentifolii*) and sample of 7% were Biotype - Q (Fidan et al., 2011; Torre et al., 2018).

Parallel to the international studies, the use of resistant varieties in the control of plant diseases is the preferred method in Turkey to reduce the negative effects of the use of chemicals in the control of plant diseases. The development of resistant varieties in breeding work is obtained as a result of long-term studies. Several resistance genes, expected in a variety in breeding studies, could result in undesirable properties along with desirable ones (Scott, 2005). The morphological determinants used in traditional breeding studies could be affected by environmental conditions, although they help distinguish the genotypes. Homozygous, dominant and heterozygous individuals cannot be identified if any one of these characters is recessive. In recent years, molecular marker assisted selection (MAS) in disease resistance has been developing rapidly (Yan et al., 2017). Thus, hundreds of plants can be selected concurrently, reliably, and rapidly, by saving time and space in the selection of disease-resistant varieties (Darling and Brickell, 1994; Barone et al., 2005; Devran et al., 2018).

The FORL-related genetic resistance is controlled by a single (*Fr1* gene) dominant gene (Roberts et al., 2001). Studies on identification of a marker associated with *Frl* demonstrated that *Frl* is located on the ninth chromosome in the tomato (Vakalounakis et al., 1997). In the study where the connections between RAPD-DNA markers and *Fr1* were determined, 1000 different RAPD primers were tested for sensitive and resistant tomato lines. In the study, *Frl* gene connection distances were examined and it was determined that the UBC 194 was the closest primer with 5.1 cM. The selection of the closest primer in breeding studies is important for the reliability of the results. Since CAPS, SCAR and Co-dominant SCAR primers, which are a further stage of RAPD primers in the identification of FORL resistance, were not developed for this disease or were not patented and published, UBC 194-RAPD primer is used by several researchers (Fazio et al., 1999).

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DOI: http://dx.doi.org/10.15666/aeer/1702_22032218

Transferring the resistance in wild varieties to culture plants is one of the frequently used methods for TYLCV resistance. For this purpose, variety breeding was conducted with TY1 resistance marker developed with *Solanum chilense* (LA1969). However, it was determined that varieties with this resistance gene do not provide the desired level of protection (Castro et al., 2007). In most Mediterranean countries, TYLCV-Israel and Mild strains are common, and TYLCV Sardinia and Sicilian strains can also be found (Fidan et al., 2011). To ensure resistance against all these strains, it was reported that tomato lines that contain both Ty3 marker, developed from *Solanum chilense* (LA1932), and Ty1 gene provide significant resistance. Similar to all viral diseases, developing resistant varieties against this viral disease, which cannot be controlled chemically and possesses high genetic variability, became one of the most important strategies in recent years (Jensen et al., 2007).

The present study aimed to identify and validate the resistance against Fusarium crown and root-rot (FORL) disease and *Tomato yellow leaf curl virus* (TYLCV / Israel, Mild and Sardinia strains (TY1 + TY3 gene) that are significant problems in tomato cultivation with molecular and classical (symptomatologic) methods. For this purpose, 418 tomato lines, which were determined to be superior in terms of yield and certain fruit quality properties and determined to be resistant to abiotic factors, were used. Thus, yield and certain fruit quality properties of line and/or variety candidates that are resistant to both diseases (FORL + TY1 + TY3) were determined.

Materials and methods

Plant material

The main material included 418 F_2 generation tomato lines procured from Cukurova University (CU, 62 lines), West Mediterranean Agricultural Research Institute (BATEM, 160 lines) and private sector (AYER, 196 lines). Fla.7781, *Solanum chilense* LA2779, Tayfun F1 and sensitive variety Hazera 5656 were used as positive controls in molecular and classical tests (symptomatologic) conducted to determine FORL and TYLCV resistance (Colak and Bicici, 2011; Kabaş et al., 2012).

Molecular Studies

Genomic DNA isolation and amplification studies

DNA Prufication Mini Kit (Thermo Scientific GeneJET Plant - K0792) was used in total genomic DNA isolation of tomato lines. For this purpose, 100 mg young leaves obtained from positive controls and tomato line during the 2-4-leaf period. The DNAs were adjusted to 40 ng using a spectrophotometer, and controlled in 0.8% agarose gel and stored at -20°C.

PCR conditions and agarose gel electrophoresis studies

PCR studies were conducted on 418 F_2 generation tomato lines for FORL and TYLCV resistant.

In the study, FORL resistance was determined with UBC194 RAPD (Fazio et al., 1999) and $SCAR_{Frl}$ (Mutlu et al., 2015) primers. In the study, PCR studies were conducted on tomato lines resistant to all three agents (FORL + TY1 + TY3) based on the new publication of $SCAR_{Frl}$ primer (*Table 1*). PCR reaction (final volume 25 µL) for FORL and TYLCV was conducted with 11 µL DreamTaq Green PCR Master Mix

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(ThermoScientific-K1082) (containing 0.5 mM Taq polymerase (2 U), 2X DreamTaq Green buffer, 0.4 mM (200 µM) of dATP, dCTP, dGTP and dTTP each and and 4 mM $MgCl_2$), 1 µL F + 1 µL R primer and 1 µL DNA (10 ng) and added 11 µL ddH2O.

PCR conditions for the FORL-UBC194 primer were as follows: 3 min pre-denaturation at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 38°C, 1 min at 72°C, and 1 cycle 10 min final elongation at 72°C min were programmed.

Table 1. *Nucleotide sequences and size of the primers used for the FORL, TY3 and TY1 resistance in the experiment*

Primer	Annealing temperature (^{0}C)	Primer sequence (5'3')	Product size (bp)	References
UBC194 (FORL)	34	5'-AGGACGTGCC-3'	590 bp resistance	Fazio et al., 1999 Morid et al., 2012
$SCAR_{Frl}$ (FORL)	53	5'-CACATTCATCATCTGTTTTAGTCTATTC3'	950 bp susceptible 1000 bp resistance 950-100 bp heterozygous resistance	Mutlu et al., 2015
P6-25-F2 (TY3)	53	5'GGTAGT GGA AAT GAT GCT GCT C-3'	320 bp susceptible 650 bp resistance	Jensen et al., 2007 Ji et al., 2007abc
P6-25-R5 (TY3)		5'-GCTCTGCCTATTGTCCCATATATAACC-3'		
JB1F (TY1)	55	5'-AACCATTATCCGGTTCACTC-3'	400 bp susceptible 450 bp resistance	Castro et al., 2007
JB1R (TY1)		5'-TTTCCATTCCTTGTTTCTCTG-3'		

PCR conditions for the FORL-$SCAR_{Frl}$, TY3 and TYl primers were as follows: 3 min pre-denaturation at 95°C, followed by 35 cycles of 30 sec at 95°C, 45 sec at 53°C, 45 sec at 72°C and 1 final elongation cycle at 72°C for 10 min were programmed. The PCR products obtained for TY1 were cut with TaqI restriction enzyme to determine their resistance. Digestion was conducted according to Thermo Scientific. Restrictions of 10 µl of the amplified products, ddH_2O µl 17, 10 × Fast Digest Green Buffer 2 µl and FastDigestenzyme 1µL were conducted at 37°C in a total volume of 30 µl with 10 U/ µl.

A GeneAmp 9700 thermocycler (Eppendorf) was used for all PCR amplifications. Digestion products were analyzed with agarose gel electrophoresis (2% agarose w/v with TBE 1buffer) and visualized by ethidium bromide staining, and the results were recorded.

Classical testing for FORL and TYLCV Pathogenicity

Classical testing were conducted on three genotypes (B26, B40, B178) from BATEM and four genotypes (A-31, A-41, A-48 A-66) from private sector were containing the 3 target resistant genes (FORL + TY1 + TY3).

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Isolate, material supply and storage

The FORL isolate was obtained from Mersin greenhouse tomato plants and it was determined that it was a molecular (PCR) FORL isolate with high virulence, and the study was conducted with the FORL isolate coded Tarsus-0 (Tarsus / Mersin isolate) and stored at -20°C (*Figure 1*) (Colak and Bicici, 2011).

Figure 1. *FORL and TYLCV symptoms in tomato plants*

In the TYLCV tests; the isolate containing all three TYLCV strains obtained from Mersin greenhouse tomato fields was used as an inoculum source (*Figure 1*) (Fidan et al., 2011). Vector whiteflies (*Bemisia tabaci*) were procured from the collection regularly produced in Adana Biological Control Research Institute Directorate (BMAE).

Classical testing for FORL resistance

The FORL isolate was developed for 7 days in PDA and inoculated to 250 mL medium that contained 100 mL of PDB (Potato Dextrose Broth) and incubated for 10 days. Seedling root immersion method was used for FORL inoculation. For this purpose, the soil on 3-4 weeks old tomato seedlings were washed out, the roots were shaved and inoculated by immersion into $1x10^6$ spores/ml FORL spore suspension for 4-5 min (Colak and Bicici, 2011). Then, the experiment was set up by planting 3 tomato seedlings from each FORL-inoculated tomato line in 5-replicates into pots (15 × 15 cm) that contained sterilized peat:perlite (1:1). Experiments were set up based on random lots experimental design. The control plants were planted after immersion into sterile water. The genetic resistance to FORL disease is controlled by a single (*Fr1* gene) dominant gene (Roberts et al., 2001). Thus, in FORL resistance tests, to determine the resistance of the lines, the plants were rooted, and the roots and vascular structures were examined, and the presence of the disease was recorded. The evaluation was conducted by observing the symptoms in the plants and browning on roots and root crowns and the scoring was conducted with a 0 - 4 scale, and resistant (0: symptom-free plants, no disease) and sensitive (>1: plants indicating symptoms, disease is present) plants were determined (Vakalounakis et al., 1997; Morid et al., 2012).

Classical testing for TYLCV resistance

Vector whiteflies (*Bemisia tabaci*) were used to infect the tomato plants in the experiment. In the cage studies, tomato plants infected with all three strains (TYLCV Israel, Mild and Sardinia strains) were used (Fidan et al., 2011). In the study, after the

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DOI: http://dx.doi.org/10.15666/aeer/1702_22032218

actual second leaf stage of the plants in the cages, 30 whiteflies per plant were released to the cages. After the whiteflies fed for 48 hours after the release, the whiteflies were terminated using an insecticide. During infection, the suction damage to the plants was prevented. For this purpose, 20 pots with 20 cm diameter containing peat:perlite (1:1) were placed in the cages (80x100x160 cm), and the experiment was set up with 4 pots per line and 3 plants per pot based on the random lots experimental design. Observations were conducted when the symptoms were obvious on the plants 15 - 21 days after the experiment was set up and maintained for 3 weeks. The plants with disease symptoms were considered sensitive and those without symptoms were considered resistant in the experiment (Lapidot and Friedman, 2002).

All classic test experiments for FORL and TYLCV were conducted at of 26 ± 2°C temperatures, 60 – 70% relative humidity, 16 hours light - 8 hours dark conditions in a climate chamber at BMAE.

Greenhouse Experiments and Determination of Fruit Quality Properties

FORL and TYLCV resistant tomato genotype seedlings were grown in the greenhouses of institutions that possessed both resistant lines. The seedlings were planted in the greenhouse with 80 cm inter-row and 50 cm intra-row distances (2500 plants/da) on March 2, 2016 (BATEM) and February 24, 2016 (AYER). All basic maintenance procedures were conducted regularly throughout the experiment (Günay, 2005).

In the study, harvested tomato fruit mean weight (g/fruit/FMW), mean yield per plant (g/plant/MYP), total yield (kg/da/TY) and certain fruit quality measures were obtained after the initial harvest. In the greenhouses in both institutions, 10 fruits were randomly selected from each line and each lot in the third tomato harvest and fruit quality properties [fruit diameter (mm / FD) and height (mm / FH), fruit water soluble dry matter (% / WSDM), fruit titratable acidity (% / TA), vitamin C (L - Ascorbic Acid) content (mg / 100g / CV), pH of fruit juice, fruit juice EC measurement (ms / cm)] were determined (Guillén et al., 2006; Cemeroğlu, 2007). Fruit diameter (mm / FD): The diameter was measured at the equatorial region of the tomato fruit by using the digital caliper (Miyutoyo 500-181-30). Fruit height (mm / FH): The longitudinal section of the tomato fruit was measured by the digital caliper (Miyutoyo 500-181-30) for fruit height. Fruit water soluble dry matter (% / WSDM): The few drops of extracted tomato juice was measured by a hand refractometer (Atago, Tokyo, Japan) for WSDM. Fruit titratable acidity (% / TA): Titratable acidity was determined by titration of 5 ml tomato juice with 0.1 N sodium hydroxide to an endpoint of pH 8.1, results are presented as % citric acid. Vitamin C (L - Ascorbic Acid) content (mg / 100g / CV): For ascorbic acid content, tomatoes were ground with a warring blender and 5 g sample was mixed with 45 ml 0.4% oxalic acid and then filtered. One ml filtrate and 9 ml 2,6-Dichlorophenolindophenol sodium salt solution ($C_{12}H_6Cl_2NO_2$-Na) mixed and then transmittance values 520 nm in a spectrophotometer (Perkin Elmer, Lambada 850 UV/Vis). Results are expressed as mg 100 g^{-1} (Ozdemir and Dundar, 2006). pH of fruit juice: The pH of the tomato juice was measured with WTW 315i model pH meter. Fruit juice EC measurement (ms /cm): The EC of the tomato juice was measured with WTW 315i model EC meter. At the end of the experiment, whether there were differences between the tomato genotypes based on the examined properties was determined with variance analysis of the obtained data using Jump software and the comparison of the means were conducted with LSD test.

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Results

Determination of FORL and TYLCV resistance of tomato genotypes with molecular studies

PCR optimizations were conducted with FORL and TYLCV resistant and sensitive controls to determine the resistance of 418 tomato line samples that constituted the study material (*Figures 2 and 3*). The PCR study conducted on 196 pure tomato lines developed by BATEM breeding department with UBC 194 primer used in FORL resistance demonstrated that 60 tomato genotypes were found FORL resistant (*Figure 2*). Again, PCRs conducted with TY3 allele used for TYLCV resistance on the same samples demonstrated that 34 out of 196 samples were resistant of TY3. It was determined that 25 samples were resistant to TYLCV as a result of the PCR conducted with TY1 locus primers that are effective on TYLCV resistance. It was determined that 3 tomato lines coded B26, B40 and B178 were resistant to FORL and both strains of TYLCV (FORL + TY1 + TY3).

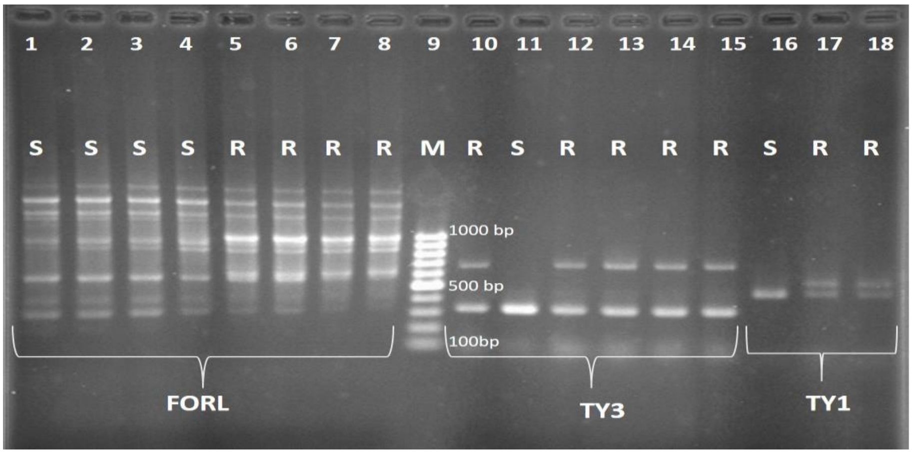

Figure 2. *PCR gel image with resistance and sensitive controls for FORL and TYLCV.* ***1,2,****3 and* ***4;*** *Hazera 5656 to FORL sensitive (S)* ***5*** *and* ***6****; FORL resistance (R) Fla.7781 genotype (590 bp)* ***7*** *and* ***8****; Tayfun F1 tomato variety to FORL resistance (590 bp double fragment)* ***9****; Thermo DNA ladder (100 bp),* ***10*** *and* ***12****; Solanum chilense LA2779,* ***13****,* ***14*** *and* ***15****; Tayfun F1 to TY3 resistance (320 and 650 bp)* ***11****; Hazera 5656 to TY3 sensitive (320 bp),* ***17****; Solanum chilense LA2779,* ***18****; Tayfun F1 to TY1 resistance (400 and 450 bp),* ***16****; Hazera5656 to TY1 sensitive (400 bp)*

As a result of the molecular studies, it was determined that 160 pure tomato lines belonging to private sector (AYER) were resistant to FORL of 42 line, 10 lines were TY1 resistant, and 12 lines were TY3 resistant. Four tomato lines, namely A31, A41, A48 and A66, were identified as resistant to all three (FORL + TY1 + TY3) among the 160 pure tomato lines belonging to AYER. It was determined that none of the 62 tomato line genotypes belong to Çukurova University were resistant to any of the three resistance resources. A total of 7 tomato lines were identified as resistant to both diseases (FORL + TY1 + TY3) as a result of the conducted PCR study at FORL resistance developed by Mutlu et al. (2015) with $\mathrm{SCAR_{Frl}}$ confirming the findings by Mutlu et al. 2015 (*Figures 3 and 4*). It was suggested that this new primer, used in resistant lines and confirmed with classical testing in the present study, was efficient in determination of FORL resistance and could safely be used in future breeding studies.

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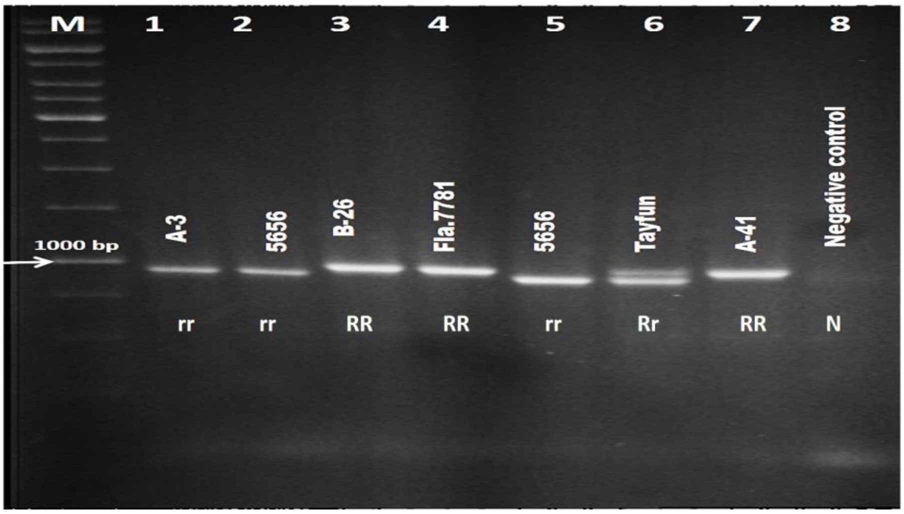

Figure 3. *Agarose gel image of SCAR$_{Frl}$ primer with resistance and sensitive controls. Thermo DNA ladder (100bp),* ***1****; A-3 tomato genotype belong to AYER,* ***2****; Hazera 5656 to FORL sensitive (950 bp),* ***3****; FORL resistance line B-26 tomato genotype belong to BATEM,* ***4****; FORL resistance Fla.7781 genotype (1000 bp),* ***5****; Hazera 5656 to FORL sensitive (950 bp),* ***6****; FORL resistance to Tayfun F1 (950-1000 bp double fragment),* ***7****; FORL resistance line A-41 tomato genotype belong to AYER,* ***8****; Negative Control (N)*

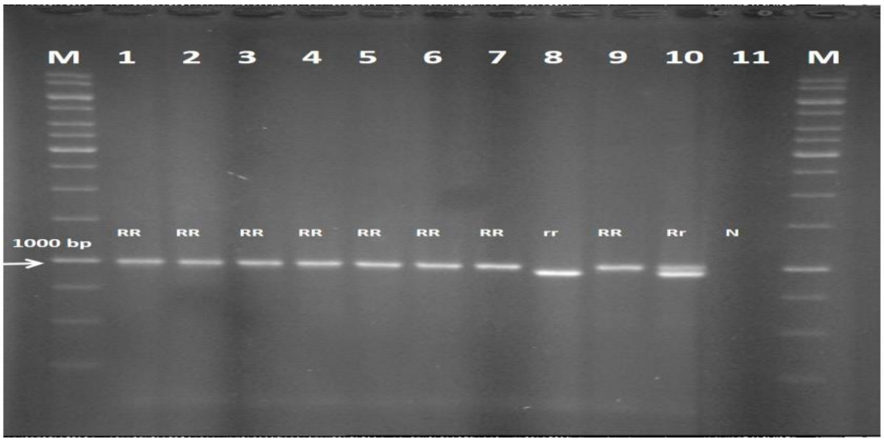

Figure 4. *A gel image showing the resistance status of the seven tomato lines identified as resistant to three diseases (FORL+ TY1+TY3) using the SCAR$_{Frl}$ primer in the study. Thermo DNA ladder (100bp),* ***1,2,3****; B-26, B-40 and B-178 tomato genotypes belong to BATEM (1000 bp);* ***4,5,6,7****; A-31, A-41, A-48 and A-66 tomato genotypes belong to AYER (1000 bp),* ***8****; Hazera 5656 to FORL sensitive (950 bp),* ***9****;* ***4****; to Fla.7781 genotype to FORL resistance (1000 bp),****10****; Tayfun F1 to FORL resistance (950-1000 bp double fragment),* ***11****; Negative Control (N)*

Classical testing results for FORL and TYLCV resistance

In classical testing for FORL resistance, the two-repeat experiments were set up with B26, B40 and B178 tomato genotypes from BATEM Institute and A31, A41, A48 and A66 tomato genotypes from AYER in BMAE controlled climate rooms. The experiments were terminated after 20 days, when foliage wilting and turgor loss, slowed growth, marked blackening and deaths in the root and root crown were observed as a result of FORL agent inoculation in the control plants (Hazera 5656 / FORL sensitive).

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It was determined that there were no symptoms of FORL induced crown and rot-root disease in all tomato genotypes in the experiment group as a result of the plant and root assessments (*Figure 5).*

Figure 5. *Classical test study of A-31 and B-40 genotypes from resistant tomato lines of FORL disease*

In classical TYLCV resistance testing studies; whiteflies fed on the plants infected with three TYLCV strains were collected with infusion tube after 2 days and released onto the tomato genotypes. TYLCV-specific symptoms such as yellowing on leaf edges and shrinkage of leaves, contraction in the leaves were observed and the leaves curled upwards starting from the margins on the 16-18th day of the experiment in sensitive control Hazera 5656 plants planted in addition to resistant line and varieties in each cage (*Figure 6*). The 7 tomato lines belonging to AYER and BATEM that were determined as TYLCV-resistant in molecular studies did not exhibit symptoms in classical testing cage study conducted with whitefly inoculation, thus both findings were confirmed. The use of molecular markers in development of disease resistance provides great advantages in terms of time, cost and reliability (Agrama and Scott, 2006; Anbinder et al., 2009; Ji et al., 2009). It is not possible to test hundreds of lines accurately and reliably with classical methods or to determine the inheritance of resistance (heterozygote or homozygote resistance).

In classical testing of resistant genotypes, breeders determine TYLCV resistance as the natural infection occurs in the fields for non-mechanical vector TYLCV. If molecular marker selection is not implemented in this viral disease with the sole vector of whiteflies, it is not always possible to wait for the natural infections through whiteflies in the field, and then conduct the analysis and it is not an accurate and reliable method (Verlaan et al., 2013). Because, it is not possible to understand whether the plants that appear resistant were exposed to the viral infection or whether they were infected by the whiteflies, but do not exhibit symptoms due to their resistance.

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Furthermore, it is not possible to set up experiments with whiteflies for hundreds of breeding lines. In the present study, the use of molecular markers reduced the number of genotypes from 418 to 7 tomato genotypes (B26, B40, B178, A31, A41, A48 and A66) that were resistant to all three factors (FORL+TY1+TY3), making it easier and more economic to verify these findings with classical tests. Although molecular markers provide a great advantage in terms of speed and cost, classic validation tests should be performed (Lee et al., 2015). This is due to the fact that it is important to perform classical validation tests (smyptomatologic) in determination of MAS-selective resistance, which taking the distances between the developed markers and the gene and human and marker-based errors into consideration (Caro et al., 2015; Scott et al., 2015).

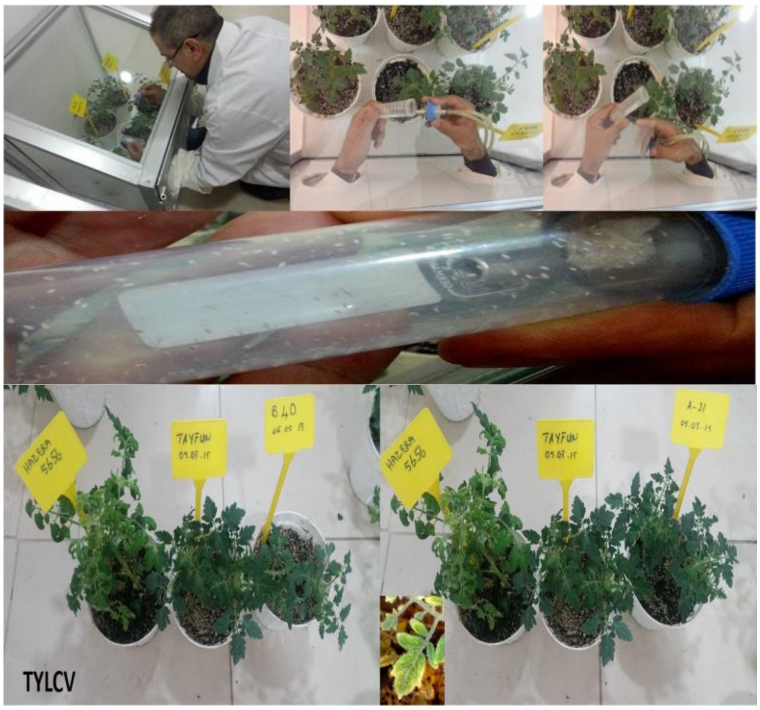

Figure 6. *The release of white flies collected on TYLCV infected plants in special cages for tomato lines taken to the TYLCV resistance test and classical test study of A-31 and B-40 genotypes from resistant tomato lines of TYLCV disease*

Determination of the yield and certain fruit quality properties in disease resistant tomato genotypes

In order to determine the yield and certain fruit quality properties of FORL and TYLCV resistant tomato genotypes, greenhouse experiments were set up in Antalya. Fruit harvest was initiated when tomato fruits ripened and turned red. Eight harvests were conducted in BATEM greenhouses and 6 harvests were conducted in AYER greenhouses. The tomato fruit yield and certain fruit quality property data throughout the cultivation season are presented in *Tables 2 and 3*.

The average yield values per plant (g/plant) and decare yield values (kg/da) were found to be statistically significant in 7 tomato genotypes determined to be resistant to FORL and TYLCV belonging to Ayer and BATEM. Among the tomato genotypes belonging to Ayer, the per plant yield ranged between 1101.8 and 2258.6 g/plant and

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the per decare yield ranged between 2754.4 and 5646.6 kg/da, the per plant yield, for tomato genotypes belonging to BATEM varied between 1206 and 2922 g/plant and per decare yield varied between 3015 and 7304.7 kg/da (*Tables 2 and 3*). In previous studies on tomato yield, it was reported that genotypes might have different responses based on their locations, climate conditions, and plant nutrition (Şen et al., 2004; Ozbay and Ateş, 2015; Demirtaş et al., 2016). The highest mean fruit weight was observed in A-48 (166.07 g/fruit) genotype among the four Ayer tomato varieties. The highest mean fruit weight was obtained in B26 (163.57 g/fruit) genotype, while the lowest mean fruit weight was obtained in B178 (98.70 g/fruit) genotype among the three tomato genotypes from BATEM. Although certain findings in the greenhouse experiments were parallel to various studies, certain differences could be observed due to varieties, ecological conditions, plant nutrition, differences in irrigation and harvest ripeness periods (Özdemir and Özer, 2016).

Table 2. *Yield and some fruit quality characteristics of tomato genotypes belong to AYER*

AYER	MYP (g/plant)	TY (kg/da)	FMV (g/fruit)	FD (mm)	FH (mm)	WSDM (%)	TA (%)	pH	EC (ms/cm)	CV (mg/100g)
A31	2258.6 a	5646.6 a	106.5 c	54.08c	59.36 a	4.4 b	0.46 a	4.6 c	4.5 a	22.54 a
A41	1337.2 b	3343.0 b	129.93 b	63.90b	56.17b	3.4 d	0.37 b	5.2 a	4.6 a	25.02 a
A48	1101.8 b	2754.4 b	166.07 a	70.49a	59.47 a	4.7 a	0.24 d	5.1 b	4.5 a	23.53 a
A66	2022.4 a	5055.9 a	98.67 d	56.42c	52.13 c	4.1 c	0.32 c	4.3 d	4.5 a	24.03 a
$Lsd_{0.05}$	327.8	819.7	5.81	2.72	1.82	0.13	0.036	0.133	0.37	2.52

Table 3. *Yield and some fruit quality characteristics of tomato genotypes belong to BATEM*

BATEM	MYP (g/plant)	TY (kg/da)	FMV (g/fruit)	FD (mm)	FH (mm)	WSDM (%)	TA (%)	pH	EC (ms/cm)	CV (mg/100g)
B26	1206 c	3015.3 c	163.57 c	69.39 a	53.65 a	5.06 b	0.30 c	4.55 a	4.0 b	26.74 a
B40	2922 a	7304.7 a	122.13 a	62.31 b	50.56 a	5.17 ab	0.39 a	4.47 a	4.5 a	25.87 a
B178	2150 b	5375.3 b	98.70 b	56.73 b	52.54 a	5.40 a	0.35 b	4.50 a	3.9 b	27.83 a
$Lsd_{0.05}$	327.3	818.3	20.34	5.64	5.19	0.236	0.035	0.1128	0.1153	5.293

The water-soluble dry matter content (WSDM) is an important quality criterion in tomato fruit, and it was determined that the highest WSDM content was in A-48 genotype with 4.7% among AYER tomato genotypes and the highest WSDM content was in B178 genotype with 5.40% among BATEM genotypes (*Table 3*). It was reported that the water soluble dry matter content in tomato fruits varies between 2.9 and 5.9% (Özbay et al., 2012). The titratable acidity (%) may vary based on the variety and ripeness period of the fruit in tomato fruit juice (Ozbay and Ateş, 2015). It was determined that the acidity rates varied between 0.46-0.24% in AYER tomato genotypes and between 0.30-0.39% in BATEM tomato genotypes.

It was found that the difference between pH values of AYER tomato genotypes was significant in the experiment and the highest pH was obtained with the A41 genotype (5.2), and the lowest pH was obtained with the A66 tomato genotype (4.3) (*Table 2*). BATEM tomato genotype pH values ranged between 4.47 and 4.55 (Bozköylü and Daşgan, 2010). There were no differences between the fruit quality properties of EC and

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vitamin C content of AYER tomato genotypes. The highest EC and vitamin C values were obtained in the A41 genotype (4.6 ms/cm-25.02 mg/100 mg). The highest EC value was obtained in the B40 genotype with 4.5 ms/cm in among BATEM genotypes. All tested varieties exceeded the limit value of 8.4 mg/100 g for Vitamin C in tomatoes (Özbahçe and Padem, 2007).

Discussion

Using molecular markers in breeding studies, the choice of the closest of the gene in the primers used in disease resistance is important in terms of the reliability of the results. Since CAPS, SCAR and co-dominant SCAR primers, which are a further step from RAPD primers in determination of FORL resistance, were not developed or patented and published for this disease, several studies, similar to the present study, have used UBC 194 RAPD primers, which is closest to the gene, to determine FORL resistance (Fazio et al., 1999). However, the fact that the RAPD markers obtained in these studies were not reproducible, the difficulties in clear definition in agarose gel and the lack of inheritance of FORL resistance (heterozygous or homozygous) demonstrated that further studies should be conducted (Tanyolac and Akkale, 2010). For this purpose, Truong et al. (2011) developed RAPD markers and transformed these into SCAR and made these available for use in breeding experiments. Mutlu et al. (2015) developed the SCAR marker into a Co-dominant SCAR, obtaining the $SCAR_{Frl}$ marker, at a distance of 0.016 cm to the gene and almost on top of the *Frl* gene, and which could demonstrate whether the inheritance is heterozygous or homozygous. It was determined in the present study once more that the co-dominant $SCAR_{Frl}$ marker is reliable for determination of the heterozygous or homozygous status of inheritance in F2 and F3 populations in breeding studies, it can be accurately and easily identified in agarose gel, reduces the hybridization study costs, saves time and promotes rapid commercialization of the lines when compared to the previous FORL resistance markers.

In the study, seven genotypically resistant lines (B26, B40, B178, A31, A41, A48 and A66) against both TY1 and TY3 were infected with whiteflies to confirm the symptomatologic results of the markers in tomato plant. Infections demonstrated that primers of the genes that provide TY1 and TY3 resistance (*Ty-1*, JB1 and *Ty-3*, P6-25) could be used in resistance studies. It was reported that the TY1 gene was not very effective alone due to the presence of several TYLCV strains and controlled with a high number of genes (Zamir et al., 1994). It was reported that the TY3 marker could be used effectively in MAS since it is located on the 6^{th} chromosome and close to the gene (0.3 cM), its Co-dominant SCAR characteristic, and could reflect heterozygous, homozygous resistant and sensitive genotypes concurrently with single PCR (Ji et al., 2007c). Although the TY4, TY5 and TY6 markers were developed since the development of TY3 marker, the present study confirmed that the most effective resistance was TY1 and TY3 resistance. The knowledge on the existing strains in Turkey and their resistance to the resistance markers and their effectiveness were determined and confirmed. The existence of different TYLCV strains, especially in Asia, revealed that different markers should be tested in these areas (Hutton et al., 2012). Israel and Mild strains of TYLCV are the most prevalent strains globally, and the TY1 and TY3 markers developed against these strains maintain the status of being the most used marker in breeding studies in the world (Lee et al., 2015).

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Conclusion

In today's conditions, there is a need for resistance against at least 3 and more disease agents depending on the production site and time. As the number of required resistances increases, breeding period gets longer, and it could even become impossible. Thanks to the present study findings, resistance to significant biotic factors (FORL + TYLCV) were determined, saving time for the line owner organization in commercialization of multi-resistance seven tomato variety candidates. This study showed that both disease resistant and argonomic as the market value of the highest new kind of candidate (A31 and B40) by determined by line with the institution will contribute greatly to quickly pass the commercialization and market presentation stages. In this study, multiple markers were tested for a gene, for the determination of FORL resistance, UBC-194 and SCARFr1 markers, TY1 and TY3 markers for TYLCV resistance have been confirmed. The results of the study will reveal the possibility that molecular markers used in the development of resistant lines and varieties of these diseases may be an alternative to classical testing and will shed light on future studies. Furthermore, with the determination of the parents who will be used as father or mother and resistant to one of the FORL and TYLCV disease, it became possible to design alternatives projects (F1 hybrid) to obtain new varieties based on the globally prominent fruit quality criterion requirements.

Acknowledgements. We would like to express our thanks to the Scientific and Technological Research Council of TURKEY (TÜBİTAK) for the financial support provided for this study within the scope of the project numbered 213 O 103.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22032218

FOREST EDGE EFFECTS ON SEEDLINGS IN MIXED ORIENTAL BEECH (*Fagus orientalis* Lipsky) - SCOTS PINE (*Pinus sylvestris* L.) STANDS

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(Received 15th Nov 2018; accepted 11th Jan 2019)

Abstract. Forest edges created by silvicultural treatment influence micro-climatic conditions and available light in forest stands. Studies regarding the impacts of forest edges on regeneration is limited in mixed Oriental beech (*Fagus orientalis* Lipsky)-Scots pine (*Pinus sylvestris* L.) stands. In this study, the influences of forest edges on height growth, root-collar-diameter growth and density of seedlings in an adjacent stand of Oriental beech-Scots pine were observed. In addition, the effects of stand basal area and height-to-crown on height growth, root-collar-diameter growth and seedlings density were monitored. The study was conducted within Samatlar Forest Planning Directorate, in Kastamonu city, Turkey. In the selected Oriental beech-Scots pine stand, twenty-five transects were installed perpendicular to the forest edge, and all the measurements were taken within these transects. There were statistically significant relationships between the distance from the forest edge and the growth of Scots pine seedlings ($p<0.05$), while the stand edge did not have any effect on the growth of Oriental beech seedlings. Density of Scots pine seedlings decreased, while Oriental beech seedling density increased from the edge into the intact stand ($p<0.05$). Stand basal area and height-to-crown did not significantly change from the edge into the intact stand ($p>0.05$). The differences in seedling growth and seedling density between the two species can be associated with their dissimilar tolerance to shade. The initial results obtained in this study point out the importance of forest edges on the regeneration of Oriental beech and Scots pine seedlings. Data suggest that regeneration of mixed Oriental beech-Scots pine should be successful using group methods rather than traditional shelterwood method within entire stand.
Keywords: *establishment, growth, regeneration, edge, silviculture*

Introduction

Forest canopy structure plays an essential role in energy fluxes between the atmosphere and the land (Smith et al., 2008). Canopy structure influence understory light availability, soil temperature and moisture, soil nutrients and several other factors in a forest (Aussenac, 2000; Xue et al., 2011). Measurement of forest canopy structure is crucial for prediction of forest productivity and development (Smith et al., 2008). Forest canopy structure is manipulated by forest managers and landowners through silvicultural practices to modify understory growth conditions (Lhotka and Stringer, 2013). Seedlings respond to natural and anthropogenic disturbances by various ratio of growth and survival (Rubin et al., 2006). Silvicultural disturbances not only influence the understory environment of the treated area, but also the microclimatic conditions of the untreated adjacent forest areas by creating forest edges in between (Baker et al., 2016).

Forest edge effect is due to the separation of the interaction between two adjacent ecosystems with a sudden transition (Murcia, 1995). Forest edges include the edges

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

formed by the forest roads, rivers, forestry practices and power lines, as well as the natural openings such as rocks (Meister, 2007). Silvicultural treatments usually create forest edges with an adjacent stand, and these edges influence micro-climatic conditions within the stand (Baker et al., 2016). Factors such as air temperature, air humidity, vapor pressure, soil moisture, soil physical and chemical properties and light intensity vary between the outer and inner parts of the forest edges (Lhotka and Stringer, 2013; Dovčiak and Brown, 2014; Wekesa et al., 2018). Thus, understanding of the edge effects is crucial in forest management and woodlands (Ouin et al., 2015). In order to quantify the spatial scale at which stands are affected, the depth of forest edge effects has been observed in several studies (Laurance, 2000; Ries et al., 2004; Lhotka and Stringer, 2013). Research shows that the edge effects can extend at least ten meter into the adjacent stand, and these differences usually disappear after the first fifty meters from the edge towards interior stand (Murcia, 1995).

Oriental beech (*Fagus orientalis* Lipsky) and Scots pine (*Pinus sylvestris* L.) are economically and ecologically important deciduous and conifer tree species with wide distribution areas in Turkey (Boydak et al., 2011; Atik, 2013). Vast acreage of mixed stands comprised of these tree species is also present in the country. The sustainability of these mixed forests is possible through the successful regeneration of these tree species. Oriental beech is known to be a shade tolerant species, while Scots pine is intolerant to shade (Kara, 2018a, 2018b). Mixed Oriental beech-Scots pine stands have been commonly managed using traditional shelterwood method in Turkey (Odabaşı et al., 2004). Shelterwood systems are high-forest systems in which seedlings are established under the parent trees, and includes the systems of successive regeneration (Matthews, 1991). However, due to the differences in their tolerance to shade, group or group shelterwood methods are considered more appropriate to manage this forest type. With group methods, the group clearings or the groups with relatively lower density create edges with the intact stand. In general, Scots pine seedlings can be attained in groups, while Oriental beech is regenerated using traditional shelterwood method within the rest of the stands using relatively higher stand density. It is also likely to obtain Scots pine seedlings outside the groups and close to edges created. Studies comparing the edge of the forest with that of the inner forest have indicated that there is a higher amount of light on the forest edge, and it usually increases tree growth and the density of understory plants at or near the edges (Gehlhausen et al., 2000; Lhotka and Stringer, 2013). However, our knowledge on the influence of forest edge, and its extend on mixed Oriental beech-Scots pine stands is limited.

Several studies have explored the effects of forest edges on understory plants within interior parts of stands (Jiquan Chen et al., 1992; Gehlhausen et al., 2000; Burton, 2002; Hamberg et al., 2009; Gatti et al., 2019). However, to improve our understanding regarding the forest edges, it has been stated that more research on the forest edges is need for different forest types in different regions (Šálek et al., 2013). To our knowledge, no such study has been conducted in mixed Oriental beech-Scots pine stands. Moreover, recently, there has been a growing concern by Turkish forester on the decreasing proportion of Scots pine trees in the mixed beech-pine stands, which would result in the conversion of these mixed stands into pure beech stands in Turkey. Thus, understanding of the forest edges in these forests will help forest managers develop more appropriate management practices for the sustainability of these mixed forests in Turkey. The determination of the edge effects will help improve the success of group methods when naturally regenerating these mixed forests types as well. Understanding of the forest edges effects on the Oriental beech and Scots pine seedlings is essential for the sustainability of mixed stands of these tree species. Therefore, in this study, the influences of forest edges on the seedling density (ha^{-1}), height growth (cm) and root-collar diameter growth (mm) of Oriental beech and Scots pine seedlings in a mixed stand were observed. It is also aimed to examine how far the edge effect is from the edge of the stand to the interior stand. Moreover, the effects of stand basal area (m^2 ha^{-1}) and height-to-crown on the height growth and root-collar-diameter growth of seedlings of both species, when moving from the edge to interior stand, were monitored.

Materials and Methods

Study area

This study was conducted in a mixed Oriental beech-Scots pine forest managed by Samatlar Forest Directorate, within the boundary of Kastamonu Forest Regional Directorate in Kastamonu, Turkey (*Figure 1*). The study area was located within the compartment 90. Total forested area is 49.149 ha, which is approximately 74% of the entire area within Samatlar Forest Directorate. Kastamonu is situated in the Euro-Siberian fito geographic region (Çolak et al., 2009). Study area is located within the natural range of Oriental beech and Scots pine species. In addition to Oriental beech and Scots pine, black pine (*Pinus nigra* subp. *pallasiana*), Calabrian pine (*Pinus brutia*), Trojan fir (*Abies equi-trojani*), juniper (*Juniperus* sp.), oaks (*Quercus* sp.) and hornbeam (*Carpinus* sp.) are other tree species of the region. Blackberry (*Rubus* spp.), rockrose (*Cistus laurifolius*), heath (*Erica* sp.), cranberry (*Cornus* sp.) and ferns (*Pteridium aquilinum*) are the common understory species within study region. Average altitude of the study area is 1340 meter above the sea level, and average slope ranges from 10 to 30% across the study area. Study area is in the transition zone between Anatolian terrestrial and Black Sea temperate climates, therefore, cold/wet winters and rainy/wet summers occur in the region. The average annual temperature is 10.9°C within the study area ranging between -1.1 and 26.6. The average annual precipitation is 570 mm ranging from 250 to 689.5 m in the last twenty years. In the study region, the vegetation period lasts approximately 137 days starting from late April through later August. Sandy clay and loamy-clay soils are usually examined in the study region. The dominant soil group is mostly brown calcareous in the study region.

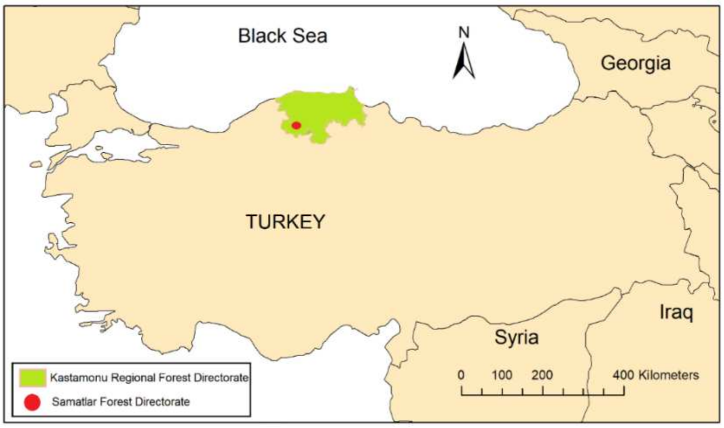

Figure 1. *Boundary of Kastamonu Regional Forest Directorate, and location of study area*

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DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

Study Design

The study was established along a mixed Oriental beech-Scots pine forest matrix that surrounded a forest opening of approximately 0.3 ha in size (*Figure 2*) in the spring of 2016. The study site is located at Longitude of 33.167075, and Latitude of 41.302296. About 90% of the stand basal area (m^2 ha^{-1}) composed of Oriental beech and Scots pine trees. The opening has been untreated to serve as a foot plot for wildlife animals for a prolonged time. The stand has been managed using the traditional shelterwood method. The topography of the opening was relatively flat, with a slope less than 10 %. This study used the edge environment around a forest opening to examine whether distance from the edge affected the root-collar-diameter growth and height growth of Oriental beech and Scots pine seedlings in a mixed stand of these tree species.

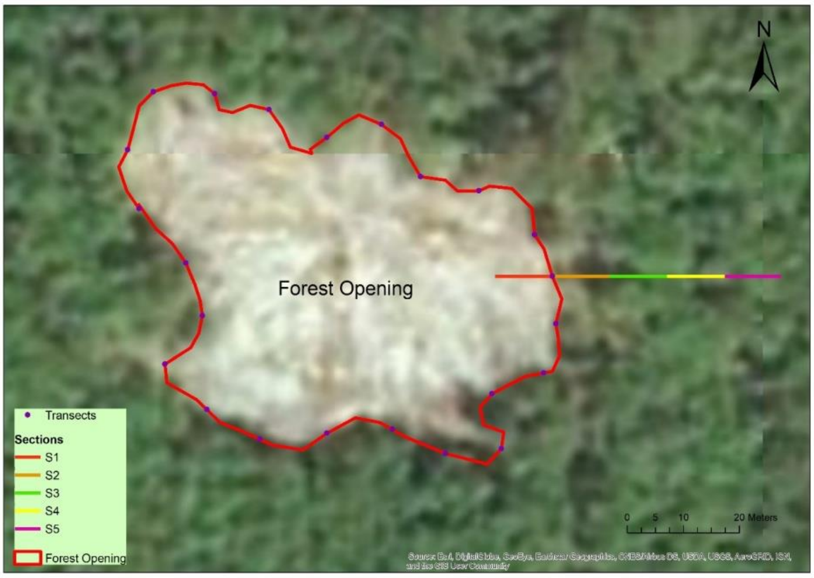

Figure 2. *Study area and a sample transect*

As suggested by Lhotka and Stringer (2013), the stand edge was determined as the border between the two nearest trees growing along the boundary of the forest opening. First, twenty-five sampling locations were chosen every ten meter along the perimeter of the forest opening (*Figure 2*) in the spring of 2016. Next, at each sampling location, twenty-five transects were installed perpendicular to the forest edge, and they extended 10 meter from the edge into the opening, and 40 meter from the edge into the stand (*Figure 2*). The width of transects was 1 meter, and there was not any canopy gaps along transects. Moreover, due to a recent understory control treatments, there was not any other species rather than Oriental beech and Scots pine understory of the study stand.

Measurements and Analysis

Measurements were taken in the summer (July and August) of 2016. Each transect was divided into five sections; one section within the opening (S1) and four sections within the stand (i.e., S2, S3, S4 and S5 for intervals of 0-10, 10-20, 20-30 and 30-40 m, respectively). In each section, all trees larger than 5 cm in diameter at breast height

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DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

(d.b.h.) were measured. Using the d.b.h. measurements, basal area of each tree was determined using the formula below, and then, it was converted to basal area per hectare.

$$\text{Tree basal area} = \text{d.b.h.}^2\ (0.0000785) \quad \text{(Eq.1)}$$

Number of seedlings for each species was counted in each section, and it was converted to calculate number of seedlings for unit area (i.e., one ha). Within each section, five-year old Oriental beech and Scots pine seedlings were individually selected in 2017. It should be noted that seedlings' age were determined by counting the whorls following DeYoung (2016). Small single branches between major whorls were not included when counting age as suggested (DeYoung, 2016). Root-collar-diameter (mm) of selected seedlings was measured with a digital caliper to monitor total root-collar-diameter growth of seedlings of both species over five-year period. For each seedling, one root-collar-diameter measurement was taken first, and next, the second measurement, which was perpendicular to the first one, was conducted. Two measurements were averaged to attain the root-collar-diameter of each seedling.

In addition, height (cm) of selected seedlings was determined with a ruler to attain the total height growth of seedlings at age five. Distance (m) of each selected seedlings to the edge border was measured using a measuring tape height-to-crown, which refers to the vertical distance (m) from the surface to the first living branch of tree (Lhotka and Loewenstein, 2008), was measured every two meter in each section with a TruPulse Laser Rangefinder (Laser Tech Inc.). Height-to-crown measurements taken in each section were averaged to obtain average height-to-crown for each section.

Lhotka and Stringer (2013) stated that the study design used in this study is not manipulated by nature, thus, each transect can be treated as "replicate" because they are treated identically. Thus, there was 25 replicates of edge effects in this study. Root-collar-diameter of seedlings at age five were averaged for each categorized distance intervals (i.e., sections). Same procedure was repeated for seedling height for each section. Log-transformation was utilized for five-year root-collar-diameter growth and height growth data to enhance residual homogeneity and normality (Mcdonald, 2013).

Mixed-effect Analysis of Variance (ANOVA) model of was utilized to define the relationships between the distance from the edge and seedling root-collar-diameter and height growth (α=0.05). The test incorporated the transects as as random effect, while the distance from the edge was treated as fixed effect. The "lmer" function of "nlme" package in R-Statistical software (R Development Core Team, 2010) was used for this analysis. Tukey's pairwise comparison method for testing statistical significance of seedling density, root-collar-diameter and height growth among the categorized distance intervals (i.e., sections) was used (α=0.05). The categorized distance intervals (i.e., sections) were the experimental units for the mixed-effect ANOVA model as suggested (Lhotka and Stringer, 2013). In addition, the influences of the distance from the edge on the number of seedlings was found using poisson regression with the "glmer" funtion in R-Statistical software, since this test is recommended for the count data such as number of seedlings per hectare. Moreover, the influences of stand basal area and height-to-crown on the total root-collar-diameter growth and total height growth of seedlings over five years were determined using the mixed-effect linear regression method for each species, in which the transect was incorporated as as random effect, while basal area and height-to-crown were treated as fixed effects. The "lme" function of "nlme"

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package in R-Statistical software (R Development Core Team, 2010) was used for this analysis. Normality and homogeneity of variance were tested with residual analysis. R-Statistical software (R Development Core Team, 2010) was utilized for all statistical analyses.

Results and Discussion

Canopy Structure

Stand basal area and height-to-crown were measured to examine canopy structure across transects. Basal area ranged from 4.73 to 65-m^2 ha^{-1} across all transects (*Table 1*). Tukey's pairwise comparison test showed that there was not significant differences between S2-S3 (p= 0.99), S2-S4 (p= 0.99), S2-S5 (p= 0.92), S3-S4 (p= 0.99), S3-S5 (p= 0.85) and S4-S5 (p= 0.59) pairs in terms of stand basal area. Due to few trees within S1, basal area was lower in this section. Basal area of S1, which was located inside the opening, was significantly different from the basal areas of sections located inside the stand (i.e., S2, S3, S4 and S5) ($p<0.05$). Even though not statistically significant ($p>0.05$), the furthest section from the edge (i.e., S5) had relatively lower basal area (26.67 m^2ha^{-1}) than the other sections located interior stand (i.e., 32.33, 32.89 and 35.47 m^2 ha^{-1} for S2, S3 and S4, respectively). This is likely due to the decreasing amount of light from the edge towards to interior stand.

Our findings on the basal area among the sections seem to be consistent with previous studies. Previous researches, which compared the edge of the forest with that of the inner forest, have found out that there were higher amount of light close to the forest edge, and it usually increased tree growth at or near the edges (Gehlhausen et al., 2000; Lhotka and Stringer, 2013). It has been also stated that amount of light in open-field conditions is usually higher than it is inside forests (Heithecker and Halpern, 2007), as we observed in this study. We found that basal area started to decrease in S5 that is at least 30 m away from the edge suggesting that forest edge had influence on overstory trees within 30 m range from the edge into the intact forest in Oriental beech-Scots pine forests. This is also supported with previous studies such as Heithecker and Halpern (2007), and Lhotka and Stringer (2013) since they revealed that the edge effects could extend 10-30 m into the adjacent stand.

Table 1. *Descriptive statistics for basal area (m^2 ha^{-1}) and height-to-crown (m). SD refers to the standard deviation of the variables*

Variables	Min.	Max.	Mean	SD
Basal area	4.7	65	31.6	17.9
Height-to-crown	0.7	17.2	6.99	*3.34*

Height-to-crown was ranged from 0.7 to 17.2 m across all transects in Oriental beech- Scots pine forests (*Table 1*). There was not statistically significant relationships among sections in terms of height-to-crown (p= 0.218, F= 1.476), that is, height-to-crown did not significantly change from the open field conditions to the interior stand. Tukey's pairwise comparison test showed that there was not any significant differences between S2-S3 (p= 0.63), S2-S4 (p= 0.88), S2-S5 (p= 0.74), S3-S4 (p= 0.98), S3-S5 (p= 0.99) and S4-S5 (p= 0.99) pairs in terms of height-to-crown. When comparing height-to-crown among sections, we attained the lowest height-to-crown (3.85 m) in S1, which

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DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

was located inside the opening. Average height-to-crowns were 6.02, 7.68, 7.10 and 7.43 m for S2, S3, S4 and S5, respectively. This is likely because the trees inside the forest opening have grown under open-grown conditions and deprived of self-pruning, and consequently, developed branches that are lower to the ground. In a similar study, Lhotka and Stringer (2013) monitored the changes in the canopy height (i.e., height-to-crown) from open-field conditions into the intact forest in a mixed deciduous forest, and discovered that height-to-crown was lowest inside the opening, and did not significantly change from edge into the stand. Given the findings in the literature, our findings regarding stand basal area and vertical canopy structure seem to be logical.

Seedling Density

The number of Oriental beech seedlings increased from open field conditions into the intact forest (*Table 2*). There was a statistically significant relationships between number of Oriental beech seedlings and distance to the stand edge ($p<0.001$). However, Tukey's pairwise comparison test showed that there was not any significant differences between S2-S3 ($p= 0.98$) and S4-S5 ($p= 0.97$) pairs in terms of number of Oriental beech seedlings per ha. In other words, only the number of Oriental beech seedlings in S1, which was located inside the opening, was significantly different from the sections located inside the stand (i.e., S2, S3, S4 and S5) ($p<0.05$). Increasing density of Oriental beech towards into the intact stand could be associated with Oriental beech's tolerance to shade, since this species can survive under overstory trees for a prolonged time (Odabaşı et al., 2004). Previous research has showed that Oriental beech seedlings can establish better under canopy than open-field conditions (Szwagrzyk et al., 2001; Parhizkar et al., 2011). Parhizkar et al. (2011) examined the effects of varying light intensities on Oriental beech stands, and reported that there was no significant differences in seedling density across the light intensities at age five. Stand basal area and height-to-crown had no significant effect on the density of Oriental beech seedlings ($p>0.05$).

Table 2. *Average number of seedlings per hectare for each species in each section*

Sections	Oriental beech	Scots pine
S1	400	7160
S2	5040	4080
S3	5320	1880
S4	7560	1240
S5	7720	680

Contrary to Oriental beech, the number of Scots pine seedlings decreased from open-field conditions towards to interior stand (*Table 2*). There was a statistically significant relationships between number of Scots pine seedlings and distance to the stand edge ($p=0.001$). It was likely that light transmittance through canopy decreased from open-field conditions to interior stand. Therefore, data showed the importance of light for Scots pine existence, which is an intolerant species to shade (Barbeito et al., 2009). Moreover, it is likely that Scots pine seedling could not compete with Oriental beech seedlings for light and nutrients under the shade of overstory trees. Previous research have explored the influence of light on the presence of Scots pine seedlings under forest canopy, substantiating the decreasing number of Scots pine seedlings attained in deeper

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DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

part of the stand (Barbeito et al., 2009; Pardos et al., 2007). Stand basal area had no significant effect on the density of Scots pine seedlings either (p>0.05).

Seedling Growth

Average height growth of Oriental beech seedlings ranged from 9.5 to 178 cm with an average of 78.34 cm, while root-collar-diameter growth ranged between 1.65 and 20.8 mm (average 10.52 mm) across all sections over five year period. In a similar study conducted in Iran, it was concluded that average seedling height and root-collar-diameter growth were 67.85 cm and 10.56 mm, respectively (Abkenar and Keshavarz, 2005). Parhizkar et al. (2011) monitored the influence of varying light intensities on Oriental beech seedlings, and found that average seedling growth ranged from 57.3 to 150 cm, while root-collar-diameter ranged from 8 to 19 mm. Our findings also fall between the ranges presented by Parhizkar et al. (2011). The distance from the stand edge did not any statistically significant influence on the height growth of Oriental beech seedlings following five year period (p=0.12) (*Figure 3*). Average height of Oriental beech seedlings did not significantly change from open-field conditions into the intact stand. In addition, there was not any statistically significant relationships between the root-collar-diameter growth of Oriental beech seedlings and the distance from the stand edge (p= 0.25) (*Figure 3*). Moreover, stand basal area had no significant influence on the height growth (p= 0.08) and root-collar-diameter growth (p= 0.13) of Oriental beech seedlings over five year period. Height-to-crown did not significantly affected the height growth (p= 0.6) and root-collar-diameter growth (p= 0.09) of Oriental beech seedlings either.

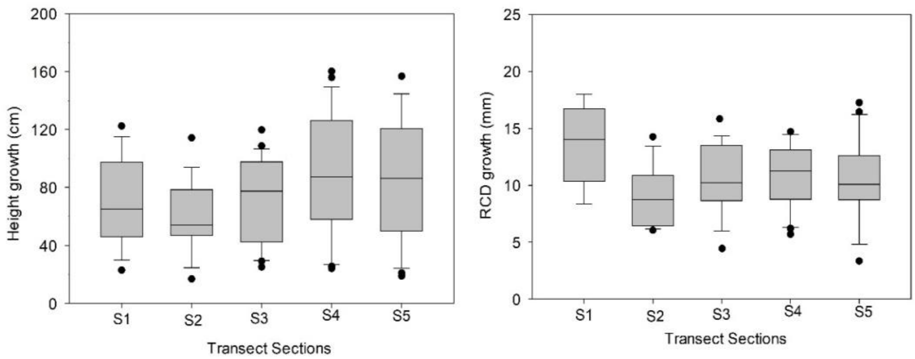

Figure 3. *The means of the height and root-collar-diameter (RCD) growth of Oriental beech seedlings under varying distances to the stand edge. Bars represent the standard deviations for the amounts of needle removal while the dots indicate the potential outliers*

The mean seedling height was 43.86 ranging from 13.3 to 98.85 cm for Scots pine seedlings, while root-collar-diameter growth ranged between 4.35 and 26.5 mm (average 10.03 mm) across all sections at age five. The growth rates of Scots pine seedlings observed in this study were consistent with previous studies. In a study conducted in Sweden, it was found that Scots pine seedlings planted under a canopy reach an average height of 40 cm at the end of the five growing seasons (Egnell and Valinger, 2003). In a different study, which monitored the effects of varying stand density on root-collar-diameter growth of Scots pine seedlings, an average of 13 mm in root-collar-diameter was determined (Gerelbaatar et al., 2015). The distance from the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

stand edge had statistically significant effect on the height growth of Scots pine seedlings following five year period (p= 0.0001) (*Figure 4*). Average height of Scots pine seedlings decreased with increasing distance from the stand edge. In addition, there was statistically significant inverse relationships between the root-collar-diameter growth of Scots pine seedlings and the distance from the stand edge (p= 0.001) (*Figure 4*). Moreover, since stand basal area did not significantly change from the edge into the stand, it had no significant effect on the height growth and root-collar-diameter growth (p>0.05) of Scots pine seedlings over five year period. Height-to-crown had also no significant effect on the height growth and root-collar-diameter growth (p>0.05) of Scots pine seedlings.

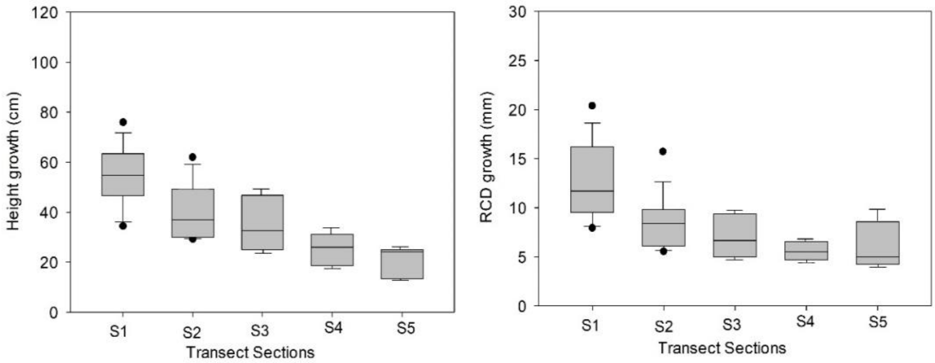

Figure 4. *The means of the height and root-collar-diameter (RCD) growth of Scots pine seedlings under varying distances to the stand edge. Bars represent the standard deviations for the amounts of needle removal while the dots indicate the potential outliers*

Understanding of the relationship between edge effects and seedling growth is essential for the establishment of understory seedlings when natural regeneration is aimed. Forest edges affect the understory light environment (Lhotka and Stringer, 2013). This study support the notion that the amount of light under the forest canopy affect seedling growth of intolerant tree species such as Scots pine. Even though stand density (i.e., basal area) and height-to-crown did not significantly change from the stand edge into the stand, Scots pine seedling growth significantly decreased towards interior stand. Thus, the decreasing seedling growth from the edge into the stand can be associated with the edge effect. Our finding suggests that forest edge was effective for light environment, within 40 m from edge, in Oriental beech- Scots pine forests. In previous research, Carus (2004) and Pardos et al. (2007) also found out that understory light is usually the most limiting factor which influence the growth of Scots pine seedlings. In a similar study, Gerelbaatar et al. (2015) examined the growth of Scots pine seedlings under varying intensities, and found higher seedling growth under higher light intensity.

The opposite pattern in Oriental beech seedlings (i.e., increasing growth of seedlings from the edge into the stand) may be related to its competition with Scots pine seedlings. Scots pine seedlings are more light-demanding species than Oriental beech seedlings. Thus, under open-field conditions, Scots pine seedlings' rapid growth likely suppressed Oriental beech seedlings, and became denser. On the other hand, Scots pine seedlings could not compete with Oriental beech seedlings interior stand due to lower amount of light, therefore, Oriental beech seedlings had more growing space and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

nutrients inside stand, and presented higher density. Data suggested that decreasing light through the edge effects did not have any negative influence on the growth of Oriental beech forests. Similarly, Abkenar and Keshavarz (2005) observed the height growth of Oriental beech seedlings under canopy and in canopy opening, and found that seedling growth did not significantly differ between the two conditions. In another study, Parhizkar et al. (2011) observed the growth of Oriental beech seedlings under varying light intensities, and revealed that there was not any relationship between light intensity and seedling growth. Given the data in previous research, our findings regarding Oriental seedling growth seem to be acceptable.

Traditional shelterwood method has been widely used in Oriental beech-Scots pine forests in Turkey Odabaşı et al. (2004), including the study area. Approximately 5000-7000 seedlings per ha at age five for each species are recommended for natural regeneration of these forests in this method (Odabaşı et. al., 2004; Calama et al., 2015). Given these numbers in the literature, our data showed that there was insufficient number of Scots pine seedlings when moving more than 10 m from the edge into the stand in the study stand. Therefore, the results suggest that shelterwood method within the entire area may be unsuccessful in these forests. Instead, Scots pine seedlings could be obtained in open-field conditions and near the edges. Groups could be created with complete clearing of vegetation, or using group shelterwood with a canopy closure of 40% in this stand type in favor of Scots pine (Odabaşı et. al., 2004). Data also showed that adequate number of Oriental beech seedlings could be obtained under the shade of overstory seedlings, thus, any partial cutting such as shelterwood or singletree selection methods outside the groups may be successful for the natural regeneration of Oriental beech in these stands. Mean height and root-collar-diameter growth of Scots pine seedlings after 10 m from the edge were lower that what observed in previous studies (Egnell and Valinger, 2003; Gerelbaatar et al., 2015) (*Figure 4*), that is, stand edge has negative effects on Scots pine seedlings after 10 m from the edge. Therefore, the size and number of groups when conducting group methods should be decided taking the range of edge effects (i.e., 10-40 m) into account in mixed Oriental beech-Scots pine stands.

Conclusions

This study presented the relationships between the distance from stand edge and growth of Scots pine seedling, while no effect of the edge on Oriental beech seedlings was observed. Data showed that the edge effect on Scots pine seedlings was present after 10 m from the edge into stand, and may extend up to 40 m into the stand adjacent to a forest opening. Study results add knowledge to the scientific understanding of the edge effects in mixed Oriental beech-Scots pine stands. Initial results suggest that Scots pine seedling can be established in canopy gaps or group clearing, while Oriental beech should be successfully attained under canopy within the rest of the stands. Traditional shelterwood method has been commonly used to regenerate mixed Oriental beech-Scots pine stands in Turkey; however, our results indicate that Scots pine seedlings may not well develop under overstory trees, and the stands may turn into pure stands of Oriental beech in long term. The influence of edges created by silvicultural treatments should be taken into account when group methods are aimed in this forest type. The data presented in this study will be helpful for forest managers who aim to enhance seedling development and establishment in mixed Oriental beech-Scots pine forests. However,

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DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

more research should be conducted on the use of group selection method in the mixed Oriental beech-Scots pine stands. In addition, this study was conducted on a single forest opening; thus, forest edge effects along varying gap sizes should be considered in the future studies. Moreover, direct light measurements thorough the sections of the lines should be taken in further research. We also stated that there has been a growing concern on the decreasing proportion of Scots pine trees in the mixed beech-pine stands and other mixed stands that contain Scots pine; therefore, similar studies should be conducted in mixed stands of Scots pine and other tree species such as oak (*Quercus*), fir (*Abies*) and spruce (*Picea*) in Turkey.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22192231

SOIL MICROBIAL COMMUNITIES AND ENZYME ACTIVITY FOR DIFFERENT RECLAMATION (ECOLOGICAL RESTORATION) PATTERNS OF ABANDONED FARMLANDS IN SHIHEZI, XINJIANG, CHINA

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(Received 21st Nov 2018; accepted 25th Jan 2019)

Abstract. Although there have been obvious land use changes in Xinjiang, China, the effect of reclamation (ecological restoration) of abandoned farmlands on soil microbial properties is rarely reported. Natural restoration of abandoned farmland was used as a control treatment, and the effects of different reclamation (ecological restoration) patterns on soil microbial properties in Shihezi, Xinjiang, China were analyzed. The results showed that there was a significant difference between different reclamation (ecological restoration) patterns in soil chemical and microbial characteristics ($P < 0.05$). These results indicate that the highest values for soil organic matter and available P were in accordance with the highest values for soil microbial communities (fungi, bacteria and total microbial amount), and the higher the value of pH, the lower the activity of urease and soil microbial biomass carbon. Land use patterns, both ecological restoration and reclamation types, were useful when only soil quality improvement was considered. Because of the advantages of improvement in soil fertility, the agroforestry pattern could be used as the preferred reclamation pattern for abandoned farmland in salinized regions. By increasing the soil water content and promoting the establishment of vegetation, the water recharge pattern could be a good choice during the early stage of ecological restoration of abandoned farmland.

Keywords: *Manas river basin, agroforestry pattern, soil biochemical properties, principal component analysis, land use change, arid regions, salinization*

Abbreviations: RRAF: reclamation (ecological restoration) of abandoned farmlands, MES: *Medicago* sp., CIS: *Citrullus lanatus* spp., AGF: agroforestry, SWR: soil water recharge, ENP: enrichment planting, ACS: *Achnatherum splendens*, NAR: natural restoration of abandoned farmlands, SOC: soil organic carbon, SOM: soil organic matter, SAN: soil available N (alkali-hydrolyzable N), SAP: soil available P, MBC: microbial biomass C, MBN: microbial biomass N, URE: urease activity, SUC: sucrase activity, PHO: phosphatase activity, CAT: catalase activity, BAC: bacteria, FUN: fungi, ACT: actinomycetes, TMA: total microbial amount

Introduction

RRAF in arid regions is critical to ensure both sustainable development of agriculture and environmental protection (Suding, 2011; Yang et al., 2016; Zhou et al., 2017). The land use changes caused by RRAF are one of the most important reasons for global environmental changes (GLP, 2005).

Artificial measures involving fertilization, irrigation and ploughing in the process of RRAF will change plant types, which eventually leads to changes in the biochemical properties of the soil (Li et al., 2013; Liu et al., 2013; Nadimi-Goki et al., 2017). Soil biochemical properties include its microbial communities and soil enzyme activity (Bastida et al., 2008; Stott et al., 2010). Soil microbial communities are an important part of soil microbial processes, which play a critical role in nutrient

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

transformation, soil health and sustaining the productivity of soil (Nannipieri et al., 2003). Soil enzyme activity is the basis for ensuring soil microbial quantity and soil physicochemical properties, and has a close relationship with the carbon and nitrogen cycle in soil (Mersi and Schinner, 1991). Moreover, soil enzyme activity can reflect the effects of plant type and crop rotation, as well as that of organic and inorganic fertilizers, etc., on soil quality (Bastida et al., 2012; Singh and Ghoshal, 2013). Soil biochemical properties are more sensitive to environmental stress than other physicochemical characteristics, which often reflect changes in soil quality, and can therefore be used to monitor soil biological processes in different land use patterns (Patra et al., 2005; Xue et al., 2008; Nadimi-Goki et al., 2017).

Unsustainable agricultural practices have resulted in the degradation and desertification of many arid ecosystems around the world (Castellanos et al., 2005), which has led to abandoned farmlands becoming a major issue worldwide (Bonet, 2004). However, there is a generalized lack of research about RRAF in arid environments (Romo-Leon et al., 2016).

In particular, inadequate use and overuse of land has contributed to serious environmental problems of land degradation in Xinjiang, northwest China (UN General Assembly, 1997). In the 50 years from 1949 to 2000, land use changes in Xinjiang were huge, indicating both abandoned farmlands caused by secondary salinization, and large-scale land reclamation caused by social and economic development (Luo et al., 2003; Fan et al., 2008; Feng et al., 2011). Land use change in the Manas river basin was especially typical in Xinjiang: the population increased from 59,000 in 1949 to 1,109,000 in 2004 (Li et al., 2008), which made it become the largest artificial oasis in Xinjiang and the fourth-largest irrigated agricultural land area in China (Feng et al., 2011). With the widespread application of drip irrigation technology since 1996, large areas of abandoned farmlands have been reclaimed, and the reclamation area exceeded the abandonment area in 1999 (Qian et al., 2006).

However, the soil physical and chemical properties, vegetation composition, history of abandonment, climatic features and the plant types of reclamation (restoration) varied with the changes to abandoned farmlands; the effects of RRAF patterns on soil biochemical properties changed in different regions, and there were even some opposing conclusions. Some researches have reported that soil microbial communities and enzyme activity increases after land reclamation (ecological restoration) (Tan and Kang, 2009; Liu et al., 2013; Guo et al., 2014). Nevertheless, other studies have found that the decline in soil organic C results in a decrease in soil bacteria, fungi and actinomycetes (Yin et al., 2013), the catabolic diversity of soil microbial communities (Degens et al., 2000) and urease activity (Raiesi and Beheshti, 2014) after RRAF.

For the Manas river basin, RRAF generally involves planting of economic crops, such as *Medicago* sp. and *Citrullus lanatus* spp., and agroforestry, etc., and RRAF generally includes water recharge, enrichment planting (native plants with resistance to salt and drought stress) or planting *Achnatherum splendens*, etc. For the Manas river basin which has drought stress and severe secondary salinization of soil, however, little is known about the effects of RRAF on soil microbial communities and enzyme activity, and which reclamation (ecological restoration) pattern is suitable for this basin.

Based on extensive investigation among existing RRAF patterns in the Manas river basin, six representative RRAF patterns were set in Shihezi, Xinjiang. The objectives

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of this paper were to: (1) compare the differences in chemical properties, microbial communities and enzyme activity of soil among different RRAF patterns; and (2) analyze correlations between microbial communities, enzyme activity and chemical properties. We expected to provide references for RRAF in drought and salinized regions.

Materials and methods

Experimental set-up and environmental conditions

The sample plot was located in Shihezi, Xinjiang, northwest China (85°35.275′E ~ 85°38.634′ E, 44°32.493′N ~ 44°33.989′ N, *Fig. 1*). Halosols are the dominant soils in plot. The plot is situated in the Manas river basin and has a temperate continental climate. Meteorological factors such as temperature, relative humidity, sunshine, precipitation and soil chemical properties are shown in *Table 1*. Six RRAF patterns, namely, planting MES and CIS, AGF, SWR, ENP and planting ACS were selected for the sample plot in 2008. According to the difference in ecosystem functions after reclamation (ecological restoration), the six RRAF patterns were classified into reclamation types including MES, CIS and AGF, and ecological restoration types including SWR, ENP and ACS. NAR was set as a control (*Fig. 2*). AGF consisted of tree (*Populus russkii* Jabl.) – wheat intercropping. SWR plots were irrigated once with 225 m^3/hm^2 in spring and autumn, respectively. ENP was set via enrichment planting of native plants, such as *Reaumuria soongorica* (Pall.) Maxim, *Suaeda salsa* L., etc., with a height of 10–15 cm and density of 20,000 individuals/hm^2. The seedling density of *A. splendens* was 3,000 individuals/hm^2 in ACS.

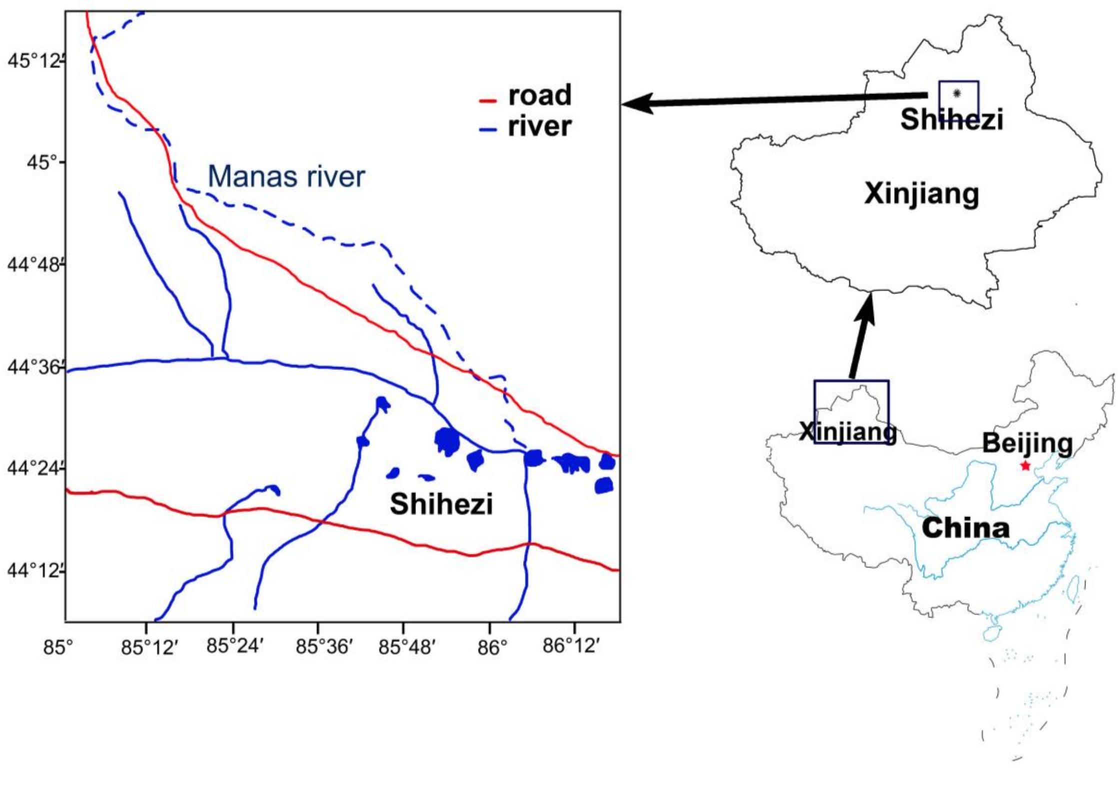

Figure 1. *Sample plot in Shihezi of Xinjiang, China*

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

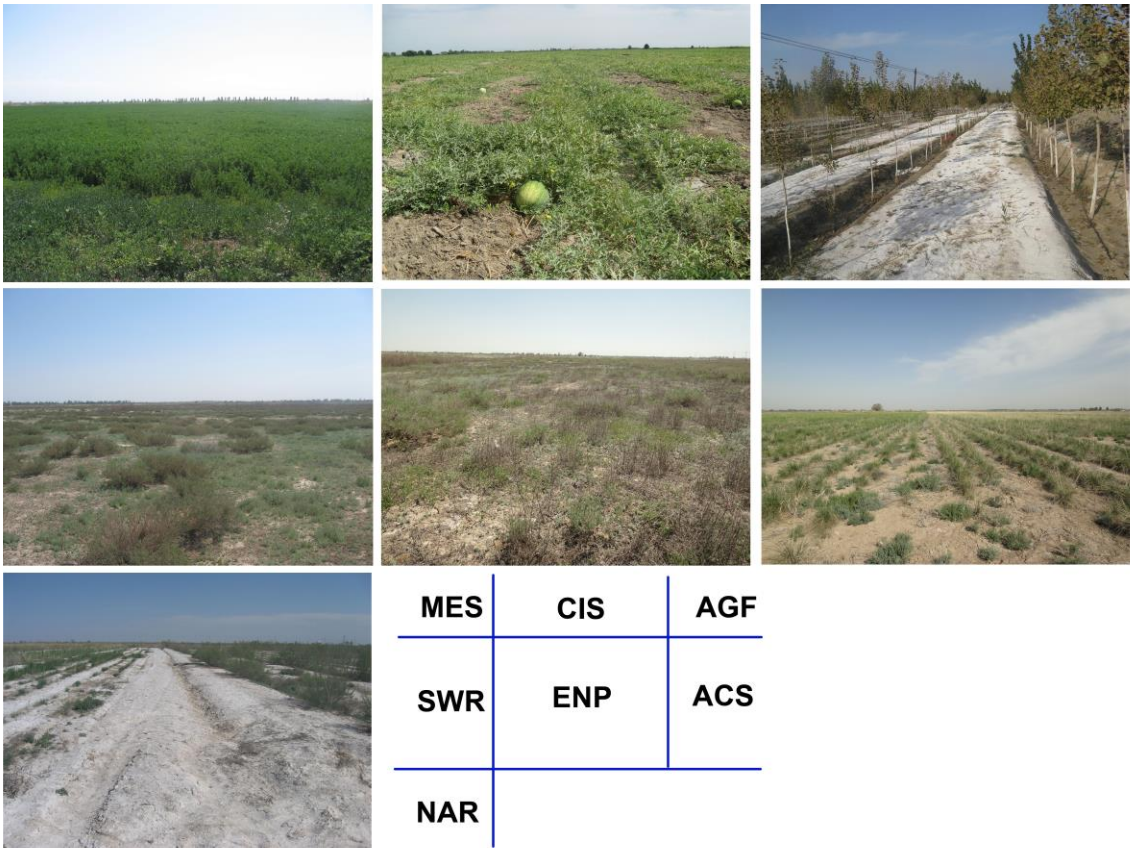

Figure 2. *Six reclamation (ecological restoration) patterns of abandoned farmlands and natural restoration*

Table 1. *Average measured parameters (vegetation and soil) of different reclamation (ecological restoration) patterns in Shihezi of Xinjiang*

Type	Control	Reclamation			Ecological restoration		
Pattern	**NAR**	**MES**	**CIS**	**AGF**	**SWR**	**ENP**	**ACS**
Climate	Arid, continental climate with annual temperature of 8.09 ℃, annual relative humidity of 63.55%, sunlight of 2769.4 h and annual precipitation of 225.3 mm						
Salt content in soil profile (g/kg)	0-20 cm	4.35 (1.14-17.15)					
	20-40 cm	3.55 (1.4-11.75)					
	40-60 cm	3.05 (1.16-9.63)					
Vegetation	The dominant species are *Kalidium foliatum* (Pall.) Moq., *Tamarix ramosissima* Ledeb, *Salsola collina* Pall., *Halostachys belangeriana* (H. caspica (Bieb.) C. A. Mey., *Limonium* spp., *Karelinia caspica* (Pall.) Less., *Melilotus officinalis* (Linn.) Pall, *Aristida adscensionis* Linn., etc.						
Soil pH	8.61 ± 0.11 a	8.04 ± 0.14 b	8.29 ± 0.10 ab	8.05 ± 0.09 b	8.32 ± 0.17 ab	8.36 ± 0.11 ab	7.97 ± 0.11 b
Soil EC (ms/cm)	5.27 ± 0.11 a	2.85 ± 0.09 d	1.89 ± 0.09 e	2.60 ± 0.15 d	3.66 ± 0.07 c	4.27 ± 0.10 b	2.68 ± 0.08 d
Soil SOM (g/kg)	3.26 ± 0.14 d	6.19 ± 0.03 c	6.95 ± 0.10 b	10.54 ± 0.20 a	6.14 ± 0.13 c	6.03 ± 0.14 c	6.23 ± 0.09 c
Soil SAN (mg/kg)	28.19 ± 0.68 d	41.51 ± 1.18 c	51.38 ± 1.36 a	54.04 ± 1.75 a	38.50 ± 0.58 c	41.86 ± 1.15 c	47.95 ± 0.61 b
Soil SAP(mg/kg)	5.52 ± 0.13 e	7.58 ± 0.21 c	8.59 ± 0.20 b	9.32 ± 0.25 a	6.35 ± 0.14 d	6.03 ± 0.19 de	6.44 ± 0.13 d
Irrigation	/	Drip irrigation	Drip irrigation	Drip irrigation	Drip irrigation	Drip irrigation	Drip irrigation
Fertilizer amount (per hm^2)	/	300 kg N	300 kg N	300 kg N, 120 kg P_2O_5 and 60 kg K_2O	/	/	/
Area (hm^2)	2.0	5.0	5.0	50.0	4.0	4.0	10.0

Data are expressed as mean ± SE, different letters indicate a significant difference among different patterns at $P < 0.05$ level. Meteorological data are collected from China Meteorological Data Network (http://data.cma.cn/)

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

Soil samples were taken in an S-shaped pattern (0–20 cm) from five points in each plot in 2011. The five soil samples were mixed, and 1 kg subsamples were extracted for analysis using the quartering method (Rasapoor et al., 2009). Three subsamples within each RRAF pattern were collected, totaling 21 subsamples.

Measurement of soil chemical properties, soil microbial communities and enzyme activity

Soil chemical properties were measured from air-dried soil samples, and soil microbial communities and enzyme activity were measured from fresh soil samples. Soil pH was measured using an Ampholine (LKB Producter AB, Stockholm, Sweden) pH meter (3310, water-to-soil ratio 2.5:1). Soil EC was determined using the electrical conductivity method (DDSJ-308A conductivity meter, water-to-soil ratio5:1, dS/m). SOC was measured by the potassium dichromate oxidation method (Vance et al., 1987). SOM was calculated according to *Equation 1:*

$$\mathrm{SOM} = {}^{\mathrm{SOC}\ (\%)}/_{58\%} \qquad \text{(Eq.1)}$$

SAN was measured using the alkaline solution diffusion method. SAP was analyzed colorimetrically using the ascorbic acid molybdate method.

Fresh soil samples were gently sieved through a 2 mm sieve and used for determining soil biological parameters. The soil microbial population (BAC, ACT, FUN and TMA) was measured by dilution plating, as described by Bulluck et al. (2002). Soil MBC and soil MBN were measured using the chloroform fumigation extraction method (Brookes et al., 1985; Vance et al., 1987). URE was measured using the indophenol blue method (Keeney and Nelson, 1982). SUC was determined using 3,5-dinitrosalicylic acid colorimetry. PHO was determined with the phenylene-disodium phosphoric acid colorimetric method (Guan, 1986). CAT was measured using the potassium permanganate titration method (Zhou et al., 2011).

Statistical analysis

All data were analyzed using SPSS 15.0 software (SPSS Inc., USA). Differences in soil variables between different RRAF methods were analyzed using a Duncan test in one-way ANOVA for multiple comparisons. Correlation analyses were carried out using Pearson two-tailed tests between the mean value by pattern of the soil chemical variables and the soil microbial properties, and between each of the soil microbial properties. The significance of the explained variation was analyzed using a generalized linear model (GLM, type III sum of squares, McCullagh and Nelder, 1989) according to *Equation 2:*

$$Y = \mu + T + P(T) + \varepsilon \qquad \text{(Eq.2)}$$

In *Equation 2,* Y is the response variable, μ is the general mean, T is the effect of the type, $P(T)$ is the effect of the pattern (within a type) and ε is the error term.

Soil microbial communities and enzyme activity were anaSlyzed using principal component analysis (PCA). The scatter plot was drawn using the scores of principal components. According to the scatter plot, combinations of soil microbial communities and enzyme activity between different RRAF patterns were analyzed.

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Results

Soil chemical properties for different RRAF patterns

There were significant differences between soil chemical properties for different RRAF patterns (P < 0.05, *Table 1*). The values of soil pH and EC were highest, and the values of SOM, SAN and SAP were lowest in NAR. The values of soil chemical properties in AGF were contrary to those in NAR (except soil EC). For other patterns, differences between soil pH in MES, CIS, SWR, ENP and ACS were not significant (P > 0.05). The difference between soil EC in MES and ACS was not significant (P > 0.05). SOM in MES, SWR, ENP and ACS was similar (P > 0.05). There was no significant difference in SAN between CIS and AGF, or between MES, SWR and ENP (P > 0.05). Differences between SAP in SWR, ENP and ACS were not significant (P > 0.05).

Variation in soil chemical properties and biochemical properties for different RRAF patterns

The GLM showed that for type factors, only SAP, CAT and ACT significantly affected soil chemical properties and biochemical properties (P < 0.01). The pattern factor significantly affected soil chemical properties and biochemical properties apart from soil pH and CAT (P > 0.05, *Table 2*). For most of the soil chemical properties and biochemical properties (13 out of a total of 15), the pattern factor significantly explained the total variation. In particular, variation was highest for the effect of pattern factor on TMA, which was up to 1670.86.

Table 2. *The sources of variation and its significance in soil chemical properties and microbial communities*

Microbial communities	Variable	Type		Source	
		Reclamation	Ecological restoration	Type	Pattern (within-type)
Soil chemical properties	pH	8.12 ± 0.69	8.22 ± 0.90	0.37 ns	2.24 ns
	EC (ms/cm)	2.45 ± 0.46	3.54 ± 0.71	4.00 ns	45.85 ***
	SOM (g/kg)	7.89 ± 2.02	6.13 ± 0.20	1.72 ns	175.08 ***
	SAN (mg/kg)	48.98 ± 2.04	42.77 ± 1.44	1.74 ns	23.96 ***
	SAP (mg/kg)	8.50 ± 0.27	6.27 ± 0.10	18.31**	11.61 ***
Enzyme activities	URE (NH_4-N mg/g soil)	1.43 ± 0.10	1.40 ± 0.07	0.02 ns	8.37 **
	SUC (glucose mg/g soil)	5.86 ± 0.31	4.87 ± 0.22	1.77 ns	44.60 ***
	PHO (phenol mg/g soil)	20.62 ± 2.23	16.39 ± 3.39	0.76 ns	53.86 ***
	CAT (0.1 mol/L $KMnO_4$ mL/g. min soil)	2.07 ± 0.09	2.90 ± 0.06	31.60 **	2.60 ns
Microbial communities	MBC (mg/kg)	163.79 ± 3.20	157.41 ± 4.56	0.41 ns	12.20 ***
	MBN (mg/kg)	19.82 ± 1.52	13.89 ± 0.51	4.06 ns	15.47 ***
	FUN (mg/kg)	3.83 ± 0.86	0.71 ± 0.06	3.35 ns	340.65 ***
	BAC (mg/kg)	79.07 ± 17.47	8.43 ± 0.57	4.11 ns	519.95 ***
	ACT (mg/kg)	13.69 ± 1.14	1.07 ± 0.08	32.65 **	45.74 ***
	TMA (mg/kg)	96.59 ± 19.31	10.21 ± 0.68	5.00 ns	1670.86 ***

Asterisks indicate significant sources at P < 0.01 (**) and P < 0.001 (***); *ns* not significant

Soil enzyme activity

For all patterns, all soil enzyme activity for NAR was at the lowest level; URE, SUC and PHO in AGF were the highest, and there was a significant difference in soil enzyme activity between AGF and NAR (P < 0.01, *Fig. 3*). URE in CIS, ENP and NAR was

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similar (P > 0.05), and URE in SWR, ACS and AGF was similar (P > 0.05). The difference between SUC in MES and AGF was not significant (P > 0.05). The difference between CAT in SWR, ENP and ACS was not significant (P > 0.05), and CAT in SWR, ENP and ACS was significantly higher than for other patterns (P < 0.05, *Fig. 3*).

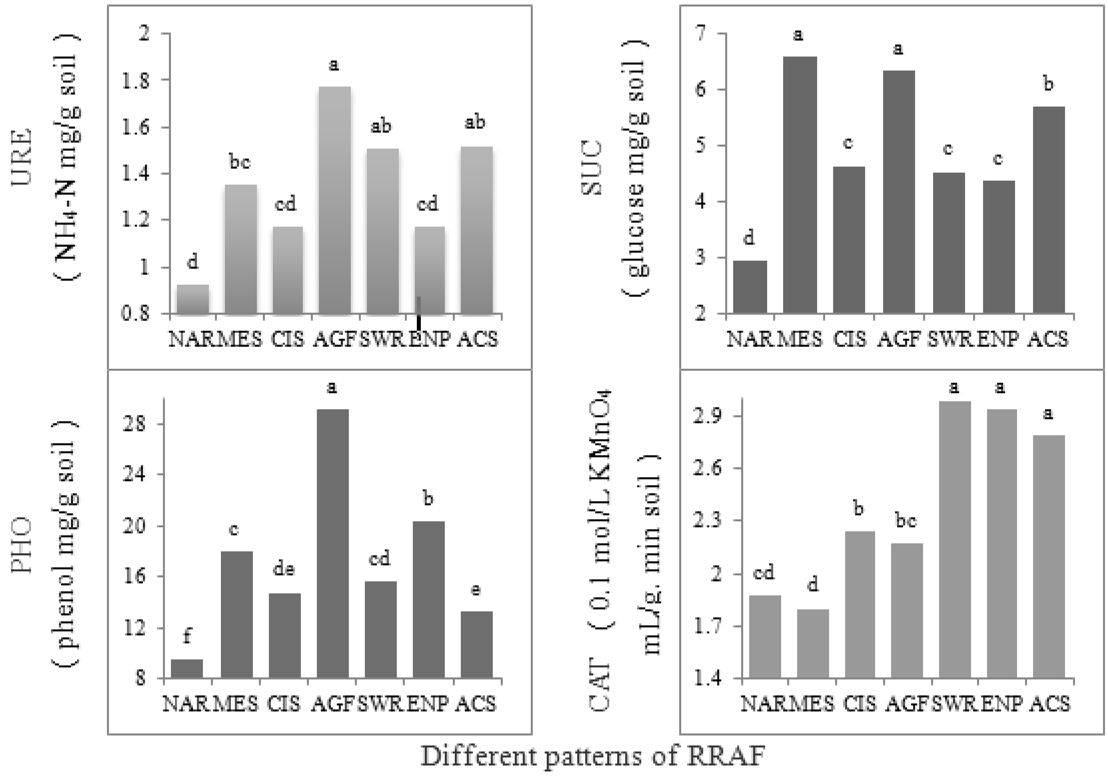

Figure 3. *A comparison of four soil microbial enzyme activities*

Soil microbial communities

Trends for soil microbial communities for different patterns were similar to those for soil enzyme activity: the values of soil microbial quantities and biomass in NAR were the lowest, while the values of soil microbial quantity and biomass in AGF were the highest. There was a significant difference between soil microbial communities in NAR and AGF (P < 0.01, *Fig. 4*). For other patterns, MBC in CIS, SWR, ENP and NAR was similar (P > 0.05); MBC in MES, ACS and AGF was similar (P > 0.05). The difference between MBN in CIS, SWR, ENP and ACS was not significant (P > 0.05). There was no significant difference in BAC between SWR, ENP and ACS (P > 0.05). The difference between ACT in SWR, ENP and ACS was not significant (P > 0.05, *Fig. 4*).

PCA of soil microbial communities and enzyme activity

Certain soil microbial communities and enzyme activity were well separated by pattern, according to soil chemical properties (*Fig. 5A*), and the classification of soil chemical properties for different patterns is shown in the PCA scatter plot (*Fig. 5B*): TMA, BAC and FUN were in accordance with the positive scores for PC1, and characterized the soil chemical properties of AGF; CAT was in agreement with the

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

negative scores for PC1, and characterized the soil chemical properties of NAR. CAT, URE and MBC were in accordance with the positive scores for PC2, and characterized the soil chemical properties of ACS; ACT, TMA and MBN were in agreement with the negative scores for PC2, and characterized the soil chemical properties of NAR.

Soil enzyme efficiency

To indicate how RRAF pattern affects soil enzyme efficiency, soil enzyme activity was normalized, and expressed as enzyme activity per gram of soil MBC or MBN (URE normalized to N, and SUC, PHO and CAT normalized to C). There were significant differences in soil enzyme efficiency between different patterns after the soil enzyme activity was normalized ($P < 0.01$, *Table 3*). When comparing normalized data with non-normalized data, no significant effects were found between enzyme activity in NAR, CIS, SWR and ENP. However, URE was significantly changed in AGF, URE now being the lowest in AGF when compared to other patterns (*Table 3*).

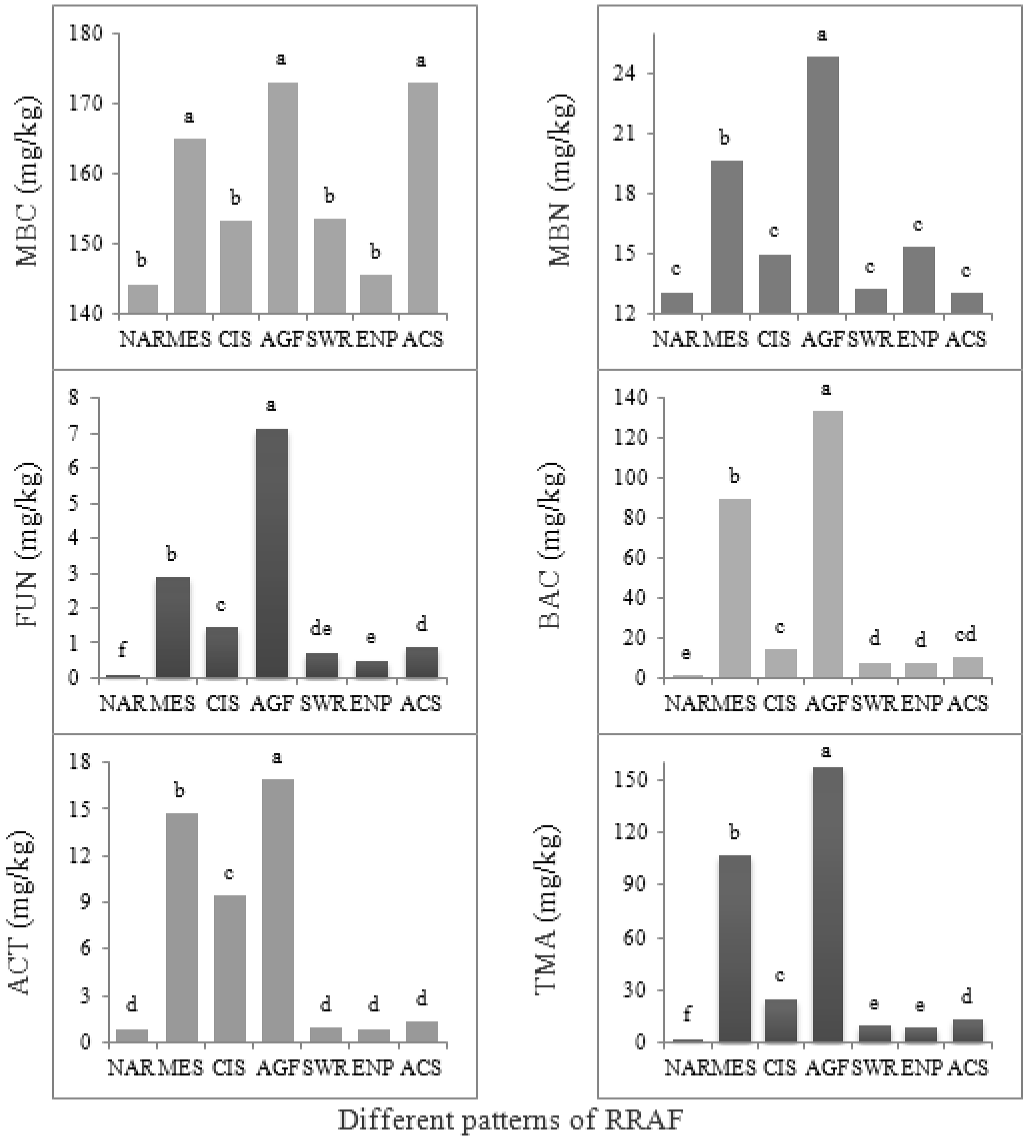

Figure 4. *Soil microbial communities in different reclamation (ecological restoration) patterns*

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

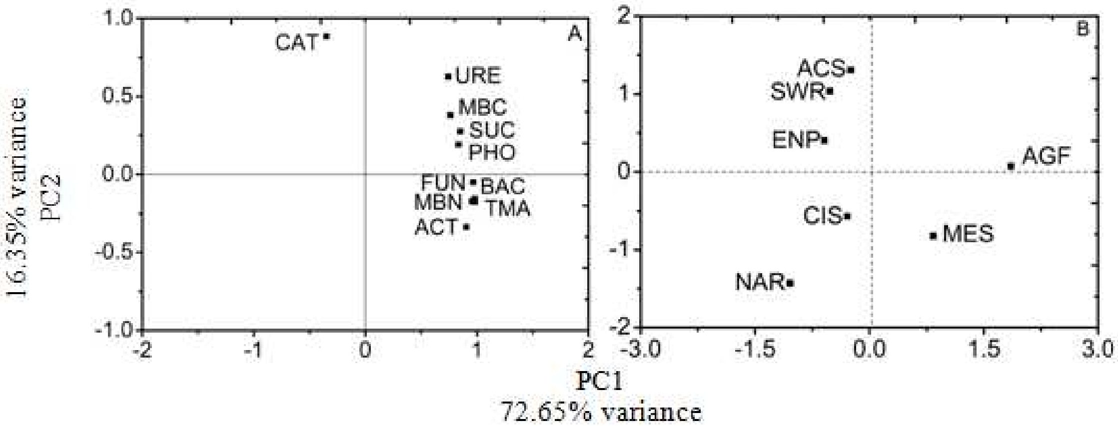

***Figure 5.** Principal component analysis (PCA) of the soil microbial communities and enzyme activities of different reclamation (ecological restoration) patterns from Shihezi of Xinjiang, China. **A** Projection of soil microbial communities and enzyme activity variables onto the component plane. **B** Scatter plot of PC1–PC2 scores, each point represents a pattern of RRAF*

***Table 3.** Soil microbial biomass C and N normalized values for soil enzyme activities in different reclamation (ecological restoration) pattern (mean ± SE)*

Type	Control	Reclamation			Ecological restoration		
Pattern	NAR	MES	CIS	AGF	SWR	ENP	ACS
URE (NH_4-N mg/g soil)	0.0703 ± 0.0020 b	0.0688 ± 0.0024 b	0.0781 ± 0.0053 b	0.0712 ± 0.0054 b	0.1139 ± 0.0047 a	0.0762 ± 0.0049 b	0.1165 ± 0.0084 a
SUC (glucose mg/g soil)	0.0205 ± 0.0009 e	0.0399 ± 0.0010 a	0.0303 ± 0.0007 d	0.0367 ± 0.0010 b	0.0295 ± 0.0008 d	0.0301 ± 0.0009 d	0.0329 ± 0.0007 c
PHO (phenol mg/g soil)	0.0653 ± 0.0012 d	0.1085 ± 0.0036 c	0.0962 ± 0.0037 c	0.1688 ± 0.0057 a	0.1018 ± 0.0039 c	0.1395 ± 0.0068 b	0.0764 ± 0.0057 d
CAT (0.1 mol/L $KMnO_4$ mL/g. min soil)	0.0130 ± 0.0004 cd	0.0109 ± 0.0004 d	0.0146 ± 0.0010 bc	0.0125 ± 0.0006 cd	0.0194 ± 0.0008 a	0.0202 ± 0.0007 a	0.0161 ± 0.0006 b

Different letters indicate a significant difference among different patterns at P < 0.01 level

Relationships between soil chemical properties and microbial variables

Regression analysis between soil microbial variables showed the following significant relationships at $P < 0.05$ level (*Table 4*): URE was positively related to SUC (r = 0.772), URE was positively related to MBC (r = 0.834), SUC was positively correlated with BAC (r = 0.774), SUC was positively correlated with TMA (r = 0.774), PHO was positively related to BAC (r = 0.804), and PHO was positively related to TMA (r = 0.799). The following significant relationships were found at $P < 0.01$ level (*Table 4*): SUC was positively related to MBC (r = 0.878), PHO was positively related to MBN (r = 0.885), and PHO was positively correlated with FUN (r = 0.863). In particular, significant relationships at $P < 0.01$ level were found between MBN, FUN, BAC, ACT and TMA (*Table 4*).

Regression analysis between soil chemical properties and microbial variables showed the following significant relationships (*Table 4*): pH was negatively correlated with URE (r = −0.786), SUC (r = −0.951) and MBC (r = −0.935); SOM was positively related to URE (r = 0.822), PHO (r = 0.894), MBN (r = 0.806), FUN (r = 0.882), BAC

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

(r = 0.763) and TMA (r = 0.769); SAP was positively correlated with MBN (r = 0.777), FUN (r = 0.831), BAC (r = 0.753), ACT (r = 0.890) and TMA (r = 0.780) (*Table 4*).

Table 4. *Correlation matrix between soil chemical and microbial variables*

	URE	SUC	PHO	MBC	MBN	FUN	BAC	ACT	TMA
pH	-0.786	**-0.951**	-0.477	**-0.935**	-0.503	-0.557	-0.591	-0.545	-0.591
SOM	0.822	0.715	**0.894**	0.67	0.806	**0.882**	0.763	0.71	0.769
SAP	0.543	0.648	0.673	0.563	0.777	0.831	0.753	**0.89**	0.78
URE		0.772	0.685	0.834	0.579	0.706	0.635	0.454	0.624
SUC			0.625	**0.878**	0.699	0.701	0.774	0.734	0.774
PHO				0.465	**0.885**	**0.863**	0.804	0.662	0.799
MBN						**0.963**	**0.976**	**0.897**	**0.977**
FUN							**0.956**	**0.866**	**0.957**
BAC								**0.912**	**0.999**
ACT									**0.931**

Significant correlations shown in underlined (P < 0.05) or in bold (P < 0.01). Soil chemical and microbiological variables not included in the table were not significantly related other variables

Discussion

Soil chemical properties, microbial communities and enzyme activity were studied for different RRAF patterns in Shihezi, Xinjiang. For most soil parameters, more variation was explained by pattern than by type (*Table 2*), which shows that soil chemical properties, microbial communities and enzyme activity are significantly affected by pattern; significant differences between soil properties for different patterns also proved the result (*Figs. 3* and *4*).

The variation in soil microbial communities (BAC, FUN, ACT and TMA) was significant for different patterns, and the variation was especially high in TMA (*Table 2*), indicating that different patterns have a significant influence on microbial communities. BAC, FUN and ACT in soil are important in regulating ecological processes such as SOM decomposition, energy flow, carbon storage, etc. (Paul and Clark, 1997). FUN, BAC, ACT and TMA for each pattern were significantly higher than for NAR (except that ACT in SWR, ENP, ACS and NAR was similar); in particular, FUN, BAC, ACT and TMA in AGF were the highest (*Fig. 4*), which indicates that AGF has the strongest effect on soil microbial communities. It may be that the growth of microorganisms in AGF has been promoted by differences in litter quality and quantity, and root exudates (Monokrousos et al., 2006; Maestre et al., 2015). The values for SOM and SAP were lowest in NAR and were highest in AGF (*Table 1*); SOM was significantly related to FUN, BAC and TMA, and SAP was significantly related to FUN, BAC, ACT and TMA (*Table 4*). These correlations indicate that different patterns significantly influence microbial communities by improving SOM and SAP, and are in accordance with the report by Tan and Kang (2009). Significant relationships were found between FUN, BAC and ACT (*P* < 0.01, *Table 4*), which indicates that microbial communities have the same response to changes in soil environment for different patterns. FUN, BAC and ACT showed significantly positive correlation with SAP, suggesting that SAP may be the dominant factor affecting the growth of microbial communities.

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Soil enzymes mainly come from microbial metabolic processes, soil animals and plant root exudates and residues (Bandick and Dick, 1999). In soil, enzyme activity is the foundation to ensure soil microbial quantity and soil physicochemical characteristics, which are closely connected to the cycles of C and N (Mersi and Schinner, 1991). As we expected, significant variation was found between soil enzyme activity for different patterns (*Table 2*). The soil enzyme activity for the six patterns was higher than for NAR (except for URE in CIS and ENP, and CAT in MES) (*Fig. 3*), showing the notable effects of different patterns on soil enzyme activity (Singh and Ghoshal, 2013; Nadimi-Goki et al., 2017). URE, SUC and PHO in AGF were the highest when compared to other patterns (*Fig. 3*), indicating that AGF can promote the utilization of N, efficiency of the C cycle and the availability of soil P (Frankenberger and Johanson, 1983; Nannipieri et al., 2002).

Many studies have shown that soil enzyme activity is positively correlated with SOM (Bastida et al., 2012; Liu et al., 2013; Liang et al., 2014; Mahajan et al., 2016; Zhang et al., 2017), because SOM serves as a precursor for enzyme synthesis (Liu et al., 2013). Our research showed that SOM was positively correlated with URE and PHO ($r = 0.822$, $P < 0.05$; $r = 0.894$, $P < 0.01$, respectively, *Table 4*). While soil pH was negatively related to URE and SUC ($r = -0.786$, $P < 0.05$; $r = -0.951$, $P < 0.01$, respectively, *Table 4*), we did not find a significant relationship between soil EC and soil enzyme activity. These correlations indicate that the effect of soil pH on URE and SUC is greater than that of EC. This may be due to the tolerance of plants in different patterns to salt stress, and the high value of soil pH was the limiting factor for URE and SUC.

Generally, soil enzyme activity represents active members of microbial communities (Bradford et al., 2008). After soil enzyme activity was normalized by MBC or MBN, we could distinguish whether the microbial communities allocated energy to microbial growth or enzyme production (Schimel and Schaeffer, 2012). Intriguingly, when enzyme activity was normalized to MBC or MBN, MBN in AGF supported lower URE (*Table 3*), but was contrary to the data for non-normalized enzyme activity (*Fig. 3*). This shows a weak N cycle in AGF when compared with SWR and ACS. Namely, URE in AGF may respond more slowly to land use changes than the other two patterns. Changes in normalized URE in AGF also showed a change of physiological function in microbial communities: allocating more energy to microbial growth rather than to enzyme production (Bradford et al., 2008; Schimel and Schaeffer, 2012).

The PCA results indicated that PC1 of AGF, with a maximum positive score, characterized the highest values of both SOM and SAP, and was in agreement with the highest values of FUN, BAC and TMA. This indicates that higher values of SOM and SAP would mean higher soil microbial biomass (FUN, BAC and TMA). The soil properties in NAR were in contrast to those for AGF. Significant positive correlations between SOM and FUN, BAC and TMA, and between SAP and FUN, BAC and TMA also prove this result (*Fig. 5; Table 4*).

PC2 of ACS, with a maximum positive score, characterized the lowest value of soil pH, which was in accordance with the highest URE and MBC, showing the high activity of soil enzymes. URE and MBC were lowest in NAR, indicating that the higher the pH value, the lower URE and MBC, as also was proved by the negative relationship between soil pH and URE and MBC (*Fig. 5; Table 4*).

In addition, variations in most soil parameters were not significant between reclamation type and ecological restoration type (*Table 2*), which shows that there was

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no significant difference between the soil properties in different types, whether the reclamation type characterized the economic benefits or the ecological restoration characterized the ecological benefits. There was a significant difference between characteristics of soil chemistry and biochemical properties were observed for different patterns. The higher the values of SOM and SAP, the greater the values of MBN, FUN, BAC and TMA, and the higher the value of soil pH in accordance with lower values of URE, SUC and MBC in soil. These correlations indicate that different plant composition, soil biochemical characteristics and human activities in different patterns have significant influences on soil biochemical properties (Lal, 2005; Singh and Ghoshal, 2013).

Conclusions

In this paper, changes in soil chemical and biochemical properties with different patterns were studied. In terms of improving soil quality, it is meaningful to use both the reclamation type and ecological restoration type in abandoned farmlands. Most soil chemical and microbial characteristics for AGF were significantly higher than for other patterns and NAR (*Table 1; Figs. 3* and *4*), demonstrating the obvious effects of AGF on soil fertility improvement (Tian et al., 2013). AGF could be used as the preferred reclamation pattern for abandoned farmland in salinized regions, because it accounts for the vulnerability of the ecological environment (arid, low temperature and salinization stress) in the sample area, as well as having both economic and ecological benefits. It should be noted that SWR could be a good choice during the early stage of RRAF, by increasing the soil water content (data not shown), and improving soil quality (particularly in soil EC, SOM, SAN, URE, SUC, PHO, CAT, FUN, BAC and TMA) when compared to NAR, thus promoting the establishment of vegetation. All patterns in our experiment have certain effects on improving the quality of soil, and this will provide references for RRAF in drought and salinized regions.

Although an optimal RRAF pattern can be selected by comparing soil chemical and biochemical properties, however, further study is needed to focus on the hydrological cycle to ensure the sustainable utilization of water resources in arid regions. Moreover, the long-term dynamic study also needed for more objective evaluation on RRAF.

Acknowledgements. This study was funded by the National Key Research and Development Program of China (2016YFC0501406), and by the Science and Technology Development & Achievements Transformation of Xinjiang Production and Construction Corps (2016AD022).

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DOI: http://dx.doi.org/10.15666/aeer/1702_22332247

CADMIUM-INDUCED CHANGES IN GROWTH AND MICRONUTRIENT COMPOSITION OF TWO PEPPER CULTIVARS

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(Received 8th Dec 2018; accepted 28th Jan 2019)

Abstract. The present research was conducted to investigate the effects of cadmium (Cd) treatments on shoot and root dry weight, SPAD values, shoot and root Cd accumulations and micronutrient compositions of two pepper cultivars (*Capsicum annuum* L. cvs. 'Demre' and 'AT58'). Plants were grown under controlled conditions with 0 and 15 µM Cd supplies. Decreasing root and shoot dry weights were observed with increasing Cd doses ($P < 0.05$). Decrease in shoot and root dry weights with 15 µM Cd supply was higher in Demre cultivar than in AT58 cultivar. Cd-induced decreases in SPAD values were greater in Demre cultivar than in AT58 cultivar. Shoot Cd concentrations were lower and root Cd concentrations were greater in AT58 cultivar than in Demre cultivar. Decreasing shoot zinc (Zn), iron (Fe), manganese (Mn) and copper (Cu) concentrations and root Zn and Mn concentrations were observed with Cd treatments. Considering the Cd uptake and transport to shoot, it was observed that there were differences in tolerance of cultivars to Cd toxicity, but it was thought that these differences were not necessarily related to microelement uptake and transport of the cultivars.
Keywords: *heavy metal, SPAD value, zinc, iron, manganese, copper*

Introduction

Cadmium (Cd) is a highly toxic heavy metal and poses significant threats to human health and environment. Developed industrial and agricultural practices lead to significant increases in soil cadmium levels (Sarwar et al., 2010). Phosphorus fertilizers, Cd-containing wastewater treatment sludge, livestock manure, wastewater effluents, various metal processing industries, cement facilities and urban traffic are considered to be the greatest sources of Cd for soil (Alloway and Steinnes, 1999; Yang et al., 2004). Although cadmium is not considered as an essential nutrient for plants, soil available Cd is easily up taken through the roots and accumulated in the plant tissues and pose serious health risks on humans through the food chain (Zhou and Qiu, 2005). High cadmium concentrations also damage plant roots, destruct photosynthetic activity, recess plant growth and development and hinder plant nutrient and water uptake from the soil (Jibril et al., 2017). High cadmium levels influence cell membrane permeability (Sengar et al., 2008) and destruct antioxidant defense mechanisms of the plants against oxidative stress conditions through elevated lipid peroxidation (Benavides et al., 2005).

Plant normal cadmium concentrations were reported as between 0.2-0.8 mg kg^{-1} and toxic levels as between 5-30 mg kg^{-1} (Allen, 1989; Kloke et al., 1984). Similar with the other stressors, Cd also hinders plant nutrient uptake, interacts with the soil available nutrients and ultimately results in imbalanced mineral nutrition (Khan et al., 2007). There is a great competition between Cd ions and the other essential plant nutrients including Ca, Mg, K, Cu, Zn, Fe, Ni and Mn required for plant growth and development (Clarkson and Luttge, 1989; Rivetta et al., 1997). In a previous study, either synergistic or antagonistic effects of cadmium were reported on plant nutrients (macro or micro) of different wheat cultivars (Zhang and Huang, 2000). In other studies, either negative

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22492256

(Bhandal and Kuar, 1992) or positive (Mitchel et al., 2000) correlations of cadmium were reported with nitrogen. Similarly, antagonistic (Li et al., 1990) and synergistic (Nan et al., 2002) interactions of cadmium were reported with zinc. Cadmium treatments increased Cu and Mo levels and decreased K, Ca, Mg and Mn levels of Birch seedlings (Gussarsson, 1994). Increasing Cd levels resulted in greater Cd, Zn, Fe, Mn and Cu accumulations in roots and slight Cd transfer to shoots (Wang et al., 2007).

Different plants have various cadmium accumulation capacities (Yang et al., 1996; Obata and Umebayashi, 1997; Yildiz, 2005). However, variations were also reported among the different cultivars of wheat (Naeem et al., 2016), barley (Tiryakioglu et al., 2006), maize (Ekmekci et al., 2008), soybeans (Shamsi et al., 2008), tomato (Hussain et al., 2015) and chilli peppers (Xin et al., 2013). Kuboi et al. (1986) classified Cd accumulation capability of the plants into three groups (high, moderate and low accumulators). Pepper is the second crop after tomato produced in greenhouses of Turkey and high-quality yield is the most critical issue in pepper cultivation in greenhouses (Abdel Latef, 2013). Therefore, the present research was implemented to investigate the Cd accumulation in two pepper (*Capsicum annuum* L.) cultivars of *Solanaceae* family. The primary target was to identify the effects of cadmium treatments on plant development and micronutrient compositions of pepper cultivars with different Cd accumulation levels.

Materials and methods

Experiment and analyses

Demre and AT58 pepper cultivars were used as the plant material of this study. The plants were grown under controlled climate conditions (26/22 °C day/night temperature, 16/8 h photoperiod, 350 µmol m^{-2} s^{-1} light intensity supplied with Osram HQI/2000/D lamps and 65-75% relative humidity). Surface-sterilized seeds with 1% (w/v) calcium hypochlorite for 10 min were rinsed through distilled water. Seeds were then sown in perlite-filled pots and allowed to germinate for 7 days. When the seedlings reached to two true-leaf stage in perlite media (12 days old), they were transferred to 2.7 L plastic pots (four seedlings per pot) filled with continuously aerated and diluted 1:3 nutrient solution for 2 days to ensure time for plant growth. Thinning was performed then as to have two seedlings in each pot. Nutrient solutions were prepared with distilled water and 2.0 mM $Ca(NO_3)_2$, 0.88 mM K_2SO_4, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.1 mM KCl, 100 µM Fe-EDTA, 10µM H_3BO_3, 0.5 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.2 $CuSO_4$ and 0.02 µM $(NH_4)_6Mo_7O_{24}$. Cadmium ($3CdSO_4$ $8H_2O$) was supplemented into nutrient solutions at five-to-six true leaf stage (25 days old) of growth for 8 days. All solutions were adjusted as to have a pH of 6.3 ± 0.1. Nutrient solutions were replaced in every 3-4 days throughout the growing period. Leaf chlorophyll contents were identified with the aid of a chlorophyll meter (SPAD-502, Minolta, Japan) before the harvest. The 32-day old plants were harvested and at harvest, roots were rinsed through 0.5 mM $CaSO_4$ and de-ionized water for 15 min, and dried at 70 °C to determine the dry weights.

Dried shoots and roots were ground; wet-digested in a microwave with 2 ml 35% H_2O_2 and 5 ml 65% HNO_3. Digested samples were then subjected to elemental analyses for Cd, Zn, Fe, Mn and Cu by an inductively coupled plasma optical emission spectrometer ICP-OES; Varian-Vista Pro) device. Measurements were checked with the readings on reference samples of National Institute of Standards and Technology (Gaithersburg, MD, USA).

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DOI: http://dx.doi.org/10.15666/aeer/1702_22492256

Statistical analysis

Experimental data were subjected to variance analysis in accordance with randomized plots design with 3 replications. Means were compared by least significant difference (LSD) test at 5% probability level.

Results and discussion

Dry matter yields, SPAD readings and cadmium in shoots and roots

Cadmium treatment (15 µM) reduced shoot dry matter yield by 37% in AT58 cultivar and by 48% in Demre cultivar and reductions in root dry matter yields were respectively observed as 21 and 29% (*Table 1*). Cd-induced regress in plant growth was also reported for wheat (Naeem, 2016), tomatoes (Kumar et al., 2015), sunflower (Azevedo et al., 2005) and pepper (Abdel Latef, 2013). In this study, more chlorosis and necrotic patches were observed over the oldest leaves of Demre cultivar than the leaves of AT58 at 15 µM Cd dose. Root browning degrees were also greater in Demre cultivar than in AT58 under Cd treatments. Generally, reduced root elongation (Dong et al., 2005) and browning (Liu et al., 1995) are the initial symptoms for cadmium toxicity in roots and chlorosis and rolling are the initial symptoms for cadmium toxicity in shoots (Weigel and Jäger, 1980).

SPAD values significantly decreased with Cd treatment in both cultivars and the decrease rates with 15 µM Cd treatment were found to be 15% in AT58 and 30% in Demre cultivar (*Table 1*). Sandalio et al. (2001) indicated the reason of Cd-induced reductions in chlorophyll content as chlorophyll degradation or destructions in chlorophyll biosynthesis and membrane integrity.

Table 1. *Effects of cadmium (-Cd = 0 and +Cd = 15 µM) treatments on shoot-root dry weights and SPAD values of AT 58 and Demre pepper cultivars*

Cultivars	Dry matter yields (mg plant^{-1})*				SPAD values	
	Shoot		Root			
	- Cd	+ Cd	- Cd	+ Cd	- Cd	+ Cd
AT58	1139 aA	723 bA	154 aB	122 bB	53 aA	45 bA
Demre	1240 aA	643 bA	234 aA	167 bA	42 aB	30 bB

*Means indicated with different small letters (between Cd treatment, in each cultivar) and by the same capital letters (between cultivars, in each Cd treatment) are significantly different at $p < 0.05$

Cadmium treatment (15 µM) significantly increased shoot Cd concentrations and contents of both cultivars (*Table 2*). Demre had higher shoot Cd concentration and content than AT58. While shoot Cd concentration and content of Demre cultivar at 15 mM Cd treatment were 148 mg kg^{-1} DW and 95.1 µg plant^{-1}, the values in AT58 cultivar were 90.5 mg kg^{-1} DW and 65.1 µg plant^{-1}, respectively. Root Cd concentrations and contents of AT58 were greater than Demre (*Table 2*). Root Cd concentration and content of AT 58 were respectively measured as 1529.7 mg kg^{-1} DW and 186.8 µg plant^{-1}. For both cultivars, roots had greater Cd concentrations and contents than the shoots (*Table 2*). Cataldo et al. (1983) pointed out that large portion of cadmium retained in plant roots and a small portion was transferred to shoots. Blum (1997) reported the greatest Cd content for roots and the least Cd content for seeds;

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22492256

stem, leaves and fruits were placed in between them. Regardless of the concentrations, both cultivars differ in their root and shoot Cd accumulation capacity. Although root Cd contents were not influenced significantly by cadmium treatments, high root Cd content of AT58 than Demre was found to be compatible with root Cd concentrations of these cultivars (*Table 2*). Previous researches tried to explain the differences in Cd-tolerance of plants with the differences in their Cd-uptake and accumulation levels (Hall, 2002; Kochian et al., 2002). Complying with these hypotheses, AT58 cultivar of the present study had greater root Cd concentration and smaller shoot Cd concentration than Demre cultivar. Such findings indicated that AT58 cultivar retained greater Cd quantities in roots.

Table 2. *Effects of cadmium (-Cd = 0 and +Cd = 15 µM) treatments on shoot-root Cd concentration and content of AT58 and Demre pepper cultivars*

Cultivars	**Cd concentrations (mg kg^{-1} DW)***				**Cd contents (µg plant^{-1})**			
	Shoot		**Root**		**Shoot**		**Root**	
	- Cd	**+ Cd**	**- Cd**	**+ Cd**	**- Cd**	**+ Cd**	**- Cd**	**+ Cd**
AT58	0.3 bA	90.5 aB	2.9 bA	1529.7 aA	0.4 bA	65.1 aB	0.5 bA	186.8 aA
Demre	0.4 bA	148.0 aA	3.7 bA	1036.1 aB	0.5 bA	95.1 aA	0.9 bA	173.0 aA

*Means indicated with different small letters (between Cd treatment, in each cultivar) and by the same capital letters (between cultivars, in each Cd treatment) are significantly different at $p < 0.05$

Shoot and root micronutrients

Cadmium treatment (15 µM) significantly decreased ($p < 0.05$) shoot Zn, Mn, Cu and Fe concentrations and contents of both cultivars (*Table 3*). Shoot Zn, Mn, Cu and Fe concentrations of AT58 cultivar decreased with Cd treatment at slightly greater rates than at Demre cultivar. The average Cd-induced decrease was 55% for Zn and Mn and 69% for Fe and Cu (*Table 3*). Cd-treatment also reduced root Zn and Mn levels significantly. Cd treatments reduced Zn and Mn levels of wheat root and shoots, but did not influence shoot and root Fe and Cu levels (Jalil et al., 1994). Cadmium toxicity mostly comes from the interactions of Cd with the other essential nutrients, especially with the same valence (Skrebsky et al., 2008). In a hydroponic experiment with barley, Cd treatments significantly decreased root and shoot Zn, Cu and Mn concentrations and shoot Fe concentrations (Wu et al., 2003).

In the present study, while root Cu content of AT58 decreased significantly with Cd treatment, root Cu content of Demre and root Cu concentrations of both cultivars did not change (*Table 3*). Iron concentration and accumulation in roots of spinach was not affected by Cd treatments (Abul Kashem and Kawai, 2007). Similar with those findings, present root Fe concentrations and contents of both pepper cultivars remained unchanged with Cd supply (*Table 3*). Kabata-Pendias and Pendias (2001) indicated strong bonds of Cu and Fe in root cells.

Present differences in shoot Zn and Cu levels were mostly attributed to Cd-induced regress in plant growth and development. Thusly, shoot Zn and Cu concentrations were not significantly different (*Table 3*). Similarly, shoot Fe and Mn concentrations-contents were not also significantly different at 15 µM Cd treatment (*Table 3*). Under Cd supply, there seems to be antagonistic relationships between root Cd concentrations-contents and root Zn concentrations-contents (*Table 3*). Under controlled conditions, Demre

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DOI: http://dx.doi.org/10.15666/aeer/1702_22492256

cultivar generally had greater quantities of Zn, Fe, Mn and Cu than AT58 cultivar (*Table 3*). Such a case may result in significantly different Zn and Cu accumulation levels under Cd supply. Differences in cultivars may be related to present Cd dose, exposure duration to this dose and micronutrient levels of the cultivars under controlled conditions.

Table 3. *Effects of cadmium (-Cd = 0 and +Cd = 15 µM) treatments on shoot-root Zn, Fe, Mn and Cu concentration and content of AT 58 and Demre pepper cultivars*

Cultivars	**Zn**		**Fe**		**Mn**		**Cu**	
	- Cd	**+ Cd**	**- Cd**	**+ Cd**	**- Cd**	**+ Cd**	**- Cd**	**+ Cd**
	*Shoot concentrations (mg kg^{-1} DW)**							
AT58	36.2 aB	15.2 bB	175.0 aA	51.1 bA	26.6 aB	11.1 bA	8.3 aA	2.4 bB
Demre	41.1 aA	19.6 bA	215.1 aA	66.0 bA	31.5 aA	14.4 bA	9.1 aA	3.2 bA
	Root concentrations (mg kg^{-1} DW)							
AT58	101.3 aA	54.7 bB	976.0 aA	1032.1aA	112.1aA	11.0 bA	36.0 aA	35.8 aA
Demre	100.8 aA	66.7 bA	708.0 aA	734.1 aB	49.8 aB	7.0 bB	31.0 aA	33.6 aA
	Shoot content (µg plant^{-1})							
AT58	41.0 aA	11.0 bA	199.3 aA	37.0 bA	30.2 aB	8.0 bA	9.5 aA	1.7 bA
Demre	51.2 aA	12.6 bA	266.2 aA	42.4 bA	39.2 aA	9.2 bA	11.3 aA	2.1 bA
	Root content (µg plant^{-1})							
AT58	15.5 aB	6.7 bB	150.6 aA	126.5 aA	17.2 aA	1.4 bA	5.5 aA	4.4 bB
Demre	23.3 aA	11.1 bA	169.7 aA	122.6 aA	12.0 aA	1.2 bA	7.3 aA	5.6 aA

*Means, indicated with different small letters (between Cd treatment, in each cultivar) and by the same capital letters (between cultivars, in each Cd treatment) are significantly different at $p < 0.05$

Conclusion

Cadmium treatment generated significant decreases in both root and shoot dry matter yields. Such decreases were greater in Demre cultivar than in AT58 cultivar. Similarly, greater decreases were observed in SPAD values of Demre cultivar than of AT58 cultivar with Cd treatment. Regardless of shoot Cd uptake and accumulation, it was observed that there were differences in Cd toxicity tolerance of the cultivars, but these differences were not attributed to differences in microelement uptake and transport of the cultivars. For Cd-polluted sites, AT58 can be recommended to reduce yield losses. Further studies are recommended to compare antioxidative defense mechanisms of AT58 and Demre pepper cultivars with different root and shoot Cd accumulation levels.

Conflict of interests. The author has not declared any conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22492256

ESTIMATION OF LIVE WEIGHT OF HOLSTEIN-FRIESIAN BULLS BY USING BODY LINEAR MEASUREMENTS

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(Received 5th Nov 2018; accepted 28th Jan 2019)

Abstract. The objective of this study was to estimate the body weight of Holstein-Friesian cattle by using multiple regression analysis. Data were collected from 29 Holstein-Friesian bulls, whose live weight, body length (BL), height at withers, chest depth, heart girth (HG), shin circumference (SC), rump height, and back rump height were measured. There was a relationship between dependent and independent variables. The estimated multiple regression equation was –431.8 + 2.438 HG + 21.21 SC + 1.041 BL, with a determination coefficient of 0.9987 and a standard error of the estimate of 5.240.
Keywords: *body weight, Holstein, heart girth, measurement, regression analysis*

Introduction

Estimating the live body weight of animals through body measurements is a practical, faster, easier, and cheaper method in rural areas where resources are scarce (Nsoso et al., 2003). Knowing the animal live weight is important for breeders, since this information allows them to estimate market prices for live animals and to determine the right dosages in drug administration and the amount of feed to be supplied for growth, maintenance, and production (Mahieu et al., 2011; Chitra et al., 2012; Tsegaye et al., 2013).

Body measurements have been widely used for estimating live weight. There is a relationship between live weight and the various body lengths, heights, and girths measured on live animals.

Using body measurements to estimate live weight with a simple measuring stick and tape may provide relative accuracy and consistency.

Live weight forms the basis for a range of research and management activities including assessment of growth rates, animal responses to different diets and environmental conditions, and determination of feed requirements. Knowing the animal weight and weight changes is also important in determining responses to genetic selection (Touchberry and Lush, 1950).

The use of linear body measurements in fattening beef cattle carries some advantages over subjective methods of cattle evaluation that involve visual assessment and scoring (Essien and Adesope, 2003). Some authors have also suggested this approach to be more reliable than weight measured with a weighing scale, since the latter can be subject to short-term effects such as gut fill, urination, and defecation (Russell, 1975).

These measurements can be taken at lower costs (when labor costs are relatively low) with a simple measuring tape and may provide relative accuracy and consistency (Guilbert and Gregory, 1952)

This is not only a sign of adult weight of the breed but also important for prediction of the daily weight gain and making the feeding programs (Tüzemen and Yanar, 2013).

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

There are several methods to estimate live weight in cattle. The polynomial regression was used to predict live weight in cattle (Assogba et al., 2017). Body weight and wither height were regressed on the other body traits. Regressions of body weight including the linear, quadratic, and cubic effects of a single independent variable (heart girth, wither height, hip width or body length) indicated that each measurement would be useful in predicting body weight (Heinrichs et al., 1992)

Digital Image Analysis was used in order to predict of body weight and carcass performance of beef cattle as well (Bozkurt et al., 2008)

However, the linear body measurements are used mostly to predict live weight in cattle and small ruminants. It is based on the measurement of heart girth, which is reported to be highly correlated with body weight in cattle (Heinrichs, 2007; Swali et al., 2008).

The objective of this study was to develop the best prediction models to estimate body weight based on linear body measurements.

Materials and methods

The experiment was conducted in Dicle University, Cattle Research Center in Diyarbakir Province, Turkey (37°57'41 N and 40°13'54 E, 650 m asl).

The study material consisted of monthly live weight and body measurement records of 29 male Holstein-Friesian bulls collected between the years 2016 and 2017. The initial weight was taken at age of 4 month. Different amounts of concentrate and roughage were provided ad libitum to the bulls considering the average live weights obtained from the monthly weighings.

A measuring stick was used to determine body length (BL), height at withers (HAW), chest depth (CD), rump height (RH), and back rump height (BRH), whereas a tape measure was used to determine heart girth (HG) and shin circumference (SC). Body measurements were taken once monthly with the bulls standing in a squeeze chute. Bulls were weighed before feeding and drinking water in the early morning.

The animals were weighed monthly using an electronic weighing scale with 1,500-kg capacity. Body measurements were taken by same observers so as to minimize bias. All the data were recorded in centimeters.

Descriptive statistics was used to present the simple means and standard deviation for all variables. Regressions of live weight (LW) on HG, CD, RH, HAW, SC, BRH, and BL were performed using simple and multiple linear regressions with the various body measurements as continuous variables. The obtained data were analyzed using Minitab 18 statistical software (Pearson’s correlation coefficients), where correlations between body weight and different body measurements were computed.

Regression analysis was employed to predict live weight from different body measurements. The best-fitting regression model was chosen based on the coefficient of determination (R^2) and standard deviation (SD). Multiple regression models were followed to estimate body weight from body measurements.

The Stepwise regression model of the measurements (all seven linear body measurements) was defined as follows (*Eq. 1*):

$$Y = \beta_0 + b_1 \beta_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + e_i, \qquad \text{(Eq.1)}$$

in which:

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Y = dependent variable (live weight),
βo = intercept,
β's = regression coefficients,
$X_{(1\ to\ 7)}$ = independent variables (HG, CD, RH, HAW, SC, BRH, and BL) and
e_i = error.

Results

In the statistical analyses, the mean and standard deviations were calculated for each attribute (*Table 1*).

Table 1. *Descriptive statistics of live weight and body measurements*

Fattening period	N	Live weight Mean±SD	Height at withers Mean±SD	Rump height Mean±SD	Chest depth Mean±SD
Initial	29	99.90±16.03	94.03±4.52	99.03±4.52	35.82±2.36
30 days	29	135.16±17.48	97.93±4.69	102.93±4.83	38.55±2.55
60 days	29	170.99±18.87	102.03±4.66	106.79±4.89	39.03±2.38
90 days	29	207.52±20.05	108.03±4.00	110.79±4.89	41.32±2.85
120 days	29	244.42±21.94	112.76±4.06	116.03±4.33	44.55±3.51
150 days	29	281.38±23.33	116.76±4.07	120.17±4.28	54.18±3.61
180 days	29	317.16±24.58	118.45±4.05	124.10±4.12	56.66±3.77
210 days	29	355.52±29.62	120.41±4.06	127.72±4.02	57.44±3.55
240 days	29	394.24±31.48	121.28±3.97	129.55±3.95	58.27±3.25
270 days	29	432.86±32.14	123.24±3.99	131.03±4.01	60.65±3.19
300 days	29	471.72±35.91	125.21±3.99	131.86±3.96	61.18±2.72
330 days	29	516.38±39.03	126.21±3.98	132.72±4.03	62.55±2.83
Final	29	553.66±34.48	127.31±4.36	133.52±4.09	64.18±3.18

Table 1. (cont.) *Descriptive statistics of live weight and body measurements*

Fattening period	N	Heart girth Mean±SD	Shin circumference Mean± SD	Body length Mean±SD	Back rump height Mean±SD
Initial	29	95.21±6.78	10.24±1.02	81.13±3.92	111.55±3.94
30 days	29	102.38±6.56	10.65±0.81	88.20±3.85	114.69±4.31
60 days	29	106.79±4.89	10.72±0.84	94.13±3.92	118.14±4.21
90 days	29	118.55±5.64	11.55±1.05	100.17±3.01	123.28±4.36
120 days	29	131.17±6.48	11.79±1.14	106.38±2.78	128.14±4.36
150 days	29	137.59±6.01	12.10±1.17	112.83±2.36	132.93±4.33
180 days	29	150.55±6.21	12.37±1.14	119.55±4.88	136.72±4.06
210 days	29	156.48±6.17	12.62±1.26	131.72±2.85	138.66±4.40
240 days	29	157.55±6.23	14.55±1.27	132.69±2.73	140.34±6.63
270 days	29	171.93±6.26	14.55±1.21	134.31±2.69	142.21±8.33
300 days	29	174.07±5.85	15.51±1.18	135.72±2.75	144.07±6.00
330 days	29	176.55±5.77	17.44±1.37	137.59±2.58	146.17±6.97
Final	29	182.83±5.93	18.82±1.97	139.21±2.83	148.52±4.31

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

All correlation coefficients were positive and highly significant (P < 0.01), and the highest correlation coefficient was determined between BW and heart girth (*Table 2*).

Table 2. *The correlation coefficients between dependent and independent variables*

	BW	HAW	RH	CD	HG	SR	BL	BRH
BW	1	-	-	-	-	-	-	-
HAW	0.956**	1	-	-	-	-	-	-
RH	0.964**	0.993**	1	-	-	-	-	-
CD	0.956**	0.987**	0.992**	1	-	-	-	-
HG	0.987**	0.981**	0.989**	0.982**	1	-	-	-
SR	0.968**	0.848**	0.854**	0.848**	0.903**	1	-	-
BL	0.978**	0.982**	0.996**	0.983**	0.988**	0.864**	1	-
BRH	0.966**	0.994**	0.996**	0.992**	0.993**	0.885**	0.990**	1

P-value < 0.01

All possible regression equations were employed in the selection of a best fitted regression equation as represented in *Table 2*. The results showed that the multiple regression equation for estimation of body weight of Holstein-Friesian bulls had tree independent variables, x_1 (heart girths), x_2 (shin circumference) and x_3 (body length) with high adjusted coefficients of determination ($R^2 = 99.87$) and low standard error of estimation (S = 5.240) in equation (Y_3; *Eq. 2*).

$$Y_3 = -431.8 + 2.438\ \mathrm{HG} + 21.21\ \mathrm{SC} + 1.041\ \mathrm{BL} \tag{Eq.2}$$

R^2 was calculated as 0.973 by using multiple linear regression analysis based on the HG to design a body-weight prediction model. When SC was added to regression model R^2 value was found as 0.9986. The best-fitting model, with the highest R^2 value (0.9987), was obtained by adding BL to the equation (*Table 3*).

Table 3. *The regression equation for live weights of Holstein bulls*

	1	2	3
Coef	**-378.3**	**-410.457**	**-431.8**
HG	4.876	3.247	2.438
T-Value	20.25	24.20	5.760
P-Value	.000	.000	.000
SR	-	20.0	21.21
T-Value	-	-13.46	14.73
P-Value	-	.000	.000
BL	-	-	1.041
T-Value	-	-	1.99
P-Value	-	-	.012
S	24.865	5.965	5.240
R^2	0.9739	99.86	99.87

$Y_1 = -378.3 + 4.876\ HG\ (R^2 = 0.9739)$
$Y_2 = -410.457 + 3.247\ HG + 20\ SR\ (R^2 = 0.9986)$
$Y_3 = -431.8 + 2.438\ HG + 21.21\ SR + 1.041\ BL\ (R^2 = 0.9987)$

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

Live weight and linear body measurements were significantly correlated with each other. Body weight had a higher correlation with HG than with any other body measurements (CD, RH, HAW, SC, BRH, and BL). In all fattening periods evaluated, the highest R^2 was obtained when the SC and BL measurements were included in the regression equations, which suggests that body weight could be more precisely estimated by the association of two or more linear measurements. However, the association of different body measurements (body length and shin circumference) would produce the best prediction equation for body weight, in this study.

The coefficients of correlation between heart girth and body weight were 0.973 for all of the 29 Holstein-Friesian bulls, and highly statistically significant. The relationship between live weight and heart girth was illustrated by graph shown as *Figure 1*.

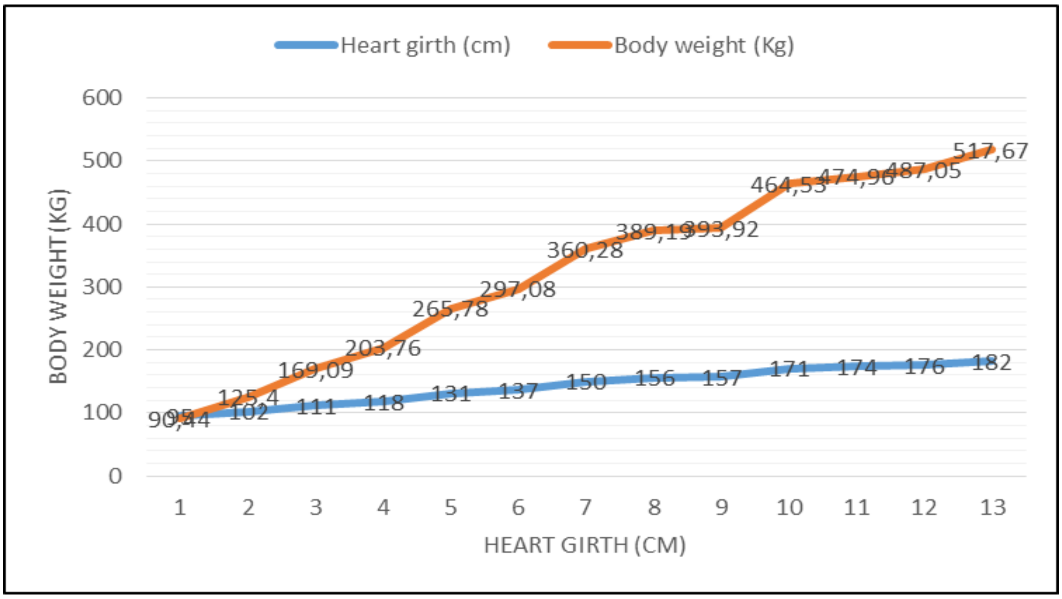

Figure 1. *Relationship between live weight and heart girth*

The regression of body weight on heart girth indicated a straight-line relationship.

The body weight of Holstein- Friesian cattle were estimated by using the multiple regression equation between the live weight and heart girth (Y_1) represented in *Table 4* and the comparison between the real values and the predicted values of body weight of Holstein-Friesian cattle was shown in *Table 5*.

Table 4. *Prediction of body weight of Holstein-Friesian cattle based on heart girth*

Heart girth (cm)	Predicted body weight (kg)	Heart girth (cm)	Predicted body weight (kg)
95	90.44	156	389.19
102	125.40	157	393.92
111	169.09	171	464.53
118	203.76	174	474.96
131	265.78	176	487.05
137	297.08	182	517.67
150	360.28		

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

Table 5. *Actual and predicted body weight*

Heart girth (cm)	Actual body weight (kg)	Predicted body weight (kg)
95	99.9	90.44
102	135.16	125.4
111	170.99	169.09
118	207.52	203.76
131	244.42	265.78
137	281.38	297.08
150	317.16	360.28
156	355.52	389.19
157	394.24	393.92
171	432.86	464.53
174	471.72	474.96
176	516.38	487.05
182	553.66	517.67

Actual and predicted body weight of Holstein-Friesian bulls were illustrated by graph shown as *Figure 2*.

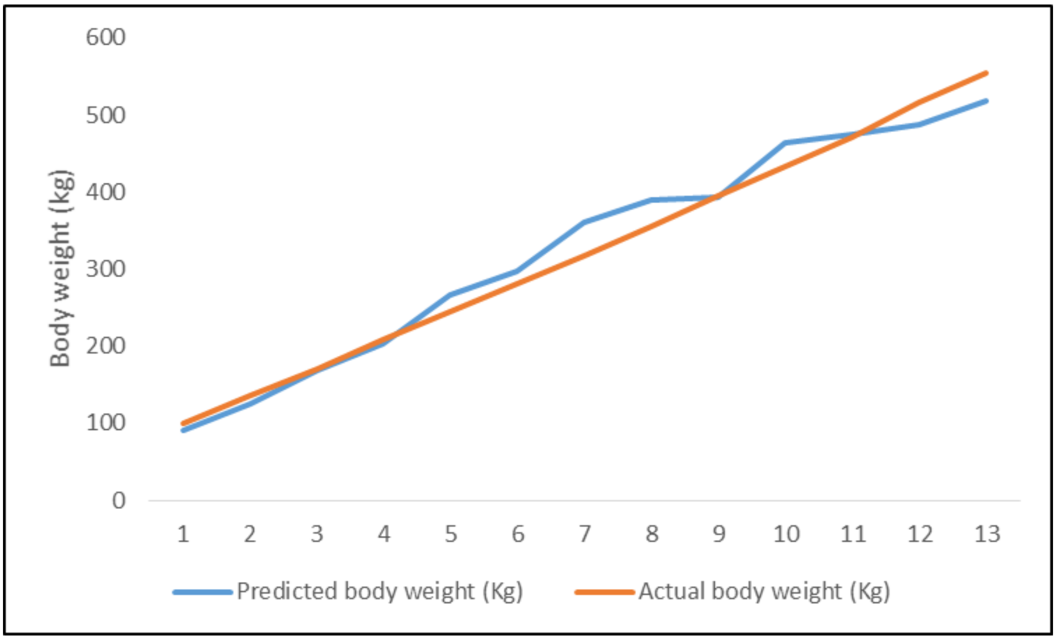

Figure 2. *Actual and predicted live weight values of Holstein bulls*

Discussion

The present findings corroborate Katongole et al. (2013), according to whom HG, WH, and body condition score (BCS) were the variables with the highest R^2 value for predicting body weight. Siddiqui et al. (2015) reported that the HG and BL were the best variables for this purpose, with $R^2 = 0.968$. Our results also agree with those published by Yan et al. (2009), who found that HG, BL, and BCS were better suited parameters for estimating body weight.

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

The highest correlation obtained in this study was between LW and HG measurement while the lowest was with CD. The high correlation between LW and HG can be attributed to the fact that, in comparison to length and HW, HG more closely reflects body condition of cows (Goe et al., 2001). This fact may also be supported by the stronger correlation observed in the present study between HG, LW and body condition scores compared to BL. Such correlations have also been reported by other workers (Francis et al., 2002; Gunawan and Jakaria, 2010; Heinrichs et al., 1992, 2007; Kashoma et al., 2011; Msangi et al., 1999; Yan et al., 2009; Lukuyu et al.2016).

The relationship between body linear measurements and LW could be exploited in designing appropriate management and selection programs in that high positive relationships among the traits suggests that an increase in one could lead to a corresponding increase in the other trait (Assan, 2013).

Body weight was highly correlated with heart girth in cattle, as concluded by Abdelhadi and Babiker (2009), Bagui and Valdez (2007) and Nesamvuni et al. (2000).

The current results are similar to those reported by Soysal and Konak (1992), Tüzemen et al. (1995), Yanar et al. (1995), Mantysaari (1996), Seokgeum et al. (1998), Adeyinka and Mohammed (2006), Koç and Akman (2007), Sawanon et al. (2011) and Mekparyup et al. (2013).

Conclusions

The high values coefficients of correlation (R^2) of the equation obtained by multiple regression analysis suggest that heart girth can be practically used alone to estimate live weight. Regression coefficients for heart girth indicate that such estimators could be used independently to estimate body weight in Holstein-Friesian bulls. Estimating the body weight of Holstein-Friesian cattle using three independent variables heart girth, shin circumference and body length appears to be a useful strategy. Linear body measurements, specifically heart girth, are useful predictors of live weight in cattle. Heart girth is the most practical parameter for predicting live weight in field conditions, especially for smallholder farmers.

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DOI: http://dx.doi.org/10.15666/aeer/1702_22572265

PREDICTING CONE PRODUCTION IN CLONAL SEED ORCHARD OF ANATOLIAN BLACK PINE WITH ARTIFICIAL NEURAL NETWORK

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(Received 14th Nov 2018; accepted 11th Jan 2019)

Abstract. Seed orchards are an important seed source because they have the most important link between tree breeding and plantation forestry. The aim of this study is to evaluate the potential of Adaptive Neuro-Fuzzy Inference Systems of artificial neural networks to predict the amount of cone in clonal seed orchards of Anatolian black pine. It was found that the coefficient of determination (R^2), the mean absolute error (MAE) and the root mean square error (RMSE) of the artificial neural network model were 0.85, 14.83 and 18.85, respectively. The amount of cone in clonal seed orchards of Anatolian black pine was predicted with high efficiency through artificial neural networks. Considering the lack of forestry studies based on the artificial neural network, this study will enable further researches to provide a new perspective.
Keywords: *ANN, Bartin, flower, forestry, Pinus nigra, Yenice-Camiyanı*

Introduction

Seed orchards are an important seed source for forest plantations. They constitute the most important link between tree breeding and plantation forestry. Considerable progress has been made in understanding the reproductive biology of conifers in seed orchards (Kang, 2001; Bilir et al., 2006). These are by far the most important outlets to forestry of breeding programs and they can be useful in gene conservation. Investment in seed orchards is often by the most cost-efficient way of increasing future forest production (Bilir et al., 2009).

Researches of seed orchard are probably the most cost efficient research possible to create better forests. It is desirable to improve the function of seed orchards. There are worries about the genetic diversity of the seed orchard crops and its impact on the future forest. Low production of sound seeds is a common problem. Seed production and collection is often expensive. Basic data on seeds and cones in seed orchards and their occurrence and variation and the possible causes of variations are desirable to get a better understanding of possible improvements of seed orchard's function, economy and impact to the forest (Bilir et al., 2009). In addition, such information is needed to establish and select seed sources and genetic conservation areas, and for future studies

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by governmental and private sectors because of potential of monumental plants in the species, and also to contribute potential theoretical studies on estimation of fertility variation and related variables such as effective number, status number and genetic diversity (Bilir et al., 2017).

Anatolian black pine (*Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe) is one of the most common and important forest tree species in Turkey due to the usefulness of its wood regarding commercial uses (Sıvacıoğlu and Ayan, 2010). The species occupies about 4.2 million ha in Turkey (Anonymous, 2015). The seed for this species is mainly supplied from the current 54 seed orchards (462.8 ha) in Turkey (Anonymous, 2018).

Artificial neural network models are a powerful empirical modelling approach and yet relatively simple compared with mechanistic models (Ji et al., 2007). Artificial neural network, possessing various types, becomes crucial especially in innovating and developing better products for society as it can solve many problems that linear system is incapable to resolve (Khairunniza-Bejo et al., 2014). Artificial neural network structure is based on the human brain’s biological neural processes. Interrelationships of correlated variables that symbolically represent the interconnected processing neurons or nodes of the human brain are used to develop models. Artificial neural network models find relationships by observing a large number of input and output examples to develop a formula that can be used for predictions (Pachepsky et al., 1996; Ji et al., 2007). Thus, the use of artificial neural network in forestry modeling can be efficient for predicting cone production of Anatolian black pine. In this study, we aimed at evaluating the potential of artificial neural networks for predicting the amount of cone for Anatolian black pine from clonal seed orchards.

Materials and methods

In this study, measurement results from Anatolian black pine seed orchard with Yenice-Camiyanı origin were used. This seed orchard has an area of 11.3 ha and is established within the boundaries of Bartın Forest Management Directorate in 1990 (Anonymous, 2018). In the study, 90 randomly selected samples from 120 experimental data were used to test the model while the remaining 30 were modeled. We used Average Female Flower Count (2015), Mean Diameter Value (2015), Average Number of Two-Year-Old Cones (2015) as input, and Average Number of Two-Year-Old Cones (2017) as output.

Adaptive Neuro-Fuzzy Inference Systems (ANFIS) is an artificial intelligence method created by combining of artificial neural networks that have parallel computing - learning capability and a fuzzy logic has syllogize ability. The functional based on the equilibrium between fuzzy logic as Takagi Sugeno Kang (TSK) and limited radial basis neural network (Lezanski, 2001). Output values calculated directly by weighting the input data according to fuzzy rules. These rules, which known databases, determined thanks to the computational algorithm based on neural networks. The learning algorithm of ANFIS is a mixed learning algorithm consisting of the least squares method and the back-propagation learning algorithm. The six-layer ANFIS structure is shown in *Figure 1*.

The structure of the ANFIS layers is as follows (Demirel et al., 2010; Gemici, 2011):

1. Layer (Input Layer): All input signals from each node directed to the next layers.

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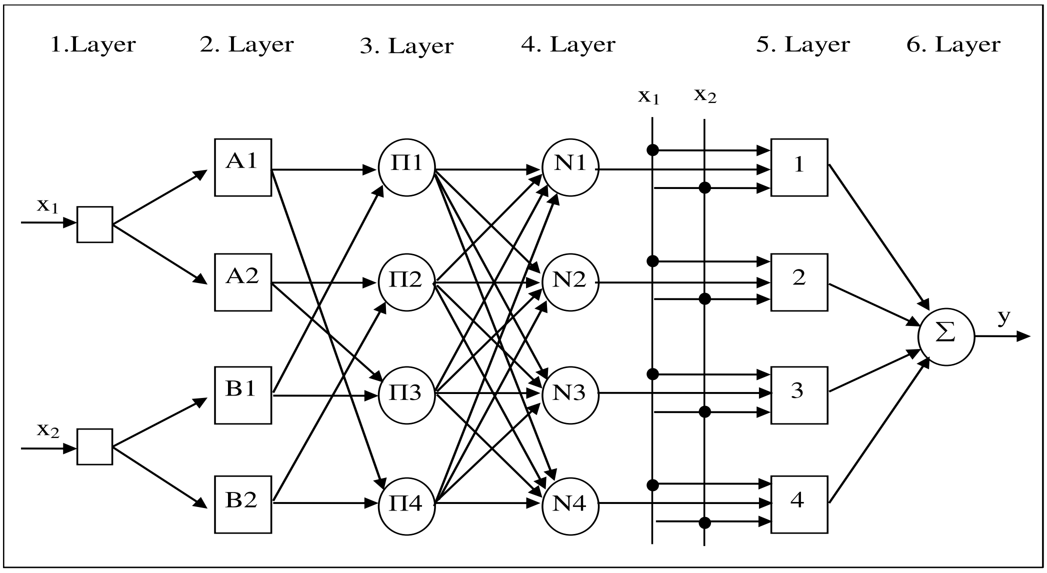

***Figure 1.** The six-layer ANFIS structure*

2. Layer (Blur Layer): Output of each node consists of the membership ratings depending on the input values and the selected membership function. The membership degrees in second layer are described $\mu_{A_j}(x)$ and $\mu_{B_j}(y)$.
3. Layer (Rule Layer): The nodes in this layer indicate the number of rules and numbers determined by the Sugeno fuzzy logic inference system. The output of each rule node (μ_i) is shown in *Equation 1,* accordingly the result of the membership degree from layer 2 is (j = 1,2) and (i = 1,…, n).

$$y_i^3 = \prod i = \mu_{A_j}(x)\ \mu_{B_j}(y) = \mu_i \quad \text{(Eq.1)}$$

At this point, y_i^3, the output values of the third layer and "n" indicates the number of nodes in this layer.

4. Layer (normalization): The nodes in this layer accept all other nodes from the rule layer as input values and calculate the normalized value of each rule. The outputs of this layer defined as the normalized firing level. Each node in this layer is an N-labeled node. The calculation of the normalized firing level found $\overline{\mu_i}$ according to *Equation 2.*

$$y_i^4 = N_i = \frac{\mu_i}{\sum_{i=1}^{n} \mu_i} = \overline{\mu_i}\ , \quad (i = 1, \dots, n) \quad \text{(Eq.2)}$$

5. Layer (Rinse Layer): In this layer calculated the weighted result values of rule. At this point, the value of node "I" calculate regarding *Equation 3*.

$$y_i^5 = \overline{\mu_i}\ [p_i x_1 + q_i x_2 + r_i]\ , \quad (i = 1, \dots, n) \quad \text{(Eq.3)}$$

(p_i, q_i, r_i) variables are the set of result parameters of the rule "i".

6. Layer (Total Layer): This layer contains only one node labelled Σ. The output value of each node summed in the layer 5 then the actual output value found.

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The function defined the output value of the system according to *Equation 4* (Özçalık et al., 2003).

$$y = \sum_{i=1}^{n} \overline{\mu_i} \, [p_i x_1 + q_i x_2 + r_i] \tag{Eq.4}$$

Results and discussion

In order to obtain a successful ANFIS model, several types of membership function types and number of membership functions should be tried and different models should be installed. In the study, triangular, trapezoidal, gauss, gauss2 and pi membership function types as membership function were applied to the model in different number of membership functions from 1 to 6. *Table 1* shows the model parameters showing the type and number of membership functions for which the best results are obtained. In order to measure the model success, it is necessary to estimate the result parameter from the model and compare it with the actual results using the input parameters that the model has never seen before. The measured 2-year-old cones values used in the testing phase and the 2-year-old cones values obtained from the model are shown in *Table 2*. The most important parameter showing the success of the ANFIS model is the going graph showing the compatibility of the measured test data shown in *Figure 2* with the model test data. The performances of the model trials measured according to the mean absolute error (MAE), square root mean square error (RMSE) and determination coefficient (R^2).

Table 1. *Parameters of the ANFIS model generated with three input data for the estimation of average two-year cone (2017)*

INPUT	OUTPUT	First input type of MF	First input number of MF	Second input type of MF	Second input number of MF	Third input type of MF	Third input number of MF	Output MF	Error					
									Training			Test		
									MAE (number)	RMSE (number)	R^2	MAE (number)	RMSE (number)	R^2
Average female flower count (2015) Mean diameter value (2015) Average number of two-year-old cones (2015)	Average number of two-year-old cones (2017)	gaussmf	1	gaussmf	1	gaussmf	5	constant	23.54	33.60	0.662	14.83	18.85	0.85

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Table 2. *The average number of two-year-old cones used as test data (2017)*

Colon 1		**Colon 2**		**Colon 3**	
Measurand	**Model**	**Measurand**	**Model**	**Measurand**	**Model**
21.3	19.51	24.1	24.88	24.5	15.58
26.7	22.55	24.7	25.34	30.8	45.90
24.5	34.37	38.4	46.95	59.2	78.50
25	34.84	44.6	57.11	84.1	67.17
37.2	50.88	63.8	74.35	105.8	120.82
32.3	72.99	81.4	62.82	164.6	130.10
44.1	46.97	117.7	148.64	210.1	199.71
53.2	53.15	169.8	131.29	130.4	121.65
73.1	85.53	100.8	74.30	76.3	92.17
99.3	61.18	73.1	72.26	108.3	126.64

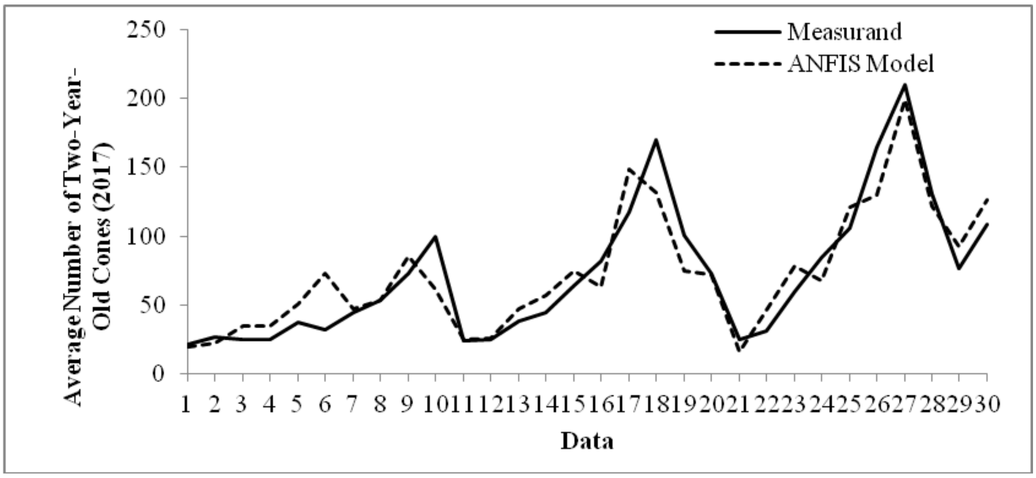

Figure 2. *ANFIS model test phase going graph*

MAE, RMSE and R^2 values found using *Equations 5–7,* respectively.

$$\text{MAE} = \frac{1}{N}\sum_{i=1}^{N}|y_{measurand} - y_{model}| \quad \text{(Eq.5)}$$

$$\text{RMSE} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(y_{measurand} - y_{model})^2} \quad \text{(Eq.6)}$$

$$R^2 = 1 - \frac{\sum_{i=1}^{N}(y_{measurand} - y_{model})^2}{\sum_{i=1}^{N}(y_{measurand} - y_{mean})^2} \quad \text{(Eq.7)}$$

Regarding the results of the test phase, R^2 = 0.850, MAE = 14.83, RMSE = 18.85 in the three input ANFIS model (*Table 1*).

This ANFIS model, in 2017 two-year old cones are estimated using the number of female flowers measured in 2015, the average diameter, and the average of two-year old cones numbers. The model estimates give significant results considering the test phase values of going and scattering graphs (*Figs. 2* and *3*).

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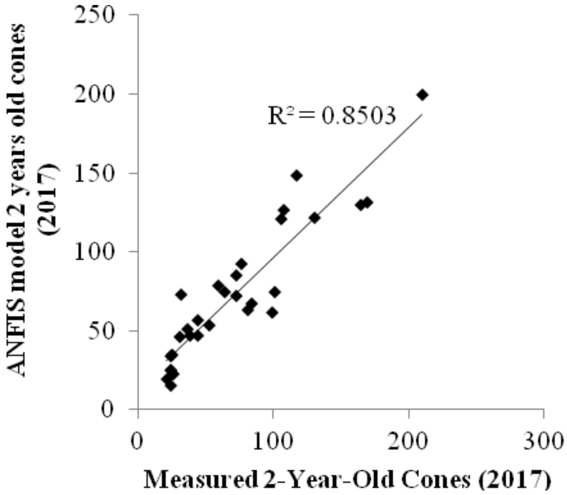

***Figure 3.** ANFIS model test phase scattering graph*

Today, it is possible to estimate the amount of cones in following two years by using the number of female flowers, mean diameter and number of two-year old cones (*Table 2*). Similarly, ANN models proved to be superior for accurately predicting rice yields under typical Fujian climatic conditions (Ji et al., 2007) and for predicting the cactus pear yield (Guimarães et al., 2018). Although the new model used by Calama et al. (2016) for annual cone production surpass the detected deficiencies of previous models, accurately predicting recent decay in cone production, Kaul et al. (2005) stressed that ANN has better predicted yield variability rather than other methods.

Conclusion

The amount of cone is crucial to sustaining forestry management. In this study, predictions of the amount of cone in clonal seed orchards of Anatolian black pine are obtained with high efficiency through Adaptive Neuro-Fuzzy Inference Systems of artificial neural networks. Considered the lack of forestry studies based on the artificial neural network, this study will enable further researches to provide a new perspective.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22672273

EFFECTS OF DIETARY SUPPLEMENTATION OF ALFALFA (*MEDICAGO SATIVA)* FIBRE ON THE BLOOD BIOCHEMISTRY, NITROGEN METABOLISM, AND INTESTINAL MORPHOMETRY IN WEANING PIGLETS

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(Received 24th Nov 2018; accepted 11th Jan 2019)

Abstract. Alfalfa (*Medicago sativa*) is a high-yielding perennial legume, cultivated worldwide with rich nutritional characteristics, and active compounds. A total of 48 crossbred (Duroc × Landrace × Yorkshire) piglets weaned at 28 days old with an average body weight of 8.42 ± 1.88 kg was used in this study. The results indicated that the supplementation of 6-12% alfalfa fibre significantly ($P < 0.05$) increased the growth performance. The digestibility of crude protein (CP) increased at higher dietary alfalfa fibre concentrations. The crude fibre (CF) and dry matter (DM) levels increased first and then decrease at higher alfalfa fibre concentration. The inclusion of alfalfa fibre increased the nitrogen (N) metabolism through increasing N digestibility and utilization and decrease fecal and urinary N content. In addition, the supplementation of alfalfa fibre increased the albumin, globulins, and total protein levels. However, dietary alfalfa fibre decreased blood glucose levels, cholesterol, triglyceride, aspartate aminotransferase, and alanine aminotransferase. There was a significant increase in the villus height (VH), crypt depth (CD), and villus height to crypt depth (VH-CD) ratio after introducing alfalfa fibre in the diet. Therefore, the inclusion of alfalfa fibre to piglet's diet is significant in improving the production performance and health in piglets.
Keywords: *Medicago sativa, gastrointestinal tract, digestibility, performance, pigs*

Introduction

The increasing consumer demand for organic food products such as natural pork and the emergence of antibiotic resistance among many pathogenic microorganisms has necessitated the search for an alternative approach to healthy production systems and the control of microbial infection in pigs (Che et al., 2018; Adams et al., 2019a, 2019b). The use of different plant base components such as dietary fibre, prebiotics, and polysaccharide seem to be promising (Griggs and Jacob, 2005; Che et al., 2018; Adams et al., 2018a). However, there are differences in the nutritional composition of different fibre containing feedstuffs (Jarrett and Ashworth, 2018). Dietary fibre can be defined as the indigestible portion of food obtained from plants which form the main component of many animals (pig) diet (Jarrett and Ashworth, 2018). Dietary fibre influences the functions of the gastrointestinal tract and the health of both animals and human (Bikker

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et al., 2006; Adams et al., 2018b). The consumption of high dietary fibre decreases the bioavailability of some key nutritional components such as some minerals and vitamins (Adams et al., 2018b) and may influence the rate of digestion of food substances and energy metabolism (Adams et al., 2018c). The intake of high-fibre diet can also increase the activities of the gut microbial composition (Varel 1987; Metzler and Mosenthin 2008), hence producing short-chain fatty acid that accounts for 10-30% of the total energy requirement of animals (Christensen et al., 1999).

Alfalfa is a high-yielding perennial legume which is cultivated worldwide with rich nutritional characteristics and active compounds (Yu et al., 2014; Chen, et al., 2016). Alfalfa fibre is primarily composed of insoluble dietary fibre, such as cellulose, lignin, and xylans, which represents more than 90% of the total dietary fibre composition of alfalfa (Bach Knudsen, 1997; Chen et al., 2013a). Alfalfa meal is high in protein and minerals as well as vitamins (Ensminger, 1992). For example, alfalfa contains vitamins such as: A, B1, B2, B6, B12, C, D, E, K, Niacin, Panthothanic acid, Inocitole, Biotin, and Folic acid with minerals such as: Boron, Calcium, Chlorine, Copper, Cobalt, Iron, Magnesium, Manganese, Molybdenum, Phosphorus, Potassium, Sodium, and Sulphur, as well as, well balanced amino acids, rich in carotenoids, and xanthophylls (Sen et al., 1998; Ponte et al., 2004; Jiang et al., 2012). Reportedly, alfalfa contains 17.5% CP, 24.1% CF, and 1,200 kcal/kg metabolizable energy (National Research Council, 1994). Wang et al. (2018) indicated that the growth of pigs on alfalfa pastures produces natural and good quality pork, which is currently gaining more attention in Europe and North America. Interestingly, for the production of better quality pork, alfalfa is used as a fresh fodder in some traditional style pig production systems in China (Wang et al., 2018). Alfalfa fibre is a high-quality protein feed hence increasing its supplementation may improve the nutritional requirement of pigs and improve the growth of piglets. Few studies reported that the supplementation of natural compounds such as flavonoids extracted from alfalfa had insignificant estrogenic impacts, which may increase the production performance, enhance antioxidant, anti-stress induction, and eradicate free radicals from animals (Xie, 2001).

Weaning in piglets is a harsh condition that is mostly associated with a growth disorder and health effects. Weaning piglets are associated with less feed consumption and low nutrient metabolism due to the immature digestive systems, stress as a result of the separation from the dam and littermates, and the sudden change from the easily digestible sow's milk to a less digestible solid feed (Chen et al., 2017). These piglets have limited susceptibility to diseases and infections from enteric bacteria accompanied with frequent diarrhea, stunted growth, low nutrient utilization efficiency, and even mortality (Kick et al., 2012; Che et al., 2018). The supplementation of alfalfa in the diet of pigs has increased in recent years due to the beneficial effects of dietary alfalfa consumption. Dietary alfalfa supplementation increased the short-chain fatty acid concentration in fecal samples of growing pigs (Chen et al., 2013a). Also, alfalfa increased the microbial activities and butyrate levels in the hindgut of the intestinal tract of pigs (Wang et al., 2018).

Therefore, we hypothesize that supplementing alfalfa fibre in the diet of weaning piglets may increase the production performance, blood biochemical composition, and improve the intestinal development and health. Hence, the aim of this study was to investigate the effects of dietary alfalfa fibre supplementation on the growth performance, nutrient digestibility, N metabolism, blood biochemical composition, and intestinal development in piglets.

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Materials and methods

Experimental site and location

The animal feeding experiment was conducted from 27th June to 21st August, 2017, involving 7 days of a pre-feeding period and a 48 days' formal test period at the animal breeding station of Jilin Agricultural University, located in Changchun city in the Jilin Province in the People's Republic of China. Jilin is found on latitude 43°42' N and longitude 126°12' E, and Changchun is on latitude 43°88' N and longitude 125°35' E. The annual rainfall ranges between 350-1000 mm (March-August) and the dry season between September – February. Winter ranges between November – March with temperatures between -8 °C (17 ºF) – -20 °C (-4.6 °F) and summer temperatures between 16 °C (61.4 °F) and 28 °C (81.2 °F) around May-July.

Sample preparation

The alfalfa was obtained from the STONE WINGSII, LLC. & SW Forage, LLC, USA. The alfalfa fibre was extracted by crushing it through a 5 mm sieve and the extract was stored in a closed vessel at room temperature prior to being incorporated into the trial diets.

Experimental animals, design and feeding management

A total of 48 crossbred (Duroc × Landrace × Yorkshire) piglets weaned at 28 days old with an average body weight of 8.42 ± 1.88 kg were used in this study (*Fig. 1*). The trials were divided into four treatment groups according to a completely randomized design with each treatment group comprised of four replicates and each replicate contains three piglets. The animals were housed in pens (1.8 × 1.2 m) with a concrete slatted floor each pen contained three piglets. The pens were cleaned on a daily basis to prevent disease outbreak in piglets. The animals were allowed to acclimatize to the diets for a week prior to the beginning of the experiment and lasted for 48 days. Four experimental diets containing 0.00% alfalfa fibre (control group, M0), 6.00% alfalfa fibre (M6), 12.00% alfalfa fibre (M12), and 18.00% alfalfa fibre (M18) were formulated according to the requirements of the National Research Council (2012). The diets contain soybean meal and cracked corn as the main sources of protein and energy respectively (*Table 1*). The piglets were fed twice a day at 08: 30 am and 05:30 pm and had *ad libitum* access to clean drinking water. Feed intake and leftover were recorded daily in the morning for each animal.

Figure 1. *Photo of experimental pigs*

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Table 1. *Composition and nutritional levels of the basal diets (g kg^{-1} as fed)*

Items	M0	M6	M12	M18
Corn	53.50	50.29	45.00	40.00
Soybean meal	28.00	26.32	24.64	22.00
Alfalfa fibre	0.00	6.00	12.00	18.00
Fish meal	3.00	2.82	3.69	5.10
Whey powder	9.00	5.50	5.50	5.00
Wheat bran	1.00	1.00	1.00	1.00
Limestone powder	1.30	1.22	1.14	0.90
Calcium hydroxide	0.80	0.75	0.70	0.66
Wheat middlings feed	2.00	1.88	1.76	1.64
Rapeseed oil	0.60	1.50	3.56	4.70
Premix	1.00	1.00	1.00	1.00
Nutritional level				
Digestive energy Mcal/kg	3.41	3.33	3.31	3.26
CP	20.10	20.02	20.06	20.27
CF	2.83	4.21	5.55	6.84
Lysine%	1.14	1.12	1.12	1.13
Methionine % + cysteine %	0.69	0.67	0.65	0.65
Threonine%	0.81	0.81	0.81	0.82
Isoleucine%	0.87	0.86	0.86	0.87
Leucine%	1.72	1.68	1.65	1.64
Proline %	0.92	0.92	0.92	0.91
Phenylalanine%	0.99	0.98	0.98	0.99
Tryptophan%	0.30	0.32	0.34	0.38
Calcium to phosphorus ratio	2:1	2:1	2:1	2:1

Premix is available per kg of feed: VA 130-396 KIU, VD2 30-124 KIU, VE 400 mg, VK2 40-150 mg, VB1 25 mg, VB2 75 mg, Cu 1500-7500 mg, Fe 1500-7500 kg, Zn 1500-3700 kg, Mn 400-3700 kg, moisture 9%, sodium chloride NaCl 6-14%, total phosphorus P 2%, lysine Lys 1.3%, Ca 10-20%, Phytase 12500U. M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet

Sample collection

The fecal samples were collected continuously from 08:30 am to 05:30 pm on day 40 to day 45 of the trial period for the determination of nutrient digestibility. The urine was collected within the same time frame as the fecal samples. After each collection, 10% hydrochloric acid was added for fixation of fecal and urine N. The fecal samples were thawed and homogenized within the group, weighed, and dried at 55 °C for 72 h. The dry fecal samples were ground through a 1-mm screen sieve and stored in -20 °C until chemical analysis. The experimental diets were sampled and stored in -20 °C for the DM, CP, and CF analysis. Blood samples were collected from the precaval vein by venepuncture at 8:30 am on day 47 after 12 h of fasting and centrifuged at 1500×g for 30 min at 4 °C. The serum samples were then isolated and frozen at -20 °C until analysis. At the end of the experiment on day 48, all the pigs were electrically stunned and killed by exsanguination. The abdominal cavity was immediately opened to remove the duodenum, jejunum, and ileum. The middle part (5 cm) of the duodenum, jejunum, and ileum was collected and fixed in 10% formalin solution for intestinal morphological

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analysis. The sample of mucosa scraped obtain from the duodenum, jejunum, and ileum was immediately frozen in liquid N, then stored in -80 °C until biochemical analysis (Wu et al., 2018).

Determination of growth performance

The piglets were weighed early in the morning prior to feeding on the first and last days of the feeding trials. The average daily feed intake (ADFI) was determined by estimating the total feed consumption per day. The average daily gain (ADG) was calculated by subtracting the initial body weight from the final body weight and divided by the days of the experiment. Feed conversion ratio (FCR) was determined by dividing the feed intake by the body weight gain.

Determination of nutrient digestion

The feed and fecal samples were analyzed for CP, CF, and DM digestibility according to the procedures described by (Hassanat et al., 2017; Che et al., 2018). Briefly, the DM content was estimated by drying feed and fecal samples in a vacuum oven at 100 °C overnight (AOAC, 1990; method 934.01). The CP (N × 6.25) was estimated using the macro-Kjeldahl procedure (AOAC, 1990; method 990.03) and the CF was determined using the procedures adapted for use in an Ankom200 Fiber Analyzer (Ankom Technology Corp., Fairport, NY).

Determination of nitrogen metabolism

The N content of all the samples was analyzed using the KjeltecTM 8400 automated Kjeldahl analyzer (PT Haes Brothers, Laboratory & Scientific Equipment). The following formula (*Eqs. 1-6*) was used to estimate the N metabolism in the diet, urine, and fecal samples.

$$\text{The N intake (g)} = \frac{daily\ feed\ intake \times CP\%\ in\ the\ diet \times 15.67}{100} \quad \text{(Eq.1)}$$

$$\text{N in feces (g)} = average\ daily\ fecal\ DM(g) \times fecal\ N(\%) \quad \text{(Eq.2)}$$

$$\text{N in urine (g)} = average\ daily\ urine\ (mL) \times N\ in\ urine\ (\%) \quad \text{(Eq.3)}$$

$$\text{N deposition (g)} = N\ intake\ (g) - fecal\ N(g) - N\ in\ urine \quad \text{(Eq.4)}$$

$$\text{Apparent N digestibility (\%)} = \frac{N\ intake - fecal\ N}{N\ intake} \times 100 \quad \text{(Eq.5)}$$

$$\text{N utilisation} = \frac{N\ intake - fecal\ N - N\ in\ urine}{N\ intake} \times 100 \quad \text{(Eq.6)}$$

Determination of blood biochemical composition of piglets

The albumin, globulin, blood glucose, urea N, triglyceride, cholesterol, aspartate aminotransferase, and alanine aminotransferase in the serum were determined by an automatic blood analyzer (7020 Clinical Analyzer, https://www.hitachi-hightech.com/global/) according to the manufacturer’s instruction.

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Piglet's intestinal morphological analysis

The intestinal segments such as the duodenum, jejunum, and the ileum was analyzed for the VH, CD, and VH - CD ratio using paraffin sectioning and the procedures described by Wu et al. (2018). Briefly, the paraffin sections were made by 1. Flushing: The trimmed tissue samples were wrapped in gauze and rinsed under running water for 24 h. 2. Dehydration: The washed tissue samples were taken out and placed on alcohol for dehydration. The concentration of alcohol as a dehydrating agent is gradually increased from 30% so that the tissue sample is gradually dehydrated, and finally dehydrated thoroughly with absolute ethanol. 3. Transparent: The dehydrated tissue sample was soaked in a solution prepared with alcohol and xylene 1:1 to replace the alcohol in the tissue sample for transparent treatment. 4. Immersion wax: After the tissue sample was treated with xylene and alcohol solution, it was preheated in an oven at 57 °C, and then placed in soft wax a, soft wax b and hard wax for 1 h each time, so that the paraffin was fully immersed in the sample. 5. Embedding: The melted paraffin was poured into a carton, the wax-impregnated tissue was placed into it, then cooled in cold water, solidify and cut into cubes. 6 Slices: The embedded wax blocks were placed on a microtome for continuous sectioning and the cut paraffin pieces were placed in hot water to be flatly attached to the glass slides and dried in an oven at about 40 °C. 7. Dewaxing: The dried chips were first placed in a xylene solution for dewaxing and then immersed in absolute ethanol at 95%, 85%, 70% and 50% alcohol in each 2 min, and finally rinsed with distilled water. 8. Hematoxylin and Eosin (H & E) staining: The dewaxed sections were first immersed in hematoxylin dye for 10 min, then rinsed with distilled water, and placed in hydrochloric acid for 5 seconds. The sections were taken out and rinsed with tap water. Put it in 50%, 70%, 85%, 95% alcohol for 2 min, and finally dye with 0.5% eosin alcohol. 9. Dehydrated and transparent: The stained slices were dehydrated by placing alcohol in different solubility and then placed in a xylene solution for transparent treatment. 10. Sealing: Neutral resin is dropped on the slice, then covered with a cover glass, and the sealed slice was observed under a microscope.

Data analysis

The data were analyzed using a one-way analysis of variance ANOVA procedure of SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). A probability value of p valve <0.05 was considered statistically significant and were differences between means was noticeable, Duncan multiple range tests (DMRT) was employed to ascertain the difference among the treatments.

Results

Growth performance of weaning piglets

As shown in *Table 2,* the addition of the M6 and M12 alfalfa fibre to the diet increased the ADG in piglets. However, there was a decrease in the ADG at M18 alfalfa fibre supplementation. There was no significant difference ($P > 0.05$) between the M0 and the other treatment groups however the M18 group was significantly lower ($P < 0.05$) than the M6 and M12. The ADFI of the M6 group was higher than that of the other treatment groups. The ADFI of the M6 group was significantly higher ($P < 0.05$) than the M0 group and the M18 group, but not significantly different ($P > 0.05$) from the M12 group ($P < 0.05$). There was no significant difference ($P > 0.05$) in the FCR

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between the M0 group and the other treatment groups. The M6 group recorded the highest FCR and the M12 group had the lowest FCR.

Table 2. *Effects of alfalfa fibre on the growth performance of piglets*

Items	M0	M6	M12	M18
ADG (kg)	0.44±0.08^{ab}	0.51±0.03^{a}	0.47±0.06^{a}	0.37±0.03^{b}
ADFI (kg)	0.96±0.18^{b}	1.18±0.11^{a}	1.02±0.14^{ab}	0.82±0.05^{b}
FCR	2.19±0.09	2.31±0.19	2.14±0.06	2.22±0.13

In the same row, values with different small letter superscripts mean a significant difference (P < 0.05), while with the same or no letter superscripts mean no significant (P > 0.05). ADG: average daily gain, ADFI: average daily feed intake, FCR: feed conversion ratio. M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet.

Nutrient digestion of weaning piglets

The effect of dietary alfalfa fibre supplementation on nutrient digestibility of piglets was shown in *Table 3*. The digestibility of CP in piglets increased with the increase in the proportion of alfalfa fibre in the diet, but there was no significant effect (P > 0.05) among treatments. The digestibility of CF increases first, and then decreases with the addition of high concentrations of alfalfa to the meal. There was no significant difference (P > 0.05) between the treatment groups. The digestibility of DM in M6 group was higher than the other treatment groups and significantly different (P < 0.05) from the M12 group and the M18 group but not different (P > 0.05) from the M0 group. The M0 group was significantly different from the M12 group.

Table 3. *Dietary supplementation of alfalfa fibre on nutrient digestibility of piglets (%)*

Items	M0	M6	M12	M18
CP	79.72±1.19	80.99±1.12	81.93±2.53	83.15±2.03
CF	47.80±1.70	49.37±0.61	47.72±0.93	46.95±5.10
DM	82.29±0.77^{ab}	82.63±1.30^{a}	79.76±0.82^{c}	80.22±1.39^{bc}

In the same row, values with different small letter superscripts mean a significant difference (P < 0.05) while with the same or no letter superscripts mean no significant (P > 0.05). CP: crude protein, CF: crude fibre, DM: dry matter. M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet.

Nitrogen metabolism of piglets

As shown in *Table 4*, the intake of N in each group varied due to the difference in feed consumption of piglets. The N intake in M12 was higher and significantly different (P < 0.05) from the M18 group but not significantly different (P > 0.05) from the M0 and M12. The fecal N content of piglets decreased with the increase in the concentration of alfalfa fibre in the diet. The fecal N content in the M0 was higher and significantly different (P < 0.05) from the M12 and M18, but not significantly different (P > 0.05) from the M6. The urine N content in the M18 was higher and significantly different (P < 0.05) from the M6 group, but not significantly different (P > 0.05) from the M0 and M12. The apparent N digestibility and N deposition were higher in the M12 in comparison with the other treatment groups. The apparent N digestibility in the M12

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was significantly different (P < 0.05) from the M0, but not significantly different from the other two treatment groups, while the N deposition in the M12 was significantly different from the M18, but not significantly different (P > 0.05) from the M0 and M6. The N deposition rate and N utilization rate in the M6 were significantly higher (P < 0.05) in comparison with the M0 and M18, but not significantly different (P > 0.05) from the M12 group.

Table 4. *Effects of alfalfa fibre supplementation on N metabolism in piglets*

Items	M0	M6	M12	M18
N intake (g/d)	44.26 ± 1.15^{ab}	45.28 ± 1.58^{ab}	47.78 ± 2.86^{a}	42.08 ± 4.22^{b}
N in feces (g/d)	4.29 ± 0.40^{a}	3.85 ± 0.08^{ab}	3.76 ± 0.20^{b}	3.64 ± 0.11^{b}
N in urine (g/d)	9.24 ± 0.31^{ab}	7.21 ± 1.16^{b}	8.92 ± 1.66^{ab}	9.52 ± 1.01^{a}
N apparent digestibility (%)	90.30 ± 1.03^{b}	91.50 ± 0.32^{ab}	92.13 ± 0.62^{a}	91.32 ± 0.62^{ab}
N deposition (g/d)	30.73 ± 1.68^{ab}	33.58 ± 1.46^{a}	34.47 ± 2.01^{a}	28.91 ± 2.68^{b}
N deposition rate (%)	69.41 ± 2.03^{bc}	74.14 ± 0.76^{a}	72.17 ± 1.99^{ab}	68.73 ± 0.54^{c}
N utilization rate (%)	76.85 ± 1.54^{b}	81.03 ± 0.79^{a}	78.33 ± 2.12^{ab}	75.27 ± 1.10^{b}

In the same row, values with different small letter superscripts mean a significant difference (P < 0.05) while with the same or no letter superscripts mean no significant (P > 0.05). N: nitrogen. M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet.

Blood biochemical parameters of piglets

As shown in *Table 5*, the concentrations of albumin, globulin, and total protein increased first and then decreases with the increase in alfalfa fibre in the diet of piglets.

Table 5. *Effects of alfalfa fibre on blood biochemical indexes of weaning piglets*

Items	M0	M6	M12	M18
Albumin (g/L)	24.0 ± 0.82^{b}	25.0 ± 2.16^{b}	29.5 ± 3.79^{a}	25.81 ± 3.19^{b}
Globulin (g/L)	32.4±0.70	33.0±1.37	32.85±4.9	32.73±3.78
Total protein (g/L)	56.4±0.98	58.0±2.60	62.35±8.47	57.48±3.70
Blood glucose (mmol/L)	3.87±0.26	3.75±0.56	3.78±0.55	3.92±0.33
Urea N (mmol/L)	4.64 ± 0.40^{a}	3.39 ± 0.06^{b}	4.08 ± 0.02^{ab}	4.29 ± 0.41^{a}
Triglyceride (mmol/L)	0.60 ± 0.03^{a}	0.56 ± 0.36^{a}	0.47 ± 0.06^{b}	0.58 ± 0.06^{a}
Cholesterol (mmol/L)	2.3±0.06	2.19±0.16	2.06±0.14	2.18±0.32
Aspartate aminotransferase (μ/L)	48.0±3.16	46.0±3.74	47.5±6.45	48.5±7.77
Alanine aminotransferase (μ/L)	42.5±2.89	39.5±2.65	41.25±4.27	40.75±4.5

In the same row, values with different small letter superscripts mean a significant difference (P < 0.05), while with the same or no letter superscripts mean no significant (P > 0.05). M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet.

The albumin concentration in the M12 group was significantly different (P < 0.05) from the other treatment groups. There was no significant difference (P > 0.05) in the globulin and total protein levels between the treatment groups. However, the highest globulin concentration was recorded in the M6, while the highest total protein level was registered in the M12. There were no significant differences (P > 0.05) in the blood glucose, cholesterol, aspartate aminotransferase, and alanine aminotransferase

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concentrations between the groups. The highest blood glucose and aspartate aminotransferase concentrations were observed in the M18 group, while the highest alanine aminotransferase was observed in the M0 group. While the highest cholesterol was observed in the M0 group. The concentration of urea N decreased with the addition of alfalfa fibre to the diet and later increased with the increase in the proportion of alfalfa fibre, but not higher than the control. The urea N level in the M6 was significantly lower ($P < 0.05$) in comparison with the M0 and M18, but not significantly different ($P > 0.05$) from the M12 group. The triglyceride concentration in the M12 was significantly lower ($P < 0.05$) in comparison with the other three groups. The M0 recorded the highest triglyceride concentration.

Intestinal development of weaning piglets

As shown in *Table 6* and *Figure 2,* the M12 group registered the highest VH in the duodenum of each treatment group and the VH of the M12 was significantly higher ($P < 0.05$) than the VH in the M18, but not significantly different ($P > 0.05$) from the M0 and M6. The CD of the duodenum was higher in the M18, but there was no significant difference ($P > 0.05$) between the treatment groups. The VH - CD ratio of the duodenum was significantly lower ($P < 0.05$) in the M0 group in comparison with the other groups. From *Table 6* and *Figure 3,* the VH in the jejunum was significantly higher ($P < 0.05$) in the M12 group in comparison with the M0, but not significantly different from the M6 and M18. There was no significant difference ($P > 0.05$) in the CD of the jejunum between the treatment groups. However, the M12 recorded the highest CD. The VH - CD ratio of the jejunum was significantly higher ($P < 0.05$) in the M18 in comparison with the M0 and M6, but not significantly different ($P > 0.05$) from the M12. From *Table 6* and *Figure 4,* the VH of the ileum first increased with the increase in the concentration of alfalfa fibre in the diet and later decreased at higher dietary alfalfa fibre levels. However, there was no significant difference ($P > 0.05$) between the treatment groups. The CD of the ileum was significantly higher ($P < 0.05$) in the M12 in comparison with the M0 and M6, but not significantly different ($P > 0.05$) from the M18. The VH - CD ratio of the ileum was higher in the M6, but there was no significant difference ($P > 0.05$) between the treatment groups.

Table 6. *Effect of alfalfa fibre supplementation on intestinal development of weaning piglet*

Item	Properties	M0	M6	M12	M18
Duodenum	VH (μm)	561.48 ± 19.36^{ab}	563.85 ± 44.35^{ab}	594.18 ± 28.78^{a}	524.19 ± 44.35^{b}
	CD (μm)	247.76±19.46	245.62±33.16	237.08±33.29	287.64±58.69
	VH:CD	1.10 ± 0.10^{b}	1.44 ± 0.22^{a}	1.57 ± 0.24^{a}	1.60 ± 0.1^{a}
Jejunum	VH (μm)	459.25 ± 40.95^{b}	489.24 ± 66.61^{ab}	538.94 ± 37.89^{a}	481.25 ± 30.18^{ab}
	CD (μm)	187.92±22.65	204.91±27.69	223.69±59.21	194.63±66.64
	VH:CD	2.47 ± 0.37^{b}	2.44 ± 0.62^{b}	2.54 ± 0.68^{ab}	2.71 ± 0.92^{a}
Ileum	VH (μm)	394.61±34.41	406.16±29.21	426.87±50.71	369.46±30.90
	CD (μm)	182.37 ± 37.81^{b}	190.60 ± 45.86^{b}	253.85 ± 15.13^{a}	244.03 ± 14.25^{ab}
	VH:CD	2.20±0.37	2.23±0.59	1.68±0.13	1.51±0.03

In the same row, values with different small letter superscripts mean a significant difference ($P < 0.05$), while with the same or no letter superscripts mean no significant ($P > 0.05$). VH: Villus Height, CD: Crypt Depth, VH:CD means the ratio of the Villus Height to Crypt Depth. M0: 0.00% alfalfa diet, M6: 6.00% alfalfa diet, M12: 12.00% alfalfa diet, M18: 18.00% alfalfa diet

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

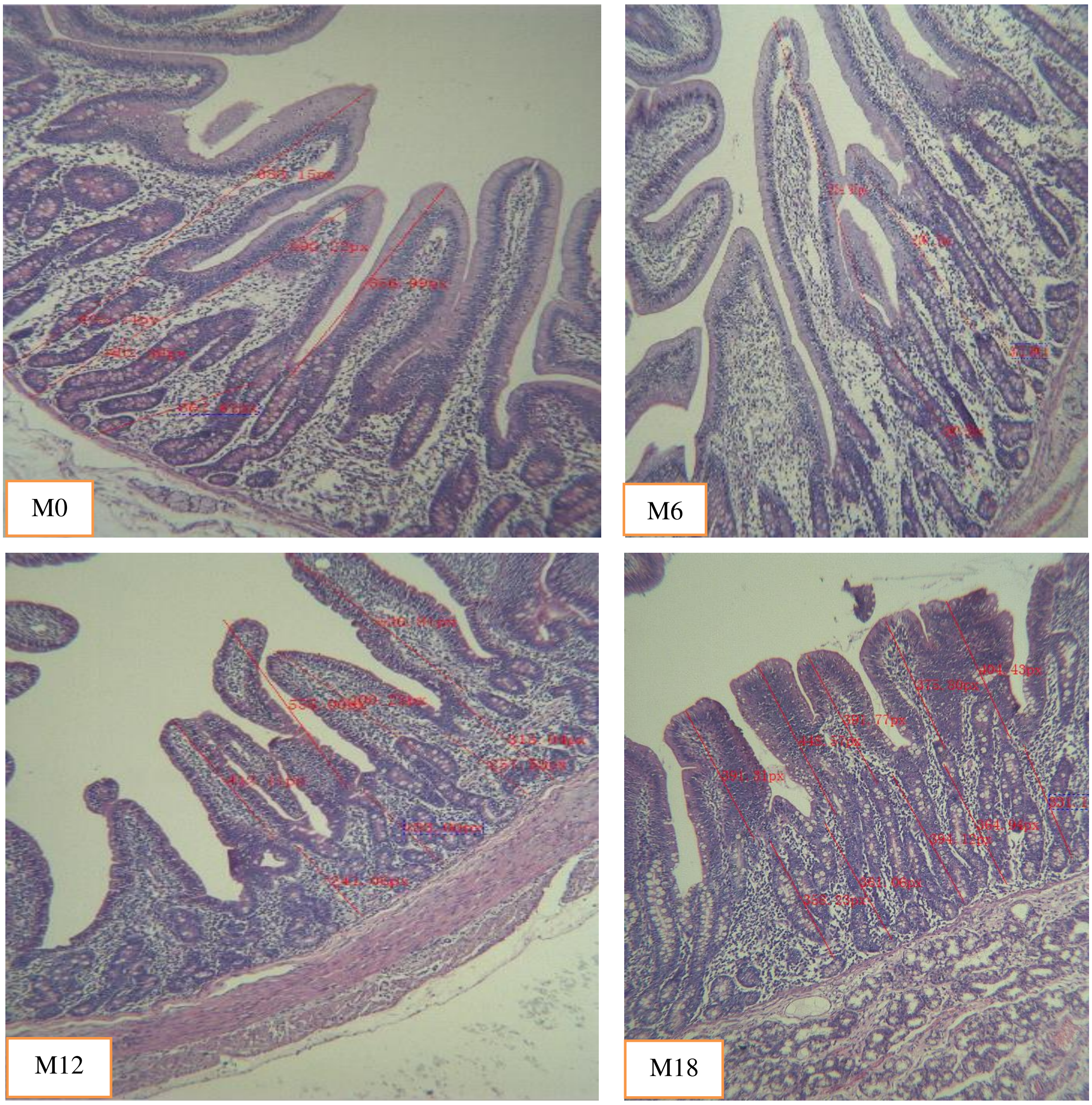
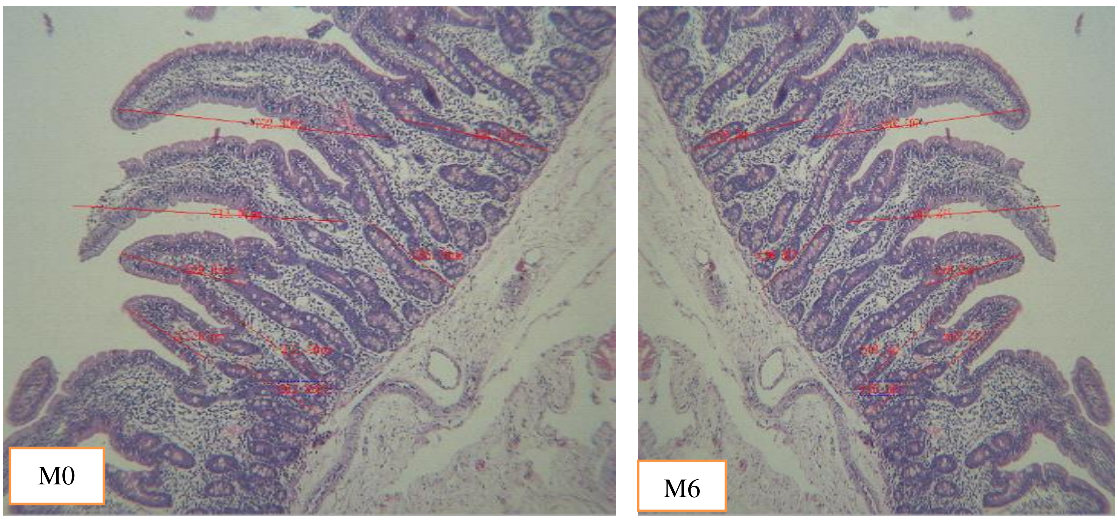


Figure 1. *Intestinal morphometry analysis of the villus height, crypt depth, and villus height to crypt ratio in the duodenum among the different treatment groups*



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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

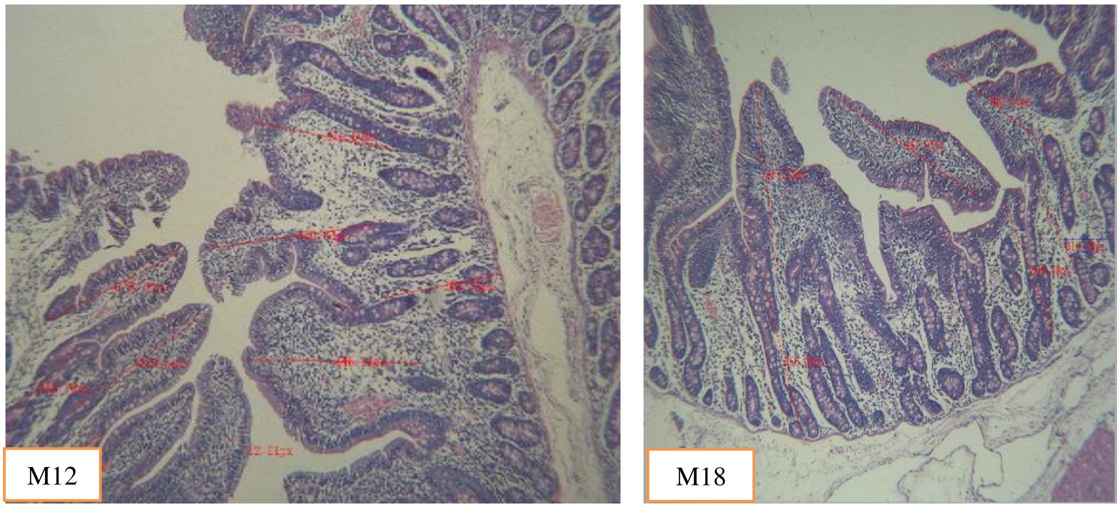

Figure 2. *Morphological analysis of the villus height, crypt depth, and villus height to crypt ratio in the jejunum among the different treatment groups*

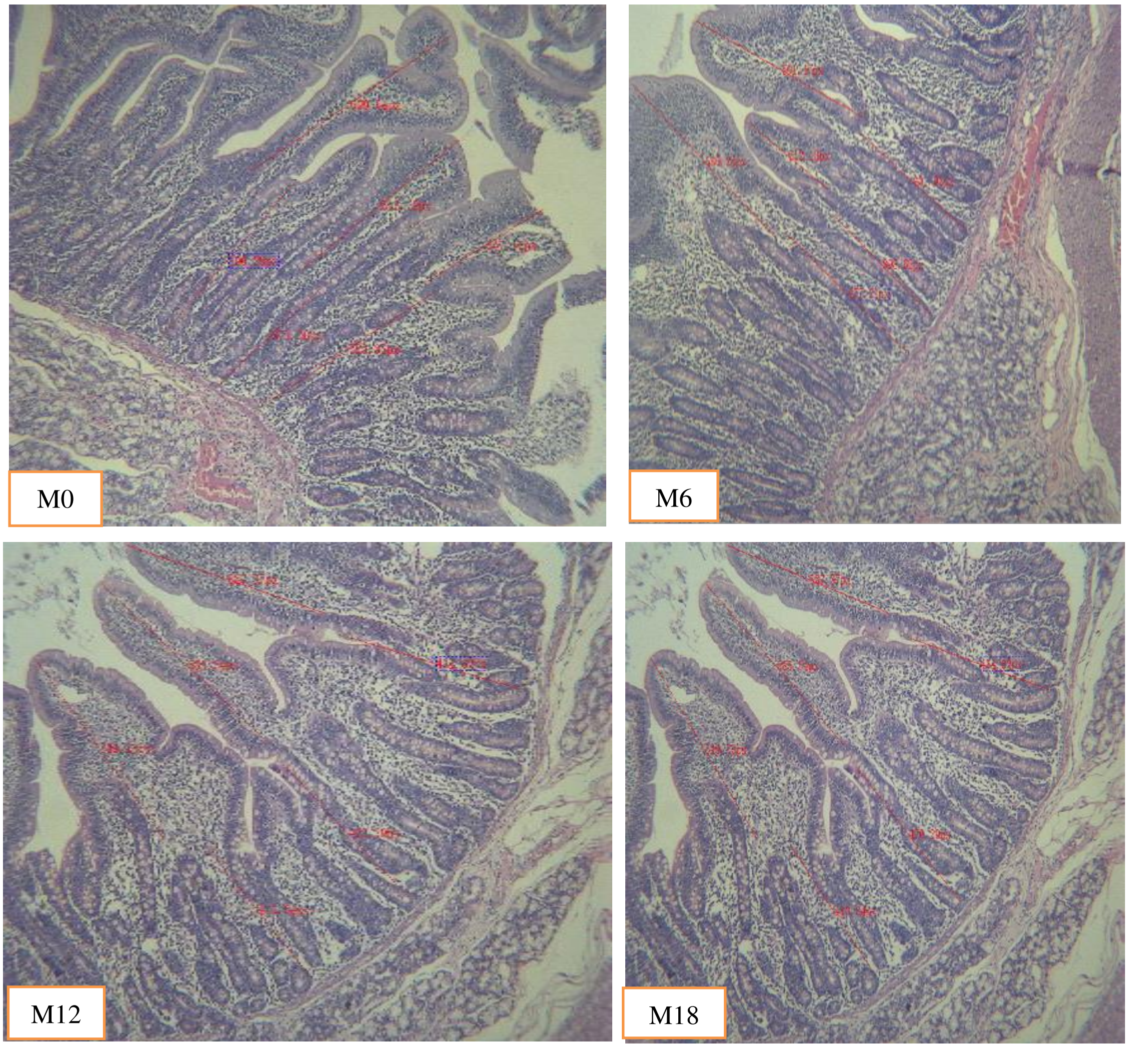

Figure 3. *Morphological analysis of the villus height, crypt depth, and villus height to crypt ratio in the ileum among the different treatment groups*

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

Discussion

Growth performance of pigs

The current study showed that the addition of different dietary concentrations of alfalfa fibre to the diet of piglets promotes their growth performance. However, at higher concentrations, there was a downward trend in the growth performance of piglets. These findings indicated that supplementing the optimum concentrations (6.00-12.00%) of alfalfa fibre to the diet of piglets will significantly promote the growth and development of piglets. Our results were in agreement with the findings of (Che et al., 2018; Adams et al., 2018a; Wang et al., 2010; Yuan et al., 2006). In supplementing *Astragalus membranaceus* fibre to the diet of weaning piglets (Che et al. 2018) and Adams et al. (2018a) observed an increase in the growth performance of piglets weaned at 28 days old. Similarly, Yuan et al. (2006) observed an increase in the growth performance of piglets after feeding on Astragalus polysaccharides. Also, after feeding on both *Astragalus membranaceus* roots powder and Astragalus polysaccharide, Wang et al. (2010) observed an increase in the growth performance of broiler chicks. Wang et al. (2016) reported an increase in the growth performance of weaning pigs fed with sugar beet pulp. Chen et al. (2016) reported that adding alfalfa flavonoid to the diet of Yangzhou geese increased their growth performance. However, supplementing higher concentration (18.00%) of alfalfa fibre will have a detrimental effect on the growth performance of weaning piglets. This is because weaning piglets cannot utilize the high fibre content present at the maximal concentrations of alfalfa fibre due to the immature gastrointestinal tract and less gut microbial composition and diversity (Che et al., 2018). Similarly, Chen et al. (2017) and Adams et al. (2018b) indicated that piglets have immature gastrointestinal systems and adapting to a less digestible solid feed such as dietary fibre seems challenging. The supplementation of dietary fibre to the diet of piglets promotes the growth and health of piglets, as these fibre components can be fermented by microorganisms in the intestinal tract to produce large volumes of short-chain fatty acid, that serves as the main energy source for weaning piglets (Christensen et al., 1999; Che et al., 2018), reduce the population of enteric bacteria in the gut that causes piglet diarrhea during weaning (Li et al., 2011; Che et al., 2018). In contrast to our findings, Wellock et al. (2008) observed a decrease in the growth performance of piglets after feeding on a dietary fibre supplemented diet. In addition, there was no effect on the growth performance of lambs after feeding on a diet supplemented with sugar beet pulp and roasted canola seed (Asadollahi, et al., 2018). Also, Jin et al. (2018) observed no change in the growth performance of growing lambs after feeding on a dietary *Urtica cannabina* supplemented diet. Moreover, dietary supplementation of alfalfa saponins had no effect on the growth performance of lambs (Liu et al., 2018). Furthermore, Wang et al. (2018) observed no effect on the ADG and ADFI in piglets after feeding on alfalfa containing diet. Jiang et al. (2012) observed no change in the growth performance of ducks fed with alfalfa meal. The difference between our findings and these studies may be due to the difference in animal species and the fibre source or alfalfa plant concentration applied. Therefore, dietary inclusion of alfalfa fibre at an optimum level can positively promote the growth performance of piglets.

Nutrient digestion of piglets

Che et al. (2018) indicated that the main importance of any nutritional supplement added to an animal's diet was as a result of its ability to be utilized by the animal.

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

Hence, the digestibility of nutritional components of a dietary supplement is a vital parameter in evaluating its inclusion in the diet. The findings of this study indicated that the supplementation of alfalfa fibre to the diet of piglets increased their nutrient digestibility. This study showed that the digestibility of CP increased with the increase in alfalfa fibre concentrations in the diet. Also, the CF and DM digestibility first increased with the addition of alfalfa fibre and then decreased as the concentration increases. Similarly, Jin et al. (2018) observed an increase in the CP digestibility after supplementing *Urtica cannabina* in the diet of growing lambs. In addition, Lara et al. (2018) observed an increase in nutrient digestibility in wethers after feeding on corn silage inoculated with *Lactobacillus plantarum* and *Bacillus subtilis*. Moreover, the supplementation of dietary alfalfa saponins in the diet of lambs increased their nutrient digestibility. Furthermore, Ebrahimi et al. (2018) reported a decrease in the nutrient digestibility of neonatal calves after feeding on *Trachyspermum ammi* and *Thymus vulgaris* oils in their diets. The supplementation of alfalfa fibre promotes the development of piglets' intestinal tract and increase the digestive function of piglets. The alfalfa fibre is a high-quality protein feed hence increasing its supplementation may increase the nutrient digestibility of piglet, especially CP digestibility. However, higher fibre content in the diet may decrease the bioavailability of some key nutritional components such as some minerals and vitamins (Adams et al., 2018b) and reduce the proportion of digestible substances in food and digestive energy (Adams et al., 2018c). In contrast to our findings, Che et al. (2018) reported a decrease in nutrient digestibility after feeding *Astragalus membranaceus* fibre to weaning piglets. Wang et al. (2016) reported a decrease in nutrient digestibility in weaning piglets after feeding on a sugar beet pulp. Also, Jørgensen et al. (1996) reported a low nutrient digestibility in pigs fed with high fibre concentrated diet. In addition, Dilger et al. (2004) fed high soy hull fibre to pigs and observed low DM digestibility. Furthermore, dietary fibre affects the function of the gastrointestinal tract, as indicated by low nutrient digestibility and accessibility of feeds with high-fibre levels (Bikker et al., 2006; Adams et al., 2018c). Pinheiro et al. (2018) reported that dietary supplementation of higher alfalfa concentrations in the diet of European adult rabbits had no effect on the digestibility of dietary components. Moreover, dietary fibre intake impacts the various methods in nutrients absorption and digestibility and this may lead to a delay in the glycemic response (Adams et al., 2018b). As reported in a review by Adams et al. (2018b, c), dietary fibre and prebiotics impact the health of the host animal by regulating blood glucose or insulin levels, stool bulking effects, enhancing the acidity of the gut, short-chain fatty acids synthesis, reducing intestinal transit time, inspiring the growth of intestinal microbes, and increase the production of blood biochemical components. Therefore, the effects of dietary fibre on nutrient digestibility may depend on the dietary fibre source or form, the nutritional component, the age of the animal, and the health of the animal.

Nitrogen metabolism and blood biochemical parameters in piglets

The difference in dietary feed consumption influence the rate of N metabolism in animals. The results of this study showed that dietary supplementation of alfalfa fibre increased the N metabolism in piglets. The piglets that were fed with 12% of alfalfa fibre (M12) had the highest N intake. Meanwhile, the fecal N content in piglets decreased with the increase in the concentration of alfalfa fibre in the diet. The highest urine N content was observed in the M18 group while the apparent N digestibility and N

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

deposition were observed in the M12 group. According to the test results, we observed that the addition of alfalfa fibre to the piglet's diet maintained the normal growth performance of piglets. Therefore, optimum dietary fibre concentration can promote the N utilization in the body, but the excessive concentration of alfalfa may reduce the time during which the chyme stays in the intestine hence reduce the N utilization rate. In addition, urinary urea levels may rapidly decompose to ammonia by exposing to the environment (Aarnink and Verstegen, 2007), causing environmental pollution. In this experiment, the addition of alfalfa fibre reduced the emission of fecal N and urinary N, hence reduced environmental pollution. Therefore, improving the fibre levels of the diet may be an important means of reducing the N emissions from pig farms.

Blood contains various proteins, sugars, lipids, and other metabolic substances such as enzymes and hormones which affect the growth and metabolism of nutrients in the body. Blood biochemical indicators influence blood levels, animal health, and impacts growth and nutrient regulation in animals. The findings of this study indicated that the supplementation of alfalfa fibre to the diet increased the albumin, globulin, and total protein concentrations in the blood of piglets. The addition of the optimum (6.00-12.00%) alfalfa fibre to the diet of piglets decreased the blood glucose, cholesterol, aspartate aminotransferase, and alanine aminotransferase in piglets, but higher alfalfa fibre increased blood glucose and aspartate aminotransferase concentration. Meanwhile, the triglyceride levels decreased with the addition of alfalfa fibre to the diet. The serum total protein, including albumin and globulin relate to the protein synthesis in the body. It influences protein concentration, synthesis, and functions in immune responses, transportation, and coagulation (Paschke et al., 2010). The results of this experiment indicated that supplementing alfalfa fibre to the diet may increase the total protein levels in pigs. Similarly, Chen et al. (2016) reported that the supplementation of alfalfa flavonoid to the diet of Yangzhou geese has no adverse effects on the total protein metabolism and liver functions. Furthermore, these authors reported that adding 150 to 450 mg/kg alfalfa flavonoids increased serum total protein concentration in geese (Chen et al., 2016). However, when the amount of alfalfa fibre reached 18%, the total protein content of the serum began to decrease. The addition of fibre to the diet has no adverse effects on the metabolic processes in piglets but excess fibre may attenuate the metabolic functions in piglets. Therefore, supplementing the optimum concentration of alfalfa fibre will promote the health and productivity of pigs. Serum urea N is the final metabolite of protein and amino acid catabolism in animals (Chen et al., 2016). Its content can accurately reflect the nutritional status of an animal and the absorption and utilization of protein in the body. Studies have shown that when serum urea N decreases, there may be a decrease in the protein metabolism of the body and increase in the N deposition (Paschke et al., 2010). Also, the findings of this experiment indicated that the urea N composition of the trial groups was lower in comparison with the M0. The M6 group had the lowest urea N content and the highest N deposition. Contrarily to our observation, Chen et al. (2016) observed an increase in the serum urea N after adding alfalfa flavonoid to the diet of Yangzhou geese. Also, Qiu et al. (2009) reported an increase in the serum urea N concentrations after feeding protein diet supplemented with dietary zinc. This further confirms the relationship between serum urea N and N deposition in animals. The blood glucose is mainly metabolized in the hepatic glycogen decomposition and intestinal absorption. It forms the main tissue components and energy metabolic substrates of the body. Therefore, changes in the blood glucose levels may influence the dynamic balance between the sugar absorption,

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

transport, and metabolism in the body. The findings of this study further showed that there was no significant effect on the blood glucose concentration in piglets after feeding on alfalfa fibre, this indicates that the rate of energy conversion between the treatment groups maintained a dynamic equilibrium. The cholesterol and triglyceride concentrations in the serum can reflect the total lipid metabolism in the body (Chen et al., 2016). The higher the cholesterol or triglyceride content, the higher the body's ability to metabolize fat. The results of this experiment indicated that alfalfa fibre may reduce the cholesterol and triglyceride concentrations in the blood of weaning piglets, hence indicating that alfalfa fibre has a certain lipid-lowering effect. Similarly, Chen et al. (2016) deemed that the addition of alfalfa flavonoid decreased the triglycerides and total cholesterol levels in Yangzhou geese. Similarly, Zhou (2011) reported that an increase in cholesterol metabolism and a reduction in the cholesterol deposition of the body, thus promoting meat quality parameters. Aspartate aminotransferase and alanine aminotransferase are involved in the metabolism of amino acids in the body and are important indicators for evaluating liver functions (Chen et al., 2016). The addition of alfalfa fibre to the diet had no significant effect on the two transaminase enzymes, hence indicating that high fibre level in the diets had no effect on the amino acid metabolism of the body. Contrarily to our observation, Chen et al. (2016) observed that the addition of alfalfa flavonoid significantly increased the levels of aspartate aminotransferase and alanine aminotransferase in comparison with the control group. Also, there was an increase in the aspartate aminotransferase and alanine aminotransferase level when rapeseeds diet was fed to geese (Wang et al., 2015a) and alfalfa diet fed to sheep (Wang et al., 2015b). The difference between our findings and that of these authors may be due to the form of alfalfa used and the experimental animal involved. Therefore, adding alfalfa fibre may promote the N metabolism, improve biochemical parameter, and no adverse effect on the amino acid metabolism.

Intestinal morphometry of piglets

Studies have indicated that dietary fibre is a valuable nutrient for the prevention of intestinal disorders and improving intestinal health in both humans and animals (Ingvar, 2004). The small intestine is the main site for digestion and absorption of various nutrients in the diet. The villi are the functional units of the small intestine and the height of the villi can affect the nutrient absorption abilities of the small intestines. The villus atrophy occurs when the cells at the top of the villi are detached or the rate of cell regeneration is decreased and the number of mature cells is reduced leading to gastrointestinal inflammatory disease (Geboes et al., 2018). The intestinal glands and its CD reflect the maturation rate of the cells and the deepening of the crypts means that the cell maturation rate decreases, therefore decreasing the rate of transport of substances from the epithelial cells to the top of the villi (Wallig, 2018). The ratio of the VH - CD influence the functions of the small intestine (Hu et al., 2018). The higher the ratio of the VH - CD, the higher the intestinal mucosal functions and the absorption capacity of the small intestine. However, lower VH - CD indicates that the intestinal mucosa is damaged and the absorption capacity of the intestine decreases (Hu et al., 2018). The results of this study showed that the VH of the duodenum, jejunum, and the ileum was higher in the M6 and M12 in comparison with the control group. In agreement with our study was the findings of Xia et al. (2005) who reported that the supplementation of copper-bearing montmorillonite to the diet of weaning piglets significantly increased the VH and the VH - CD ratio in the small intestinal mucosa

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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compared to the control group. Meanwhile, Xia and colleagues observed no significant effects on the small intestinal morphology after supplementing $CuSO_4$ to the diet of piglets (Xia et al., 2005). Similarly, Jiang et al. (2012) reported that there was a significant difference in the VH, CD, and VH - CD ratio in the duodenum, jejunum, and ileum of ducks fed with different concentrations of alfalfa meal. Broiler chickens fed with wet diet had high VH and low CD in the duodenum, jejunum, ileum, cecum, and the colon in comparison with those fed with dry diet (Yasar and Forbes, 1999). High VH was observed in the duodenum after feeding birds with *Bacillus subtilis* (Samanya and Yamauch, 2002) and yeast cell wall (Zhang et al., 2005). Also, Hedemann et al. (2006) observed that feeding pigs with high-insoluble fibre diets may guard the small intestine against pathogenic bacteria by increasing the VH. In addition, Chen et al. (2013b) indicated that piglets that were fed with wheat bran fibre had a higher VH in the ileum than those fed with soybean fibre. These same authors found that the supplementation of wheat bran fibre and pea fibre in the diet of weaning piglets increased the number of mucosal goblet cells in the colon (Chen et al., 2013b). Furthermore, Xu et al. (2003) indicated that feeding fructooligosaccharide (0.4%) to broilers increased the ileal VH, CD, and VH - CD ratio. We observed that the VH of the duodenum and the ileum decreased at higher concentration of alfalfa fibre (M18) in comparison with the other groups. Xia et al. (2005) indicated that the digestive tract accommodates some stresses that relatively causes a deviation in the intestinal mucosa of piglets due to the close proximity of the mucosal surface of the intestinal components. The supplementation of dietary fibre levels in the diet promotes the development of the intestinal villi in piglets, but higher fibre content may lead to atrophy of the villi. The variations in the intestinal morphology such as shorter villus and deeper crypts have been related to the presence of toxins and other chemical agents (Xu et al., 2003). Gerritsen et al. (2012) showed that dietary fibre levels affect the changes in the intestinal morphology of animals, mainly due to the changes in the surface area of the intestines such as the height and number of villi. Kleessen et al. (2003) reported that feeding high fibre diets to pigs increased the VH of the ileum and jejunum, but had no significant effect. Also, the ileal VH - CD ratio in piglets fed with wheat bran fibre was higher than piglets that were fed with maize bran fibre (Chen et al., 2013a). In this study, the ratio of the VH - CD in the duodenum and jejunum increased with the increase in the supplementation of alfalfa fibre in the diet while in the ileum the highest VH - CD ratio was recorded in the M6 group. The above test results showed that a piglet fed diet with alfalfa as the main fibre source can promote the development of the small intestinal villi and improve nutrient digestibility and utilization rate. In contrast to our study, Nabizadeh (2012) reported that dietary supplementation of inulin in the diet of broiler chicks had no effect on VH, CD, and the VH - CD ratio in the duodenum and jejunum on day 42 of age. In addition, cellulose supplementation significantly reduced the VH in both the jejunum and ileum (Chen et al., 2014). However, inulin inclusion significantly increased ileal VH in broiler chickens at 42 days of age (Chen et al., 2014). Studies have shown that the level of feed intake affects the height of the small intestinal villi and the depth of the crypt. The increase in feed intake promotes the development of the small intestine. The reason may be that some fibre components can regulate the composition of microorganisms in the intestine through fermentation to increase the intestinal VH and CD of animals (Jiang et al., 2012). The supplementation of fibre in the diet may stimulate volatile fatty acids produced by intestinal microbial fermentation, while butyric acid promotes the intestinal

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

cell production, thereby deepening CD (Gidenne et al., 2002). Early weaning of piglets can cause stress induction in piglets which may cause changes to the intestinal morphology, atrophy of the small intestinal villi, and deepening of the crypts, hence affecting the body's absorption ability and utilization of nutrients and decreased immunity. Therefore, piglets weaning stress may be alleviated by appropriately raising the fibre level of the diet to maintain the intestinal health of piglets.

Conclusion

This experimental study showed that adding 6-12% of alfalfa fibre to the diet of weaning piglets may improve the growth and nutrient digestibility of piglets, reduce N emissions, and improve N utilization in piglets. Also, adding 6-12% of alfalfa fibre to the diet of piglets may promote the utilization of protein and maintain the balance in glucose metabolism. At the same time improve the intestinal morphological structures and improve the digestion and absorption of the small intestine. Therefore, further research is recommended to elucidate the effects of alfalfa fibre on the gut microbial function with reference to nutrient digestibility and N metabolism in piglets.

Acknowledgements. This work was financially supported by the national key research and development program of China (2017YFD0502104), and the scientific project of Jilin province (20170309003NY & 20180101023JC).

Conflict of interests. The authors declare that there is no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_22752295

ASSEMBLAGES OF NECROPHILOUS CARRION BEETLES (COL., SILPHIDAE) IN AGRICULTURALLY USED AREAS

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(Received 19th Oct 2018; accepted 2nd Jan 2019)

Abstract. This study on Silphidae fauna was carried out at different sites forming part of an agricultural landscape. Catches were carried out in two selected habitats (Borek Stary and Widna Góra) in the Subcarpathian region (south-eastern Poland) during three growing seasons (2009-2010 and 2014) using Barber pitfall traps. It included potato, fodder beet and cereal crops (Widna Góra) as well as field margins (Borek Stary and Widna Góra). Four traps were placed at each of the sites studied then were emptied on average every two weeks. As a result of the observations, a total of 5491 beetles from 13 species were collected.
Keywords: *Silphidae, Thanatophilus sinuatus, Nicrophorus vespillo, crop culture, field margins*

Introduction

Most representatives of carrion beetles (Col., Silphidae) are necrophagous and/or predatory. Herbivorous species are a minority (Anderson and Peck, 1985; Sikes, 2008). Alongside ground beetles (Col., Carabidae) and rove beetles (Col., Staphylinidae), beetles from this family are an important component of epigeic entomofauna of agricultural landscape from the ecological point of view (Tischler, 1955). Necrophagous carrion beetles perform important ecological functions, which can be particularly seen in the agricultural aspect.

Biochemical transformations that accompany the decomposition of dead animals lead to an increased amount of mineral nutrients and humus in the soil and, as a further consequence, they affect soil fertility regeneration. Overall, they contribute to the cycling of elements in nature. In the aspect of agricultural crops that are at risk of outbreaks of many pests, among others gastropods (Gastropoda: Pulmonata: Stylommatophora), it is important to notice the positive biological role of carrion beetles. *Silpha carinata* is a predator of gastropods, both shelled snails and slugs (Dekeirsschieter et al., 2011). *P. atrata atrata* and *A. laevigata laevigata*, in which the characteristically elongated head facilitates shell penetration, exhibit a morphological adaptation for hunting shelled snails. Hunting strategies in these species have been described by Heymons and Lengerken (1932), Plate (1951), Linssen (1959), and Barronio (1974).

Agricultural progress and modern industrialization that aim to improve production in the food sector, given the growing human population, do not guarantee the sustainability of ecosystems. What is more, the loss of identity of ecosystems leads to the impoverishment of biodiversity (Wink et al., 2005). Anthropogenic pressure on agricultural landscape, which includes various agronomic practices, the use of fertilizers and crop protection chemicals as well as different plant and animal production systems, exerts an adverse impact on the diversity of zoocenoses occurring in agrarian landscape

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DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

(Boone et al., 1999; Stoate et al., 2001; Porhajašová et al., 2015). Invertebrates, especially insects, are an animal group that is particularly sensitive to landscape changes. As a numerous and diverse component of different biocenoses, they perform important functions in ecosystems (Konieczna and Krupa, 2013), and a decline in their numbers may have a negative cascade effect (Coleman and Hendrix, 2000; Nichols et al., 2007).

The impact of agronomic practices on beneficial coleopterofauna has been identified quite well with regard to, among others, ground beetles (Col., Carabide) (Holland and Luff, 2000; Honěk and Jarošík, 2000; O'Rourke et al., 2008; Bukejs and Balalaikins, 2008; Bukejs, 2009; Eyre et al., 2012; Lemic et al., 2017; Schwerk and Dymitryszyn, 2017) and rove beetles (Col., Staphylinidae) (Bohac et al., 1999; Balog and Markó, 2006; Ghahari et al., 2009). As predatory entomophages, these both beetle families are frequently analyzed comprehensively (Andersen et al., 1983; Andresen, 1997; Frank and Reichhart, 2004; Tamutis et al., 2004). It should be indicated that the cited publications are only selected examples. However, as far as carrion beetles of arable fields are concerned, research is fragmentary. The studies conducted in the Czech Republic (Petruška, 1968; Novák, 1961, 1962; Kočárek and Benko, 1997; Kočárek, 1997; Růžička, 1994; Jakubec and Růžička, 2012; Stanovský et al., 2005), Austria (Juen et al., 2003), Luxembourg (Schlechter, 2008), Lithuania (Tamutis et al., 2007), and Poland (Kamińska, 1989; Konieczna et al., 2014) can be considered to be the most important publications presenting the occurrence of Silphidae in agriculturally used areas.

Apart from providing biological pest control, ground beetles (Arus et al., 2012; Renkema et al., 2014) are commonly considered to be bioindicators of environmental changes (Rainio and Niemelä, 2003; Agvin and Luff, 2010). Referring to the definition of McGeoch (1998), not only Carabidae, but also trophically different beetle families (Staphylinidae, Nitidulidae, Scarabaeidae, Scotylidae) can be regarded as indicator organisms (Beroiz et al., 2010). According to a study by dos Santos Fernandes et al. (2011), Silphidae can also be considered as potential biomarkers of environmental quality.

In analyzing the occurrence of beetles in agriculturally used areas, research on both areas that are subject to direct agricultural use (crop fields) and land that is excluded from such agricultural use (field boundary strips, field margins, and adjacent meadows) is worth undertaking. The need to conduct comparative studies was highlighted by Huruk (2006). Their results can be much more interesting and can allow us to infer on the impact of human activity on the natural environment. At the same time, it is worth noting that the negative impact of agricultural practices on organisms seems to be less noticeable in field margins (Marshall and Moonen, 2002). Taking this into account, the aim of this study was the following: (i) to identify the species composition of carrion beetles occurring in arable fields and field margins, (ii) to determine the dominance system of carrion beetles at such sites, and (iii) to compare the structure of assemblages of beetles caught.

Materials and methods

Study sites

This study on assemblages of necrophilous carrion beetles (Col., Silphidae) was conducted in south-eastern Poland in the years 2009, 2010, and 2014 (*Fig. 1*). Catching

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DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

of entomofauna was carried out in Borek Stary (UTM EA73) and Widna Góra (UTM FA13/FA23). In Widna Góra, the study included the following crops: (i) a mixed cereal crop for animal feeding (at equal proportions of *Avena sativa* L., *Hordeum vulgare* L., and *Triticum aestivum* L.), (ii) fodder beet (*Beta vulgaris* L., semi-sugar type, cv. 'Zentaur Poly'), and (iii) potato (*Solanum tuberosum* L.), including the fields margins adjacent to these crops (iv, v, vi). In Borek Stary, the study was carried out on (vii) a field margin separating a mixed cereal crop for animal feeding (spring cereals: *Avena sativa* L. and *Hordeum vulgare* L.) and pastures (*Fig. 2a-g*).

Methods for describing the study sites

The field margins were characterized based on relevés made using the Braun-Blanquet method. Due to the limited surface area of the field margins, the relevé area was 20 m^2. To determine the cover-abundance of each of the identified species, the Braun-Blanquet seven-point scale was used (1964), in which a value of 5 corresponds to cover above 75%, 4 – 50-75%, 3 – 25-50%, 2 – 5-25%, and 1 – about 5%. The symbol "+"denotes rare species (2-5 specimens), while r – sporadic species (1 specimen) (Pawłowski, 1977; Wysocki and Sikorski, 2014). In the agriculturally used areas (the potato, fodder beet, and mixed cereal crops), due to the homogeneity of the crop stands, cover of layer C was accepted as 100% (100% culture purity).

Catching of carrion beetles

This study was conducted over three growing seasons (2009-2010 and 2014). Beetles were captured using Barber pitfall traps, which were emptied at 14-day intervals. The pitfall traps were made of plastic containers with a diameter of 9 cm, placed flush with the ground level, and filled with ethylene glycol solution to $^1/_3$ of their volume. Four traps were placed at each of the sites studied. They were monitored from the first 10 days of May to the third 10 days of October. Poultry slaughterhouse waste was used as bait. 11 harvests were carried out in 2009, whereas in 2010 and 2014 – 12 in either of these years. The content of one trap from one harvest at one site was treated as one sample. Over the three-year study period, a total of 380 samples were taken for analysis. The captured entomological material was analyzed under laboratory conditions.

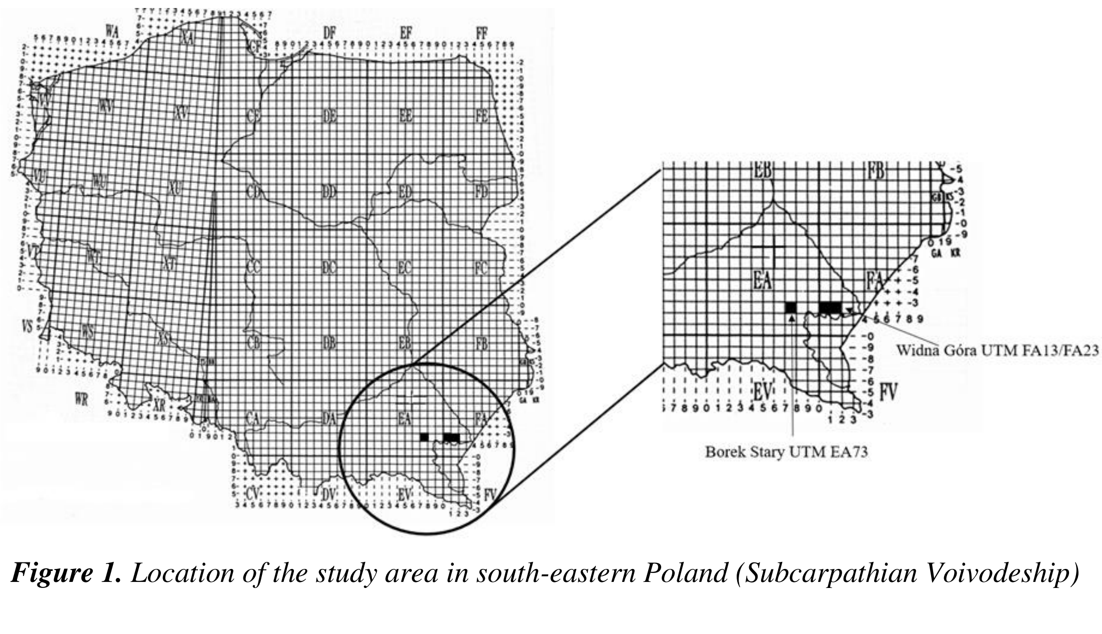

***Figure 1.** Location of the study area in south-eastern Poland (Subcarpathian Voivodeship)*

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Figure 2. *Study sites: 2a- field margin between cereal crop and pasture; 2b- field margin between potato crop and meadow; 2c- field margin between fodder beet crop and cereal crop; 2d- field margin between cereal crop and meadow; 2e- potato crop; 2f- fodder beet crop; 2g- cereal crop*

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DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

Treatments

Basic agronomic treatments, such as ploughing, harrowing, and fertilization, were done in the crop fields included in the entomological survey. In the potato crop field, the soil was fertilized with farmyard manure and a compound fertilizer, NPK(S) 6-20-30-(7). The following foliar herbicides were applied: 70% metribuzin and quizalofop-P-ethyl 50 g/l. To control the Colorado potato beetle (*Leptinotarsa decemlineata* Say), an insecticide containing zeta-cypermethrin was used. A concentrated boron fertilizer was additionally applied before row closure. The soil for the fodder beet crop was fertilized with a compound fertilizer, NPK(S) 6-20-30-(7). To improve the soil nutrient availability, a nitrogen fertilizer with boron was additionally applied. To control weeds, two herbicides were used: S-metolachlor 960 g/l and 43.0% chloridazon. After fodder beet emergence, the following foliar fertilizers were applied: a nitrogen-magnesium concentrate and boron ethanolamine. In the mixed cereal crop, a foliar herbicide containing S-metolachlor 960 g/l was used.

In the field margins separating the crop fields, the only agronomic treatment was single grass cutting after harvest of the crops in the adjacent fields.

Nomenclature and statistical analyses

Beetles were identified using the keys of Mroczkowski (1955) and Šustek (1981). The qualitative and quantitative characteristics of Silphidae caught were analyzed using zoocenological indices such as the dominance index (D), the Shannon-Wiener species diversity index (H'), and the Pielou species evenness index (J') (Shannon and Weaver, 1949; Pielou, 1966; Górny and Grüm, 1981). To evaluate the significance of differences in the number of species and individuals of carrion beetles (Col., Silphidae) trapped at the individual sites, a Chi-squared test was used (χ^2). To determine similarities between the assemblages of beetles caught, the following was used: (i) cluster analysis using the Ward agglomeration method, in which the Euclidean distance was accepted as a measure of similarity, and (ii) faunistic similarity using the Sørensen formula (P) (Magurran, 2004). The level of significance (the maximum allowable probability of type I error) was set at $\alpha \leq 0.05$. The obtained results were statistically analyzed using Statistica v.12 and Excel 2007 software.

Results

Results of investigation of the floral composition

The results of the phytosociological analysis of the field margins are shown in *Table 1*.

Generally, the number of plant species identified in the field margins was high, with the highest number in the field margin located next to the mixed cereal crop in Borek Stary. The percentage of plants in ground cover was there as much as 95%, while 66% of the species can be described as characteristic of hay meadows. These were predominantly the following grasses with varying fodder value: *Festuca rubra, Holcus lanatus, Agrostis capillaris, Dactylis glomerata,* and *Arrhenatherum elatius,* among which the following dicotyledonous herbs were found, among others: *Geranium pratense, Leontodon autumnalis, Plantago lanceolata*, and *Taraxacum officinale.* The percentage of papilionaceous plants was low and they accounted for 11% of the total plant cover.

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Table 1. *Floral composition of the field margins studied*

Study site				
	Borek Stary	**Widna Góra**		
Cover	**Field margin in cereal crop**	**Field margin in potato crop**	**Field margin in fodder beet crop**	**Field margin in cereal crop**
A	-	-	-	-
B	-	-	-	-
C	100% *Festuca rubra* 4, *Holcus lanatus* 3, *Agrostis capillaris* 2, *Dactylis glomerata* 2, *Potentilla anserina* 2, *Vicia sepium* 2, *Arrhenatherum elatius* 1, *Equisetum arvense* 1, *Bromus hordeaceus* +, *Capsella bursa-pastoris* +, *Cirsium arvense* +, *Convolvulus arvensis* +, *Deschampsia caespitosa* +, *Epilobium hirsutum* +, *Erigeron annuus* +, *Festuca arundinacea* +, *Geranium dissectum* +, *Geranium pratense* +, *Geum urbanum* +, *Leontodon autumnalis* +, *Luzula campestris* +, *Plantago lanceolata* +, *Poa pratensis* +, *Potentilla erecta* +, *Setaria viridis* +, *Stellaria graminea* +, *Taraxacum officinale* +, *Trifolium dubium* +, *Trifolium pratense* +, *Trifolium repens* +, *Urtica dioica* +, *Vicia cracca* +, *Vicia grandiflora* +, *Vicia tetrasperma* +, *Solidago gigantea* r.	95% *Arrhenatherum elatius* 4, *Dactylis glomerata* 3, *Achillea millefolium* 2, *Agrostis capillaris* 2, *Elymus repens* 2, *Glechoma hederacea* 2, *Holcus lanatus* 2, *Galinsoga parviflora* 1, *Taraxacum officinale* 1, *Aegopodium podagraria* +, *Apera spica-venti* +, *Artemisia vulgaris* +, *Calamagrostis epigejos* +, *Cerastium holosteoides* +, *Convolvulus arvensis* +, *Equisetum arvense* +, *Erigeron annuus* +, *Fallopia convolvulus* +, *Festuca arundinacea* +, *Galium mollugo* +, *Geranium dissectum* +, *Geum urbanum* +, *Gnaphalium uliginosum* +, *Leontodon hispidus* +, *Lolium perenne* +, *Plantago major* +, *Ranunculus repens* +, *Setaria viridis* +, *Solidago gigantea* +, *Thlaspi arvense* +, *Trifolium pratense* +, *Urtica dioica* +, *Vicia cracca* +.	80% *Elymus repens* 3, *Artemisia vulgaris* 2, *Fallopia convolvulus* 2, *Holcus lanatus* 2, *Chenopodium polyspermum* 1, *Agrostis capillaris* +, *Arrhenatherum elatius* +, *Chenopodium album* +, *Cirsium arvense* +, *Convolvulus arvensis* +, *Dactylis glomerata* +, *Erigeron annuus* +, *Festuca rubra* +, *Galinsoga parviflora* +, *Geranium dissectum* +, *Gnaphalium uliginosum* +, *Lolium perenne* +, *Phleum pratense* +, *Polygonum aviculare* +, *Polygonum persicaria* +, *Setaria viridis* +, *Stellaria media* +, *Tanacetum vulgare* +, *Brassica napus* var. *napus* r.	100% *Calamagrostis epigejos* 4, *Elymus repens* 3, *Urtica dioica* 3, *Artemisia vulgaris* 2, *Convolvulus arvensis* 2, *Solidago gigantea* 2, *Festuca arundinacea* 1, *F. rubra* 1, *Geum urbanum* 1, *Holcus lanatus* 1, *Achillea millefolium* +, *Aegopodium podagraria* +, *Agrostis capillaris* +, *Anthriscus sylvestris* +, *Armoracia rusticana* +, *Cirsium arvense* +, *Daucus carota* +, *Epilobium parviflorum* +, *Erigeron annuus* +, *Galeopsis tetrahit* +, *Galinsoga parviflora* +, *Glechoma hederacea* +, *Hypochaeris radicata* +, *Lamium album* +, *Lysimachia nummularia* +, *Phleum pratense* +, *Plantago lanceolata* +, *Polygonum aviculare* +, *Setaria glauca* +, *Taraxacum officinale* +.

A- tree layer, B- shrub layer, C- herb layer

The vegetation found in the field margin bordering the potato crop in Widna Góra also showed certain relationships with hay meadows. *Arrhenatherum elatius* and *Dactylis glomerata* were the dominant species there, whereas among dicotyledonous species the following grew most frequently: *Achillea millefolium, Glechoma hederacea*, and *Taraxacum officinale.* The percentage of segetal species in the ground cover of the studied patch was only 13%. These were typical weeds accompanying root crops.

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The vegetation found in the field margins adjacent to the fodder beet and mixed cereal crops in Widna Góra was distinguished by a different floristic composition. There, segetal and ruderal plants were found to have a distinctly higher percentage, both in terms of the number of species and cover. Most segetal species grew in the field margin located in the vicinity of the fodder beet crop, and these were the following, among others: *Elymus repens, Galinsoga parviflora, Chenopodium polyspermum, Ch. album, Fallopia convolvulus, Cirsium arvense, Polygonum persicaria*, and *P. aviculare*. They accounted for as much as 62% of the ground cover of the studied patch. The percentage of plants typical of hay meadows was small and they were primarily represented by *Holcus lanatus.*

The vegetation found in the field margin next to the mixed cereal crop was characterized by the highest presence of ruderal plants. Their percentage in the total number of species was 27%, while in the cover it was as much as 57%. Attention should be paid to *Solidago gigantea*, a species considered to be invasive in Poland, which occurred in large numbers (Tokarska-Guzik et al., 2012; Otręba and Michalska-Hejduk, 2014) and which was accompanied by *Calamagrostis epigejos,* a species that grows in great numbers in set-aside meadows and fields (Barabasz-Krasny, 2011; Kryszak et al., 2006). Among ruderal species, the following occurred: *Urtica dioica, Erigeron annuus, Geum urbanum, Anthriscus sylvestris*, and *Epilobium parviflorum*, while as regards field weeds, the following grew most frequently: *Elymus repens, Convolvulus arvensis*, and *Artemisia vulgaris*. Plants typical of hay meadows were found in small numbers.

Results of investigation of carrion beetles

A total of 5491 beetle individuals belonging to 13 Silphidae species were collected, and the highest number of beetles was found in the field margin in Borek Stary (*Table 2*). Statistically significant differences in the number of individuals trapped were found ($p < 0.05$), but no differences were found in the number of species ($p = 0.8228$). The subfamily Silphinae, with 4035 individuals and 7 species, was represented in greatest numbers. The sites where all identified species were found were the following: the field margin in Borek Stary, the field margin next to the potato crop, and the field margin adjacent to the mixed cereal crop in Widna Góra. At the same time, these were the sites that were characterized by the highest similarity (P = 100.0%). It was also observed that, in faunistic terms, the field margins investigated showed a greater similarity to one another than in the case of the field margin – adjacent crop field system (*Table 3*).

T. sinuatus was the species that was found in all the sampling areas and which dominated the cover-abundance distribution (3229 individuals) (*Fig. 3a*). Another species that was found in large numbers at all the sites was the eurytopic species *N. vespillo* which, likewise *T. sinuatus*, belonged to the eudominant class (*Table 3*; *Fig. 3b*). Moreover, a third eudominant was found to be present in the field margin next to the potato crop and in the margin next to the mixed cereal crop. These species were *S. obscura obscura* and *O. thoracicum,* respectively. *N. sepultor* was a taxon that showed the lowest numbers (11 specimens). The lowest number of identified species was found in the fodder beet crop (8 species).

To evaluate the species diversity, the commonly applied indices were used (*Table 2*). Generally, these coefficients showed higher values for the field margins than for the crop fields adjacent to them. The Shannon-Weaver index exhibits higher values for the less numerous assemblages, but with a good structure. The highest value of this index

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

(H′=0.83) was recorded in the field margin bordering the mixed cereal crop. It was the site where the highest number of species was found (13), but at the same time an even distribution of the dominance class (*Table 4*) and the lowest number of individuals (628 beetles). At this site, the evenness of species distribution also showed the highest value (J′ = 0.22).

Table 2. *Species composition, abundance, and species diversity index of Silphidae caught in crop fields (F) and field margins (M)*

Species	Borek Stary		Widna Góra					
	2009	2010	2014					
	Cereal		Potato		Fodder beet		Cereal	
	M	M	F	M	F	M	F	M
Oiceoptoma thoracicum L.	60	37	1	5	6	10	18	67
Phosphuga atrata atrata L.	14	7	0	2	5	3	30	11
Silpha carinata Hbst.	2	1	5	2	0	1	19	23
Silpha obscura obscura L.	76	53	16	66	41	13	30	42
Silpha tristis Ill.	16	29	0	10	0	4	0	3
Thanatophilus rugosus L.	17	13	5	7	4	15	5	12
Thanatophilus sinuatus F.	977	685	355	129	302	262	310	209
Nicrophorus humator Gleditsch	10	15	0	2	0	2	21	10
Nicrophorus interruptus Steph.	5	3	0	2	6	1	31	21
Nicrophorus investigator Zett.	0	2	2	4	0	1	0	14
Nicrophorus sepultor Charp.	0	1	1	3	0	0	2	4
Nicrophorus vespillo L.	243	231	69	87	103	64	194	170
Nicrophorus vespilloides Hbst.	26	21	3	8	7	12	13	42
Total – number of specimens	1446	1098	457	327	474	388	673	628
Total – number of species	11	13	9	13	8	12	11	13
Shannon-Weaver index (H')	0.59	0.54	0.34	0.70	0.47	0.50	0.68	0.83
Pielou's evenness index (J')	0.17	0.15	0.11	0.19	0.16	0.14	0.20	0.22

Table 3. *Similarity of Silphidae communities (%) at the study sites*

Study site			Borek Stary	Widna Góra					
			Cereal	Potato		Fodder beet		Cereal	
			M	F	M	F	M	F	M
Borek Stary	Cereal	M	-	63.6	100.0	76.2	96.0	91.7	100.0
Widna Góra	Potato	F	63.6	-	81.2	70.6	76.2	80.0	81.2
		M	100.0	81.2	-	76.2	96.0	91.7	100.0
	Fodder beet	F	76.2	70.6	76.2	-	70.0	84.2	76.2
		M	96.0	76.2	96.0	70.0	-	87.0	96.0
	Cereal	F	91.7	80.0	91.7	84.2	87.0	-	91.7
		M	100.0	81.2	100.0	76.2	96.0	91.7	-

F- crop fields, M – field margins

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

Table 4. *Dominance of Silphidae caught in crop fields (F) and field margins (M)*

Species	Borek Stary		Widna Góra					
	Cereal		Potato		Fodder beet		Cereal	
	M	M	F	M	F	M	F	M
Oiceoptoma thoracicum L.	SD	SD	SR	R	R	SD	SD	ED
Phosphuga atrata atrata L.	SR	SR	-	SR	R	SR	SD	R
Silpha carinata Hbst.	SR	SR	R	SR	-	SR	SD	SD
Silpha obscura obscura L.	D	SD	SD	ED	D	SD	SD	D
Silpha tristis Ill.	R	SD	-	SD	-	R	-	SR
Thanatophilus rugosus L.	R	R	R	SD	SR	SD	SR	R
Thanatophilus sinuatus F.	ED	ED	ED	ED	ED	ED	ED	ED
Nicrophorus humator Gleditsch	SR	R	-	SR	-	SR	SD	R
Nicrophorus interruptus Steph.	SR	SR	-	SR	R	SR	SD	SD
Nicrophorus investigator Zett.	-	SR	SR	R	-	SR	-	SD
Nicrophorus sepultor Charp.	-	SR	SR	SR	-	-	SR	SR
Nicrophorus vespillo L.	ED	ED	ED	ED	ED	ED	ED	ED
Nicrophorus vespilloides Hbst.	R	R	SR	SD	R	SD	R	D

ED- eudominants, D- dominants, SD- subdominants, R- recedents, SR- subrecedents

Figure 3. *Most numerous Silphidae. 3a- representative of Silphinae - T. sinuatus; 3b- representative of Nicrophorinae - N. vespillo*

In describing the assemblages of beetles, an important element is to analyze the dominance structure (*Table 4*). Among all the study sites, five dominant groups were only found in the field margin in Borek Stary in 2009 and in the field margin next to the

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cereal crop in Widna Góra. No dominant class was found at the other sites, except for the fodder beet crop. In turn, the highest number of subdominants was recorded in the mixed cereal crop. These were the following species: *O. thoracicum*, *P. atrata atrata*, *S. carinata*, *S. obscura obscura*, *N. humator*, and *N. interruptus*. Generally, the recedent and subrecedent classes were the most numerous groups as regards the species grouped in them.

The dendrogram of the species composition similarity of the study sites displayed significant differences (*Fig. 4*). Two main agglomerations that grouped the sites from the same localities were distinguished. Evidently, the field margins in Borek Stary distinguished themselves from the other study areas. The fodder beet crop and the field margin adjacent to it were the sites in the agriculturally used area in Widna Góra which were characterized by the greatest similarity.

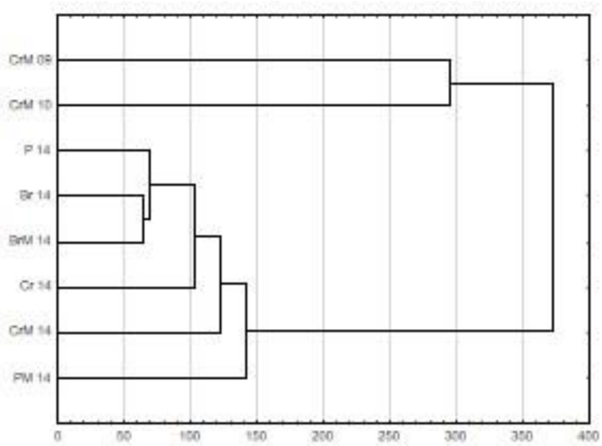

Figure 4. *Dendrogram of similarity of Silphidae caught at the study sites. CrM- field margin in cereal crop, BrM- field margin in fodder beet crop, PM- field margin in potato crop, Cr- cereal crop, P- potato crop, Br- fodder beet crop, years: 09- 2009, 10- 2010, 14- 2014*

Discussion

In the case of studies directly focused on the family Silphidae, bait traps are usually used (Novák, 1961, 1962; Petruška, 1968; Kamińska, 1980; Růžička, 1994; Kočárek and Benko, 1997). Due to the trophic structure and reproductive behavior of carrion beetles, such methodology gives better results of catches (Coyle and Larsen, 1998; Kočárek, 2000; Brousseau et al., 2010). This can be confirmed by the catches in the potato crop. 9 Silphidae species, represented by 457 individuals, were recorded in Widna Góra. On the other hand, in a previous cross-sectional study of the Subcarpathian region which was conducted using unbaited traps, a significantly lower number of Silphidae was trapped, both in qualitative and quantitative terms (Konieczna et al., 2014).

In the potato crop (similarly to all the other sites), the eudominant species were *T. sinuatus* and *N. vespillo*. Another important species in the dominance structure was *S. obscura obscura*, which made up the subdominant class (no dominants were found). Juen et al. (2003) found the above-mentioned species to be present in organic potato crops, but with a different order of dominance, since *S. obscura obscura* proved to be the most numerous species. But *T. sinuatus*, whose number was 355 specimens in Widna Góra, showed a very low number (3 ex.) in the study by Juen et al. (2003). In

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total, the above cited authors recorded 4 species in the imaginal stage (104 ex.) and 5 species in the pre-imaginal stage (86 ex. +11 ex. *Thanatophilus* sp.) in the organic potato crop. Both in qualitative and quantitative terms, the results of the investigations carried out in Widna Góra were distinctly different from the results of Juen et al. (2003), which may be associated with the use of bait traps. A publication by Aleksandrowicz (2002), which relates to the effect of spraying with the insecticide Decis on beetle assemblies occurring in potato crops, draws similar conclusions. In this research, *S. obscura obscura* was also the most numerous representative of Silphidae. *N. vespillo*, in turn, which had the status of eudominant in Widna Góra, was classified as a subrecedent in the study by Aleksandrowicz (2002).

In the fodder beet crop site, the lowest number of species (8 taxa) was identified among all the sampling areas. The number of representatives of the genus Nicrophorus was also lowest. The following three taxa were observed: *N. interruptus*, *N. vespillo*, and *N. vespilloides*. Different results were obtained by Novák (1961, 1962) and Petruška (1968). Their research was carried out in Czechoslovakia and related to the genus *Nicrophorus*. Novák (1961) showed 8 species to be present in a fodder beet crop. In a subsequent study, 7 taxa were found (Novák, 1962; Petruška, 1968). Compared to the study conducted in Widna Góra, the three mentioned species were also found in the studies of the above-mentioned authors. Both in the above-mentioned publications and in the present study, the dominant species was *N. vespillo*.

A study on the impact of application of organic fertilizers on beneficial entomofauna in sugar beet crops was conducted by Porhajašová et al. (2015). The Silphidae was the third largest beetle family found in this study (5.23% of the total assemblage), but it was dominated by Carabidae (63.86%) and Staphylinidae (20.07%). In this case, it can also be presumed that the absence of bait was one of the factors determining the catches of carrion beetles.

Among the crop fields studied, the site where the highest numbers of Staphylinidae beetles were found was the mixed cereal crop, where 673 individuals classified in 11 species had been trapped. As far as the number of taxa is concerned, Kamińska (1980) obtained different results. In wheat and barley growing fields, she demonstrated only 3 carrion beetle species (554 individuals). It is worth adding that, similarly to the study done in Widna Góra, in the research conducted by Kamińska (1980) the dominant species were *T. sinuatus* and *N. vespillo*. The same species were the most numerous group in terms of dominance in a study conducted by Tamutis et al. (2007). Their observations were focused on the occurrence of epigeic coleopterofauna in conventional and organic barley crops. This research revealed that, depending on the agricultural production system, the Silphidae catches were different. Under conventional cropping conditions, 6 carrion beetle species were found, represented by 660 individuals. In the organic crops, 5 species were found, but with a twice lower number of individuals (326). In a study carried out by Varvara and Zamfirensu (2009) in a wheat plantation, the trapped Silphidae accounted for 20.62% of the entire assembly of beetles caught, thus constituting the second largest family of this order. Shah et al. (2003), on the other hand, obtained completely different results for winter cereal crops. Only one species was recorded there – *N. vespillo*, represented by 6 individuals.

Monocotyledonous crops are characterized by a high and dense canopy, and mechanical agronomic treatments are limited to soil preparation before seeding and direct harvest. Such a habitat structure can create more favorable living conditions for beetles (even for temporary living). On the other hand, a lower number of individuals

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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was found at the other crop sites (the potato crop and the fodder beet crop), which may indicate the unattractiveness of the habitat, due to both the nature of cropping (a low crop canopy, monoculture, the lack of floristic diversity, a lower probability of finding food) and the intensity of agronomic operations performed.

As a result of the catches conducted in the field margins, the highest numbers of beetles were shown in the field margin next to the mixed cereal crop in Borek Stary. Over the two-year study period in this area, a total of 2544 specimens representing 13 species were captured. The same number of identified taxa was determined in the field margin adjacent to the potato crop and in the field margin next to the mixed cereal crop in Widna Góra, but with a lower number of individuals.

Field margins that exhibit both floristic and structural diversity promote increased insect biodiversity (Thomas and Marshall, 1999). Field margins, field boundary strips or parts of arable fields excluded from agricultural production are biodiversity refuges, while predatory entomofauna inhabiting there may contribute to biological pest control (Fry, 1995; Wratten, 1998). In the field margins bordering the mixed cereal crop, the faunistic similarity was 100.0% (Borek Stary and Widna Góra). The high numbers of Silphidae trapped at these sites can be associated with the nature of the adjacent crops.

The publications of Růžička (1994) as well as of Kočárka and Benko (1997) can be mentioned among the few studies regarding the occurrence of carrion beetles in field margins. Růžička (1994) described open spaces excluded from agricultural use. His observations were carried out, among others, in a field margin overgrown with *Urtica* and *Cirsium* and bordering a maize crop as well as on an edge of a barley crop bordering a hornbeam (*Carpinus*) coppice. At these sites, a total of 13 species were found, out of which 11 species were common with the species captured in the field margin in Borek Stary as well as in the field margin adjacent to the potato crop and the field margin adjacent to the mixed cereal crop in Widna Góra. The study by Růžička (1994) demonstrated the presence of *Aclypea opaca* (1 ex.) and *Dendroxena quadrimaculata* (1 ex.), which did not occur in this study. On the other hand, in our study two species were recorded which Růžička (1994) did not confirm to occur. These were the following species: *S. obscura obscura* and *S. carinata*.

In a field margin separating a barley crop and a maize crop, in turn, Kočárek and Benko (1997) found the presence of 226 individuals from 10 species, out of which 9 occurred both in Borek Stary and in Widna Góra. In their study, the most frequently occurring species were *T. sinuatus* and *N. vespillo*.

Conclusion

When analyzing the qualitative and quantitative structure of the Silphidae caught in the field margins relative to the crop fields adjacent to them, a general trend was noticed towards the occurrence of a higher number of individuals and a lower number of species at the environmentally poorer sites (the crop fields). In the field margins bordering the crops, on the other hand, lower numbers of individuals were recorded, but a higher number of taxa identified. Such a distribution of beetles confirms Thienemann's biocenotic rule (1920), according to which the increased diversity of a habitat entails a decline in the total number of individuals. On the other hand, the proportion of recedents and subrecedents, that is, individuals that are few in number but which are important in the aspect of biodiversity preservation, increases.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

Acknowledgements. We would like to thank Tomasz Olbrycht, PhD Eng, from Department of Agroecology, Faculty of Biology and Agriculture, Rzeszów University, for help to prepare the pictures of Silphidae.

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DOI: http://dx.doi.org/10.15666/aeer/1702_22972313

CHANGES IN CARBOHYDRATE CONTENTS IN TABLE POTATO TUBERS UNDER THE INFLUENCE OF SOIL CONDITIONER UGMAX

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(Received 31st Oct 2018; accepted 11th Jan 2019)

Abstract. The content of total sugars and reducing sugars in potato tubers is one of the most important quality features determining the suitability for direct consumption and for processing into food products. The objective of the study was to determine the effect of the soil conditioner UGmax on total sugars, reducing (simple) sugars and sucrose in potato tubers. Experimental material consisted of tubers of second early table potato grown in a field experiment conducted in central-eastern Poland. Following chemical analyses, statistical calculations were performed and they demonstrated a significant effect − an application of the soil conditioner UGmax contributed to an increase in the tuber content of reducing sugars compared with control potato tubers. The highest simple sugar content was found after three applications of the soil conditioner at the rate of 1.0 and 2.0 dm^3 ha^{-1}. The remaining carbohydrates determined in the study did not respond to UGmax application. Cultivars grown in the study differed significantly in terms of reducing sugar content only, more sugars being accumulated by Tajfun rather than Satina.
Keywords: *cultivars, reducing sugars, Solanum tuberosum, sucrose, total sugars*

Introduction

Plant production intensification and specialisation, which have led to reduced soil tillage, monoculture, intensive chemical protection, use of heavy machines/tools, shortage of natural manures, result in worsened physical, chemical and microbiological soil properties as well as limited availability of nutrients for plants (Malero et al., 2006). Soil environment is impoverished and lower yields are often produced. Due to this, the need arises to reduce the unfavourable influence and seek eco-friendly solutions. The idea of environment and consumer protection is becoming increasingly popular and seems to become a necessity (Mrówczyński and Roth, 2009). The soil conditioner UGmax, produced by 'Bogdan' Trade-Service Co, Ltd., is a microbiological product available on the Polish market and recommended for application in crop cultivation. It is an extract obtained from a special compost and used to enhance soil fertility. Microorganisms contained in the conditioner boost soil biological activity, increase the solubility of mineral compounds which plants find difficult to take up and promote humus formation (Trawczyński and Bogdanowicz, 2007; Dębska et al., 2016). It results in beneficial changes in soil physical and water status-related properties, pH and structure as well as better rooting system development (Kaczmarek et al., 2008; Piotrowska et al., 2012; Dębska et al., 2016) and, in consequence, increased higher

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

quality yields of crops (Dinesh et al., 2010; Kołodziejczyk, 2014; Pobereżny et al., 2015; Kowalska, 2016).

Potato is one of major agricultural plants which is a staple food for people worldwide and a crop which is classified as one of key plants responsible for maintaining food security. Potato is consumed fresh or processed in both developing and developed countries (Camire et al., 2009; Lisińska et al., 2009; King and Slavin, 2013). Potato tubers are highly nutritious as they contain plenty of starch, protein characterised by high dietetic value, vitamin C, B_3, B_6, minerals, in particular potassium, phosphorus, magnesium and iron. The crop is also a valuable source of health-promoting compounds such as dietary fibre, phenols and carotenoids (Slavin, 2013; Andre et al., 2014). Tuber chemical components are cultivar-related but are also affected by agronomic practices, water availability and thermal conditions (Abong et al., 2009; Grudzińska et al., 2014; Ozturk and Polat, 2016). As it has been suggested that plant protection products, fertilisers, biostimulants and microbiological products may cause changes in plant chemical composition, studies have been undertaken to check their effect on the nutritional value of agricultural produce (Sawicka and Pszczółkowski, 2005; Gugała et al., 2013). Hence the present study aimed at determining the effect of the soil conditioner UGmax on total sugars, reducing sugars and sucrose in table potato tubers.

Materials and methods

Experimental and Agronomic Management

Research results were obtained from a three-year field experiment conducted at the Zawady Experimental Farm of Siedlce University of Natural Sciences and Humanities (52°03′N; 22°33′, central-eastern Poland) on a soil classified as Luvisol, whose pH ranged from slightly acidic to acidic (4.81-5.91 pH in KCl) and which was characterised by a high to very high available phosphorus content, moderate to high potassium content and low magnesium content (*Table 1*) (Marcinek and Komisarek, 2011). The experiment was set up as a split-plot arrangement with three replicates.

Table 1. *Selected chemical properties of soil on which the experiment was conducted*

Study years	Acidity (pH in KCl)	Organic matter (g kg^{-1})	Available nutrients (mg kg^{-1} soil)		
			P	K	Mg
2008	4.99	15.4	90.64	124.5	41.0
2009	4.81	16.8	76.12	173.4	34.0
2010	5.91	18.1	73.48	112.1	45.0

The first factor was two mid-early cultivars of table potato – Satina and Tajfun (*Table 2*).

Table 2. *Description of potato cultivars used in the study*

Cultivars	Registration year	Origin	Maturity	Yield (t ha^{-1})	Utilization
Satina	2000	Germany	second early	43.9	canned, frozen, salads, boiled
Tajfun	2004	Poland	second early	49.8	boiled

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

The second factor was five methods of an application of the soil conditioner UGmax:

1. Control object - without the use of UGmax spraying with water.
2. UGmax - application before planting, at a dose of 1.0 $dm^3 \cdot ha^{-1}$.
3. UGmax - application before planting, at a dose of 0.5 $dm^3 \cdot ha^{-1}$ and double foliar application (10-15 cm potato plant height) at a dose 0.25 $dm^3 \cdot ha^{-1}$, (flowering) at a dose 0.25 $dm^3 \cdot ha^{-1}$.
4. UGmax - application before planting, at a dose of 1.0 $dm^3 \cdot ha^{-1}$ and double foliar application (10-15 cm potato plant height) at a dose 0.5 $dm^3 \cdot ha^{-1}$, (flowering) and at a dose 0.5 $dm^3 \cdot ha^{-1}$.
5. UGmax - double foliar application (10-15 cm potato plant height) at a dose 0.5 $dm^3 \cdot ha^{-1}$, (flowering) and at a dose 0.5 $dm^3 \cdot ha^{-1}$.

The conditioner contained: lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas,* yeast, actinomycetes as well as the following amounts (mg l) of macroelements and microelements: potassium − 2905, nitrogen − 1200, sulphur − 1000, phosphorus − 220, sodium − 200, magnesium − 100, zinc − 20, manganese − 0.3 - produced by Bogdan, Trade-Service Co., Poland (Trawczyński, 2007; Zarzecka and Gugała, 2013). Farmyard manure was applied at a rate of 25.0 t ha^{-1} in addition to mineral fertilisers used at the following rates: 44.0 kg ha^{-1} P (in the form of 46% triple superphosphate - Group Azoty Chemical Establishments Police SA, Poland), 124.5 kg ha^{-1} K (in the form of 60% potassium salt – Establishments Trading Commodities Siarkopol Sp. z o.o. Tarnobrzeg, Poland) and 100 kg N per 1 ha (in the form of 34% ammonium salt - Group Azoty Nitrogen Establishments Pulawy SA, Poland) applied in spring. Potatoes were planted at the spacing of 0.675 x 0.370 m in mid-April and harvested at physiological maturity in the first half of September. Weeds were controlled by means of a mixture herbicides: chlomazon (Command 480 SC - FMC Chemical sprl, Belgium) at a dose of 0.2 dm^3 ha^{-1} and linuron (Afalon Dispersive 450 SC – Agan Chemical Manufactures Ltd., Israel) at a dose of 1.0 dm^3 ha^{-1} applied 5-7 days prior to potato plant emergence. During the period of crop growth and development, potato blight was controlled using fungicides: metalaksyl-M 3.8% and mankozeb 64% (Ridomil Gold MZ 68 WG - Syngenta Crop Protection AG, Switzerland) – 2.0 kg ha^{-1} and mankozeb (Dithane 455 SC - lndofil Industries Limited, Republic of India) – 3.0 dm^3 ha^{-1}, and Colorado potato beetle was controlled using insecticides: chlotianidyna (Apacz 50 WG - Sumitomo Chemical Takeda Agro Co., Japan) at a dose 0.04 kg ha^{-1} and tiametoksam (Actara 25 WG - Syngenta Crop Protection AG, Switzerland) at a dose 0.08 kg ha^{-1}.

Determination of carbohydrates

Before harvest, tuber samples from 10 plants per plot were collected in all experimental units and used for chemical analyses. The analyses were carried out each year in triplicate, representing a total of 90 samples. Total sugars and reducing sugars were determined after harvest in the fresh matter of unpeeled potato tubers by means of the Schoorl-Luff method (Krełowska-Kułas, 1993). The method involves reduction, under alkaline conditions, of copper sulphate by reducing sugars contained in Luff solution at the boiling temperature. Basic solution (hydrolysate) was prepared. 12.5 g of potato tubers were grated, made up with water and boiled for 3 minutes. After cooling, the solution was transferred to a 300 ml flask and 5 ml potassium ferrocyanide as well as 5 ml aluminium sulphate were added. After stirring, filtration was performed using a soft filter.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

Total sugars were determined by Schoorl-Luff method. 25 ml of hydrolysate was placed in a 300 ml flask, 2.5 ml of concentrated HCl was added and the mixture was heated for 5 minutes at 68-72°C (hydrolysis of sugars to simple sugars occurred). The solution was cooled and, after adding potassium hydroxide KOH until constant pH was obtained in the presence of phenolphthalein, made up to 25 ml. Next, 12.5 ml Luff solution was added and followed by 12.5 ml hydrolysate, 25 ml acetic acid, 12.5 iodine solution and 27.5 ml hydrochloric acid. Then, the excessive iodine was titration titrated using sodium thiosulfate until light blue colour was obtained.

Determination of reducing sugars was performed by Schoorl-Luff method. 12.5 ml Luff solution and 12.5 ml hydrolysate were transferred to a 300 ml flack (no hydrolysis was conducted). Then, the procedure was the same as for total sugars.

Sucrose content was calculated by subtracting reducing sugars from total sugars after hydrolysis x 0.95. Analyses were performed at the Chemical Laboratory of the Department of Agrotechnology, Siedlce University of Natural Sciences and Humanities in Siedlce.

Statistical analysis

The results of the study were subjected to statistical analysis using the analysis of variance, and the significance of differences was assessed using Tukey test at the significance level of P = 0.05. Statistical calculations were performed in Excel using the authors' own algorithm based on the split-plot mathematical model.

Weather conditions

Weather conditions in individual study years, presented in *Figure 1* and *Table 3*, varied. Precipitation was the highest in 2010 when it was higher than the long-term sum by as much as 144.8 mm. The lowest rainfall was in 2009 when it was also unevenly distributed throughout the individual months of the growing season. By contrast, the year 2008 saw the most favourable rainfall pattern whereas 2010 was the warmest. The remaining growing seasons had temperatures similar to the long-term mean.

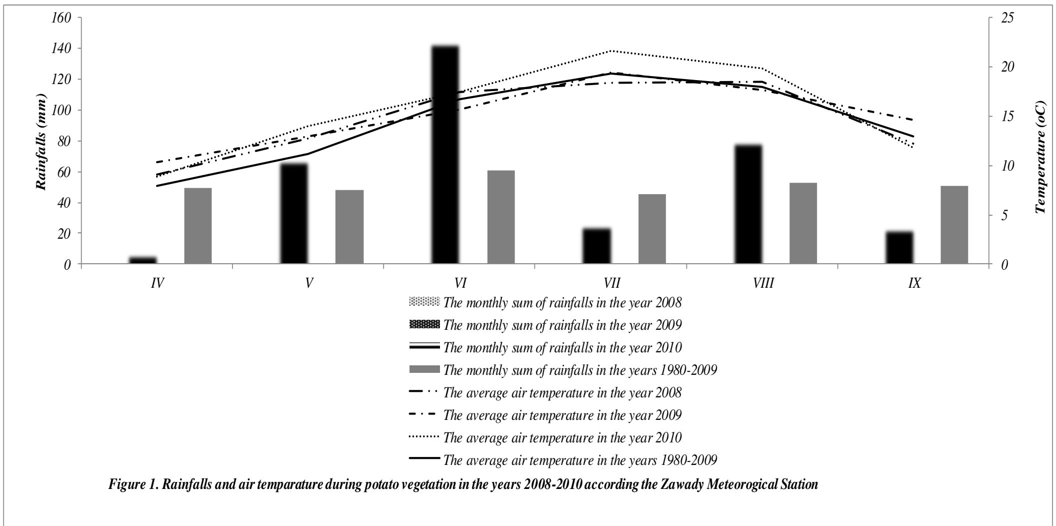

***Figure 1.** Rainfalls and air temparature during potato vegetation in the years 2008-2010 according the Zawady Meteorogical Station*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

Table 3. *Climatic conditions during the growing seasons according to the Zawady Meteorological Station*

Study years	Deviations from the long-term (1980–2009) mean						
	Total rainfall (mm)						
	IV	V	VI	VII	VIII	IX	IV-IX
2008	-21.4	+37.4	-11.7	+24.1	+22.4	+12.7	+56.5
2009	-41.5	+20.7	+84.5	-19.3	+27.9	-25.8	+39.5
2010	-38.9	+45.0	+1.9	+31.3	+53.3	+59.2	+144.8
	Air temperature (°C)						
	IV	V	VI	VII	VIII	IX	IV-IX
2008	+1.2	+1052	+0.7	-0.9	+0.5	-0.8	+0.3
2009	+2.4	+1.7	-1.0	+0.1	-0.3	+1.6	+0.7
2010	+1.0	+2.8	+0.7	+2.3	+1.8	-1.2	+1.2

Results

Total sugars

Statistical analysis of the obtained results demonstrated that total sugar content was significantly affected by the weather conditions throughout the study years (*Tables 4, 5*).

Table 4. *Total sugars content in potato tubers depending on UGmax and cultivars (g kg^{-1} fresh matter) (mean of years)*

Treatments (I)	Doses (dm^3 ha^{-1})	Cultivars (II)		Mean
		Satina	Tajfun	
1. Control – without UGmax	–	6.54	6.50	6.52
2. UGmax – before planting	1.0	6.55	6.59	6.57
3. UGmax – before planting and double foliar application	1.0	6.57	6.60	6.57
4. UGmax – before planting and double foliar application	2.0	6.61	6.64	6.63
5. UGmax – double foliar treatment	1.0	6.54	6.56	6.55
Mean		6.56	6.57	6.57

LSD at: 0.05 for: treatments (I) = ns; cultivars (II) = ns; interaction (I x II) = ns
ns – not significant

Table 5. *Total sugars content in potato tubers depending on UGmax and weather conditions (g kg^{-1} fresh matter) (mean of cultivars)*

Treatments (I)	Doses (dm^3 ha^{-1})	Years (III)			Mean
		2008	2009	2010	
1. Control – without UGmax	–	6.46	6.22	6.88	6.52
2. UGmax – before planting	1.0	6.49	6.25	6.98	6.57
3. UGmax – before planting and double foliar application	1.0	6.49	6.25	7.01	6.59
4. UGmax – before planting and double foliar application	2.0	6.50	6.29	7.09	6.63
5. UGmax – double foliar treatment	1.0	6.47	6.23	6.96	6.55
Mean		6.48b	6.25b	6.98a	6.57

LSD at: 0.05 for: treatments (I) = ns; years (III) = 0.31; interaction (I x III) = ns
ns – not significant

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

It averaged 6.57 g kg^{-1} fresh matter (0.657%), the value being lower than 1%, which is favourable from the standpoint of consumers. The accumulation of total sugars was the smallestt in tubers harvested in 2009 (6.25 g kg^{-1}) which was optimal in terms of hydrological conditions. In 2008, total sugar content was slightly but insignificantly higher compared with the 2009 growing season. An accumulation of total sugars was the highest in the wet and the warmest 2010, on average 6.98 g kg^{-1}, the differences being significantly higher compared with tubers harvested in 2008 and 2009. The cultivars grown in this experiment contained similar amounts of total sugars. In the study described here, a tendency was observed for tubers harvested from UGmax-treated plots to slightly increase total sugars. However, the values were very small and ranged from 0.03 to 0.05 g kg^{-1}.

Reducing sugars

In the present study, reducing sugars content ranged from 3.41–3.90 g kg^{-1} fresh matter and were significantly affected by cultivars, an application of the soil conditioner UGmax and weather conditions during the study years (*Tables 6, 7*).

Table 6. *Reducing sugars content in potato tubers depending on UGmax and cultivars (g kg^{-1} fresh matter) (mean of years)*

Treatments (I)	Doses (dm^3 ha^{-1})	Cultivars (II)		Mean
		Satina	Tajfun	
1. Control – without UGmax	–	3.44	3.47	3.46c
2. UGmax – before planting	1.0	3.51	3.51	3.51abc
3. UGmax – before planting and double foliar application	1.0	3.52	3.55	3.53ab
4. UGmax – before planting and double foliar application	2.0	3.57	3.57	3.57
5. UGmax – double foliar treatment	1.0	3.47	3.51	3.49bc
Mean		3.50b	3.52a	3.51

LSD at: 0.05 for: treatments (I) = 0.06; cultivars (II) = 0.01; interaction (I x II) = ns
ns – not significant

Table 7. *Reducing sugars and sucrose content in potato tubers depending on UGmax and weather conditions (g kg^{-1} fresh matter) (mean of cultivars)*

Treatments (I)	Doses (dm^3 ha^{-1})	Years (III)			Mean
		2008	2009	2010	
1. Control – without UGmax	–	3.45	3.74	3.18	3.46c
2. UGmax – before planting	1.0	3.52	3.80	3.22	3.51abc
3. UGmax – before planting and double foliar application	1.0	3.53	3.84	3.23	3.53ab
4. UGmax – before planting and double foliar application	2.0	3.55	3.90	3.27	3.57a
5. UGmax – double foliar treatment	1.0	3.47	3.79	3.22	3.49bc
Mean		3.50b	3.81a	3.22c	3.51

LSD at: 0.05 for: treatments (I) = 0.06; years (III) = 0.18; interaction (I x III) = ns
ns – not significant

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

Cv. Tajfun accumulated more reducing sugars than Satina. The soil conditioner promoted an accumulation of reducing sugars, significant differences being detected for treatment 3 and 4, that is when UGmax had been applied three times. Under the weather conditions of 2010, the concentration of these sugars was the lowest and so the most favourable compared with the remaining study years. It was mainly due to the air temperature which was by 2.3 and 1.8°C higher towards the end of the growing season (July and August).

Sucrose

Sucrose content in potatoes was significantly affected by weather conditions in the study years (*Tables 8, 9*). The concentration of this disaccharide was the lowest in 2009 whereas in the remaining years it was significantly higher. Cultivars grown in the present study accumulated a similar amount of sucrose. An interaction of cultivars and soil conditioner application regime was found, which indicates a different response of the cultivars to spraying with UGmax. There was also observed an interaction of UGmax application regime and study years. The least sucrose was recorded in 2009 in tubers harvested from UGmax-treated plots which were sprayed three times at the rate of 2.0 dm^3 ha^{-1}.

Table 8. *Sucrose content in potato tubers depending on UGmax and cultivars (g kg^{-1} fresh matter) (mean of years)*

Treatments (I)	Doses (dm^3 ha^{-1})	Cultivars (II)		Mean
		Satina	Tajfun	
1. Control – without UGmax	–	2.95a	2.87a	2.91
2. UGmax – before planting	1.0	2.89b	2.92a	2.91
3. UGmax – before planting and double foliar application	1.0	2.90ab	2.90a	2.90
4. UGmax – before planting and double foliar application	2.0	2.89b	2.91a	2.90
5. UGmax – double foliar treatment	1.0	2.92ab	2.90a	2.91
Mean		2.91ab	2.90a	2.91

LSD at: 0.05 for: treatments (I) = ns; cultivars (II) = ns; interaction (I x II) = 0.05
ns – not significant

Table 9. *Sucrose content in potato tubers depending on UGmax and weather conditions (g kg^{-1} fresh matter) (mean of cultivars)*

Treatments (I)	Doses (dm^3 ha^{-1})	Years (III)			Mean
		2008	2009	2010	
1. Control – without UGmax	–	2.86a	2.35a	3.52b	2.91
2. UGmax – before planting	1.0	2.82a	2.33a	3.58ab	2.91
3. UGmax – before planting and double foliar application	1.0	2.81a	2.30a	3.60ab	2.90
4. UGmax – before planting and double foliar application	2.0	2.81a	2.27a	3.63a	2.90
5. UGmax – double foliar treatment	1.0	2.86a	2.32a	3.56ab	2.91
Mean		2.83b	2.31c	3.58a	2.91

LSD at: 0.05 for: treatments (I) = ns; years (III) = 0.12; interaction (I x III) = 0.09
ns – not significant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

Discussion

In potato production, the most important characteristics which determine fresh tuber consumption suitability and processability include: dry matter content, total sugars (glucose + fructose + sucrose) and reducing sugars (glucose + fructose) which are also called simple sugars. Tubers ought to contain 18-22% dry matter and up to 1% total sugars (when this level, is exceeded, tubers taste sweet) (Lisińska et al., 2009). The results of the research showed that the total sugar content depended only on weather conditions. Total sugars and sucrose content were the highest in tubers harvested in 2010 when both precipitation and temperature during the growing period were the highest, the lowest values for both the traits being recorded in 2009 when precipitation was the lowest. A similar response of tubers to weather conditions, in terms of total sugar accumulation, was observed by other authors (Sawicka and Pszczółkowski, 2005; Zarzecka and Gugała, 2009). Cultivars (Satina and Tajfun) grown in the experiment discussed here contained similar amounts of total sugars, which is consistent with results reported by other workers (Gugała et al., 2013) although Bhattacharjee et al. (2014) found significant differences between cultivars being compared as to their tuber content of total sugars. There is no published literature concerning the effect of UGmax on total sugars, reducing sugars or sucrose in potato tubers.

The concentration of reducing sugars in the tubers of potato for processing for foodstuffs is one of the most important qualitative characteristics, and also called a priority. As a result of long-term studies conducted in Poland and worldwide (Coop et al., 2000; Lisińska et al., 2009; Zgórska, 2013; Grudzińska et al., 2014), the following baseline and optimal concentrations of reducing sugars in potato tuber fresh matter have been determined: not more than 0.25% (optimum level: up to 0.15%) for potatoes destined for chips production, not more than 0.50% (optimum level: up to 0.25%) for French fries varieties, and not more than 0.50% (optimum level: up to 0.25%) for dried products and fresh consumption. Tubers of potato which contain increased amounts of reducing sugars (glucose and fructose) turn brown when heated. In the present studies, reducing sugars content depended significantly on the cultivar, soil conditioner UGmax application and moist-thermal conditions during potato vegetation.The effect of potato cultivar on this characteristic was reported by other authors (Abong et al., 2009; Bhattacharjee et al., 2014; Grudzińska et al., 2014). On the contrary, Sawicka and Pszczółkowski (2005), Gugała et al. (2013) found no such influence. Grudzińska et al. (2014) reported that reducing sugar content was primarily affected by the air temperature 10 days before potato harvest. Moreover, the authors (Grudzińska et al., 2014) found, based on correlation coefficients which they had calculated, that the higher temperature was observed 10-20 days before harvest, the lower concentration of reducing sugars was recorded. A statistically significant effect of hydrothermal conditions on an accumulation of reducing sugars was also noted by other authors (Gugała et al., 2013; Brazinskiene et al., 2014). Cultivars grown in the present study accumulated similar amount of sucrose as in the findings reported by other authors (Sawicka and Pszczółkowski, 2005; Brazinskiene et al., 2014; Czerko et al., 2016).

Many authors have shown that the UGmax soil conditioner had a desirable, beneficial effect on the total and commercial yield of potato tubers, tuber weight per potato (Trawczyński and Bogdanowicz, 2007; Kowalska, 2016; Zarzecka and Gugała, 2016), increased starch and vitamin C content (Zarzecka et al., 2018) and improved soil chemical properties (Trawczyński and Bogdanowicz, 2007, Dębska et al., 2016) as compared to the control.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

Conclusion

The application of soil conditioner UGmax significantly increased the content only of reducing sugars in potato tubers, whereas the total sugars and sucrose content did not change. However, the largest, significant influence on the content of carbohydrates (total sugars, reducing sugars, sucrose) had weather conditions during the years of research. The cultivars grown in this experiment can be used in direct consumption, for dried products and for processing into French fries and soil conditioner UGmax used did not cause adverse changes in the chemical composition of tubers.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23152324

STATUS OF DIEBACK OF *JUNIPERUS PROCERA* (AFRICAN PENCIL CEDAR) IN NATURAL STANDS AND PLANTATION IN ALSOUDA HIGHLANDS, SAUDI ARABIA

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(Received 8th Nov 2018; accepted 11th Jan 2019)

Abstract. The natural habitat of *Juniperus procera* is a unique ecosystem in Saudi Arabia. The ecosystem of Aseer highlands is an important habitat for flora and fauna. Alsouda Highlands, situated in the south-western part of Saudi Arabia, is claimed to be the highest peak in Saudi Arabia. These forests suffered varying degrees of degradation. Juniperus trees in these highlands have experienced widespread decline with substantial dieback for 35 years. The aim of the study is to evaluate the status of *J. procera* dieback in Alsouda natural stands (2400-2950 m). The results showed that >80 % *J. procera* trees within the natural stands had symptoms of dieback but >65% was not severely affected. Tree recovery signs of new lateral shoots, leaves, sprouts and flowering were evidently observed even among the severely affected trees. The plantation showed no symptoms of dieback, but their growth rate was relatively low. The NDVI showed an increasing trend of greenness with time, especially in the seasons with high rainfall. The effect of slope aspect was evident in the severity of dieback and greenness of the vegetation as detected by the NDVI data. The west facing slopes were higher in NDVI values than the east-facing slopes, due to higher precipitation in the west – facing slopes by the southwestern monsoons. The results indicate significant effect of seasonal rainfall with respect to tree health.
Keywords: *African pencil cedar, natural stands, plantations, new regeneration, NDVI, Aseer region*

Introduction

Juniperus procera Hochst. ex. Endl. (Cupressaceae), known as African pencil cedar and Arar in Arabic, is the world's largest juniper, evergreen, dioecious and wind pollinated forest tree species (Farjon, 2005; Couralet and Bakamwesiga, 2007; Negash and Kagnew, 2013). It spreads naturally in the highlands above 1800 m.a.s.l., but can occurs in a broader range of 1000–3500 m.a.s.l., with an average annual temperature range from 5 °C to 20 °C (Couralet and Bakamwesiga, 2007). It is listed in the IUCN red list of the threatened species due to overexploitation and global decline in its populations (Farjon, 2013).

The species is the dominant forest tree in the southwestern highlands of Aseer Region Kingdom of Saudi Arabia (KSA) at elevations from 2000 to 3000 m.a.s.l (El-Juhany et al., 2008; El-Juhany, 2015), and the density decreases in the subsequent lower elevations (El-Juhany and Aref, 2013). In addition to aesthetic values, the Juniper natural stands are important component of the highland ecosystems in Aseer (Aref et al., 2013) as they enhance biodiversity, reduce soil erosion, conserve rainfall water, and provide wood products (Aboulfatih et al., 1989).

Dieback symptoms and low rate of natural regeneration of *J. procera* were observed in Alsouda, Aseer Region during the early 1980's (El-Juhany, 2015). Symptoms were

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DOI: http://dx.doi.org/10.15666/aeer/1702_23252338

up to 50% among trees in some parks, in addition to absence of new regeneration (Barth and Horst Strunk, 2000). It was attributed to climate change (Fisher, 1997), soil erosion and water run (Aref et al., 2013; El Atta et al., 2013), deprived eroded-soil from litter and mycorrhiza (Barth and Horst Strunk, 2000) and human interferences (Gardner and Fisher, 1994). Highlands in Aseer region had experienced warming trends (Krishna 2014) and decreasing annual rainfall (Hosny and Almazroui, 2015). Dieback were reported in Juniper ecosystems in the Arabian Peninsula and south-west Asia (Fisher, 1997), and parts of Africa (Borghesio et al., 2004).

Globally, Forest dieback and decline are significant threat to biodiversity in forest ecosystems with serious economic and aesthetic impacts (McKinneya et al., 2014. Climate and weather stresses were identified prime causes for water stresses and tree declines (Auclair, 1993; Rice et al., 2004; Jurskis, 2005). Climate change was reported to l affect *J. procera* future distribution in northern Ethiopia (Abrha et al., 2018). Natural pests and diseases causes can affect managed- forest condition and stability (Freer-Smith, 2007). Biotic factors are generally considered as contributing secondary factors, however they were primary causes for dieback in English woodlands (Woodward and Boa, 2013), Spruce forest (Kana et al., 2013), and Juniper forests (Borghesio et al., 2004). Study in Ethiopia showed that *J. procera* trees were tolerant and less affected by cypress aphid compared to *cupressus lusitanica* (Demeke, 2018).

The Juniper Forest in Asir is the only dense forest in the Kingdom and is particularly important from the point of view of conservation. Of particular concern is the widespread decline of *Juniperus procera* on exposed lower slopes with some stands consisting mainly of dead or dying trees. Hence, the aim of this study was to evaluate the health status of *J. procera* and extent of dieback in southwestern highlands of KSA. The objectives were to determine the severity of dieback within natural stands and plantations in Alsouda area, and temporal trends of tree health using satellite images in relation to annual rainfall and slope aspects.

Materials and methods

Study area

The study was conducted in Alsouda Area in Aseer Region, in the southwestern highlands of KSA, which is an important touristic area with the richest plant biodiversity in Aseer Region and KSA (Vincent, 2008). The study site is elongated in shape and dissected by small valleys with a total area of 291 km^2 (*Fig. 1*) and dominated by *J. procera* trees. The satellite image of Landsat depicted in FCC (False color composite (RGB) in order to observe the features clearly. The site was delineated based on topography and maximum stocking density of Juniper trees. It is located between latitude of 18° 8' 43.81" N and 18° 27' 32.00" N and longitude of 42° 18' 0.559"E and 42° 28' 58.317"E and elevations 2400 to 2950 m.a.s.l. As part of the Arabian shield in the western part of the Saudi Arabia, the topography is undulating and characterized by natural geological erosion and sedimentation phenomena (Mallick et al., 2014; Youssef et al., 2016). The dominate type of soil classes are loamy sand, sandy loam, and loam (Mallick, 2016). Alsouda receives the highest amount of annual rainfall in KSA by southwestern monsoons, with variable annual patterns (Vincent, 2008). Precipitation is mainly from March to June during spring and summer growing seasons (Wheater et al., 1989), and minimum and maximum temperature are 19.3 °C and 29.70 °C, respectively.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23252338

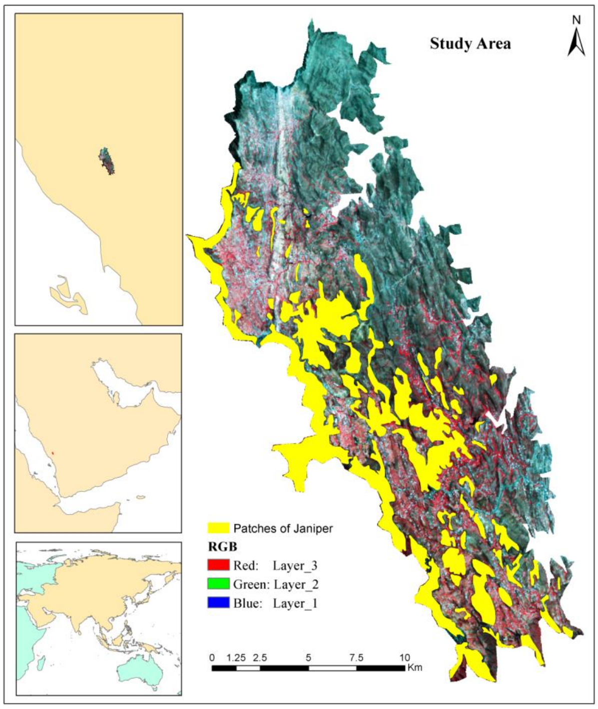

Figure 1. *Study area*

Data collection

Site-based surveys and remote sensing techniques were used as proven valuable tools to vegetation condition across a spatial and temporal range (Vogelmann et al., 2012).

Field survey

Ground surveys were conducted during October 2017 to March 2018 for the *J. procera* natural stands and plantations. According to an initial reconnaissance survey, the natural stands were divided into two aspects as east and west-facing slopes. Systematic random sampling was carried out along six transect lines in each aspect, with a total of 188 trees in the east slope and 201 in the west aspect. Tree dieback was evaluated at sampling point by the first author on ordinal scale from "1" healthy tree without obvious symptoms to "6" severely affected or dead tree (*Fig. 2*). Trees in the four plantation plots, numbered from 1 to 4, were measured for dieback symptoms, tree height and crown width. The nursery raised-seedlings were planted in 2006 with a spacing of about 3 m × 3 m. The number of measured trees 51, 73, 33 and 54 in plots 1, 2,3 and 5, respectively.

Figure 2. *Ordinal scale of 1 to 6 (1= healthy, no signs of dieback and 6 = severe or dying)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23252338

Meteorological data

Rainfall data was obtained from the meteorological station situated within the study area (Al-Sooda Station no. 00028, 18° 15' 08" N (latitude) and 42° 24' 15.7" E (longitude). Whereas temperature data, the meteorological station located towards the 20 km of south-west. (Abha Station no. 41112, 18° 14' 25" N (latitude) and 42° 39' 11" E (longitude). The data related to the rainfall and temperature were operated and maintained by Ministry of Environment, Water and Agriculture and Presidency of Meteorology and Environment (PME), Saudi Arabia respectively. The elevation of both meteorological stations is 2585 and 2125 m from the m.s.l. respectively. The rainfall dataset analysed for the period 1965–2017, whereas temperature datasets for 1978-2017.

Remote sensing data processing

Satellite datasets of LANDSAT TM and LANDSAT-8 level-1TP (Level 1TP product contains systematically, radiometrically, geometrically, and topographically corrected using GCPs) over Aseer region were used for the preparation of the Normalized Difference Vegetation Index (NDVI) and Land use and land cover (LULC) maps. Digital number (DN) data converted to at-sensor radiance for LANDSAT data (https://landsat.usgs.gov/landsat-8-l8-data-users-handbook). ENVI-FLASSH (Fast Line-of-sight Atmospheric Analysis of Spectral Hypercubes) an atmospheric correction module was used to retrieve spectral reflectance.

Conversion of DNs to physical units

At-sensor radiances measured at a wavelength spectrum which captures the image in DNs values, which have no unit and any physical implication; therefore, it is essential to coverts to radiance and reflectance.

OLI and TIRS at sensor spectral radiance

Satellite images were processed in units of absolute radiance using 32-bit floating-point calculations and the values were converted to 16-bit integer values. It was then converted to spectral radiance using the radiance scaling factors provided in the metadata file of LANDSAT-8 (*Eq. 1*):

$$L_\lambda = M_L * Q_{cal} + A_L \quad \text{(Eq.1)}$$

where:

L_λ = Spectral radiance ($Wm^{-2}sr^{-1}\mu m^{-1}$)

M_L = Radiance multiplicative factor for the band (RADIANCE_MULT_BAND_n from the metadata).

A_L = Radiance additive factor for the band (RADIANCE_ADD_BAND_n from the metadata).

Q_{cal} = Pixel value in DN

Atmospheric correction

ENVI-FLASSH (Fast Line-of-sight Atmospheric Analysis of Spectral Hypercubes) an atmospheric correction module was used to retrieve spectral reflectance. The

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standard atmospheric (tropical atmosphere) and the geometric parameters (such as aerosol, CO_2, azimuth, zenith angle, square slit, etc.) were calculated using the FLAASH module (Detail could be found in the weblink). The atmospheric parameters (such as water vapor and Aerosol Optical Depth (AOD)) procured from the AERONET (AErosol RObotic NETwork) and incorporated into the FLAASH module.

Normalized difference vegetation index (NDVI)

NDVI is one of the band ratios that are highly correlated with vegetation parameters such as green leaf biomass and green leaf area. It quantifies vegetation by measuring the difference between near-infrared (which vegetation strongly reflects) and red light (which vegetation absorbs). NDVI values ranges from -1 to +1. As shown below, NDVI uses the NIR and red channels in its formula:

$$NDVI = (Infrared – Red)/(Infrared + Red)$$

Moreover, it reduces variation due to surface topography and compensates for variation in radiance as a function of sun elevation for different parts of the scene, which is highly valuable in regional studies. To demonstrate the utility of Landsat data for monitoring vegetation status at the regional scale, random temporal plots are selected and analyzed for the NDVI values. The maximum NDVI is calculated to determine the index of greenness of the vegetation at the time of peak growth season. It is calculated to represent the maximum foliage cover in the study period. Overall, NDVI is a standardized way to measure healthy vegetation, where higher NDVI values indicate healthier vegetation.

Table 1 shows the satellite data acquisition and its related information. The NDVI data were converted into monthly values using the maximum value composite method to reduce cloud disturbance and increase the overall quality of the dataset (Fensholt and Proud, 2012).

Statistical analysis

Trends of annual rainfall and temperature series data were analyzed using Mann-Kendall trend test, and Mann-Whitney U test was used for the dieback ordinal data. Differences among the four plantation plots were analyzed using one-way analysis of variance procedure.

Table 1. *Characteristics of satellite data used in the study*

Satellite/sensor	Date of acquisition	Path/row	Spatial resolution	Information extracted
Landsat TM	1988-06-12	167/47	30 m (Band3 and Band4)	NDVI
Landsat TM	1993-04-07; 1993-06-26	167/47	30 m (Band3 and Band4)	NDVI
Landsat TM	1998-03-20; 1998-04-21; 1998-05-07; 1998-06-08;	167/47	30 m (Band3 and Band4)	NDVI
Landsat TM	2000-03-09; 2000-04-10; 2000-04-26; 2000-05-28; 2000-06-13	167/47	30 m (Band3 and Band4)	NDVI
Landsat-8	2016-03-05; 2016-03-21; 2016-04-06; 2016-04-22; 2016-05-08; 2016-05-24; 2016-06-09	167/47	30 m (Band4 and Band5)	NDVI

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Results

Dieback of J. procera in natural stands

Most of the trees in the west and east facing slopes had shown dieback symptoms dieback with varying extent of severity (*Fig 3*). The effect of slope aspect was significant (Pr = 0.03), and the west-facing slopes were less affected than the east-facing slopes. The west-facing slopes had significantly fewer trees with severe dieback symptoms (4%), and more trees without obvious symptoms (17%) compared to the east facing slopes (8% and 11%; respectively) (*Fig. 3*).

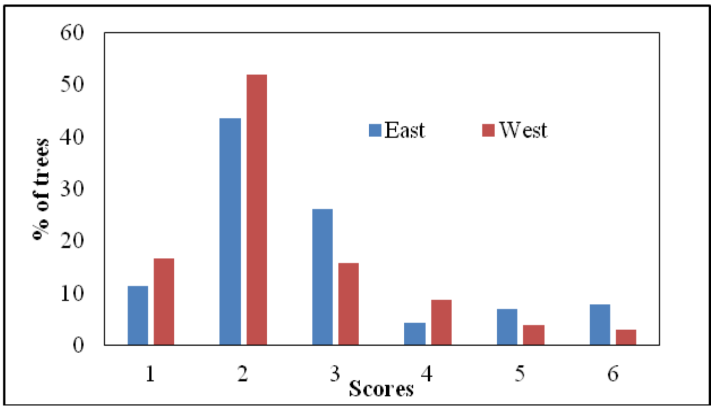

Figure 3. *Percent of J. procera according dieback symptoms in the west and east facing slopes in Alsouda (1 = no symptoms to 6 severely affected dying or dead trees, significant difference between east and west facing slopes Pr = 0.03 using Mann-Whitney U test)*

All of the trees with scores 2 to 5 and some of severely affected trees showed recovery signs and healthy lateral branches. The recovery signs include new lateral shoots, new needles and flowers at different stages (*Fig. 4*). Also, new regenerates were spotted in the well- stocked Juniper stands.

Figure 4. *Recovering J. procera trees from dieback in Alsouda natural stands: A and B) new shoots and leaves C) sprouts and D) new regenerates*

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J. procera plantation plots

The 12- year's trees showed no signs of dieback symptoms in the four plantation plots in Alsouda (*Fig. 5*). Difference among the four plots was significant (Pr < 0.001) in height and crown diameter, where plot 2 had significantly shorter trees and smaller crown diameters than the other 3 plots plants (*Table 2*).

Table 2. *Tree height and crown width (cm) of 12-years J. procera plantations in Alsouda, Aseer region*

Plot number	Number of trees	Tree height and SE	Crown width and SE	Dieback symptoms
1	51	254 cm ± 9.5 A	191 cm ± 6.9 A	No symptoms
2	73	193 cm ± 7.6 B	153 cm ± 6.6 B	No symptoms
3	33	234 cm ± 12.3 A	173 cm ± 8.8 A	No symptoms
4	97	246 cm ± 8.5 A	180 cm ± 6.7 A	No symptoms

Figure 5. *Row of planted J. procera from nursery raised seedling in 2006, Alsouda National Park, Aseer Region*

Meteorological data

Total annual rainfall was variable among the years from 1965 to 2017, and the trend was not significant (*Fig. 6a*). However, maximum, minimum, and annual temperature showed increasing trend (Pr < 0.01) during the period of 1978 to 2017 (*Fig. 6b*).

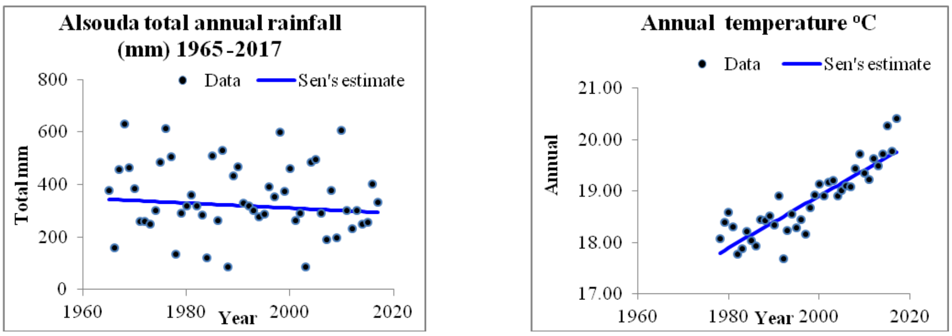

Figure 6. *A) Alsouda annual amount of rainfall during 1965 to 2017 (Pr > 0.05), and B) Abha annual average temperature during 1978 to 2017 (highly significant increasing trend)*

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NDVI results

Figures 7 and *8* illustrate an increasing of NDVI values from 1988 to 2016, with a significant drop in 2000 that witnessed drier rainy season. The pattern of NDVI class distribution showed greener narrow western strips *J. procera* (NDVI > 0.51) that extend from south to north with gradual decrease in the NDVI values from west to east. Dissecting the area according to aspect illustrates clear and marked trend of higher values in the west facing slopes (*Tables 3* and *4*).

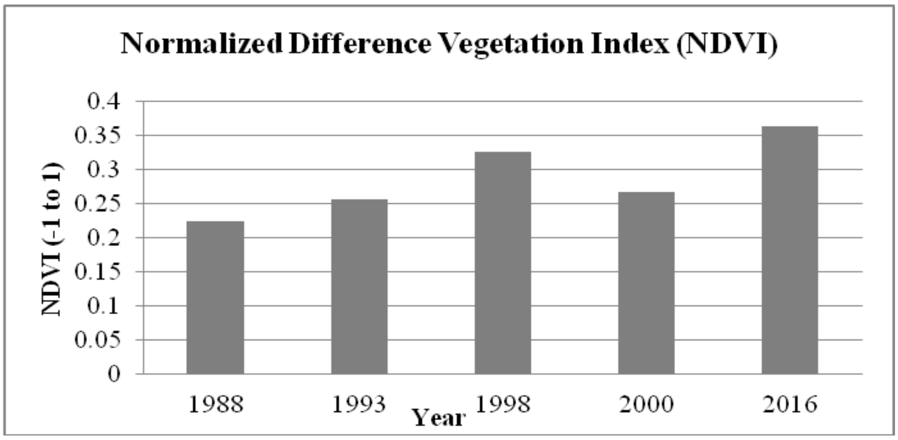

Figure 7. *Mean NDVI value of the study area in 1988, 1993, 1988, 2000 and 2016*

Discussion

J. procera is the most important indigenous tree species in the forest ecosystems of the southwestern highlands (above 2000 m.a.s.l) KSA for its ecological, economically and aesthetic values. It is an essential component in ecotourism attractions of Alsouda, Aseer Region, which is the most frequented touristic site during summer time (June to August) in KSA.

Tree health and stocking density are of vital importance, and dieback is the main problem facing forest ecosystems in KSA. El-Juhany (2015) estimated that 11.5% of the *J. procera* trees in Aseer were affected by dieback. The results of this study showed that many trees have dieback symptoms (84% and 89% in the West and east facing- aspect, respectively). However, the severely affected trees were 7% and 15% in the west and east facing slopes, respectively. Tree recovery signs were in most of the affected trees with new needled, branches, and sprouts. Unlike (Barth and Horst Strunk, 2000), natural regeneration was detected in the well-stocked and remote slopes, indicating the importance of nursing trees (Senbeta and Teketay, 2001). The NDVI pattern and classes confirmed recovering trends Juniper Natural stands.

The 12-year trees in the four plantation plots were free from dieback symptoms, and reached an average height of 2.0-2.5 m and an average crown diameter of 1.7-1.9 m. Tree height is less than the 7-year old plantation in the highlands of Ethiopia (Negash and Kagnew, 2013). This comparatively slow growth may be due low mean annual rainfall of Alsouda (350) compared to 1140 mm in Ethiopia highlands.

Topography and aspect of slopes have significant influences on the microclimate and soil moisture content. Måren et al. (2015) attributed differences in vegetation of *Pinus* and *Juniperus* species to soil characteristics between slope aspects in high altitude of semiarid environments.

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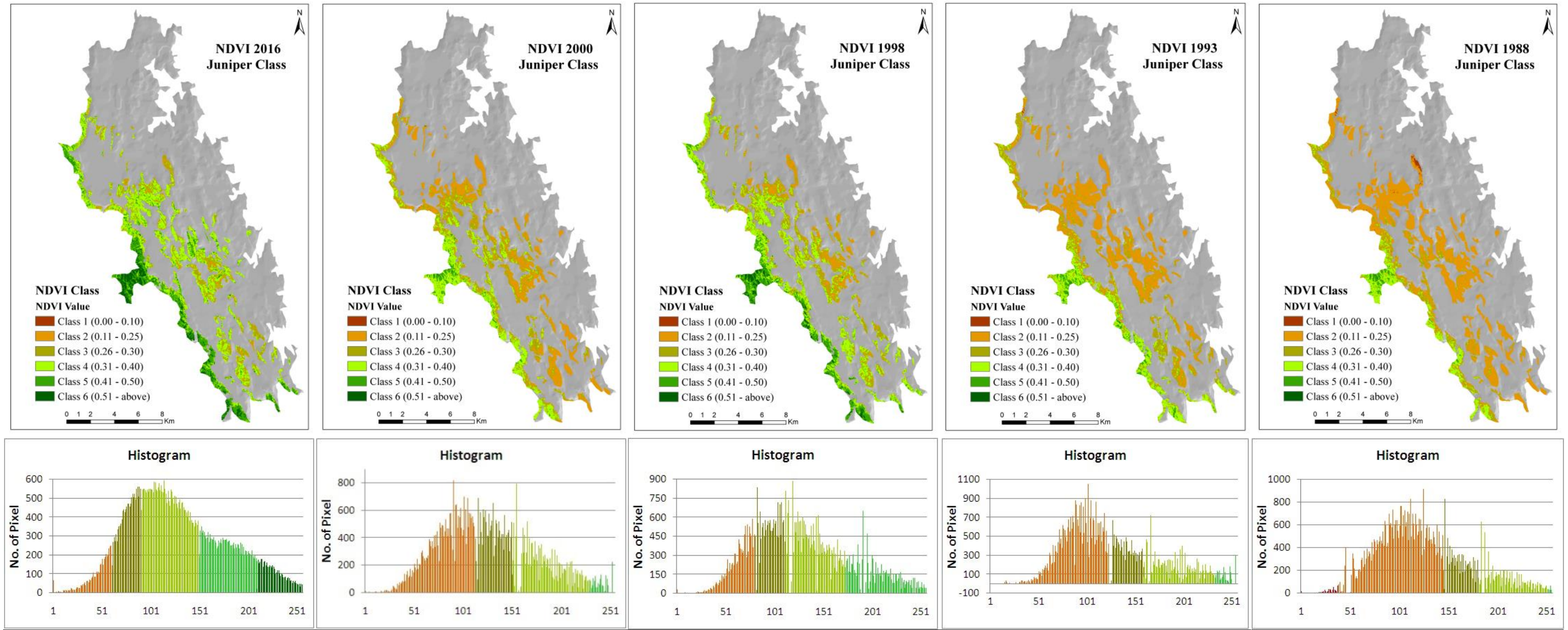
DOI: http://dx.doi.org/10.15666/aeer/1702_23252338



Parameters	Values	Parameters	Values	Parameters	Values	Parameters	Values	Parameters	Values
NDVI Min	0.080	NDVI Min	0.048	NDVI Min	0.042	NDVI Min	0.011	NDVI Min	0.003
NDVI Max	0.693	NDVI Max	0.531	NDVI Max	0.654	NDVI Max	0.524	NDVI Max	0.518
NDVI Mean	0.364	NDVI Mean	0.268	NDVI Mean	0.326	NDVI Mean	0.257	NDVI Mean	0.225
NDVI Std.	0.086	NDVI Std.	0.068	NDVI Std.	0.083	NDVI Std.	0.070	NDVI Std.	0.072
Cumm. Rainfall (March, April, May and June) 2016	324.5mm	Cumm. Rainfall (March, April, May and June) 2000	85.8mm	Cumm. Rainfall (March, April, May and June) 2000	368.5mm	Cumm. Rainfall (March, April, May and June) 2000	64.8mm	Cumm. Rainfall (March, April, May and June) 2000	10.0mm
Elevation	2400-3000m	Elevation	2400-3000m	Elevation	2400-3000m	Elevation	2400-3000m	Elevation	2400-3000m

Figure 8. *Analysis of NDVI data (1988, 1993, 1998, 2000 and 2016)*

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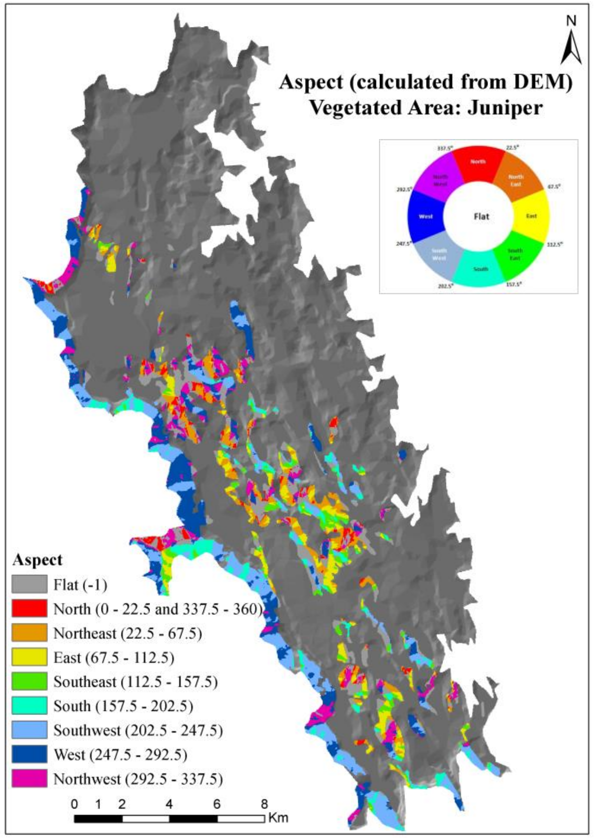
Table 3. *Slope Aspect (calculated from DEM) Juniper Vegetated Area*



Year		2016		2000		1998		1993		1988	
	Area	Mean NDVI	Std. Dev.	Mean NDVI	Std. Dev.	Mean NDVI	Std. Dev.	Mean NDVI	Std. Dev.	Mean NDVI	Std. Dev.
Flat (-1)	11.28	0.336	0.072	0.258	0.060	0.302	0.070	0.235	0.054	0.216	0.064
North (0 - 22.5 and 337.5 - 360)	2.23	**0.376**	0.093	**0.291**	0.073	**0.345**	0.093	**0.275**	0.072	**0.245**	0.090
Northeast (22.5 - 67.5)	5.53	**0.328**	0.061	**0.255**	0.049	**0.299**	0.059	**0.229**	0.045	**0.208**	0.047
East (67.5 - 112.5)	5.01	**0.331**	0.072	**0.251**	0.059	**0.300**	0.071	**0.242**	0.061	**0.214**	0.061
Southeast (112.5 - 157.5)	3.08	0.345	0.087	0.260	0.064	0.308	0.089	0.251	0.076	0.221	0.073
South (157.5 - 202.5)	4.51	0.369	0.092	0.268	0.067	0.333	0.091	0.260	0.081	0.233	0.074
Southwest (202.5 - 247.5)	12.19	**0.396**	0.087	**0.278**	0.066	**0.355**	0.089	**0.285**	0.076	**0.238**	0.070
West (247.5 - 292.5)	11.31	**0.388**	0.088	**0.286**	0.070	**0.347**	0.083	**0.266**	0.068	**0.241**	0.077
Northwest (292.5 - 337.5)	5.39	0.363	0.087	0.275	0.070	0.332	0.087	0.256	0.073	0.234	0.084

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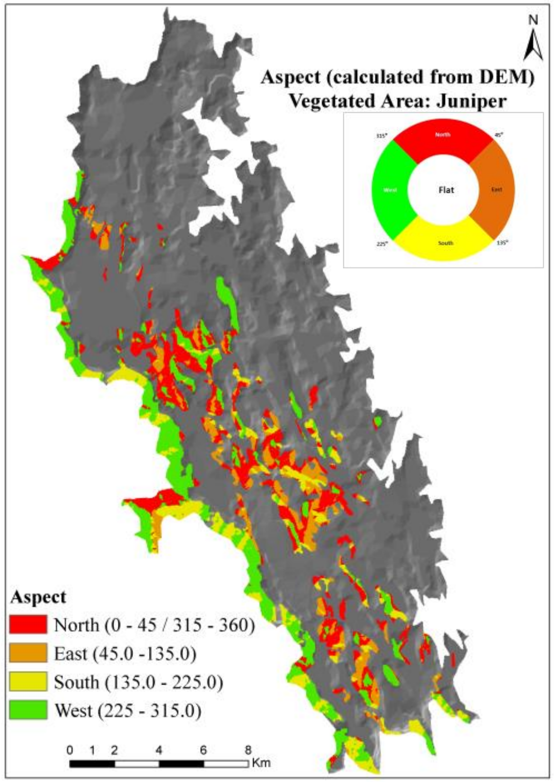
Table 4. *Slope Aspect (four side orientation) (calculated from DEM) Juniper Vegetated Area*



Year		2016		2000		1998		1993		1988	
	Area	**Mean NDVI**	**Std. Dev.**	**Mean NDVI**	**Std. Dev.**	**Mean NDVI**	**Std. Dev.**	**Mean NDVI**	**Std. Dev.**	**Mean NDVI**	**Std. Dev.**
North ($0 - 45^0$ and $315 - 360^0$)	18.57	0.343	0.078	0.263	0.063	0.311	0.077	0.240	0.060	0.215	0.071
East ($45^0 - 135^0$)	9.57	0.331	0.071	0.254	0.058	0.300	0.070	0.239	0.059	0.213	0.059
South ($135^0 - 225^0$)	11.23	**0.381**	0.090	**0.271**	0.066	**0.339**	0.092	**0.271**	0.081	**0.233**	0.072
West ($225^0 - 315^0$)	21.15	**0.387**	0.089	**0.282**	0.069	**0.348**	0.085	**0.278**	0.071	**0.239**	0.075

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In this study, the west-facing *J. procera* had low impact from dieback and had greener vegetation NDVI classes than the east-facing slopes. The west- facing slopes receive higher rainfall higher by the southwestern monsoons precipitation patterns in Alsouda (Vincent, 2008), and consequently higher soil moisture content than the east-facing slopes. Similarly, El-Atta et al. (2013) demonstrated significantly higher leaf water potential (Ψw), chlorophyll content, and net photosynthetic ability of *J. procera* trees under increased soil moisture content in Alsouda.

Conclusions

Extent and severity of dieback was quantified among *J. procera* natural stands in Alsouda, Aseer Region, which was first reported in the early 1980's. Most of the trees have shown symptoms of dieback (> 80%), however they were not seriously affected and showed recovery signs. The recovery signs include new shouts, leaves and flowering even within the severely affect trees. In addition, the new natural regeneration in the well- stocked slopes indicates the importance of tree nursing effect to enhance restoration of Juniper stands. The 12-years plantations showed no symptoms of dieback, but growth rate is relatively low as compared to plantation in other regions that have higher annual rainfall. The meteorological data showed variable rainfall pattern without significant trend over time, while temperature showed significant increasing trends. The effect of slope aspect was evident in the severity of dieback and greenness of the vegetation as detected by the NDVI data. The west facing slopes were higher in NDVI values (greener vegetation) than the east-facing slopes, which may be due to higher precipitation in the west – facing slopes by the south-western monsoons (Vincent, 2008). The tree recovery, new regeneration and absence of dieback in the plantation plots, in spite of the warming trend and variable rainfall, may indicate that climate change is not the prime cause for the dieback phenomenon in the south-western highlands of KSA. The results indicate the importance of annual rainfall in the general health and greenness of the trees. Further studies are needed to quantify differences in rainfall between east and west-facing slopes, and elucidate effect of nursing trees on enhancing natural regeneration of *J. procera.* It is recommended that the plantations efforts should continue, especially among trees within *natural* stands to benefit from the nursing effect.

Acknowledgements. The authors extend their appreciation to the Deanship of Scientific Research, King Khalid University for funding this work through General Research Project under grant number (G.R.P-424-38).

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APPLICATION OF ARTIFICIAL NEURAL NETWORKS AND PARTICLE SWARM OPTIMIZATION FOR TIMBER EXTRACTION WITH CABLE CRANE

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(Received 13th Nov 2018; accepted 11th Jan 2019)

Abstract. The fact that forest areas in Turkey and the world are located at high and steep mountainous areas makes it more difficult to extraction the wood raw material. Therefore, the retreat of forest areas to mountainous areas has brought cable crane to the forefront. Thus, it has become possible to solve complex problems by way of artificial intelligence techniques. The purpose of this study was to determine the impact of factors related with timber extraction via URUSMIII cable crane on total time via Artificial Neural Network (ANN), Particle Swarm Optimization (PSO) and Multiple Regression Analysis (MRA). The data were obtained from oriental spruce timbers which were acquired from spruce stands in the Artvin Forest Directorate, located at NE Turkey. The factors with impact on total time (ground slope, line slope, lateral pull, number of logs, diameter of logs, length of logs, log volume, yarding distance) were measured along with the total time. Determination coefficient (R) and the expressions that indicate error variance (MSE, RMSE and MAE) were taken into consideration for determining the model with the best results. PSO model was determined as the best structure (R = 0.85 MSE = 0.0143, RMSE = 0.1194, MAE = 0.0839): in this study according to the obtained results. The results indicate that PSO had the best performance in the study followed by ANN and finally MRA with the lowest performance. The PSO model can be used for similar conditions on the planning of forest operation, the control of applications and the determination of unit of price for forest workers.
Keywords: *forest operations, timber extraction, total time, artificial neural networks, particle swarm optimization, multiple regression analysis*

Introduction

The need for timber continues to increase in Turkey every day; however forest areas continue to decrease rapidly. The transport stage is quite difficult, expensive and time-consuming in forest management. It is an important issue that the product is extracted with the minimum damage to itself and the environment as well as with minimum loss of quality.

Cable crane systems are used for extraction purposes in many mountainous regions in the world. Since majority of the forests in countries such as Austria, Czech Republic, Japan, Norway and Scotland are located in mountainous areas, cable-crane systems are frequently used for extraction purposes (Russell and Mortimer, 2005; Huber and Stampfer, 2015; Proto and Zimbalatti, 2015)

As is known, forest cable-crane systems are manufactured in whole and are used for carrying heavy forest assets from the air over distances of 300-2000 m. According to yarding distance, these are called as short (< 300 m), middle (300-800 m) and long (800 <) distance skylines (Acar et al., 2005).

URUSMIII cable crane was started to be used in our country during the late 1970s. Although it is not very widespread throughout the country, it is used extensively for timber extraction in the Artvin region (Acar and Şentürk, 2000).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23392355

Cable cranes are quite different in comparison with other extraction methods. The volume is limited for manual extraction. Sliding is one-directional and can be carried out in case the incline is high. Animal-powered extraction cannot be performed from the top to the bottom in steep areas. Whereas for tractor extraction there are various disadvantages such as the issue that transportation cannot be made from the top to the bottom as well as short cable length.

Artificial Neural Networks (ANN) are used as a popular method by many different researchers in different engineering applications. ANN is a structure that based on the learning model of the human brain which contains neurons and is comprised of different layers by combining these neurons using different weights (Haykin, 1999). ANN is a very strong method especially for data modelling when regression coefficients are low (Esteban et al., 2009).

Heuristic optimization is used recently in algorithms in addition to artificial neural networks for improving the success and/or speed of artificial neural networks (Bağış and Çetin, 2009; Liu et al., 2008; Özbeyaz, 2010). Particle Swarm Optimization (PSO) which is one of the heuristic methods has been used successfully in many areas due to reasons such as the ease with which it can be implemented, the low number of parameters that need to be adjusted and the fact that it operates with real numbers. One of these is the training of artificial neural networks (Tamer and Karakuzu, 2006; Delice, 2008; Hema et al., 2008; Gu et al., 2009).

Various studies have been carried out on timber extraction operations during which time studies have been carried out for cable crane URUSMIII (Aykut et al., 1997; Çağlar, 2002; Baldini and Pollini, 1998; Heinrich, 1998; Trzesnowski, 1998; Ledoux and Huyler, 2000; Rieger, 2001; Krpan et al., 2001; Acar et al., 2005; Öztürk and Demir, 2007; Zimbalatti et al., 2009; Zimbalatti and Proto, 2010; Çalışkan, 2015).

The use of Artificial Neural Networks (ANN) and Particle Swarm Optimization (PSO) in timber extraction is at a starting stage in Turkey. Hence, there are no studies in literature which compare the performance of ANN and PSO with other models.

The purpose of this study was to examine the impact of factors related with the interaction of timber extraction with URUSMII cable crane on total time via ANN, PSO and MRA. The data were obtained from oriental spruce timbers from spruce stands in Artvin Forest Directorate, in NE Turkey.

Materials and methods

Study area

This study was carried out in the Ardanuç forest planning unit covering an area of approximately 76817.0 ha of forest with the growing stock 5.9 million m³ in the Artvin province in the northeastern Black Sea region of Turkey. The area was located between 41° 12’ 34” North, and 41° 48’ 55” East. Dominant tree species used for production purposes are natural oriental spruce (*Picea orientalis* Link.), and nordmann fir (*Abies nordmanniana* Stew) and oriental beech (*Fagus orientalis* Lipsky). The climate in Artvin is mild with humid summers. Annual precipitation and temperature averages approximately 700 mm and 13 °C, respectively (Akman, 1990). Felling and delimbing operations were used to be carried out via chainsaws. Cable crane Urus (MIII) is mostly represented as off-road machines and have been widely used (*Fig. 1*). Urus MIII cable crane is generally used in uphill yarding operations, ranging from 500 to 600 m and combining with Mercedes Benz Unimog U1500 truck. Four workers are employed in

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operating the cable crane. The set up duration of cable crane is between 10 and 16 h and pull up duration is between 4 and 8 h depends on terrain conditions. The number of safety ropes ranges between 2 and 4. The basic characteristics of the URUSMIII were given in (Çağlar et al., 2007; Öztürk and Şentürk, 2016).

Figure 1. *Urus MIII cable crane*

Field data collection

Timber extraction was carried out using cable crane. Measured data for the timber operations have been recorded in study forms. Time values for each stage have been measured as 1/100 min (PM) using a chronometer, the amount of work done has been determined in units of m^3, factors that affect the work done (ground slope, line slope, lateral pull, number of logs, diameter of logs, length of logs, log volume, yarding distance) have also been recorded in the study form. No intervention was made on the workers regarding issues such as starting and stopping of work, breaks, pauses, dealing with other operations.

Variables that were considered to have an impact on the work time for skidder timber extraction operations have been evaluated as X_{ii} (X_{11}-X_{18}) and expressed in numerical values. These variables have been briefly explained in *Table 1*.

Repetition time measurement method was used for time measurements and the work phases, total turn time and waiting times were determined using a digital chronometer. The measured time values were obtained for a two person working group in units of 1/100 min. Work phases and related time values have been expressed as Y_{ii} (Y_{11}-Y_{19}) for cable crane timber extraction operations and related time values have been briefly explained in *Table 1*.

Measurements and observations were carried out for timber extraction operations for spruce trees using cable crane (Hauling back of empty carriage (Y_{11}), Descending of bundle hook (Y_{12}), Pulling of hook to logs (Y_{13}), Pulling of loaded hook to carriage (Y_{14}), Moving of loaded carriage to landing site (Y_{15}), Descending of loaded hook to ground (Y_{16}), Unhooking of loads (Y_{17}), Pulling backwards of empty carriage (Y_{18}), Nonworking time (which covers spare and delay time of workers (Y_{19}), Total activity time (y_{fa}).

Total activity time (*Eq. 1*):

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$$y_{fa} = y_{11} + y_{12} + y_{13} + y_{14} + y_{15} + y_{16} + y_{17} + y_{18} + y_{19} \quad \text{(Eq.1)}$$

The ANN-PSO models were generated using Matlab software. Statistical analyses were carried out via “SPSS 21.0” software.

Artificial neural network (ANN)

Artificial neural networks are mathematical models inspired by the structure and behavior of the human brain (Olden et al., 2008). The ANN network includes three main layers: input, hidden and output. These layers are used for data input, data transmission and data output respectively. The hidden layer function is used to transfer the results to the output layer (Fausett, 1994; Haykin, 1994). The output of each neuron can be written as (*Eq. 2*):

$$y_i = f\left(\sum w_{ij} x_i\right) \quad \text{(Eq.2)}$$

where *yi* represents the input that a single node j receives. The function f can be a simple threshold, sigmoid or hyperbolic tangent function. The weights between the nodes i and j are denoted as w_{ij}: x_i represents the output from node i.

Whereas the input layer in this study was comprised of 8 neurons as ground slope, line slope, lateral pull, number of logs, diameter of logs, length of logs, log volume and yarding distance; there was only 1 neuron of Total activity time (y_{fa}) in the output layer. The number of neurons in the hidden layer varies according to the characteristic of the problem.

Back propagation algorithm (BPA) is used for training ANN networks in this study since it is easy to understand and prove mathematically. The back propagation artificial neural network models have already been described and are used widely (Rumelhart et al., 1986; Fausett, 1994; Haykin, 1994; Özçelik et al., 2010).

The hyperbolic tangent sigmoid (tansig) transfer function presented by the following equation was used between the input and hidden layers in the model in this study (Fausett, 1994). A linear (pureline) transfer function was used between the hidden and output layers.

NET: Weighted sum (*Eq. 3*)

$$f\left(NET_i\right) = \frac{1}{1+e^{-NET_i}}\ ,\ f\left(NET_i\right) = NET_i \quad \text{(Eq.3)}$$

BPA uses two parameters that control the speed at which training takes place. The learning coefficient determines the amount of change in the weights. It was observed that values ranging between 0.2 and 0.4 are generally used and that the value of 0.6 yields the most successful results (Öztemel, 2003). The momentum coefficient has an impact on training performance. It was observed that selecting a value ranging between 0.6 and 0.8 would be best (Öztemel, 2003).

Particle swarm optimization (PSO)

Particle Swarm Optimization (PSO) was first developed by Kennedy and Eberhart (1995) inspired by the behaviors of bird swarms when trying to find food. The

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population is defined as swarm in this algorithm, whereas each individual is defined as particle. These particles are accepted to be flowing in multi-dimensional space. PSO can be applied successfully in many areas such as artificial neural network training (Zhao et al., 2005; Awad, 2006).

PSO is started with a group of random values (particle swarm) and the optimum solution is tried to be determined by way of iterations. Particle positions are updated according to the best two values in each iteration. The first value includes the coordinates that provides the best solution for that particle until that point. This value is defined as "pbest" and should be stored in memory. Whereas the other best value includes the coordinates that provides the best solution for all particles in that population until that moment. This is the global best value and is denoted as "gbest". For example, let us assume that there are n particles comprised of D parameters.

Every particle has its own position, velocity and best solution. The PSO method can be presented as follows (Elbeltagi et al., 2005; Al_Janabi et al., 2018):

The swarm position in a D space of the *i*th particle can be presented by (*Eq. 4*):

$$X_{\bar{I}} = \left(X_{i1}, X_{i2}, X_{i3}, ..., X_{iD}\right), \quad i = 1,2,3,..,N \quad \text{(Eq.4)}$$

Whereas velocity of each particle can be represented as (*Eq. 5*):

$$V_{\bar{I}} = \left(V_{i1}, V_{i2}, V_{i3}, ..., V_{iD}\right), \quad i = 1,2,3,..,N \quad \text{(Eq.5)}$$

Each particle maintains a memory of its previous best position. The best swarm position is given by (*Eq. 6*):

$$pbest_{\bar{I}} = \left(pbest_{i1}, pbest_{i2}, pbest_{i3}, ..., pbest_{id}\right), \quad i = 1,2,3,..,N \quad \text{(Eq.6)}$$

gbest is unique for all particles in each iteration (*Eq. 7*):

$$gbest_{\bar{I}} = \left(gbest_{i1}, gbest_{i2}, gbest_{i3}, ..., gbest_{id}\right), \quad i = 1,2,3,..,N \quad \text{(Eq.7)}$$

A particle velocity should be updated and the following equation can be used (*Eqs. 8-9*):

$$V_i^{k+1} = V_i^k + c_1 r_1^k.\left(pbest_i^k - x_i^k\right) + c_2 rand_2.\left(gbest_i^k - x_i^k\right) \quad \text{(Eq.8)}$$

$$Fitness = \sum_i \left(Y_i - X_i V\right)^2 \quad \text{(Eq.9)}$$

where the parameters of c_1, c_2 are constants and r_1, r_2 are random numbers ranging between 0–1. $pbest_i$ is the best local solution of the *i*th particle for the iteration number up to the *i*th iteration. The best global solution of all particles is g_{best}. The "inertia weight" w controls the effect of the previous velocity of the particle on the current one. If the value of w is greater than 1, it means that the particle favored searching over exploitation; whereas a w value of less than 1 is an indication that the particle gave more importance to the current best positions (Al_Janabi et al., 2018).

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Network architecture, training rate and momentum factor have been determined in our study after examining different combinations. Several different ANN models were developed and tested in order to determine the optimal number of neurons in the hidden layer by trial and error estimates. A single hidden layer was used to significantly reduce the computational time. The general structure of ANN is shown in *Figure 2*.

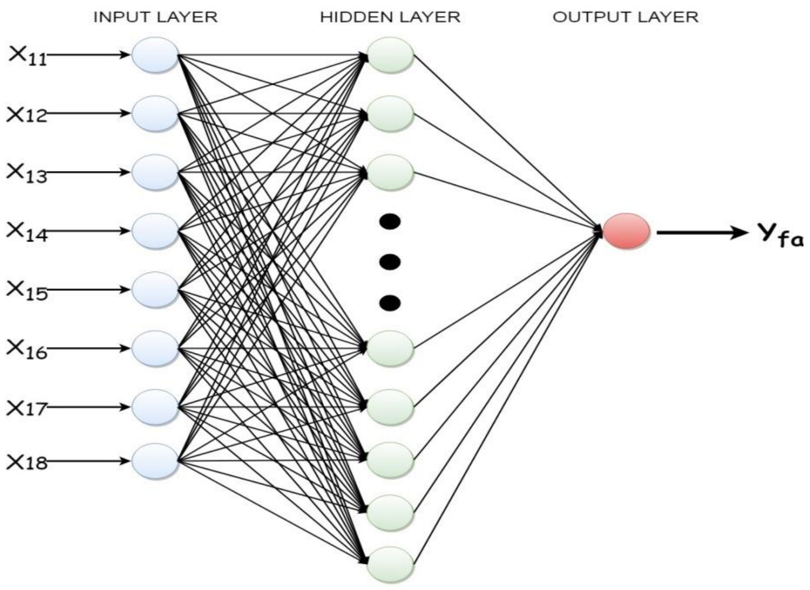

Figure 2. *Schematic diagram of ANN model*

Multiple regression analysis (MRA)

Regression is one of the methods used for testing whether there is a relationship between two or more variables and to express the relationship between the variables by way of linear or curvilinear equations (Öztürkcan, 2009). Regression analysis using more than one independent variable is called multiple regression analysis.

The general structure of the equation in cases when there is more than one independent variable $\left(\text{such as } X_1, X_2, X_3, ...\right)$ (*Eq. 10*):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + ... + \beta_n x_n \pm \varepsilon \quad \text{(Eq.10)}$$

where Y: dependent variable, *Xi*: independent variable $\left(i = 1, 2, 3, .., N\right)$ β_i: regression parameters $\left(i = 1, 2, 3, .., N\right)$, ε : random error and *n*: number of unknown parameters.

In this study, ground slope, line slope, lateral pull, number of logs, diameter of logs, length of logs, log volume, yarding distance are selected as the independent variables and total time is selected as the dependent variable.

Statistical calculations on variables that are independent from (x_{ii}) and dependent on (y_{ii}) measurement results have been carried out as:

- Calculation of the average and deviations,
- Examination of the variables that are effective on the actual time spent for each work phase or the unit time value,

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- Examination of the relations between variables,
- And determination of the impact of independent variables on the total time spent for work phases

Model evaluation criteria

In this study, the performance of different predictive models is evaluated by using statistical measures including the; Corrected determination coefficient (R), Mean Squared Error (MSE), Root Mean Square Error (RMSE) and mean absolute error (MAE) were used as criteria for comparing ANN, PSO and MRA. Accordingly, high R and low MSE, RMSE and MAE values indicate the best model. MAE and MSE values were close to 0 and the R value was close to 1, thereby indicating that the predicted value strongly converges to the right (Hocking, 1976; Law, 1999; Cho, 2003; Arıkan, 2014).

a) The correlation coefficient (R) (*Eq. 11*):

$$R = \frac{\sum\left(X_i - \bar{X}\right)\left(Y_i - \bar{Y}\right)}{\sqrt{\sum\left(X_i - \bar{X}\right)^2\left(Y_i - \bar{Y}\right)^2}} \qquad \text{(Eq.11)}$$

b) Mean squared error (MSE) (*Eq. 12*):

$$MSE = \frac{1}{n}\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)^2 \qquad \text{(Eq.12)}$$

c) Root mean square error (RMSE) (*Eq. 13*):

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - y_i\right)^2}{n}} \qquad \text{(Eq.13)}$$

d) Mean absolute error (MAE) (*Eq. 14*):

$$MAE = \frac{\sum_{i=1}^{n}\left(x_i - y_i\right)}{n} \qquad \text{(Eq.14)}$$

where X_i and Y_i are the observed and predicted data, respectively; $\bar{X}$ and $\bar{Y}$ are the mean of the observed and predicted and n the number of observations in the dataset.

Results and discussion

The arithmetic average, standard deviation, max and min values for the actual time values measured in units of 1/100 min as the variables of observed values regarding the work phases via cable crane have been calculated and presented in *Table 1*.

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Single input variance analysis was used to examine whether the impact of the correlation matrix indicating the relationship between the variables and x_{ii} groups on the values of y_{ii} was statistically significant or not.

Table 1. *Work phases and their descriptive statistics*

Work phases	*Average*	*Std. dev.*	*Min.*	*Max.*	Work phases	*Average*	*Std. dev.*	*Min.*	*Max.*
y_{11}: Hauling back of empty carriage	33.00	17.76	15.00	111.0	y_{fa}: Total activity time	619.0	253.1	275.0	1422
y_{12}: Descending of bundle hook	32.0	21.04	10.00	114.0	x_{11}: Ground slope	70.33	1.69	68.00	73.00
y_{13}: Pulling of hook to logs	167.0	107.2	31.00	500.0	x_{12} : Line slope	51.97	0.23	51.00	52.00
y_{14}: Pulling of loaded hook to carriage	69.22	71.15	60.00	499.0	x_{13}: Lateral pull distance	20.76	0.64	10.00	35.00
y_{15}: Moving of loaded carriage to landing site	233.0	97.55	101.0	447.0	x_{14}: Yarding distance	140.92	61.72	70.00	250.00
y_{16}: Descending of loaded hook to ground	10.00	3.52	4.00	230.0	x_{15}: Diameter of logs	33.76	8.78	22.00	61.00
y_{17}: Unhooking of loads	29.65	23.79	5.00	218.0	x_{16}: Length of logs	5.24	1.05	3.00	8.00
y_{18}: Pulling backwards of empty carriage	7.44	1.43	4.00	12.00	x_{17}: Number of logs	3.15	0.98	1.00	6.00
y_{19}: Nonworking time	36.00	43.90	8.00	314.4	x_{18}: Log volume	1.49	0.69	0.57	3.65

The data were first normalized (0-1) in this study and 70% and 30% of the complete data was used for training and testing respectively. Therefore, 27 testing and 64 training data sets were randomly selected from all land data. The training set adjusts the connection weights and the parameters of the model and the testing set evaluates the trained ANN performance and generalization power (Ghajar et al., 2012a, b).

A network with eight inputs, one hidden layer, and one output was selected in this study. The inputs were: ground slope (x_{11}), line slope (x_{12}), lateral pull distance (x_{13}), extraction distance (x_{14}), diameter of logs (x_{15}), length of logs (x_{16}), number of logs(x_{17}), and log volume (x_{18}); while the output was Total activity time (Cable crane, yfa).

An 8 layer artificial neural network was established in this study and a back-propagation algorithm (BPA) was used as a learning algorithm. The hidden layer and the number of neurons in this layer were determined through trial and error. In this regard, one hidden layer with fifteen (15) neurons was included in the model. Neurons with numbers ranging from 1 to 30 were given to this layer in order to determine the number of neurons that would be included in the hidden layer and each model was tested 10 times to determine the best model for our study. The sigmoid transfer functions were used in input, hidden and output layers in the model. The most suitable model was identified as the model with a network structure of 8-15-1.

Each combination of learning rates and momentum factors were tested for different numbers of hidden neurons. The network was trained in variable epochs via ANN learning algorithm with a learning rate of 0.001 and a momentum coefficient of 0.2. This was the best combination that conducts to the smaller values of R, MSE, RMSE and MAE in *Table 2*. Regression values for the data used in the training and testing of the ANN have been given in *Figure 3*.

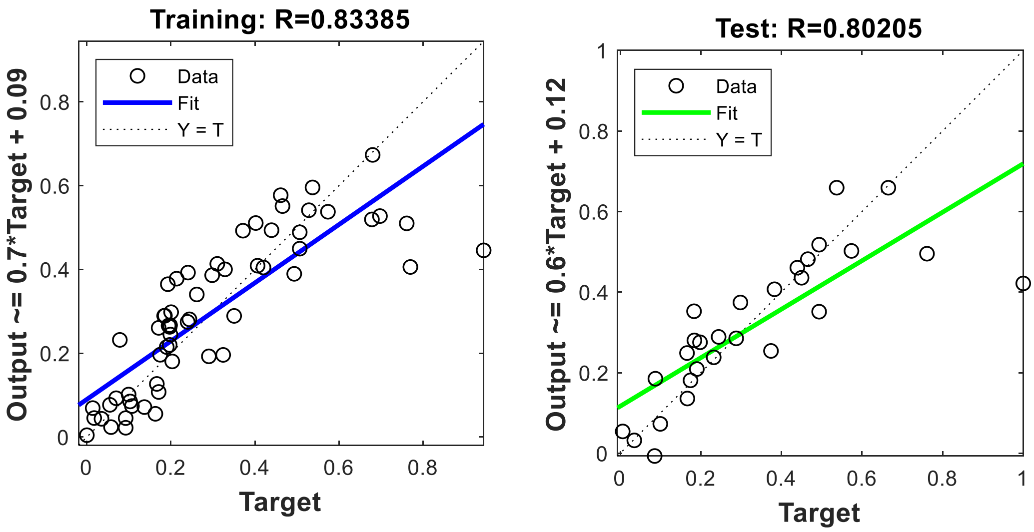

Figure 3. *Training and test, distribution graphs for the ANN prediction model*

Figure 3 shows the R values graph for the training and test stages of the studied model. The values determined were R = 0.8338 for the learning stage and R = 0.8020 for the test stage of the model. *Figures 4* and *5* respectively show the graphs comparing the model predictions and observed values for the ANN model.

It can be observed from *Figures 4* and *5* that the values of the total time during cable crane operations were generally predicted to be close to the observed value.

As mentioned, PSO was used as the artificial neural network algorithm in this study and MSE was applied as a fitness function. The objective of this algorithm is decreasing MSE. The number of data used for training and testing were 64 and 27, respectively.

ANN learning process was started by randomly generating the weights that hold the numerical value of the connections between the layers. These weights express the particle values for PSO. Whereas the number of connections between the layers represents the particle size. The network was setup for each particle and training samples were sent to the network in order. Total error (MSE) was calculated after all values were presented to the network and the obtained value was accepted as the fitness value. This fitness value is determined as the pbest value in the first step; whereas the best fitness value among the particles is determined as gbest. If the fitness value that is the error is not at an acceptable value, the particles are updated with pbest and gbest values. The network was re-established according to the new particle values, the values were resent to the network and fitness value calculation was carried out. These operations are carried out until the best fitness value (gbest) reaches the desired value (very close to 0) or until the maximum number of iterations.

Test process is started if the error is at an acceptable level. This time the network is setup according to the (gbest) particle values, test samples are sent to the input layer in order and the acquired values are provided as the sample output. The last acquired gbest value gives the classification performance of the network when no threshold is applied on the network output.

As a result of the experiments; the optimal parameters for ANN-PSO were determined as; number of hidden neurons = 18, number of particles = 25, number of iterations = 500, (c_1 and c_2 = 2). Particle numbers generally range from 20 to 40 (Tamer

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and Karakuzu, 2006). It was stated in the experiments conducted by the researchers on this algorithm that c1 = c2 = 2 gave good results (Shi and Eberhart, 1998; Liping et al., 2005).

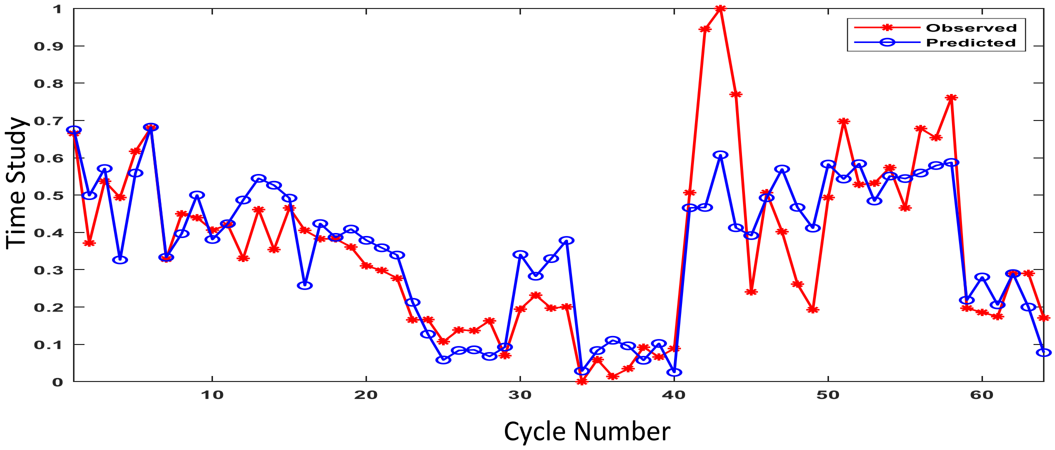

Figure 4. *Comparison of predicted and observed values for training sets using ANN*

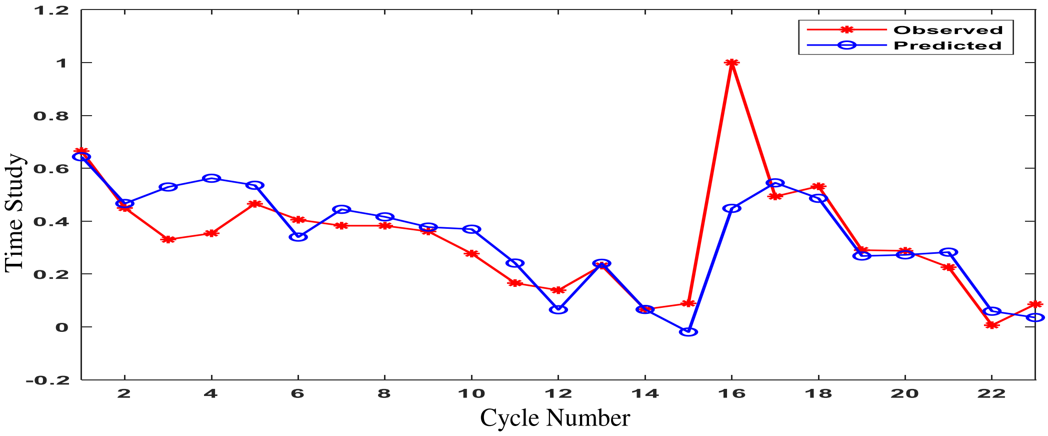

Figure 5. *Comparison of predicted and observed values for test sets using ANN*

This was the best combination that conducts to the smaller values of R, MSE, RMSE and MAE in *Table 2*. *Figures 6* and *7* show the graphs in comparison with the model predictions and observed values for the PSO model.

It can be observed from *Figures 6* and *7* that the values of the total time cable crane operations are generally predicted close to the observed value.

Alternative equalities have been generated via multiple regression analysis. Operations carried out for the timber extraction total activity time with cable crane (y_{fa}) have been given in detail.

Regression equalities based on the $y_{fa} = f\left(X_{11}, X_{12}, X_{13}, X_{14}, X_{15}, X_{16}, X_{17}\right), X_{18})$ relationship have been given in *Table 2*. Consistency of the equation was tested using the coefficients obtained from the regression equation and test data. Graphs that compare the model predictions obtained from the MRA model and the observed values have been given in *Figure 8*.

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It can be observed from *Figure 8* that the values of the total time during cable crane operations are generally predicted close to the observed value.

Table 2. *Regression equalities for calculating the total activity time of timber extraction via cable crane*

Nu	Total time	*b*	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	R-sq
1	y_{fa}	-6.14	0.19	0.39	0.13	0.02	-0.01	0.14	-1.5	-0.21	0.699
2	y_{fa}	-6.23	0.19	0.39	0.13	0.02		-0.14	0.16	-0.22	0.698
3	y_{fa}	13.80	0.18		0.13	0.22		0.14	0.49	-0.21	0.697
4	y_{fa}	11.62	0.16		0.14	0.22		0.19	0.27		0.695
5	y_{fa}	10.66	0.15		0.14	0.22			0.17		0.690
6	y_{fa}	10.63	0.14		0.13	0.22					0.685
7	y_{fa}	-0.11			0.13	0.24					0.677

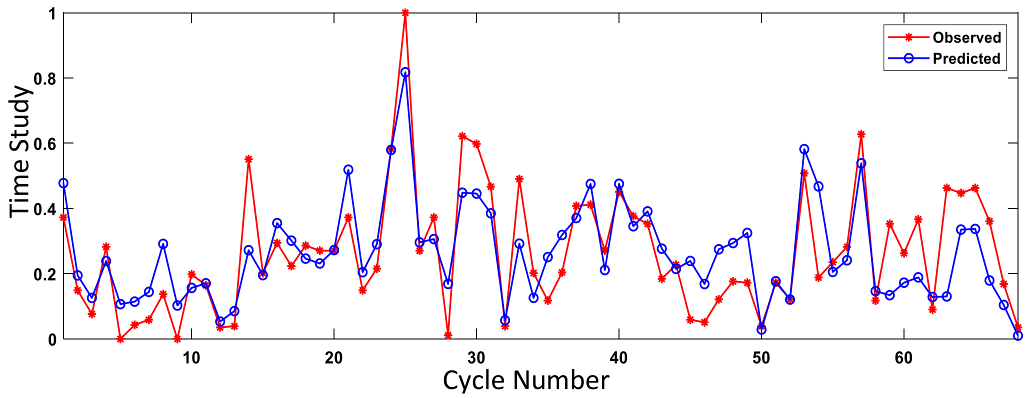

Figure 6. *Comparison of predicted and observed values for training sets using PSO*

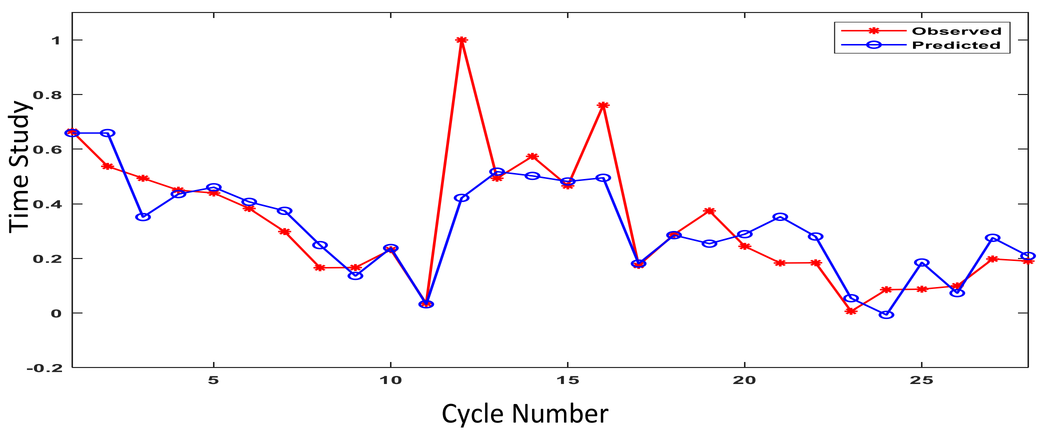

Figure 7. *Comparison of predicted and observed values for test sets using PSO*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23392355

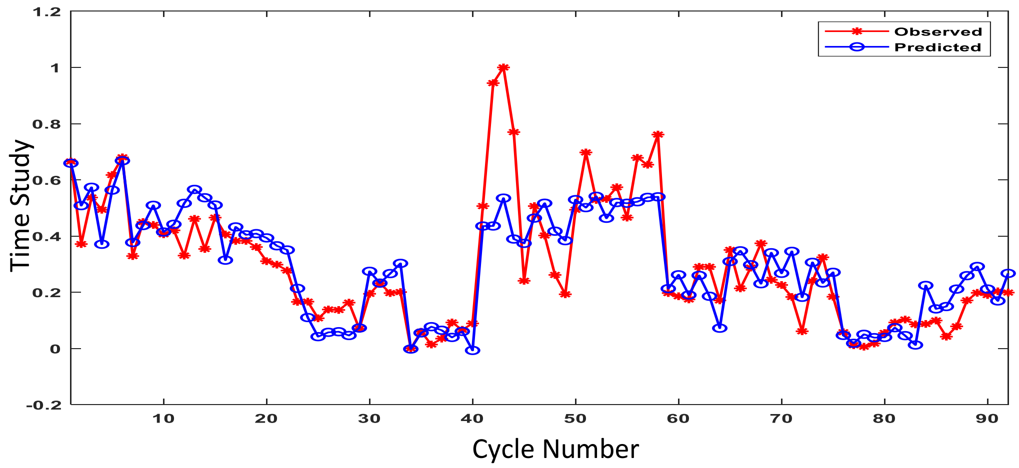

Figure 8. *Comparison of predicted and observed values using MRA*

The performances for predicting total activity time for cable crane are compared using three techniques as ANN, PSO and MRA. The values of performance measures are given in *Table 3*.

Table 3. *A comparison of the ANN, PSO and MRA, models*

Model	R	MSE	RMSE	MAE
PSO	0.85	0.0143	0.1194	0.0839
ANN	0.83	0.0152	0.1232	0.0907
MRA	0.69	0.0194	0.1434	0.1186

Values of R, MSE, RMSE and MAE were compared at the end of the study for determining the algorithm with the best performance. Based on the results obtained from this study, the PSO has been found to be better than the ANN and MRA models, because the PSO has a higher ability in global search than in ANN and MRA models.

ANN and PSO have been applied successfully in the field of forest modeling. Specifically, artificial neural network approach has been carried out for many objectives such as modeling individual tree survival probabilities (Guan and Gertner, 1995), diameter growth based climatic variables (Zhang et al., 2000); wildlife planning issues (Bettinger et al.,2002), forecasting wood demand (Güngör et al., 2004*)* tree volume (Diamantopoulou, 2005a; Diamantopoulou and Milios, 2010; Özçelik et al., 2008, 2010), tree stem diameters (Diamantopoulou, 2005b, 2006; Leite et al., 2011), predicting forest fire burn areas (Cortez and Morais, 2007), forest planning (Pukkala, 2009), tree felling times (Karaman and Çalışkan, 2009), tree heights (Özçelik et al., 2013), prediction of skidding time (Naghdi and Ghajar, 2012), trunk volume estimates ((Bayati and Najafi; 2013), predicted forest fire burn areas (Safi and Bouroumi, 2013), forest feature extraction (Li et al., 2014), prediction of winching time (Bayati and Najafi; 2015), tree diameter increments (Ercanlı et al., 2016) and describing diameter distribution (Bolat et al., 2016), predicting forest fire (Al_Janabi et al., 2018).

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Conclusion

In this study, the impact of factors interacting during timber extraction via URUSMIII cable crane on total time was determined by using ANN, PSO and MRA. The data were obtained from oriental spruce timbers from spruce stands in Artvin Forest Directorate located at NE Turkey. In the study, 70% and 30% of the complete dataset were used for training and testing, respectively. Therefore, 27 testing and 64 training data sets were randomly selected from all land data.

Determination coefficient (R) and the expressions that indicate error variance (MSE, RMSE and MAE) were taken into consideration for determining the model with the best results. The R values obtained in the study were determined to vary between 0.85 and 0.83 for PSO and ANN respectively and as 0.69 when MRA was used.

Based on the results obtained from this study, it was determined that the performance of PSO model was better compared to ANN and MRA. The study also indicates that PSO may be used efficiently in forestry operations.

Acknowledgements. The author wishes to thank student Mr. Kamil BİLGİN and employees of Ardanuç Forest Enterprise who contributed to this study. I also thank Dr. Yusuf Sevim for his support and contributions in Matlab. Moreover the author wishes to thank two anonymous reviewers for their useful comments and advice.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23392355

THE RELATIONSHIPS BETWEEN THE LITTERFALL AND THE CANOPY CLOSURE OF ULUDAĞ FIR (*ABIES NORDMANNIANA* (STEV.) SUBSP. *BORNMULLERIANA* (MATFF.)) FORESTS

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(Received 20th Nov 2018; accepted 11th Jan 2019)

Abstract. The litter is the organic layer on the forest ground and has a variety of ecological functions. The litterfall is the main source of litter. The structure of forest canopy affects most ecologic processes such as the net primary production and litterfall in forest ecosystems. This research study focused on the exploration of the changes of litterfall based upon canopy closure of Uludağ fir forests. The study area was selected from Bolu Aladağ forests. Ten litterfall traps were systematically emplaced under the stand. The litterfall accumulated in each trap was collected monthly. The hemisphere photographs of the canopy closures above the stands were analysed with Gap Light Analyzer (GLA) software. The canopy closures were calculated separately for each hemisphere photographs with angles expanded in the multiples of 10° angle of view. The highest correlation coefficient ($r = 0.359$) was found between the amount of total litterfall (333.5 $g \cdot m^{-2} \cdot y^{-1}$) and the canopy closure at 20° angle of view. The canopy closure values observed at 20° angle of view varied between 6.4% and 83.3%. Different mathematical models were developed to estimate the amount of litterfall fractions based on the canopy closure values at 20° angle of view. The coefficients of determination (R^2) for the models developed as part of this study are 0.93 for foliar, 0.82 for seed and 0.83 for wood fraction. Practitioners can estimate the amounts of litterfall components, spreading distances and the accumulating amounts based on the distance by using the mathematical models developed in this study.
Keywords: *Bolu-Aladağ forests, effective closure, hemisphere photograph, litterfall trap, spreadable litterfall*

Introduction

The litter is the organic layer pooled by the dead plant and animal residues on the forest ground. The litter has variety of ecological functions such as soil protection (Enez et al, 2016), water infiltration (Koralay et al., 2015), nutrient deposits (Dündar, 1988), carbon sequestration (Duyar, 2010) and habitat for soil fauna (Duyar and Makineci, 2016) besides some other indirect ecological functions (Sariyildiz and Savaci, 2017). As stated by Dubber et al. (2017), the amount of litterfall is closely related to the net primary production in forest ecosystems. While the litterfall of deciduous trees is predominantly realized in autumn (Pitman et al., 2010), the litterfall of evergreen coniferous trees befalls through the year (Duyar, 2014).

The structure of forest canopy controls both ecologic and ecophysiological processes. The crown width (Duyar, 2014), self-pruning of branches (Mäkinen and Colin, 1999; Wang et al., 2015), the amount of precipitation on the ground (Kiniş et al., 2011), the amount of snowbank (Duyar and Aydın, 2016), and understory vegetation (McDonald et al., 2015; Chandler and McGraw, 2017) are some of the many examples.

Measurements techniques for canopy closure were studied. Various methods were developed (Mailly, 2017) since 1940s as the effects of canopy closure on stand dynamics were pronounced (Garrison, 1949). The most widely used system of today is the Digital Hemisphere Photograph (DHP) of canopy processed with the computers (Chianucci and Cutini, 2013; Macfarlane et al., 2014; Origo et al., 2017). Numerous

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

data such as crown canopy, Woody Area Index (WAI), Leaf Area Index (LAI) and solar radiation penetrating to the stand can be generated with the use of DHP (Liu and Jin, 2017; Mailly, 2017). The litterfall components such as foliar, seed and wood with respect to the stand structure (Liu et al., 2015) and dynamics of decomposition (Zalamea et al., 2012) are estimated based on the established relationships between the canopy data and the ground data obtained from the litterfall collection methods on the ground.

The amount, phenology and the composition (foliar, seed and wood) of litterfall from the trees are under the influence of the mean stand age and height (Atay, 1971), canopy closure (Lopez et al., 2008) and LAI (Liu and Jin, 2017) besides the features of tree species and climate characteristics. The forest practitioners use these data in order to estimate and evaluate the amount, timing and extent of seed cover (Dassot and Collet, 2015) as well as the process of nutrient flow (Magalhães et al, 2014) and carbon sequestration (McGovern and Pasher, 2016) within a stand structure (Leblanc and Fournier, 2017).

Uludağ fir (*Abies nordmanniana* (Stev.) subsp. *bornmuelleriana* (Matff.)) is an endemic subspecies of *Abies nordmanniana* (Stev.) and indigenous to the mountains in the Western Black Sea Region (between Uludağ mount and Kızılırmak river) in Anatolia (Anşin and Özkan, 1997). It occurs at altitudes of 900–2000 m on mountains with the cool and humid weather. Its optimum habitat is about 1550 m (Kantarcı, 1979). The uneven aged management of fir forests (Sıvacıoğlu et al., 2007) create a multiple layered forest structure (Topaçoğlu et al., 2008; Genç et al., 2012) with various sizes of gaps. Fir is a typical shade tolerant tree species (Çalışkan, 1992), and its green crown protrudes up till lower branches (Şevik et al., 2010).

In principle, the litterfall dynamics are critical in the sustainable management of forest ecosystems including the soil properties, nutrient regimes, carbon cycle and some other monitoring studies. Furthermore, litterfall collection method is quite time consuming, exhausting and costly. Developing mathematical models are the most appropriate and an economic method for monitoring studies such as litterfall dynamics and prediction of amount and spreading distances of litterfall components. There are few scientific studies about the dynamics of litterfall in forest ecosystems in Turkey. However, there seems no particular research endeavor designed to investigate the relationships between the amount of litterfall and the canopy closure determined by the DHP in Uludağ fir forest ecosystems. We propose and expect that establishing the relationships between the litterfall collected with the traps and the canopy closure of fir trees determined by the DHP and the exploration of the associated estimation models and methods based on the driven data would contribute to the basic ecological studies in forestry and fill the gaps in the literature as far as fir forest ecosystems are concerned. Thus, the amount and spreading distance of litterfall components will be estimated based on canopy closure ratios in the forest.

This research focuses on the exploration of the changes and the correlation of litterfall in relation to the canopy closure of Uludağ fir forests, determined with the DHP. Based on the results, different mathematical models were investigated for the prediction of the spatial distribution of litterfall, amount of spreadable and collected litterfall.

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Materials and methods

The study area

The study was carried out in the Şerif Yüksel Research Forests managed by the Western Black Sea Research Institute in the Aladağ forests located in the southern part of Bolu province in the Western Black Sea Region in Turkey. The research plot lied on a southern aspect with < 5% slope wand an average elevation of 1570 m (40°37'12.2"N and 31°35'59.5"E) (*Fig. 1*).

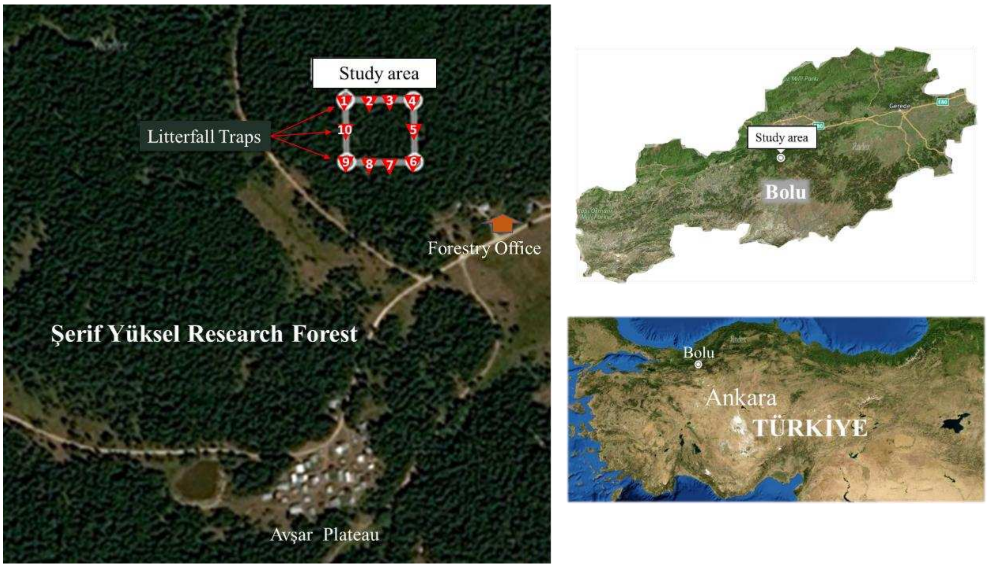

Figure 1. *Map of study site and location of plot (Google earth, 2017)*

The climate of the area

According to Thornthwaite climate classification system (Thornthwaite, 1948), based on the calculations using the long-term data (1960–2012) at Bolu meteorological station (742 m asl), the site had a climate type A C_2' r b'_4 at 1600 m elevation, characterized with (A) a relatively maritime condition, (C_2') without any water shortages or a small shortage, (r) low temperature and (b'_4) high moisture (Duyar, 2014). This plateau sitting at 1600 m of elevation is cooler and rainier compared to the city of Bolu (Tolunay, 1997).

Stand characteristics of the area

The case study area presents multi layered fir stands mixed with relatively few Scots pine (*Pinus sylvestris* L.) trees and managed by uneven aged management system. All of trees on 20 m × 20 m area were measured in the middle of study area, for the determination of the stand characteristics of the study area. The average height of the stand was nearly 21.7 m and the average width or diameter of crown was about 5.1 m (*Table 1*).

Table 1. *The stand characteristics of the study area*

The average diameter at breast height (cm)	Basal area (m²/ha)	Crown diameter (m)	Average height (m)	Dominant height (m)	Number of trees (number/ha)
35.2±12.5	63.5±5.0	5.1±0.9	21.7±6.4	29.0±1.9	608±201

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Litterfall sampling

The sampling was conducted between 2011 and 2014 years. Ten litterfall traps were systematically emplaced in an interval of 25 m × 50 m over the one ha of the area (100 m × 100 m) (*Fig. 1*). The litterfall samples were collected once a month by using the 50 cm depth and 0.25 m² (50 × 50 cm) litterfall traps (ICP Forests, 2004) (*Fig. 2*). These are located 1.30 m above the forest floor (Martius et al., 2004). The collected total litterfall ($Total_{Lf}$) biomass was manually separated into three fraction groups; foliar, seed and wood fraction and oven-dried at 65 °C for dry weight determination. The foliar fraction consists of the needles of fir in fir forest ecosystems. Seeds refer to the reproductive organs such as flowers, fruits, seeds, catkins and pieces of cones. Wood fractions are woody pieces thinner than 2 cm in diameter such as twig, branch and bark. It is assumed that branches over 2 cm in diameter are deadwood, not litterfall fraction (Pitman et al., 2010).

Figure 2. *Litterfall traps*

Determining canopy closures with digital hemisphere photographs (DHP)

The canopy DHP over the litterfall traps were taken with a high resolution (12 megapixels) fisheye objective digital camera mounted on the tripod. The default adjustments were selected automatically (Macfarlane et al., 2014). Canopy closures rates were determined with DHP Gap Light Analyzer (GLA) software (Frazer et al., 1999). Each one of the DHP processed with DHP GLA software was sliced into 18 circles with large viewing zenith angles expanded in the multiples of 10°. The largest zenith angle was recorded to be 89°. GLA software computes the percentage of open sky seen (% canopy openness) based on the angles from the hemispherical photographs (Frazer et al., 1999). Thus, the canopy closure ratio (% canopy closure) of each circle is separately determined by dividing the closure area by the circle area (Gonsamo et al., 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

Determination of canopy closure areas

The average stand height is about 21.7 m (*Table 1*) and the height at which the traps are located away from the forest floor is 1.3 m. The distance between the trap and the start of the canopy closure (HD) is 20.4 m. The diameters of canopy cover (CD) viewed from the traps were determined with the tangent (α) values of the perpendicular trap centered zenith angles expanded in the multiples of 10° (*Eq. 1*).

$$CD = HD \times \tan(\alpha) \qquad \text{(Eq.1)}$$

The area of canopy closure viewed from the stated angle was determined with the circle area (A) (*Eq. 2*).

$$A = CD^2 \times \pi/4 \qquad \text{(Eq.2)}$$

The most appropriate correlation was determined at the angle values with the highest correlation based on the angular closure of litterfall components. Then, mathematical models were developed based on the relationships between the amount of litterfall component and the values of canopy closure viewed at the angles above the traps.

Statistical analyses

The litterfall samples and their components collected monthly from the litterfall traps were evaluated in $g \cdot m^{-2}$ unit. The samples were statistically analyzed using IBM SPSS statistics program (SPSS Inc., Chicago, IL, USA). The correlation and the distribution of the samples with respect to the traps and closure are evaluated at $\alpha = 0.05$ confidence level. The ANOVA test was used in order to calculate the statistical distribution of samples and Duncan test was used to determine the differences among the samples.

Results

Distribution of litterfall components

Mean 333.5 (min = 137.8 – max = 526.2) $g \cdot m^{-2} \cdot y^{-1}$ amount of litterfall was accumulated within 10 litterfall traps with different canopy closures. It was found wide variation among the amount of litterfall collected within the traps due to their locations and variations of canopy closure. The foliar litterfall has the largest share (72%) of the total amount of litterfall. The rates of seed and wood litterfall were almost similar to each other (14%). There were significant differences between the amount of $Total_{Lf}$, foliar and wood components accumulated within the traps ($P < 0.01$). The annual amount of collected litterfall components were 2401 $kg \cdot ha^{-1} \cdot y^{-1}$ for foliar, 472 kg $ha^{-1}y^{-1}$ for seed and 462 $kg \cdot ha^{-1} \cdot y^{-1}$ for woody parts (*Table 2*).

Canopy closure

As seen in the hemisphere photographs, some traps coincided with gap areas under loose canopy closure while others coincided with dense areas under high density canopy of trees. There were also differences in perpendicular canopy closures right over the traps. Additionally, the differences in canopy closures were quite distinct from the center of the traps towards outside. Moreover, the amount of energy and sun light

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

penetrating to the crown varies due to the canopy structure of the trees. Such reality influences the size of canopy and the amount of the vegetative and generative organs of the trees. The self-thinning of branches decreased in the trees with plenty of light and located around the large gaps as depicted in *Figure 3*. The green or live canopy starts at a lower height and cover larger areas.

Table 2. *The distribution of litterfall components to the traps*

Litterfall traps	Foliar		Seed		Wood		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	25.35bc	24.62	4.81	11.69	13.70b	34.56	43.85d	43.61
2	12.00a	16.82	1.85	3.31	0.81a	1.57	14.67a	17.35
3	25.90bc	26.77	4.53	7.68	5.10a	7.88	35.53bcd	30.76
4	10.03a	12.53	1.99	5.3	0.76a	1.78	12.78a	14.18
5	26.47bc	30.2	4.65	9.58	2.81a	4.79	33.93bcd	30.16
6	26.45bc	27.17	5.37	7.46	6.52a	13.38	38.33cd	34.87
7	7.79 a	8.47	2.01	4.45	1.68a	3.9	11.48a	11.82
8	18.41abc	18.39	4.88	12.61	1.51a	3.58	24.79abc	21.87
9	30.16c	30.29	6.33	15.08	3.95a	5.66	40.44d	34.00
10	17.55ab	23.32	2.9	4.7	1.66a	4.17	22.11ab	25.32
Total	20.01	23.93	3.93	9.02	3.85	12.82	27.79	30.05
P	0.000		0.330		0.000		0.000	


a

b

c

d

Figure 3. *Hemisphere photos of canopy closure (Traps: a 2; b 3; c 8; d 9)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

Significant differences were found among the average canopy closure rates of the canopy angularly observed above the litterfall traps (P < 0.01) (*Table 3*). The crown rates show an increasing trend from the narrow angle of 10° (40%) towards hemisphere 178° (81%). However, certain differences in crown rates in each hemisphere photos enlarging with 10° zenith angles among the traps were also observed. For example, the average canopy closure (89%) at the narrowest angle (10°) was higher than the canopy closure (85%) at the largest angle (178°) as some traps (trap 6, 3 and 1) coincided with dense canopy closure. The crown rates determined under 10° zenith angles ranged from 0.13% to 93.22% with a Standard Deviation (SD) of 39.82%. Thus, certain differences in the crown rates represented by the traps were also found. The extreme values decreased as the evaluated crown area expanded with respect to the increase of zenith angles. As a result, the crown rates stabilized (mean = 81.4%, SD = 4.87% for 178°) (*Table 3*).

Table 3. *The canopy closure rates over the traps by the angles*

Angles	Traps canopy closure (%)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**	**SD**
10	84.05	0.66	88.27	2.09	47.45	93.22	0.13	18.00	64.32	1.73	39.99	39.82
20	80.10	20.89	83.29	12.83	63.31	94.26	6.40	27.33	66.56	19.48	47.44	33.22
30	68.55	40.38	80.41	28.79	64.37	87.47	18.12	35.38	67.08	51.22	54.18	23.02
40	66.31	51.18	75.06	42.90	61.46	80.39	31.41	46.77	68.92	63.89	58.83	15.38
50	66.97	56.22	67.50	53.24	57.31	75.32	41.07	55.48	65.40	63.85	60.24	9.60
60	67.03	60.09	63.98	59.26	58.02	74.70	47.80	60.84	64.19	62.49	61.84	6.87
70	66.93	63.54	64.40	62.90	58.62	76.47	54.49	63.49	64.77	63.83	63.95	5.63
80	67.94	67.18	64.92	65.05	60.06	78.70	59.28	65.60	64.86	65.23	65.88	5.28
90	70.27	71.05	65.44	66.80	60.46	80.01	63.34	67.34	64.99	66.47	67.62	5.33
100	72.54	74.68	66.20	69.16	61.42	81.24	65.84	68.46	65.91	68.23	69.37	5.57
110	74.40	77.62	67.71	71.42	63.52	82.48	67.95	69.26	66.57	69.85	71.08	5.65
120	76.17	79.57	69.47	73.36	65.43	83.95	69.43	70.32	67.89	71.69	72.73	5.67
130	77.80	81.10	71.44	75.30	67.00	85.30	71.07	71.50	69.10	73.91	74.35	5.65
140	79.32	82.52	73.32	76.98	68.18	86.47	72.58	72.71	70.20	75.60	75.79	5.66
150	80.70	83.64	74.81	78.53	68.99	87.27	73.88	73.87	71.06	77.23	77.00	5.67
160	82.01	84.73	76.39	79.92	70.14	87.88	75.17	75.01	72.21	78.80	78.23	5.56
170	83.56	86.00	78.32	81.54	72.24	88.70	77.00	76.70	74.15	80.62	79.88	5.21
178	84.87	87.12	80.01	82.98	74.30	89.56	78.73	78.39	75.90	82.15	81.40	4.87
Mean	74.97	64.90	72.83	60.17	63.46	84.08	54.10	60.91	68.00	63.13	66.65	17.96

The relationship between canopy closure and litterfall

Based on the relationships between the amount of litterfall for each component of litterfall and the values of canopy closure viewed at the angles above the traps, relatively the highest positive correlation (r = 0.225**) for the woody part was found within the canopy closure at 10° angle of view. In the meantime, the highest correlations for the foliar component (r = 0.285**) and for the seed component (r = 0.136**) of litterfall were attained within the canopy closure at 20° angle of view.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

The levels of correlations show a decreasing trend towards negative values from 30° angle onwards (*Table 4*).

The closer the canopy closure of litterfall is to the traps horizontally, the higher the amount of litterfall collected at the traps is. According to the correlation values indicated in *Table 4,* the amount of litterfall collected in the traps has a limited relationship with the canopy closure viewed at certain angles from the traps. Additionally, *Figure 4* shows that canopy closure values observed within the distance from the center towards the areas viewed until 20° angle of view contribute the highest to the collected litterfall according to the physical and aerodynamic features of litterfall components. However, they indicate decreasing trend after 30° angle.

Table 4. *The relationships of the litterfall fractions at various angles*

Litterfall fractions	Correlations (N = 360)										
	10°	20°	30°	40°	60°	80°	100°	120°	140°	160°	178°
Foliar	0.273**	0.285**	0.284**	0.274**	0.214**	0.101	-0.00	-0.03	-0.04	-0.06	-0.06
Seed	0.135*	0.136**	0.126*	0.119*	0.104*	0.056	0.00	-0.02	-0.03	-0.04	-0.04
Wood	0.225**	0.214**	0.180**	0.160**	0.164**	0.116*	0.09	0.08	0.08	0.08	0.08
Total	0.354**	0.359**	0.341**	0.322**	0.272**	0.146**	0.03	0.00	-0.01	-0.02	-0.02

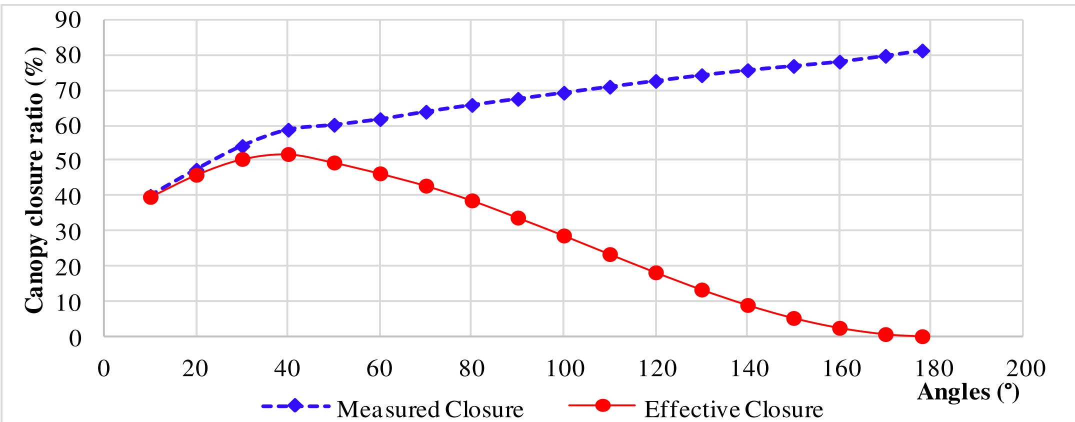

Figure 4. *Measured and the effective canopy closure determined by the angles*

Effective canopy closure for litterfall

The diameter and the area of crown viewed at angular side from the traps were determined with the model developed (*Eqs. 1* and *2*) by the value of zenith angle centered with the traps and the average height of the stand in question (*Table 5*).

Modeling the litterfall with canopy closure

The most appropriate correlation is usually determined at the angle values with the highest correlation based on the angular closure of litterfall components. The amount of woody parts of the litterfall at 10° angular closure is estimated with an exponential function. However, the amount of seed and foliar components as well as the $Total_{Lf}$ at 20° angular closure are estimated with second degree polynomial functions (*Table 6*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

Table 5. *The fractions and the effective closure viewed from the angle based on the tree height*

Angle (°)	Viewed diameter (m)	Viewed area (m²)	Measured closure (%)	Cosα² adjustment value	Effective closure (%)
10	3.6	10.0	39.9915	0.9924	39.688
20	7.2	40.6	47.4432	0.9698	46.013
30	10.9	93.9	54.1781	0.9330	50.549
40	14.8	173.2	58.8295	0.8830	51.948
50	19.0	284.3	60.2363	0.8214	49.478
60	23.6	435.8	61.8401	0.7500	46.380
70	28.6	641.0	63.9457	0.6710	42.908
80	34.2	920.5	65.8828	0.5868	38.662
90	40.8	1307.4	67.6184	0.5000	33.809
100	48.6	1856.9	69.3675	0.4132	28.661
110	58.3	2666.6	71.0778	0.3290	23.384
120	70.7	3922.2	72.7278	0.2500	18.182
130	87.5	6012.6	74.3515	0.1786	13.280
140	112.1	9869.1	75.788	0.1170	8.866
150	152.3	18209.8	76.9958	0.0670	5.158
160	231.4	42050.6	78.2253	0.0302	2.359
170	466.3	170807.4	79.8845	0.0076	0.607
178	2337.4	4291086.5	81.4013	0.0003	0.025

Table 6. *The mathematical models to estimate the litterfall fractions*

Litterfall fractions	Angle of effective closure	Mathematical models	Coefficients of determination (R²)
Foliar	20°	$-0.005x^2 + 0.6976x + 3.1006$	0.93
Seed	20°	$-0.0011x^2 + 0.1422x + 0.7365$	0.82
Wood	10°	$1.0997 \times e^{0.0213x}$	0.83
$Total_{Lf}$	20°	$-0.0051x^2 + 0.8386x + 4.7628$	0.92

The model representing woody parts shows a convex curve and increases gradually with respect to the increase of 10° angular closure values. However, the functions of foliar, seed and $Total_{Lf}$ show a concave curve with respect to the increase in canopy closure values. Thus, the trend of the collected litterfall increases up to a certain point (seed 65%; foliar 70%) and decreases right after that point (*Fig. 5*).

The measured data obtained from the prediction of the spatial distribution of litterfall collected basically from the crown of the trees that are the principle sources of the litterfall were extrapolated. The spatial (horizontal) distribution of litterfall was simulated based on the annual average annually collected litterfall (333.5 $g \cdot m^{-2}$) in an area with 3.6 m in diameter at 20° angle of view. The horizontal sizes were assumed to be the heights of the trees in order to ease the prediction in practice. The $Total_{Lf}$ estimated with the extrapolation of the theoretical sources of the litterfall was distributed to the horizontal areas according to the tree height (TH) criteria. The horizontal distribution of litterfall decreases with respect to the zenith angles $(Cos\alpha)^2$.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

The Amount of Spreadable Litterfall (ASL) model was able to best represent the relationships (*Eq. 3*). It was estimated that the amount of $Total_{Lf} = 13976$ g·y⁻¹ in the litterfall source decreases gradually from the center towards outside further down to the 7-tree heights (7 × 20.4 m = 142.8 m) to 280 g·y⁻¹ (2%).

$$ASL = Total_{Lf} \times (Cos(Arctan(TH)))^2 \quad \text{(Eq.3)}$$

Amount of Accumulative Litterfall (AAL) in a unit area was modelled with an exponential function based on the departure from the center (*Eq. 4*). The amount of litterfall to be collected at two-tree distance (40.8 m) from the litterfall source was estimated to be 0.54 $g \cdot m^{-2} \cdot y^{-1}$ (*Table 7*).

$$AAL = 197.47e^{-2.129AB} \quad \text{(Eq.4)}$$

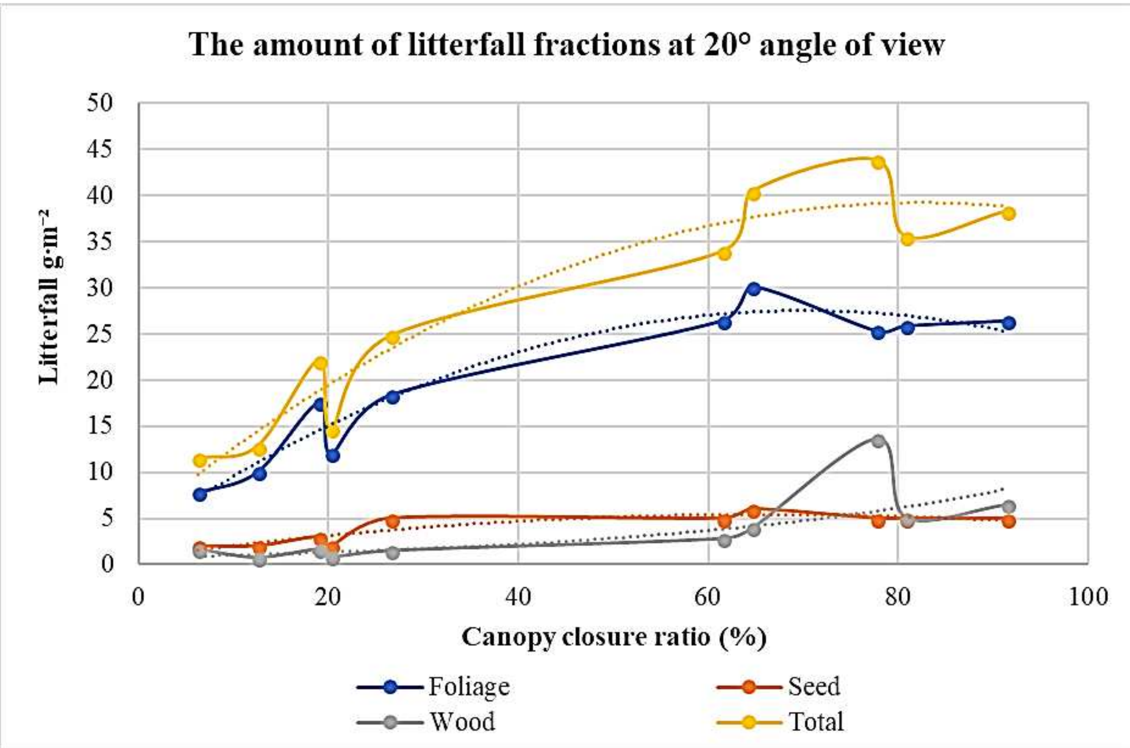

***Figure 5.** The change of litterfall based on the canopy closure*

Discussion

The average amount of litterfall collected yearly in the traps is about 333.5 $g \cdot m^{-2} \cdot y^{-1}$, 72% of which is leaves and the rest (28%) consists equally of seed and woody pats of litterfall fractions. A similar study conducted in China by Yang et al. (1991) in a different Fir stands (*Abies fabric*) indicated that the amount of litterfall was about 286 $g \cdot m^{-2} \cdot y^{-1}$. The 17% of the $Total_{Lf}$ was classified for wood fractions, 73% was for leaves and the rest for the seed fraction. Also, Dündar (1988) found that the amount of litterfall of Scotch pine stands was about 468 $g \cdot m^{-2} \cdot y^{-1}$ in Bolu-Aladağ, in Turkey. Edwards et al. (2017) investigated the proportions of litterfall components and concluded that the rate of foliar is nearly 63.5%, the rate of wood is 27.7% and the rate of seed is about

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

8.8% in northeastern Australia. While the rates of leaves litterfall fraction are similar in both studies, the rates of seed and woody parts differed from each other. The underlying reasons may be attributed to the fact that the parts of trees would be different as well as the frequency or interval of seed-rich years based on the case study period and the time of the seed maturity would be dissimilar to each other. As the firs are cone rich trees, the parts of the cones assumed to be the part of seed contributed to the litterfall as much wood parts as possible. Kucuk et al. (2014) showed that the average 41% of canopy litterfall was allotted to the needle, 13% to the fine branch, 20% to the medium branch, 18% to thick branch and 8% to the very thick branch in young black pine stands.

Table 7. *The theoretical distribution of litterfall at the horizontal distances*

Distribution distance	**Distribution distance for 20.4 m stand height**	**Amount of spreadable litterfall**	**Ratio of $Total_{Lf}$**	**Spread area**	**Amount of accumulative litter**
Tree height	**m**	**$g \cdot y^{-1}$**	**%**	**m^2**	**$g \cdot m^{-2} \cdot y^{-1}$**
0	Source of litterfall	13965	99.9	1	13962
1/8	2.55	13761	98.5	20	674
Quarter	5.1	13154	94.1	82	161
Half	10.2	11181	80.0	327	34
1	20.4	6988	50.0	1307	5.345
One and half	30.6	4300	30.8	2942	1.462
2	40.8	2795	20.0	5229	0.535
3	61.2	1398	10.0	11766	0.119
4	81.6	822	5.9	20918	0.039
5	102	538	3.8	32684	0.016
6	122.4	378	2.7	47065	0.008
7	142.8	280	2.0	64061	0.004
8	163.2	215	1.5	83671	0.003

The collected litterfall did not show a steady increasing linear trend with respect to the values of canopy closure. The amount of collected litterfall was found to be relatively higher in the stands with loose canopy closures over the traps. Chianucci et al. (2014a) warned that one would incline to make more mistakes in the stands with larger gaps compared to the stands with dense canopy closure. The lower degrees of canopy closure cause the lower layers of the stands to have more sun light energy (Lopez et al., 2008). It is believed that the self-thinning of branches starts and proceed slower, and the amount of accumulated vegetative and generative biomass increase as intense light energy penetrates into the stands. Therefore, more energy penetrates into the stands and the carbon sequestration increases. The amount of light energy penetrating inside the canopy decreases in the high dense stands. As a result, self-thinning process accelerates among the trees and the crowns of the trees develop narrower and start from high above the ground. Thus, volume of the crown of the trees around the gaps are quite bigger and the vegetative and generative organs are plenty in the stands with loose canopy closure as compared to the dense stands. Lopez et al. (2008) indicated that such situation is more important in the forest with mostly deciduous stands and the early plant species within the understory of stands are able to find opportunities to store plenty of light energy before the blooming of leaves in the spring season (before the initiation of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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closure). That the rate of canopy closure is relatively lower in the forests with shade tolerant species like fir influences positively the development of seedlings and the maintenance of the multiple layered and un-even aged stand structure.

The rates of the canopy closure above the litterfall traps show a gradual increasing trend with respect to the increase in zenith angle. The same trend was also observed in some other Pinus, Cupressus, Quercus and Carpinus stands (Gonsamo et al., 2013; Chianucci et al., 2014b). A similar decreasing trend of gaps in canopy closure with respect to the increase in zenith angle was also observed by Gonsamo et al. (2013) and Chianucci et al. (2014b)

In this study, while there were significant differences between the canopy closures above the traps in narrow angles (< 40° = 20° zenith angle) in our case study, such differences between the canopy closures are gradually eliminated with the expansion of the angles (40° <). Therefore, it becomes difficult to establish a correlation between the canopy closure and litterfall and develop the associated mathematical models when a study is designed to use larger angle. Thus, it is important to decide at the beginning the appropriate angle interval that allows the best relationships with the litterfall collected manually on a certain interval in order to estimate the litterfall based on the canopy closure of stands. The best correlation was found at 20° angle for foliar and seed and at 10° angle for woody parts of the litterfall. The canopy closure that indicated the best correlation with the litterfall at 20° angle was used as a basis in developing the mathematical models in this study. Based on this reality, the mathematical models were thus developed for each fraction of litterfall at these angles. The R^2 for the models developed as part of the study are 0.93 for foliar, 0.82 for seed, 0.83 for woody fractions and 0.92 for the $Total_{Lf}$. Liu and Jin (2016) showed that rate of the relationships R^2 between the collected litterfall and the LAI determined by the DHP ranges from 0.42 to 0.76 in coniferous forests in China. As well, they developed models to estimate the seasonal amount of litterfall based on the effective LAI determined at 45-60° zenith angles.

Although LAI of forest canopy closure could be determined with optical methods, errors may be encountered due to the storage of leaves and trunk's silhouettes on top of each other based on the viewing angles. Similarly, a certain zenith angle needs to be used, instead of a whole area, in determining the values of LAI as an alternative method to estimate the litterfall. The DHPs were analysed at different zenith angles (0-45°, 30-60°, 45-60° and 0-75°) for effective measurements of LAI in deciduous-coniferous mixed forest. The highest correlation was found with the effective LAI viewed at 45-60° zenith angle based on the litterfall collecting method (Liu et al., 2015). However, errors issuing from the DHP can be removed using appropriate indices (Frazer et al., 2017).

In this study, the effective spreading distance was found to be 7.2 m diameter viewed at 20° zenith angle since the parts of the cones were included with the seed fraction of litterfall. Pamay (1962) indicated that the effective seed spreading distance of the seed trees in the edges of stands is 10-40 m for Anatolian pine (*Pinus nigra* Arnold) and Scotch pine and 20-40 m for Spruce (Picea sp. Mill.). The spreading distance of the seeds are generally dependent on the height of the trees, the weights and the flying capabilities of the seeds. However, the dry, hot and windy weather conditions help increase the flying distances of the seeds. In the meantime, the flying distance of the seeds can reach up to 400 m in Anatolian pine, 480 m for Scotch pine and 200 m in fir

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23572372

trees according to (Atay, 1971). These flying distances include the wing shaped seeds detached from the carpel and cones.

Understanding the litterfall dynamics within the forest ecosystems are critical in the sustainable management of forest ecosystems including the nutrient cycle, carbon sequestration and some other monitoring studies. However, litterfall collection is quite time consuming, laborious and expensive process. One of the most appropriate, practical and economic methods in determining the amount of litterfall and its spreading distances is to develop mathematical models. In this study, we have developed some mathematical models based on canopy closure of the stands using the data obtained from the litterfall collection in Uludağ fir stands. However, the developed models can be rectified with the further data to accommodate various site conditions. Besides, new studies may need to be designed in order to determine the amount and the spreading distances of $Total_{Lf}$ and its fractions in the gaps of the edges of the stands. The necessary mathematical models can be developed for the other commercial trees in our forest ecosystems based on the sampling method and the models developed in this study.

Conclusions

In conclusion, this study has developed statistical relationships for estimating canopy cover based on the litterfall in Uludağ fir forest stands. These results obtained in Bolu-Aladağ (Turkey) can also be used for similar ecological conditions of Uludağ fir. The main findings indicated that the highest correlation was found between the amount of $Total_{Lf}$ and the canopy closure at 20° angle of view (a circle with the diameter of 1/3 tree height at horizontal) using DHP. The amount of litterfall, spreading distance and the collecting amount based on the distance were estimated by the mathematical models developed as part of this study.

The data of fall and spread of the litter are very important for forestry sciences such as ecology, silviculture and management. Therefore, it would be necessary for similar studies to be carried out for other broad-leaved and coniferous tree species in all forest ecosystems. But, litterfall collection studies are long-term and effortful. Due to these reasons, the planed litterfall researches should be designed appropriate for mathematical modeling in the future. Thus, it may be possible to use the obtained results in wider areas. Practitioners can estimate the amounts of litterfall components, spreading distances and the accumulating amounts by using these mathematical models.

Acknowledgements. This work is supported by the Scientific Research Project Fund of Karabuk University under the project number KBUBAP-17-BM-385.

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INVESTIGATION OF CHANGES IN CLIMATE DATA USING CHECKERBOARD: THE CASE OF AKARÇAY BASIN

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(Received 24th Oct 2018; accepted 20th Dec 2018)

Abstract. Water resources planning requires very good data set and accurate analyses, one of which is a trend identification procedure. Different trend analyses are available in the literature and one of the most commonly used one is the Mann-Kendall trend analysis. The biggest problem of this analysis is that trends change with the selection of different time periods. Although many researchers have tried to find different solutions to this problem, in this study, trends are set for all combinations of the available data to cover at least 10-year periods and the results are displayed on a 3-dimensional checkerboard. For the application precipitation and temperature data are considered from 5 meteorology stations in the Akarçay, Turkey.
Keywords: *Afyon, period, trend, rainfall, temperature*

Introduction

Rapid population growth and industrialization have a huge impact on global climate. Particularly in recent decades, the concept of climate change has received growing attention among the researchers. Studies especially address the amount of precipitation (Paul et al., 2017), precipitation intensity (Olive et al., 2017), mean temperature (Khan et al., 2017), extreme temperature conditions (Zhao et al., 2017), amount of evaporation (Xu et al., 2007), stream flow (Saplioglu et al., 20014) and groundwater level fluctuations (Gebert et al., 2007). These are also mathematical (Sing et al., 2016), statistical (Zeleňáková, 2017; Kristo et al., 2017; Onyutha, 2016) and graphical (Cui et al., 2017; Sen, 2012) description studies about trend analysis.

Statistical models are crucial for trend detections. Many researchers conducted statistical studies on trends and proposed different methods (Saplioglu, 2015). One of the most widely used methods is the Mann-Kendall test, which has been employed to analyze seasonal and annual precipitation trends in Bangladesh (Baria et al., 2016), precipitation and drought trends of the Aegean region, Turkey (Bacanli, 2016), precipitation trends in Sicily (Cannarozzo et al., 2006), around the Nile River (Onyutha et al., 2016), in Turkey (Partal and Kahya, 2006), Florya, Istanbul (Guclu et al., 2018), Córdoba, Argentina (Casaa and Nasello, 2010), in the Iberian peninsula (Bustins, 2008), in three different regions of Japan (Yue and Hashino, 2003), in Brazil (Carvalhao et al., 2014), in Sudan (Goanstera et al., 2015), precipitation and temperature trends in the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23732384

Northeastern United States (Karmesha, 2012), stream trends and the impact of precipitation and subsurface waters on these trends in India (Kumar et al., 2009), changes in hourly precipitation in Peninsular Malaysia (Syafrina et al., 2018), temporal and spatial precipitation changes and estimation in Mongolia (Kim et al., 2011) and 6-minute precipitation intensity trends in Australia's southern regions (Kamruzamana et al., 2016).

Some researchers have applied the Mann-Kendall test for different time periods and observed that the results were different. For example, Baria et al. (2016), analyzed 50-year data in northern Bangladesh and observed no trends except for one station. They also found different downward trends in all stations for 1990 and beyond. Cannarozzo (2006) conducted a study on 247 stations leading to different trends for three different periods as 1921-2000 (170 trends), 1931-1960 (65 trends) and 1961-1990 51 trends. Although Carvalhao et al. (2014) identified a significant trend from 1899 to 1970 they did not detect any trend from 1970 and beyond. Goanstera et al. (2015), investigated precipitation amount to identify trends from 1950 to 2009 and 1970 to 2009. They observed statistically significant trends during the former part of the records, but no statistically significant trends along the latter part. Seo et al. (2012), used the Mann-Kendall test for different time periods and displayed the confidence intervals of the trends on a checkerboard. Studies suggest that using the Mann-Kendall test for different periods yields different results.

In this study of temperature and precipitation trends are analysed in which is one of Turkey's 25 sub-basins Akarçay. The Mann-Kendall test was used for trend analysis. However, the entire data set was scanned at least 10 years during this process. The results are presented as 3D graphics. The most important aim of the study is to show that the trends may change when the period of the selected period changes rather than determining the current trend.

Materials and methods

This study investigates possible precipitation and temperature trends in Akarçay basin, Turkey.

Akarçay basin features

Akarçay is one of the 25 basins in Turkey and it has a total drainage area of 7,337 km^2 along the closed depression basin extending between Uşak-Banaz in the West and Konya-Doğanhisar in the East. It length is parallel to the tectonic line between the Taurus Belt and Afyon Metamorphics with orientation along 38.7380-39.0098 NW-30.5604-31.4217 SE direction its length – 160 km with 70 km width (*Fig. 1*).

Tables 1 and *2* present statistical parameters of annual homogenized precipitation records and temperature records, respectively

Methods

It is emphasized in this study that the start and end years are crucial in the classical Mann-Kendall tests. In any analysis, trends vary according to start and end years. In the same time series record there may be more than one trend subsequently with different slopes. For this reason, data set is sub-divided into minimum 10-year periods and the

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DOI: http://dx.doi.org/10.15666/aeer/1702_23732384

Mann-Kendall test is performed on each period and the results are presented on 3D checkerboard graphics.

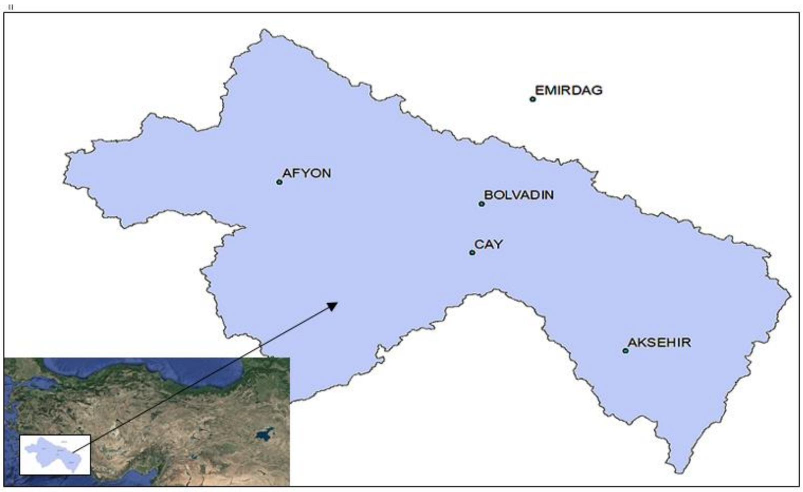

Figure 1. *Site location map of Akarçay basin*

Table 1. *Statistical analysis of data from precipitation measurement stations of Akarçay basin*

	Afyon	Akşehir	Bolvadin	Çay	Emirdağ
Altitute	1034	1002	1018	996	983
Latitude	38.7380 N	38.3688 N	38.7268 N	38.5903 N	39.0098 N
Longitude	30.5604 E	31.4297 E	21.0477 E	31.0315 E	31.1463 E
Mean (mm)	434.70	615.42	384.03	526.23	401.18
Standard deviation	91.98	130.20	60.29	98.84	99.34
Kurtosis	0.10	0.12	-0.17	-0.32	0.55
Skewness	0.22	0.39	-0.31	0.30	0.17
Maximum (mm)	238.20	371.30	217.30	333.80	161.70
Minimum (mm)	679.10	942.70	484.20	751.30	698.70
Initial data	1929	1941	1957	1967	1953
Number of data	81	69	46	40	57
Confidence level (95.0%)	20.34	31.28	17.90	31.61	26.36

Table 2. *Statistical analysis of data from temperature measurement stations of Akarçay basin*

	Afyon	Akşehir	Bolvadin	Çay	Emirdağ
Mean (°C)	11.19	11.94	11.01	11.80	11.47
Standard deviation	0.72	0.85	0.75	0.70	0.74
Kurtosis	-0.09	2.32	0.02	1.38	-0.13
Skewness	-0.12	-0.66	0.09	-0.04	0.32
Maximum ($^{\circ C}$)	9.28	8.78	9.22	10.14	9.93
Minimum ($^{\circ C}$)	12.84	13.78	12.79	13.07	13.24
Initial data	1929	1941	1969	1969	1965
Number of data	81	64	41	41	45
Confidence level (95.0%)	0.16	0.21	0.24	0.36	0.22

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Mann-Kendall test statistic

The Mann-Kendall test (Mann, 1945; Kendall, 1970) is a nonparametric procedure, which does not make any assumption regarding the data distribution. The aim of the Mann-Kendall test is to verify whether a trend exists in a time series. If the hypothesis is rejected, it means that there is a trend. During any analysis, time series are divided into two groups; x1, x2, ..., xn, (xi, xj). If i < j for each xi < xj, then P value is increased by one, in the opposite case, that is, for … xi > xj, M value then the test statistics, S = P – M, is defined (Saplioglu et al., 2017).

Kendall correlation coefficient for n > 10 is calculated as follows (*Eqs. 1-2*).

$$\mu_s = 0 \; ve \; \sigma_s = \sqrt{n(n-1)(2n+5)/18} \quad \text{(Eq.1)}$$

$$z = \left.\begin{matrix} \frac{s-1}{\sigma_s} & s > 0 \\ 0 & s = 0 \\ \frac{s-1}{\sigma_s} & s < 0 \end{matrix}\right] \quad \text{(Eq.2)}$$

If the Z-score is less than α corresponding to Z/2 in a normal distribution, then the hypothesis is accepted, indicating that there is no trend. If the Z-score (*Eq. 2*) is greater than α corresponding to Z/2, then the hypothesis is rejected, suggesting that there is a trend. If S is positive, then it is an upward trend, on the other hand, if S is negative, then it is a downward trend. The Mann-Kendall test method is practical as it does not make any assumption regarding the data distribution (Saplioglu et al., 2014).

Applied method

The Mann-Kendall test statistic generally analyzes trends in series between a start and end points. This method iteratively arranges the start and end points and allows one to calculate trends in different periods (at least 10 years). These trend changes are displayed on a checkerboard. The x axis represents the start years, the y axis is for end years and the z axis stands for the confidence interval of the trend, and hence, this axis assumes values between -1 and 1. The range from 0 to 1 indicates a downward trend while from -1 to 0 is for an upward trend. Generally at a confidence interval of 95%, the results are considered as statistically significant indicating the presence of a trend. The blue color scale (-1-0) shows a downward trend while the red color (0-1) implies an upward trend. Software was generated using MATLAB in order to apply the method quickly and automatically (*Fig. 2*). This software was generated embedding the Mann-Kendall statistical subprogram (*Fig. 3*) into MATLAB.

Results and discussion

In the Akarçay basin, stations with data set suitable for the Mann-Kendall test (5 for precipitation and 5 for temperature) were selected and analyzed.

Precipitation trends

Mann-Kendall tests are performed in at least 10 different combinations on the precipitation data set for 5 stations. All results are summarized in tables and graphs. In the classic Mann-Kendall tests, only one analysis is performed at the start and end times of a data set. 2628,

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1830, 990, 561 and 1176 Mann-Kendall tests are performed for Afyon, Akşehir, Bolvadin, Çay and Emirdağ, respectively (*Table 3*).

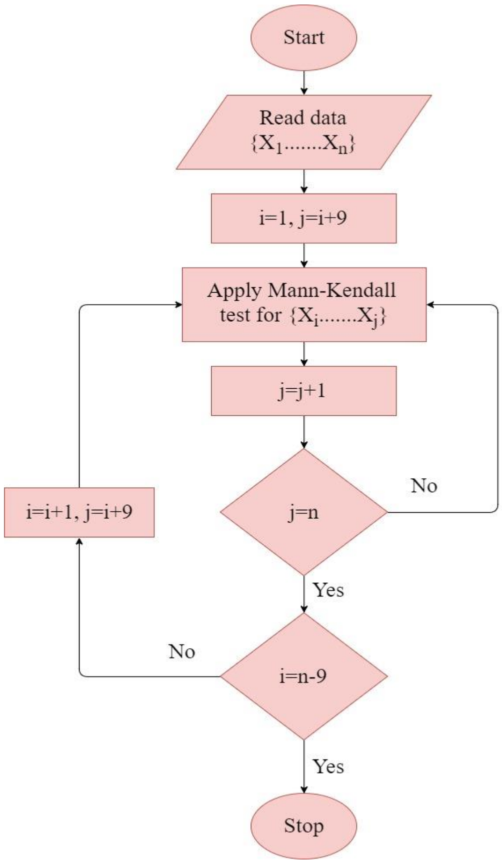

Figure 2. *Flow diagram of the software for the model*

Algorithm 1 Mann-Kendall Function

```
procedure MANN-KENDALL(a)
    L = length(a)
    l = 0
    u = 0
    for i ← 1 to L − 1 do
        for i ← i + 1 to L do
            if a[i] < a[j] then
                l = l + 1
            end if
            if a[i] > a[j] then
                u = u + 1
            end if
        end for
    end for
    s = (u − l)
    z = (s−1)/(σs)
end procedure
```

Figure 3. *Algorithm of the Mann-Kendall test used in the software*

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Table 3. *Trend statistics for precipitation data*

	Iteration	Negative trend	Negative rate	Positive trend	Positive rate
Afyon	2628	524	19.94	112	4.26
Akşehir	1830	446	24.37	235	12.84
Bolvadin	990	3	0.30	2	0.20
Çay	561	42	7.49	1	0.18
Emirdağ	1176	46	3.91	64	5.44

Trend analyses for different periods are visually presented on the checkerboard. Out of 2,628 Mann-Kendall tests applied to the Afyon precipitation data, 524 revealed a negative trend while 112 indicated a positive trend (*Fig. 4*). All data between the initial and final points indicate the presence of a negative trend. However, this can sometimes be misleading. For example, the analysis of Afyon station data over the period from 1929 to 2009 shows a trend at a confidence interval of 95%, however, no trend is observed in the analysis of the data over the period from 1929 to 2008 or from 1930 to 2009. Similar examples can be found in the literature (*Fig. 4*).

On the other hand, out of 1,830 Mann-Kendall tests on the Akşehir precipitation data (1941-2011), 446 (24.37%) had a negative trend, whereas 235 (12.84%) with a positive trend. Most of the positive trends are concentrated in the upper left corner of the figure, indicating an upward trend (*Fig. 5*).

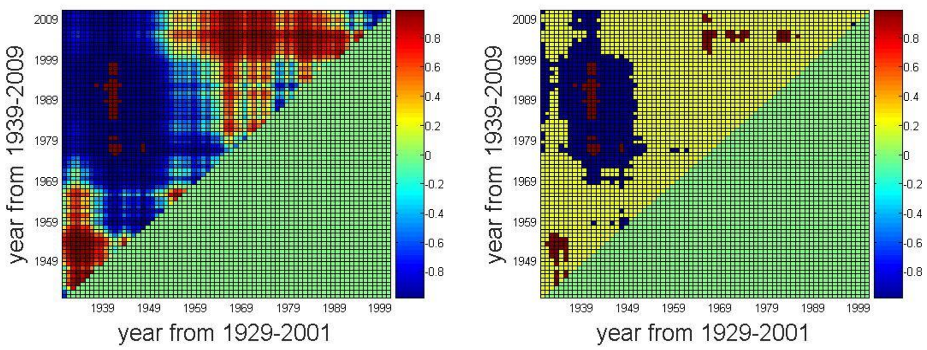

Figure 4. *Afyon precipitation trend analysis results a-) all confidence intervals b-) 95% confidence interval*

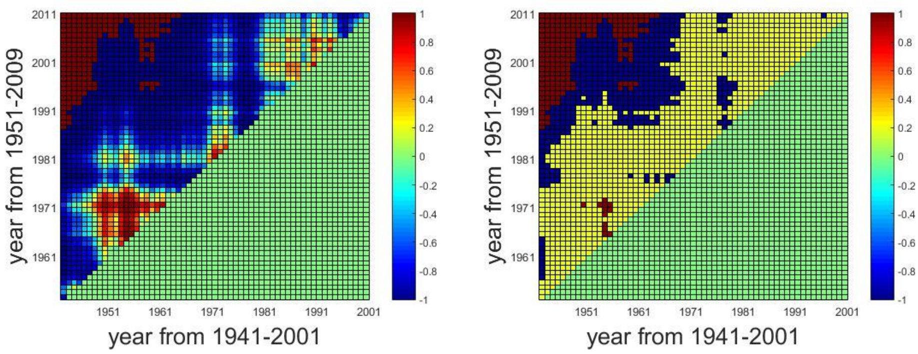

Figure 5. *Akşehir precipitation trend analysis results a-) all confidence intervals b-) 95% confidence interval*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23732384

Mann-Kendall tests conducted 990 times on the Bolvadin precipitation data (1957-2009), 3 (0.30%) exhibited a negative trend and 2 (0.20%) exhibited a positive trend, with the remaining cases no trend (*Fig. 6*).

Out of 561 Mann-Kendall tests performed on the Çay precipitation data (1964-2006), 42 (7.49%) showed a negative trend while 1 (0.18%) showed a positive trend. Most of the negative trends are concentrated in the upper left corner of the figure, suggesting that although the number of trends is low, the negative trend has accelerated in recent years (*Fig. 7*).

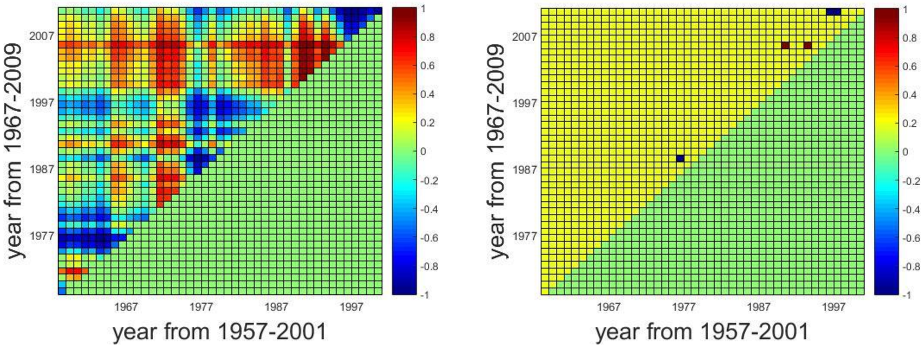

Figure 6. *Bolvadin precipitation trend analysis results a-) all confidence intervals b-) 95% confidence interval*

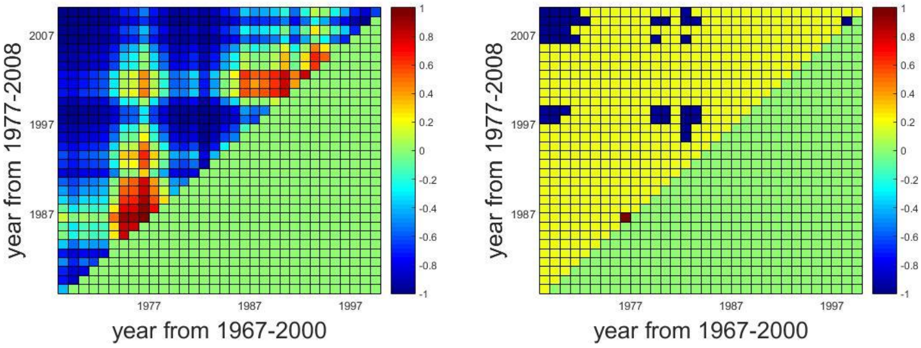

Figure 7. *Çay precipitation trend analysis results a-) all confidence intervals b-) 95% confidence interval*

Temperature trends

Mann-Kendall tests are also performed at least for 10 different combinations on the temperature data set at 5 stations. In the classic Mann-Kendall tests, only one analysis is performed at the start and end times of a data set. 2628, 1941, 528, 528 and 666 Mann-Kendall tests are performed for Afyon, Akşehir, Bolvadin, Çay and Emirdağ, respectively. Trend statistics are given in *Table 4.*

Similarly, 1,176 Mann-Kendall tests carried out on the Emirdağ precipitation data (1953-2009), 46 (3.91%) had a negative trend, whereas 64 (5.44%) showed a positive trend. The positive trends are concentrated in the lower left corner of the figure, while the negative ones are in the center of the figure, indicating that there has been no trend in recent years (*Fig. 8*).

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Table 4. *Trend statistics for temperature data*

	Iteration	Negative trend	Negative rate	Positive trend	Positive rate
Afyon	2628	245	9.34	94	3.58
Akşehir	1941	11	0.57	79	4.07
Bolvadin	528	0	0.00	153	28.98
Çay	528	0	0.00	32	6.06
Emirdağ	666	33	4.95	46	6.91

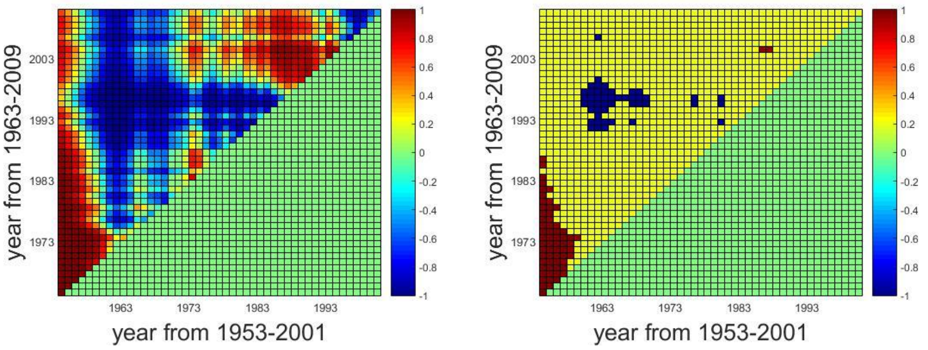

Figure 8. *Emirdağ precipitation trend analysis results a-) all confidence intervals b-) 95% confidence interval*

Trend analyses performed for different periods are visually presented on the checkerboard. Out of 2,628 Mann-Kendall tests on the Afyon temperature data, 245 (9.34%) had a negative trend, while 94 (3.58%) had a positive trend (*Fig. 9*).

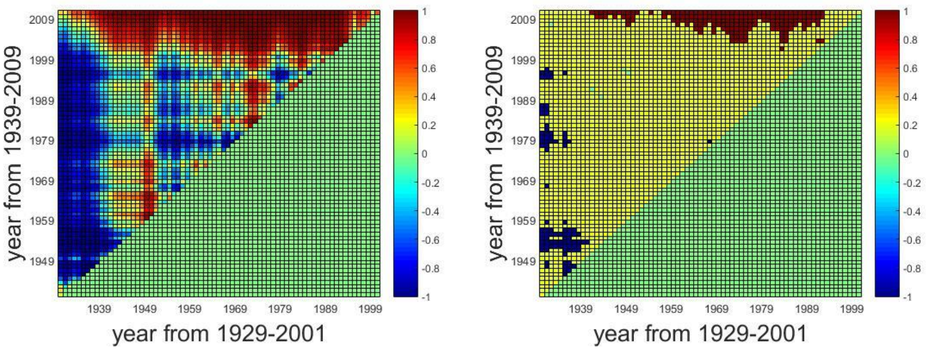

Figure 9. *Afyon temperature trend analysis results a-) all confidence intervals b-) 95% confidence interval*

On the other hand, 1941 Mann-Kendall tests performed on the Akşehir temperature data (1953-2009), yielded 11 (0.57%) negative trends and 79 (4.07%) exhibited a positive trend. The lack of positive trends, especially on the left side of the figure, indicates that the trend in this region is not pronounced (*Fig. 10*).

Similarly, out of 528 Mann-Kendall tests conducted on the Bolvadin temperature data (1969-2009), 0 (0%) had a negative trend with 153 (28.98%) positive trend. The concentration of positive trends at the top of the figure suggests that trends in this region

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have increased. However, when the classical Mann Kendall test is applied, only one value would be expected to be as 0.05 (the only cell in the upper left corner of the figure, and therefore, no trend would be reported (*Fig. 11*).

Additionally, out of 666 Mann-Kendall tests carried out on the Çay temperature data (1969-2009), 33 (4.59%) and 46 (6.91%) exhibited a negative and positive trend, respectively. The distribution of cells on the figure does not suggest that there is no trend (*Fig. 12*).

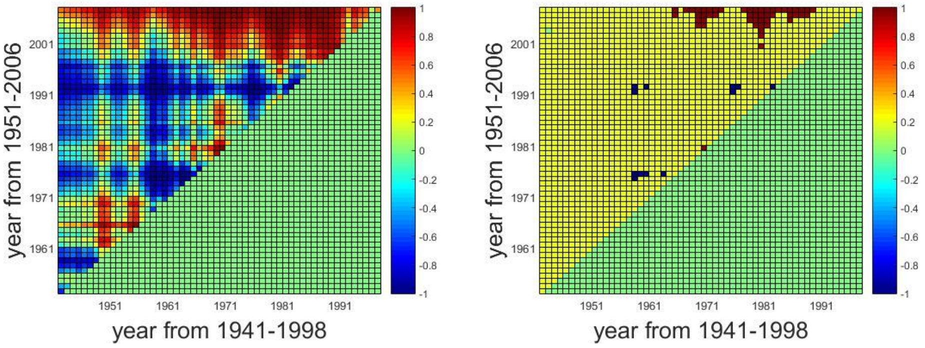

Figure 10. *Akşehir temperature trend analysis results a-) all confidence intervals b-) 95% confidence interval*

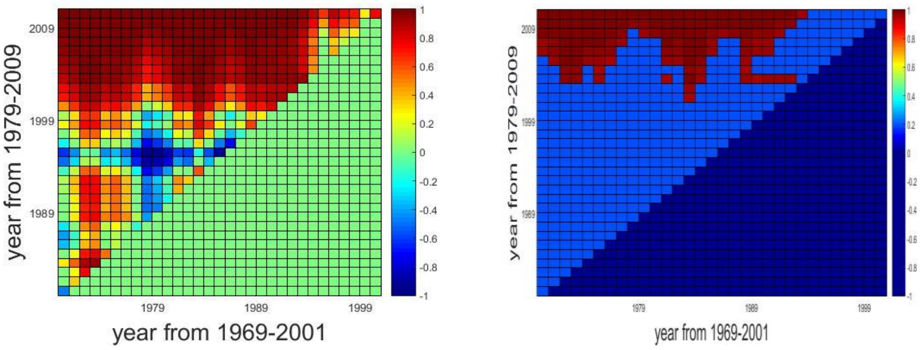

Figure 11. *Bolvadin temperature trend analysis results a-) all confidence intervals b-) 95% confidence interval*

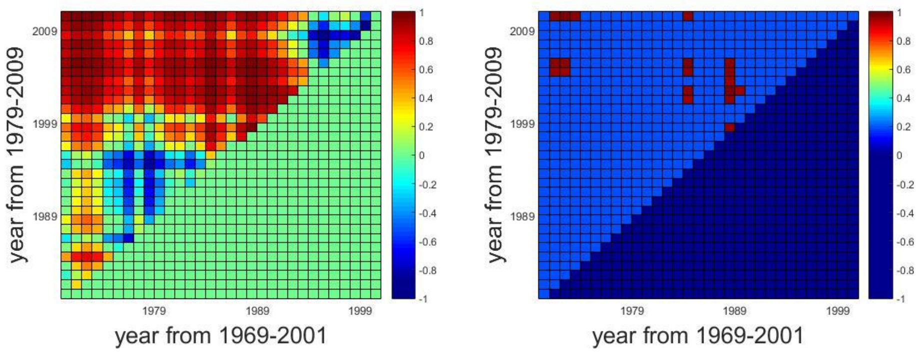

Figure 12. *Çay temperature trend analysis results a-) all confidence intervals b-) 95% confidence interval*

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Finally, out of 666 Mann-Kendall tests applied to the Emirdağ temperature data (1965-2009), 46 (3.91%) revealed a negative trend with 64 (5.44%) positive trend. *Figure 13* shows that all the negative trends are associated with the 1965-1995 data and that recent data do not indicate the presence of a trend (*Fig. 13*).

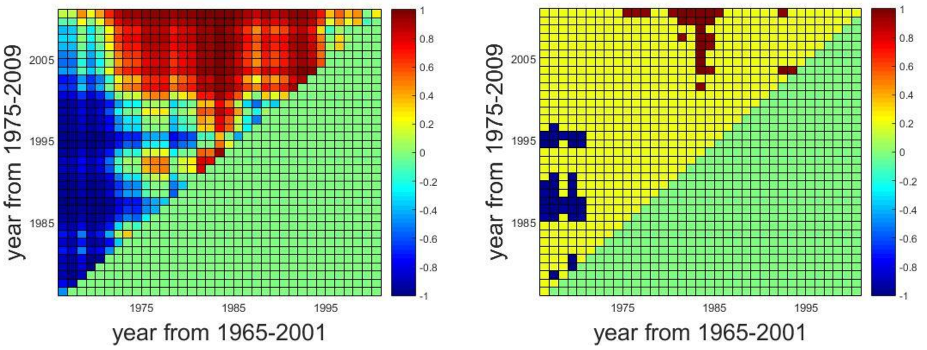

Figure 13. *Emirdağ temperature trend analysis results a-) all confidence intervals b-) 95% confidence interval*

In many studies, the initial and end values of the data are available (Saplioglu et al., 2014; He et al., 2017; Quan et al., 2018). These dates are the dates when records started and records were reached. In this study, it was tried to get answers to questions such as how different the trend would be if the record started to be recorded at a later date than the records started to be recorded, or whether the study would affect the trend values that were made n years ago or n years later. (n-10) (n-9) / 2 studies are possible since a minimum period of 10 years is selected. In this study, all possibilities for all stations are found and shown on checkerboard. For example, the first data from the Emirdağ temperature station were taken in 1973, and in 2008 the researcher who was working did not observe the trend formation, but in 2009, another researcher in the researcher determined the positive trend.

Conclusion

Trend identification is as important as data analysis for the assessment of water resources. The Mann-Kendall test is one of the most commonly used methods for trend analysis. However, changing the start or end time of a period can result in different trend values. In this study, Mann-Kendall tests are performed on all subsets (each having 10 or more year’s data). Confidence intervals of z values are determined and displayed three-dimensionally on a checkerboard.

Data from five precipitation and five temperature stations on Akarçay basin in Turkey, are evaluated and their trends are presented on the checkerboard. Results indicate that the precipitation tends to decrease in Akşehir station and that the temperature significantly increases in Bolvadin station.

This study has eliminated the problem of “time interval selection” for the Mann-Kendall test. For example, Afyon meteorology station precipitation data indicate the presence of a trend between 1929 and 2009 at a confidence interval of 95%. However, no trend is detected in the data if the start year is 1930 or the end year is 2008. Similarly, although the increasing trend in recent years in the Bolvadin temperature data

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is conspicuous on the checkerboard, the classical Mann-Kendal test fails to detect a trend at a confidence interval of 95%. The analyses in this study are performed for each time interval, and therefore, trend analysis is better analyzed graphically. In addition, using software to analyze the data allowed obtaining individual trend results for all time periods in a very short time. The study is important in terms of showing that different trends can be found in the same station at different periods.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23732384

ACCUMULATION OF SELENIUM IN RYE PLANTS (*SECALE CEREALE* L.) AT DIFFERENT STAGES OF DEVELOPMENT AND GRAIN QUALITY DUE TO SELENATE SOIL SUPPLEMENTATION

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(Received 23rd May 2018; accepted 2nd Jan 2019)

Abstract. The accumulation of selenium by rye at different stages of development depending on the dosages of selenium and pH of soil was investigated. The findings testify that in case of increased selenium content in the soil selenium concentration in the plant also increased. Moreover, it was revealed that in the process of vegetation total selenium content in the rye increases; however the intensity of selenium accumulation by plants decreases. The positive effect of low concentrations of selenium on biomass accumulation of rye plants was found. The total protein content in rye grain was higher by application of selenium. The results of this work show the possibilities of applying of the seleniferous fertilizers for the increasing in the content of micronutrient in the grain plants. The recommended optimal concentrations of selenium additive are 0.01-0.05 mg kg^{-1} of soil.
Keywords: *cereals, seleniferous fertilizers, microelement, yield, proteins*

Introduction

Rye (*Secale cereale* L.) is one of the most important cereals in middle, eastern, and especially north Europe. Among the cereals, rye can be grown under extreme climatic and poor soil conditions. On a global scale rye is a minor crop, its production being about 5% that of wheat or rice (Korzun et al., 2001). However, recently, interest in this crop has increased because of its dietary value. Rye is especially rich source of phytochemicals such as phenolic acids, lignans, alkylresorcinols and benzoxazinoids (Pihlava et al., 2018).

So far scientists do not know for certain whether selenium is an essential microelement for growth and development of plants. Some researchers admit necessity of selenium for plants others believe that selenium is not an essential element for normal life history of vegetative organism. However, there are a number of facts confirming positive influence of selenium on various life processes. Thus, it has been established that selenium increases antioxidant potential and stress-resistance of plants, stimulates their growth in the conditions of oxidation stress caused by UV-radiation (Hartikainen and Xue, 1999; Xue and Hartikainen, 2000; Pennenen et al., 2002; Germ et al., 2007; Golob et al., 2018), herbicides, hypothermia (Seppaenen et al., 2003; Xu et al., 2003), drought stress (Nawaz et al., 2015a; Aissa et al., 2018), plant's ageing (Xue et al., 2001; Djanaguiraman et al., 2004, 2005), salt stress (Kong et al., 2005; Diao et al., 2014; Jiang et al., 2017). There has been noted increase of yield level of some plants further to application of low concentrations of selenium (Graham et al., 2005; Thavarajah et al., 2015; Nawaz et al., 2015b). Positive effect of selenium is also

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revealed in increased germinating capacity and improved survival potential of plantlets in stress conditions (hypo- and hyperthermia, hypoxia, soil salinity and acidulation etc.) In the presence of selenium lipid membrane peroxidation processes in conditions of high light intensity and low temperatures are reduced, testifying to the effect that selenium has an influence on oxidation-reduction cell status (Zhu et al., 2009).

Selenium intake by plants from soil depends on many factors, for instance, on soil pH, humus content, chemical form of selenium (Zhao et al., 2005; Hawrylak-Nowak et al., 2015). Other important factor able to influence selenium quantity in plants is plant species. There are certain selenium accumulating plants, which accumulate from 100 to several thousand mg Se per kg of dry substance, in other plants including cereals selenium content is 0.01-1.00 mg kg^{-1} on the average (Terry et al., 2000; Wangeline et al., 2011; Pilon-Smits, 2017). Lyons et al. (2005) showed that the main reason for variability in the content of selenium in some cereals (wheat, barley, rye) is the selenium concentration in the soil, but not genotypic variation. Similar findings were also obtained in studies with three varieties of wheat (Sharma et al., 2015).

Analysis of selenium accumulation pattern is very important both for study of biochemical role of this microelement for plants and to study application potential of selenium-containing fertilizers in order to increase selenium supply of animals and humans.

Purpose of this paper was to study selenium accumulation by rye (*Secale cereale* L.) at various ontogenesis stages subject to application of selenium additive to the soil and the impact of this on the yield and grain quality.

Materials and methods

Plant cultivation

The pot experiment was conducted in Kaliningrad, Russia (54°43'N, 20°30'E), from 12 April till 17 July, 2015, in a glass covered greenhouse of the Institute for Chemistry and Biology at Immanuel Kant Baltic Federal University. The greenhouse was not heated and we followed the natural light cycle, no additional lighting was supplied. Common rye (*Secale cereale* L.) cv. Pukhovchanka was used for experiments. The rye was grown in culture pots in permanent-set soil having pH 5.4 and pH 6.6. The dimension of pot was 29 × 20 cm (height × radius) and each pot contained 10 kg of soil. Selenium was applied in the soil in the form of a sodium selenate solution (Na_2SeO_4) in concentrations 0.01 mg, 0.05 mg, 0.1 mg, 0.5 mg (in terms of 1 kg of the soil). Selenium effect on the plants was compared to control treatment (without selenium supplementation). Initial selenium content in the soil was 0.084 mg/kg. Moreover, following basic fertilizers were applied: 0.17 g of nitrogen in terms of ammonium nitrate, 0.13 g of P_2O_5 in terms of potassium hydrophosphate, 0.28 g of K_2O in terms of potassium hydrophosphate and sulfate and 0.044 of MgO in terms of magnesium sulfate per kg of the soil. Solutions of fertilizers and sodium selenate were applied in such manner so that they cannot mix with each other. First in extreme points of soil surface square basic microelements were applied, whereas sodium selenate was applied in the central point. After the soil dried out it was mixed and the rye was planted. Into each pot were planted 20 plants. During experiments the plants were watered with distilled water.

The analysis of plant were performed at three plant growth stages (GS): at tillering stage (GS 26, main shoot and 6 tillers), booting stage (GS 45, mid-boot stage: boots just

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visibly swollen) and milk development stage (GS 75, medium milk) according to Zadoks' scale (Zadoks et al., 1974).

Four replications for each treatment (selenium suplements, soil pH, and stage of development) were condacted. There were 120 pots in total.

Plant analysis

Selenium was determined by atomic absorption spectrometry (Unicam M Series, Software Solaar, Thermo Scientific) with mineralization of vegetative samples through autoclave decomposition under pressure (Kurkova et al., 2008).

The extraction of reserve proteins was performed by deionized water (for albumin), 5% NaCl (for globulin), 60% ethanol (for prolamin), and 0.4% NaOH (for glutelin). Protein concentration was determined according to Bradford, using BSA as the standard (Bradford, 1976).

Statistical analysis

Statistical analysis was performed using the SigmaPlot 12.3 (Systat Software GmbH, Erkrath, Germany). The mean of four independent samples were taken to represent the result of each replicate. The results were reported as mean ± standard deviation (SD). To identify the difference in means the one-factorial ANOVA was conducted for each factor (selenium application, growth stage, pH of soil, organs of plant) separately Difference among means were determined by Tukey's test at a significance level of $p \leq 0.05$.

Results

Accumulation of selenium in rye at different stages of development

In order to study selenium accumulation by the rye the plants were grown in the soil enriched with this microelement in concentrations 0.01–0.5 mg Se kg^{-1} of soil. Findings on selenium content in vegetative specimen were compared to the plant without selenium supplementation. Initial selenium content in the soil was 0.084 mg kg^{-1}. Selenium was identified in above-ground part of the plant at three stages of vegetation. Results of study of relation between selenium content in all plant and quantity of selenium applied in the soil at different stages of the plant ontogenesis are shown in *Figure 1*.

The findings testify that in case of increased selenium content in the soil selenium concentration in the plant also increased. Thus, after application of 0.05 mg of selenium per kg of soil, 4.74 ± 0.06 µg of selenium was identified in the plant (at milk development stage), while after application of 0.5 mg of selenium per kg of soil selenium content in the plant was almost 16 times higher – 74 ± 2 µg. Moreover, it was revealed that in the process of vegetation total selenium content in the rye increases, however as it is obvious on the picture intensity of selenium accumulation by plants decreases.

In order to study intensity of selenium accumulation by the rye at different ontogenesis stages total selenium content in the plant was calculated per gram of vegetating mass. Dependence of selenium concentration in the plant on selenium concentration in the soil is illustrated in *Figure 2*.

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The study shows that tillering stage was characterized by maximum selenium concentration, as the plant advanced intensity of its accumulation progressively decreased. Maximum selenium concentration in the rye at tillering stage was 59.3 ± 0.4 μg g^{-1}, at booting stage – 31.7 ± 0.3 μg g^{-1} and at milk development stage – only 11.8 ± 0.3 μg g^{-1}.

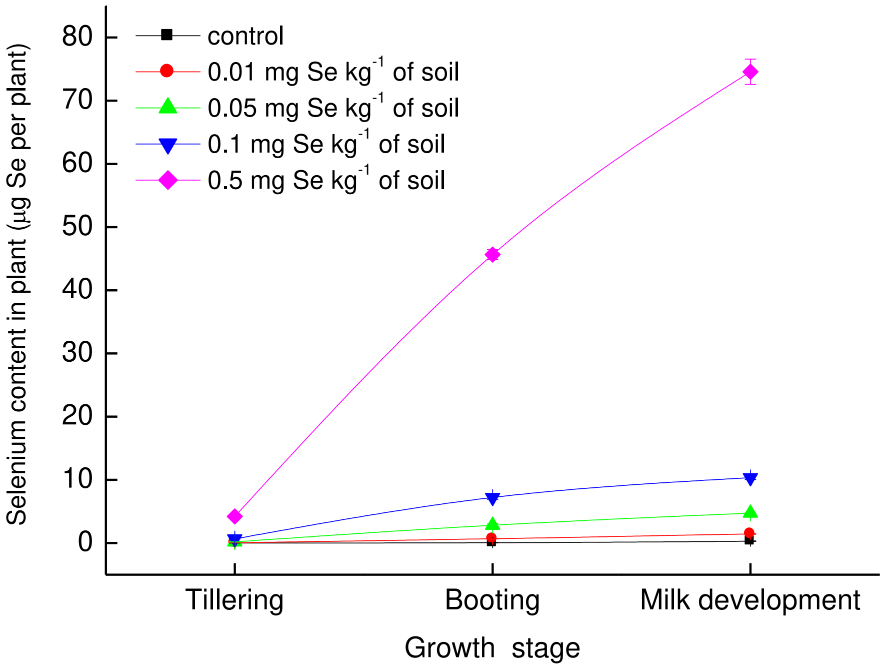

Figure 1. *Selenium content in rye plants at different stages of ontogenesis in depending on the amount of selenium supplements (soil pH 6.6)*

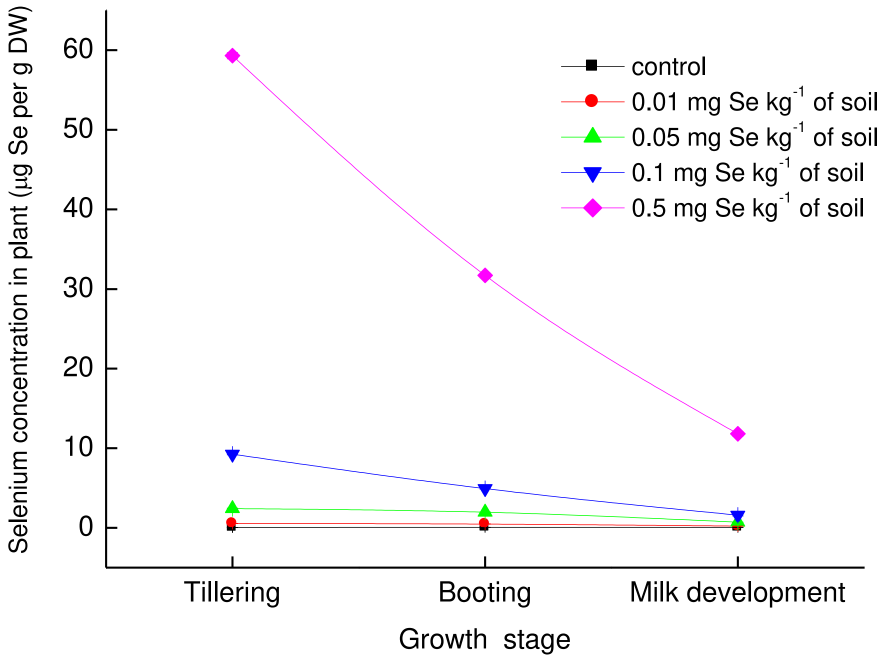

Figure 2. *Selenium concentration in rye plants at different stages of ontogenesis in depending on the amount of selenium supplements (soil pH 6.6)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

Influence of soil pH on the selenium accumulation in rye

As seen from the provided data, soil acidity difference being investigated influenced selenium intake by the plant. And considerable difference was obvious for the plants grown in the soil enriched with this microelement (*Table 1*). The significant higher concentration of selenium in rye plant growing on less acidic soil (pH 6.6) was determined. However, at tillering stage in plant growing on soil with pH 5.4 and without selenium supplementation the concentration of selenium was higher. At the later stages of plant development (booting and milk development), no significant difference in the selenium concentrations in plants growing on the soil with different acidity and without selenium additives was found.

At the same time, it was revealed that nature of selenium accumulation by the rye in the process of vegetation does not depend on the soil acidity: at the early stage of development – at tillering stage the microelement was received by the plant intensively, at the same time total selenium content in the plant increased.

Table 1. *Selenium concentration in rye plants (µg g^{-1}) in depending of soil acidity and selenium addition concentration*

Stage of ontogenesis	Selenium addition concentration mg Se kg^{-1} soil	pH 5.4	pH 6.6
Tillering	0	0.042±0.002^{a}	0.031±0.002^{b}
	0.01	0.58±0.03^{a}	0.55±0.02^{a}
	0.05	2.2±0.1^{b}	2.41±0.07^{a}
	0.1	7.4±0.1^{b}	9.24±0.08^{a}
	0.5	56.8±0.7^{b}	59.3±0.4^{a}
Booting	0	0.048±0.004^{a}	0.051±0.003^{a}
	0.01	0.42±0.01^{b}	0.47±0.03^{a}
	0.05	1.81±0.04^{b}	1.97±0.04^{a}
	0.1	4.38±0.05^{b}	4.94±0.08^{a}
	0.5	24.9±0.1^{b}	31.7±0.3^{a}
Milk development	0	0.046±0.002^{a}	0.047±0.001^{a}
	0.01	0.167±0.005^{b}	0.212±0.004^{a}
	0.05	0.65±0.02^{b}	0.71±0.01^{a}
	0.1	1.42±0.07^{b}	1.6±0.1^{a}
	0.5	10.4±0.6^{b}	11.8±0.3^{a}

Means within rows followed by different lower-case letters stand for significance different at $p \leq 0.05$

Selenium distribution in different organs of the rye plant

Study of selenium distribution in different organs of the rye plant testified that selenium content in the grain was higher than in the stem (*Table 2*). It was particularly evident in case of low selenium content in the soil; in case of higher selenium additives in the soil difference between its content in the stem and in the ear became not so evident. Thus, there was no significant difference between the content of selenium in the stem and grain of plants grown with the addition 0.05 mg selenium per kg soil and by soil acidity of 5.4.

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Table 2. *Selenium content in grain and stem (µg per plant) of rye plants depending on selenium supplements concentration and pH of the soil*

Soil pH	Selenium addition concentration mg Se kg^{-1} soil	Grain	Stem
5.4	0	0.21±0.01[a]	0.12±0.01[b]
	0.01	0.75±0.08[a]	0.42±0.04[b]
	0.05	2.7±0.1[a]	1.59±0.09[b]
	0.1	6.4±0.5[a]	3.4±0.5[b]
	0.5	35.4±1.3[a]	33.1±1.7[a]
6.6	0	0.18±0.04[a]	0.11±0.01[b]
	0.01	0.82±0.05[a]	0.52±0.03[b]
	0.05	2.93±0.03[a]	1.75±0.04[b]
	0.1	6.1±0.2[a]	4.5±0.2[b]
	0.5	38.3±1.2[a]	35.1±0.6[b]

Means within rows followed by different lower-case letters stand for significance different at $p \leq 0.05$

Effect of soil applied selenium concentration on biomass accumulation of rye

This paper studies effect of applied selenium soil addition on accumulation of biomass by the rye plants at various ontogenesis stages (*Table 3*). The findings show that optimal for the rye plant is selenium additive in concentration of 0.01-0.05 mg kg^{-1} of soil (soil pH is 6.6). Under these conditions at all stages of development the plants built up maximum biomass both compared to the reference specimen and to the specimen with higher selenium concentrations. At booting stage positive effect of selenium additive in the form of sodium selenate was fixed for all plants irrespective of concentration of selenium applied in the soil. In the case of soil pH 5.4 selenium effect on the plant was more toxic. Positive effect was fixed at tillering stage after application of 0.01 mg Se kg^{-1} of soil and at booting stage after application of 0.05-0.1 mg Se per kg of soil, whereas at milk development stage selenium effect was purely toxic irrespective of concentration.

Table 3. *Influence of selenium concentration in soil on the biomass (g) of rye plants at different ontogenesis stages*

Stage of ontogenesis	Soil pH	Selenium addition concentration, mg Se kg^{-1} soil				
		0	0.01	0.05	0.1	0.5
Tillering	5.4	0.074±0.004[a]	0.081±0.002[a]	0.075±0.004[a]	0.077±0.005[a]	0.073±0.004[a]
	6.6	0.072±0.005[b]	0.083±0.005[a]	0.078±0.003[ab]	0.072±0.001[b]	0.071±0.001[b]
Booting	5.4	1.39±0.024[b]	1.42±0.016[b]	1.47±0.018[a]	1.48±0.024[a]	1.38±0.020[b]
	6.6	1.22±0.038[b]	1.44±0.031[a]	1.43±0.018[a]	1.46±0.046[a]	1.44±0.036[a]
Milk development	5.4	6.73±0.029[a]	6.54±0.049[b]	6.42±0.065[c]	6.28±0.057[d]	6.24±0.048[d]
	6.6	6.41±0.080[bc]	6.79±0.047[a]	6.68±0.069[a]	6.47±0.049[b]	6.32±0.022[c]

Means within rows followed by different lower-case letters stand for significance different at $p \leq 0.05$

Moreover, we studied selenium effect on accumulation of biomass separately by the ear and stem of the rye (*Table 4*). Application of lower concentrations of selenium at

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soil pH 6.6 promoted increase of biomass both of the stem and of the ear. At this, for the ear stimulating effect of selenium was fixed for all its concentrations, while maximum ear mass was fixed after application of 0.05 mg Se kg^{-1} of soil. In more acid soil we revealed mass decrease both for the ear and the stem after enriching the soil with selenium.

Table 4. *Influence of selenium concentration in soil on the biomass (g) of different organs of rye plants at milk development stage*

Plant part	Soil pH	Selenium addition concentration, mg Se kg^{-1} soil				
		0	0.01	0.05	0.1	0.5
Stem	5.4	2.67±0.042^{a}	2.61±0.044^{ac}	2.63±0.023^{ab}	2.54±0.031^{c}	2.59±0.031^{bc}
	6.6	2.62±0.034^{b}	2.84±0.041^{a}	2.54±0.030^{c}	2.53±0.022^{c}	2.41±0.021^{d}
Ear	5.4	4.02±0.030^{a}	3.90±0.030^{b}	3.83±0.053^{bc}	3.75±0.065^{cd}	3.67±0.040^{d}
	6.6	3.79±0.049^{c}	3.96±0.039^{b}	4.14±0.044^{a}	3.87±0.052^{bc}	3.84±0.034^{c}

Means within rows followed by different lower-case letters stand for significance different at $p \leq 0.5$

Influence of selenium on quantitative composition of reserve protein fractions in rye

Quantitative composition of reserve protein fractions was depending on selenium supply concentration to the soil (*Table 5*). The total protein content was higher by application of selenium. However, only the contents of globulin and glutelin in the rye grains increased with increasing Se fertilization. For albumin maximum of content was shown for low selenium concentrations (control and 0.01 mg kg^{-1}). Prolamin concentrations were not affected by selenium concentration in soil.

Table 5. *Influence of selenium in soil (pH 6.6) on quantitative composition of protein fractions in the grain of rye plants*

Selenium addition concentration, mg Se kg^{-1} soil	Total protein content, %	Reserve proteins, % of total protein content			
		Albumins	Globulins	Prolamins	Glutelins
0	12.4±0.4^{b}	24.7±0.7^{ab}	19.2±0.2^{d}	25.4±0.4^{a}	16.5±0.2^{d}
0.01	13.9±0.6^{a}	25.3±0.4^{a}	19.8±0.6^{d}	24.7±0.7^{ab}	17.8±0.6^{c}
0.05	14.3±0.3^{a}	23.9±0.9^{b}	21.6±0.6^{c}	25.1±0.8^{a}	18.2±0.4^{c}
0.1	13.8±0.3^{a}	21.8±0.6^{c}	23.4±0.4^{b}	23.4±0.6^{b}	19.7±0.8^{b}
0.5	13.6±0.5^{a}	20.1±0.4^{d}	25.9±0.8^{a}	24.2±0.9^{ab}	23.4±0.7^{a}

Means within rows followed by different lower-case letters stand for significance different at $p \leq 0.05$

Discussion

The study of selenium accumulation by the rye plants at different growth stages in depending on selenium soil application in concentrations 0.01 – 0.5 mg Se kg^{-1} was conducted. There are four main methods for selenium application to plants: (1) adding Se to the soil; (2) foliar or fruit spraying; (3) soaking seeds in a Se solution before sowing; and (4) hydroponic cultivation with a nutrient solution containing Se (Puccinelli et al., 2017). The first two methods are most widely used due to their efficient and convenient. Despite a number of advantages of foliar processing, it is important to take into account the structural and chemical characteristics of the plant

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

leaves. In the case of cereals, it is very difficult to produce true foliar processing, some fertilizer is deposited on the soil and extracted by the plant from there. In addition, the application of selenium into the soil before planting, allows us to estimate its accumulation into the plant at different stages of development.

The findings on selenium accumulation by the rye plants display in general the picture typical for cereals, when some elements (especially those participating in synthesis of labile organic compounds) are absorbed by plants at early ontogenesis stages with a rate exceeding dry substance accumulation. Later, after completion of tillering stage stem starts its intensive growth, which leads to sharp decrease of relative content of these elements in dry substance as a result of “dilution” effect.

The one reason of increased intensity of selenium accumulation by the rye in ontogenesis in the course of our experiment was probably exhaustion of the soil in the culture pot as a consequence of absorption of most part of selenium from the soil at initial stages of the plant development. However, as computation shows selenium content in the soil after tillering stage decreased by 1-2%, while selenium concentration per gram of dry vegetating material decreased by 20%. Therefore, the reason of decreased intensity of selenium accumulation by the plants in vegetation process could not be only soil exhaustion with this microelement.

High selenium content in vegetating material at tillering stage was probably associated first of all with physiological functions of selenium. At tillering stage metabolic processes in nurslings are active resulting in intensive generation peroxides of hydrogen and organic molecules, by-products of many biochemical reactions. Hypothetically selenium microelement in plants, same as in mammal organisms, is an essential component of antioxidant system contributing to cell protection against destructive effect of active forms of oxygen. Antioxidant action of selenium may be explained by its influence on activity of glutathione-peroxidase – ferment having foremost significance in protection of plant cell from continuously generated hydroperoxides (Durán et al., 2016; Wu et al., 2017). Thus, intensive selenium accumulation by rye plants at tillering stage testifies to important role of this microelement for normal growth of rye especially at early stages of the plant development.

Assimilation of selenium by the plant is influenced by the physicochemical factors of the soil, such as redox status, pH and microbiological activity (Mehdi et al., 2013). In the present study the rye plant growing on less acidity soil accumulated more selenium. Increase of selenium content in the plants grown in less acid soil can be explained, on the one hand, by the fact that in the soil of high acidity anionic adsorption increases therefore most part of selenium is in absorbed condition, on the other hand, in case of low soil pH, hexavalent selenium is more prone to restorative process, and consequently in acid soil it is mostly in the form of selenite-ion which is not easily accessible for the plants. Lack of trustworthy difference in selenium intake by the plants depending on soil acidity in the case of small concentrations of the microelement is most probably conditioned by the fact that absorption and restorative processes insignificantly influence total pool of bioavailable selenium in the soil.

The study indicated that selenium predominantly accumulates in the grain, in comparison with the stem of rye. This result conforms to literary facts received earlier for winter wheat (Schulz et al., 1998). Selenium concentration in the grain was conditioned, most of all, by its physiological role in the plant, in particular, by selenium ability to substitute sulfur in most important amino acids (Sors et al., 2005; Reynolds et

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al., 2017; El Mehdawi, 2018). Moreover, predominant selenium accumulation in the ear may indirectly evidence necessity of this microelement. Such result is also of practical importance and should be taken into account when enriching cereals with selenium in order to increase selenium supply of animals and humans.

One of the peculiar features of selenium microelement is its dual nature, i.e. possible manifestation of both antioxidant and pro-oxidant features. Basic factor conditioning one or another selenium action is its concentration.

Positive effect of selenium on growth processes of the rye may be connected with its antioxidant features, i.e. ability to liquidate excessively accumulated free radicals. Thereupon excessive quantities of selenium, on the contrary, lead to intensification of generation processes of active oxygen forms. Some authors attribute positive effect of selenium on plant growth processes to its influence on hormonal status, in particular, under effect of sodium selenite increase of indoleacetic acid and decrease of abscisic acid were observed (Wang et al., 2018).

Negative effect of high concentrations of selenium on accumulation of biomass by the rye plants may also be attributed to substantial disturbance of the cell amino acid balance. Rye pertains to the plants of the 3rd group, i.e. plants not accumulating selenium, for which excessive substitution of cysteine or methionine in proteins by selenium-cysteine or selenium- methionine is particularly typical. At soil pH 5.4 selenium effect on the plant was more toxic. Toxic effect of selenium for the plants grown on more acid soil may be attributed to the fact that at this pH selenate-ion is more prone to restorative processes and may transform to selenite-ion form being more toxic for plants.

The influence of selenium on protein metabolism has been shown in this work. The content of the total protein was higher by the addition of selenium, in particular by increasing the proportion of globulins and glutelin. Earlier a number of authors also noted that the biological role of selenium in plants can be associated with its effect on the metabolism of nitrogen (Poluboyarinov and Golubkina, 2015; Reis et al., 2018).

Conclusions

The research shows that selenium is more intensively accumulated by the rye plants at initial stage of development, it is mainly concentrated in the plant's ear, and in small concentrations selenium exerts simulating effect on biomass accumulation. Thus, the findings certify to the important biological importance of selenium for growth and development of plants. However, mechanisms of protection effect of selenium in vegetative organisms remain unclarified. One of possible mechanisms of such effect is selenium antioxidant features which were proved in respect with humans and animals.

In addition, this study identified the optimum concentration of selenium supplements (0.01-0.05 mg Se kg^{-1} soil). By these concentrations not only increase of studied plants biomass, but also achieved the necessary level of selenium (100 μg g^{-1}) in cereals, which guarantees the security of this trace element humans and animals were observed. Thus, the data allow more fully uncover the function of this trace element in plants, and thus create the basis for the use of selenium-containing fertilizers is not only to increase the availability of selenium for humans and animals, but also to increase the productivity of crop production.

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

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APPENDIX

One way analysis of variance

Data source: Tillering_0

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.0420	0.00200	0.001000
pH 6.6	4	0	0.0310	0.00200	0.001000

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.000242	0.000242	60.500	<0.001
Residual	6	0.0000240	0.00000400		
Total	7	0.000266			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5. vs. pH 6.6	0.0110	2	11.000	<0.001	Yes

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One way analysis of variance

Data source: Tillering_0.01

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.580	0.0300	0.0150
pH 6.6	4	0	0.550	0.0200	0.01000

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00180	0.00180	2.769	0.147
Residual	6	0.00390	0.000650		
Total	7	0.00570			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.147).

Power of performed test with alpha = 0.050: 0.196

The power of the performed test (0.196) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One way analysis of variance

Data source: Tillering_0.05

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	2.200	0.1000	0.0500
pH 6.6	4	0	2.410	0.0700	0.0350

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0882	0.0882	11.839	0.014
Residual	6	0.0447	0.00745		
Total	7	0.133			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.014).

Power of performed test with alpha = 0.050: 0.790

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.210	2	4.866	0.014	Yes

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One way analysis of variance

Data source: Tillering_0.1

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	7.400	0.1000	0.0500
pH 6.6	4	0	9.240	0.0800	0.0400

Source of Variation	DF	SS	MS	F	P
Between Groups	1	6.771	6.771	825.756	<0.001
Residual	6	0.0492	0.00820		
Total	7	6.820			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	1.840	2	40.639	<0.001	Yes

One way analysis of variance

Data source: Tillering_0.5

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	56.800	0.700	0.350
pH 6.6	4	0	59.300	0.400	0.200

Source of Variation	DF	SS	MS	F	P
Between Groups	1	12.500	12.500	38.462	<0.001
Residual	6	1.950	0.325		
Total	7	14.450			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.999

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	2.500	2	8.771	0.001	Yes

One way analysis of variance

Data source: Booting_0

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.0480	0.00400	0.00200
pH 6.6	4	0	0.0510	0.00300	0.00150

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0000180	0.0000180	1.440	0.275
Residual	6	0.0000750	0.0000125		
Total	7	0.0000930			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.275).

Power of performed test with alpha = 0.050: 0.084

The power of the performed test (0.084) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One way analysis of variance

Data source: Booting_0.01

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.420	0.01000	0.00500
pH 6.6	4	0	0.470	0.0300	0.0150

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00500	0.00500	10.000	0.020
Residual	6	0.00300	0.000500		
Total	7	0.00800			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.020).

Power of performed test with alpha = 0.050: 0.712

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.0500	2	4.472	0.020	Yes

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

One way analysis of variance

Data source: Booting_0.05

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	1.810	0.0400	0.0200
pH 6.6	4	0	1.970	0.0400	0.0200

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0512	0.0512	32.000	0.001
Residual	6	0.00960	0.00160		
Total	7	0.0608			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).

Power of performed test with alpha = 0.050: 0.997

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6		0.160	2	8.000	0.002

One way analysis of variance

Data source: Booting_0.1

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	4.380	0.0500	0.0250
pH 6.6	4	0	4.940	0.0800	0.0400

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.627	0.627	140.944	<0.001
Residual	6	0.0267	0.00445		
Total	7	0.654			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.560	2	16.790	<0.001	Yes

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

One way analysis of variance

Data source: Booting_0.5

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	24.900	0.1000	0.0500
pH 6.6	4	0	31.700	0.300	0.150

Source of Variation	DF	SS	MS	F	P
Between Groups	1	92.480	92.480	1849.600	<0.001
Residual	6	0.300	0.0500		
Total	7	92.780			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	6.800	2	60.821	<0.001	Yes

One way analysis of variance

Data source: Milk development_0

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.0460	0.00200	0.001000
pH 6.6	4	0	0.0470	0.001000	0.000500

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00000200	0.00000200	0.800	0.406
Residual	6	0.0000150	0.00000250		
Total	7	0.0000170			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.406).

Power of performed test with alpha = 0.050: 0.049

The power of the performed test (0.049) is below the desired power of 0.800.
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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

One way analysis of variance

Data source: Milk development_0.01

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.167	0.00500	0.00250
pH 6.6	4	0	0.212	0.00400	0.00200

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00405	0.00405	197.561	<0.001
Residual	6	0.000123	0.0000205		
Total	7	0.00417			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.0450	2	19.878	<0.001	Yes

One way analysis of variance

Data source: Milk development_0.05

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	0.650	0.0200	0.01000
pH 6.6	4	0	0.710	0.01000	0.00500

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00720	0.00720	28.800	0.002
Residual	6	0.00150	0.000250		
Total	7	0.00870			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.993

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.0600	2	7.589	0.002	Yes

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One way analysis of variance

Data source: Milk development_0.1

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	1.420	0.0700	0.0350
pH 6.6	4	0	1.600	0.1000	0.0500

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0648	0.0648	8.698	0.026
Residual	6	0.0447	0.00745		
Total	7	0.110			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.026).

Power of performed test with alpha = 0.050: 0.643

All pairwise multiple comparison procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	0.180	2	4.171	0.026	Yes

One way analysis of variance

Data source: Milk development_0.5

Group Name	N	Missing	Mean	Std Dev	SEM
pH 5.4	4	0	10.400	0.600	0.300
pH 6.6	4	0	11.800	0.300	0.150

Source of Variation	DF	SS	MS	F	P
Between Groups	1	3.920	3.920	17.422	0.006
Residual	6	1.350	0.225		
Total	7	5.270			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006).

Power of performed test with alpha = 0.050: 0.927

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
pH 5.4 vs. pH 6.6	1.400	2	5.903	0.006	Yes

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One way analysis of variance

Data source: Organ_5.4_0

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	0.210	0.01000	0.00500
Stem	4	0	0.120	0.01000	0.00500

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0162	0.0162	162.000	<0.001
Residual	6	0.000600	0.0001000		
Total	7	0.0168			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem	0.0900	2	18.000	<0.001	Yes

One way analysis of variance

Data source: Organ_5.4_0.01

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	0.750	0.0800	0.0400
Stem	4	0	0.420	0.0400	0.0200

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.218	0.218	54.450	<0.001
Residual	6	0.0240	0.00400		
Total	7	0.242			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem		0.330	2	10.436	<0.001

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One way analysis of variance

Data source: Organ_5.4_0.05

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	2.700	0.1000	0.0500
Stem	4	0	1.590	0.0900	0.0450

Source of Variation	DF	SS	MS	F	P
Between Groups	1	2.464	2.464	272.287	<0.001
Residual	6	0.0543	0.00905		
Total	7	2.519			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem	1.110	2	23.336	<0.001	Yes

One way analysis of variance

Data source: Organ_5.4_0.1

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	6.400	0.500	0.250
Stem	4	0	3.400	0.500	0.250

Source of Variation	DF	SS	MS	F	P	
Between Groups	1	18.000	18.000	72.000	<0.001	
Residual	6	1.500	0.250			
Total	7	19.500				

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem	3.000	2	12.000	<0.001	Yes

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One way analysis of variance

Data source: Organ_5.4_0.5

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	35.400	1.300	0.650
Stem	4	0	33.100	1.700	0.850

Source of Variation	DF	SS	MS	F	P
Between Groups	1	10.580	10.580	4.620	0.075
Residual	6	13.740	2.290		
Total	7	24.320			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.075).

Power of performed test with alpha = 0.050: 0.353

The power of the performed test (0.353) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One way analysis of variance

Data source: Organ_6.6_0

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	0.180	0.0400	0.0200
Stem	4	0	0.110	0.01000	0.00500

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.00980	0.00980	11.529	0.015
Residual	6	0.00510	0.000850		
Total	7	0.0149			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.015).

Power of performed test with alpha = 0.050: 0.778

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem	0.0700	2	4.802	0.015	Yes

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One way analysis of variance

Data source: Organ_6.6_0.01

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	0.820	0.0500	0.0250
Stem	4	0	0.520	0.0300	0.0150

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.180	0.180	105.882	<0.001
Residual	6	0.0102	0.00170		
Total	7	0.190			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem		0.300	2	14.552	<0.001

One way analysis of variance

Data source: Organ_6.6_0.05

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	2.930	0.0300	0.0150
Stem	4	0	1.750	0.0400	0.0200

Source of Variation	DF	SS	MS	F	P
Between Groups	1	2.785	2.785	2227.840	<0.001
Residual	6	0.00750	0.00125		
Total	7	2.792			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem		1.180	2	66.751	<0.001

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One way analysis of variance

Data source: Organ_6.6_0.1

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	6.100	0.200	0.1000
Stem	4	0	4.500	0.200	0.1000

Source of Variation	DF	SS	MS	F	P
Between Groups	1	5.120	5.120	128.000	<0.001
Residual	6	0.240	0.0400		
Total	7	5.360			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem	1.600	2	16.000	<0.001	Yes

One way analysis of variance

Data source: Organ_6.6_0.5

Group Name	N	Missing	Mean	Std Dev	SEM
Grain	4	0	38.300	1.200	0.600
Stem	4	0	35.100	0.600	0.300

Source of Variation	DF	SS	MS	F	P
Between Groups	1	20.480	20.480	22.756	0.003
Residual	6	5.400	0.900		
Total	7	25.880			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003).

Power of performed test with alpha = 0.050: 0.975

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
Grain vs. Stem		3.200	2	6.746	0.003

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One way analysis of variance

Data source: Tillering_5.4

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	0.0740	0.00400	0.00200
0.01	4	0	0.0810	0.00200	0.001000
0.05	4	0	0.0750	0.00400	0.00200
0.1	4	0	0.0770	0.00500	0.00250
0.5	4	0	0.0730	0.00400	0.00200

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.000160	0.0000400	2.597	0.079
Residual	15	0.000231	0.0000154		
Total	19	0.000391			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.079).

Power of performed test with alpha = 0.050: 0.371

The power of the performed test (0.371) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

One way analysis of variance

Data source: Tillering_6.6

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	0.0720	0.00500	0.00250
0.01	4	0	0.0830	0.00500	0.00250
0.05	4	0	0.0780	0.00300	0.00150
0.1	4	0	0.0720	0.001000	0.000500
0.5	4	0	0.0710	0.001000	0.000500

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.000427	0.000107	8.754	<0.001
Residual	15	0.000183	0.0000122		
Total	19	0.000610			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.983

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

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Comparison	Diff of Means	p	q	P	P<0.050
0.01 vs. 0.5	0.0120	5	6.871	0.002	Yes
0.01 vs. 0.1	0.0110	5	6.299	0.004	Yes
0.01 vs. 0	0.0110	5	6.299	0.004	Yes
0.01 vs. 0.05	0.00500	5	2.863	0.302	No
0.05 vs. 0.5	0.00700	5	4.008	0.080	No
0.05 vs. 0.1	0.00600	5	3.436	0.161	Do Not Test
0.05 vs. 0	0.00600	5	3.436	0.161	Do Not Test
0 vs. 0.5	0.001000	5	0.573	0.994	Do Not Test
0 vs. 0.1	0.000	5	0.000	1.000	Do Not Test
0.1 vs. 0.5	0.001000	5	0.573	0.994	Do Not Test

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One way analysis of variance

Data source: Booting_5.4

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	1.390	0.0240	0.0120
0.01	4	0	1.420	0.0160	0.00800
0.05	4	0	1.470	0.0180	0.00900
0.1	4	0	1.480	0.0240	0.0120
0.5	4	0	1.380	0.0200	0.01000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.0331	0.00828	19.418	<0.001
Residual	15	0.00640	0.000426		
Total	19	0.0395			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.1 vs. 0.5	0.100	5	9.685	<0.001	Yes
0.1 vs. 0	0.0900	5	8.717	<0.001	Yes
0.1 vs. 0.01	0.0600	5	5.811	0.007	Yes
0.1 vs. 0.05	0.01000	5	0.969	0.957	No

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0.05 vs. 0.5	0.0900	5	8.717	<0.001	Yes
0.05 vs. 0	0.0800	5	7.748	<0.001	Yes
0.05 vs. 0.01	0.0500	5	4.843	0.027	Yes
0.01 vs. 0.5	0.0400	5	3.874	0.094	No
0.01 vs. 0	0.0300	5	2.906	0.289	Do Not Test
0 vs. 0.5	0.01000	5	0.969	0.957	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One way analysis of variance

Data source: Booting_6.6

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	1.220	0.0380	0.0190
0.01	4	0	1.440	0.0310	0.0155
0.05	4	0	1.430	0.0180	0.00900
0.1	4	0	1.460	0.0460	0.0230
0.5	4	0	1.440	0.0360	0.0180

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.160	0.0401	32.633	<0.001
Residual	15	0.0184	0.00123		
Total	19	0.179			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.1 vs. 0	0.240	5	13.696	<0.001	Yes
0.1 vs. 0.05	0.0300	5	1.712	0.746	No
0.1 vs. 0.5	0.0200	5	1.141	0.924	Do Not Test
0.01 vs. 0.01	0.0200	5	1.141	0.924	Do Not Test
0.01 vs. 0	0.220	5	12.555	<0.001	Yes
0.01 vs. 0.05	0.01000	5	0.571	0.994	Do Not Test
0.01 vs. 0.5	0.000	5	0.000	1.000	Do Not Test
0.5 vs. 0	0.220	5	12.555	<0.001	Yes
0.5 vs. 0.05	0.01000	5	0.571	0.994	Do Not Test
0.05 vs. 0	0.210	5	11.984	<0.001	Yes

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One way analysis of variance

Data source: Milk development_5.4

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	6.730	0.0290	0.0145
0.01	4	0	6.540	0.0490	0.0245
0.05	4	0	6.420	0.0650	0.0325
0.1	4	0	6.280	0.0570	0.0285
0.5	4	0	6.240	0.0480	0.0240

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.640	0.160	61.475	<0.001
Residual	15	0.0391	0.00260		
Total	19	0.679			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0 vs. 0.5	0.490	5	19.205	<0.001	Yes
0 vs. 0.1	0.450	5	17.637	<0.001	Yes
0 vs. 0.05	0.310	5	12.150	<0.001	Yes
0 vs. 0.01	0.190	5	7.447	<0.001	Yes
0.01 vs. 0.5	0.300	5	11.758	<0.001	Yes
0.01 vs. 0.1	0.260	5	10.190	<0.001	Yes
0.01 vs. 0.05	0.120	5	4.703	0.032	Yes
0.05 vs. 0.5	0.180	5	7.055	0.001	Yes
0.05 vs. 0.1	0.140	5	5.487	0.011	Yes
0.1 vs. 0.5	0.0400	5	1.568	0.800	No

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

One way analysis of variance

Data source: Milk development_6.6

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	6.410	0.0800	0.0400
0.01	4	0	6.790	0.0470	0.0235
0.05	4	0	6.680	0.0690	0.0345
0.1	4	0	6.470	0.0490	0.0245
0.5	4	0	6.320	0.0220	0.0110

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.608	0.152	46.792	<0.001
Residual	15	0.0488	0.00325		
Total	19	0.657			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.01 vs. 0.5	0.470	5	16.486	<0.001	Yes
0.01 vs. 0	0.380	5	13.329	<0.001	Yes
0.01 vs. 0.1	0.320	5	11.225	<0.001	Yes
0.01vs. 0.05	0.110	5	3.858	0.096	No
0.05 vs. 0.5	0.360	5	12.628	<0.001	Yes
0.05 vs. 0	0.270	5	9.471	<0.001	Yes
0.05 vs. 0.01	0.210	5	7.366	<0.001	Yes
0.01 vs. 0.5	0.150	5	5.262	0.015	Yes
0.01 vs. 0	0.0600	5	2.105	0.585	No
0 vs. 0.5	0.0900	5	3.157	0.221	No

One way analysis of variance

Data source: Stem_5.4

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	2.670	0.0420	0.0210
0.01	4	0	2.610	0.0440	0.0220
0.05	4	0	2.630	0.0230	0.0115
0.1	4	0	2.540	0.0310	0.0155
0.5	4	0	2.590	0.0310	0.0155

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.0371	0.00928	7.543	0.002
Residual	15	0.0185	0.00123		
Total	19	0.0556			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.959

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0 vs. 0.1	0.130	5	7.413	<0.001	Yes
0 vs. 0.5	0.0800	5	4.562	0.039	Yes
0 vs. 0.01	0.0600	5	3.421	0.164	No
0 vs. 0.05	0.0400	5	2.281	0.512	Do Not Test
0.05 vs. 0.1	0.0900	5	5.132	0.018	Yes
0.05 vs. 0.5	0.0400	5	2.281	0.512	No
0.05 vs. 0.01	0.0200	5	1.140	0.925	Do Not Test
0.01 vs. 0.1	0.0700	5	3.992	0.081	No
0.01 vs. 0.5	0.0200	5	1.140	0.925	Do Not Test
0.5 vs. 0.1	0.0500	5	2.851	0.305	Do Not Test

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One way analysis of variance

Data source: Stem_6.6

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	2.620	0.0340	0.0170
0.01	4	0	2.840	0.0410	0.0205
0.05	4	0	2.540	0.0300	0.0150
0.1	4	0	2.530	0.0220	0.0110
0.5	4	0	2.410	0.0210	0.0105

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.408	0.102	109.266	<0.001
Residual	15	0.0140	0.000932		
Total	19	0.422			

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.01 vs. 0.5	0.430	5	28.164	<0.001	Yes
0.01 vs. 0.1	0.310	5	20.304	<0.001	Yes
0.01 vs. 0.05	0.300	5	19.649	<0.001	Yes
0.01 vs. 0	0.220	5	14.410	<0.001	Yes
0 vs. 0.5	0.210	5	13.755	<0.001	Yes
0 vs. 0.1	0.0900	5	5.895	0.006	Yes
0 vs. 0.05	0.0800	5	5.240	0.016	Yes
0.05 vs. 0.5	0.130	5	8.515	<0.001	Yes
0.05 vs. 0.1	0.0100	5	0.655	0.990	No
0.1 vs. 0.5	0.120	5	7.860	<0.001	Yes

One way analysis of variance

Data source: Grain_5.4

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	4.020	0.0300	0.0150
0.01	4	0	3.900	0.0300	0.0150
0.05	4	0	3.830	0.0530	0.0265
0.1	4	0	3.750	0.0650	0.0325
0.5	4	0	3.670	0.0400	0.0200

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.292	0.0729	34.943	<0.001
Residual	15	0.0313	0.00209		
Total	19	0.323			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0 vs. 0.5	0.350	5	15.323	<0.001	Yes
0 vs. 0.1	0.270	5	11.821	<0.001	Yes
0 vs. 0.05	0.190	5	8.318	<0.001	Yes
0 vs. 0.01	0.120	5	5.254	0.015	Yes

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

0.01 vs. 0.5	0.230	5	10.070	<0.001	Yes
0.01 vs. 0.1	0.150	5	6.567	0.003	Yes
0.01 vs. 0.05	0.0700	5	3.065	0.244	No
0.05 vs. 0.5	0.160	5	7.005	0.001	Yes
0.05 vs. 0.1	0.0800	5	3.503	0.149	No
0.1 vs. 0.5	0.0800	5	3.503	0.149	No

One way analysis of variance

Data source: Grain_6.6

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	3.790	0.0490	0.0245
0.01	4	0	3.960	0.0390	0.0195
0.05	4	0	4.140	0.0440	0.0220
0.1	4	0	3.870	0.0520	0.0260
0.5	4	0	3.840	0.0340	0.0170

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.303	0.0758	39.000	<0.001
Residual	15	0.0292	0.00194		
Total	19	0.332			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.05 vs. 0	0.350	5	15.878	<0.001	Yes
0.05 vs. 0.5	0.300	5	13.610	<0.001	Yes
0.05 vs. 0.1	0.270	5	12.249	<0.001	Yes
0.05 vs. 0.01	0.180	5	8.166	<0.001	Yes
0.01 vs. 0	0.170	5	7.712	<0.001	Yes
0.01 vs. 0.5	0.120	5	5.444	0.012	Yes
0.01 vs. 0.1	0.0900	5	4.083	0.072	No
0.1 vs. 0	0.0800	5	3.629	0.128	No
0.1 vs. 0.5	0.0300	5	1.361	0.868	Do Not Test
0.5 vs. 0	0.0500	5	2.268	0.517	Do Not Test

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One way analysis of variance

Data source: Total protein content

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	12.400	0.400	0.200
0.01	4	0	13.900	0.600	0.300
0.05	4	0	14.300	0.300	0.150
0.1	4	0	13.800	0.300	0.150
0.5	4	0	13.600	0.500	0.250

Source of Variation	DF	SS	MS	F	P
Between Groups	4	8.240	2.060	10.842	<0.001
Residual	15	2.850	0.190		
Total	19	11.090			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.997

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.05 vs. 0	1.900	5	8.718	<0.001	Yes
0.05 vs. 0.5	0.700	5	3.212	0.208	No
0.05 vs. 0.1	0.500	5	2.294	0.507	Do Not Test
0.05 vs. 0.01	0.400	5	1.835	0.697	Do Not Test
0.01 vs. 0	1.500	5	6.882	0.002	Yes
0.01 vs. 0.5	0.300	5	1.376	0.863	Do Not Test
0.01 vs. 0.1	0.1000	5	0.459	0.997	Do Not Test
0.1 vs. 0	1.400	5	6.424	0.003	Yes
0.1 vs. 0.5	0.200	5	0.918	0.964	Do Not Test
0.5 vs. 0	1.200	5	5.506	0.011	Yes

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

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One way analysis of variance

Data source: Albumins

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	24.700	0.700	0.350
0.01	4	0	25.300	0.400	0.200
0.05	4	0	23.900	0.900	0.450
0.1	4	0	21.800	0.600	0.300
0.5	4	0	20.100	0.400	0.200

Source of Variation	DF	SS	MS	F	P
Between Groups	4	74.848	18.712	47.253	<0.001
Residual	15	5.940	0.396		
Total	19	80.788			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.01 vs. 0.5	5.200	5	16.527	<0.001	Yes
0.01 vs. 0.1	3.500	5	11.124	<0.001	Yes
0.01 vs. 0.05	1.400	5	4.449	0.045	Yes
0.01 vs. 0	0.600	5	1.907	0.667	No
0 vs. 0.5	4.600	5	14.620	<0.001	Yes
0 vs. 0.1	2.900	5	9.217	<0.001	Yes
0 vs. 0.05	0.800	5	2.543	0.410	No
0.05 vs. 0.5	3.800	5	12.077	<0.001	Yes
0.05 vs. 0.1	2.100	5	6.674	0.002	Yes
0.1 vs. 0.5	1.700	5	5.403	0.012	Yes

One way analysis of variance

Data source: Globulins

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	19.200	0.200	0.1000
0.01	4	0	19.800	0.600	0.300
0.05	4	0	21.600	0.600	0.300
0.1	4	0	23.400	0.400	0.200
0.5	4	0	25.900	0.800	0.400

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DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

Source of Variation	DF	SS	MS	F	P
Between Groups	4	120.032	30.008	96.179	<0.001
Residual	15	4.680	0.312		
Total	19	124.712			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.5 vs. 0	6.700	5	23.990	<0.001	Yes
0.5 vs. 0.01	6.100	5	21.842	<0.001	Yes
0.5 vs. 0.05	4.300	5	15.396	<0.001	Yes
0.05 vs. 0.1	2.500	5	8.951	<0.001	Yes
0.1 vs. 0	4.200	5	15.038	<0.001	Yes
0.1 vs. 0.01	3.600	5	12.890	<0.001	Yes
0.1 vs. 0.05	1.800	5	6.445	0.003	Yes
0.05 vs. 0	2.400	5	8.593	<0.001	Yes
0.05 vs. 0.01	1.800	5	6.445	0.003	Yes
0.01 vs. 0	0.600	5	2.148	0.567	No

One way analysis of variance

Data source: Prolamins

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	25.400	0.400	0.200
0.01	4	0	24.700	0.700	0.350
0.05	4	0	25.100	0.800	0.400
0.1	4	0	23.400	0.600	0.300
0.5	4	0	24.200	0.900	0.450

Source of Variation	DF	SS	MS	F	P
Between Groups	4	9.968	2.492	5.065	0.009
Residual	15	7.380	0.492		
Total	19	17.348			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.009).

Power of performed test with alpha = 0.050: 0.802

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0 vs. 0.1	2.000	5	5.703	0.008	Yes
0 vs. 0.5	1.200	5	3.422	0.163	No
0 vs. 0.01	0.700	5	1.996	0.630	Do Not Test
0 vs. 0.05	0.300	5	0.855	0.972	Do Not Test
0.05 vs. 0.1	1.700	5	4.847	0.026	Yes
0.05 vs. 0.5	0.900	5	2.566	0.401	Do Not Test
0.05 vs. 0.01	0.400	5	1.141	0.925	Do Not Test
0.01 vs. 0.1	1.300	5	3.707	0.116	No
0.01 vs. 0.5	0.500	5	1.426	0.848	Do Not Test
0.5 vs. 0.1	0.800	5	2.281	0.512	Do Not Test

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance

Data source: Glutelins

Group Name	N	Missing	Mean	Std Dev	SEM
0	4	0	16.500	0.200	0.1000
0.01	4	0	17.800	0.600	0.300
0.05	4	0	18.200	0.400	0.200
0.1	4	0	19.700	0.800	0.400
0.5	4	0	23.400	0.700	0.350

Source of Variation	DF	SS	MS	F	P
Between Groups	4	112.432	28.108	83.160	<0.001
Residual	15	5.070	0.338		
Total	19	117.502			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All pairwise multiple comparison procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
0.5 vs. 0	6.900	5	23.737	<0.001	Yes
0.5 vs. 0.01	5.600	5	19.265	<0.001	Yes
0.5 vs. 0.05	5.200	5	17.889	<0.001	Yes
0.5 vs. 0.1	3.700	5	12.728	<0.001	Yes

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0.1 vs. 0	3.200	5	11.008	<0.001	Yes
0.1 vs. 0.01	1.900	5	6.536	0.003	Yes
0.1 vs. 0.05	1.500	5	5.160	0.017	Yes
0.05 vs. 0	1.700	5	5.848	0.007	Yes
0.05 vs. 0.01	0.400	5	1.376	0.863	No
0.01 vs. 0	1.300	5	4.472	0.044	Yes

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_23852421

AN INVESTIGATION ABOUT HEAVY METAL POLLUTION OF DUDEN AND GOKSU STREAMS (ANTALYA, TURKEY)

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(Received 5th Oct 2018; accepted 2nd Jan 2019)

Abstract. The cities are also expanding with growth in construction rapidly, depending on the population growth. This development causes a lot of environmental problems. Pollution in water is one of the main problems and the aim of this paper is to evaluate water pollution in the study area. There are some statistical methods developed for the measurement and evaluation of water pollution; heavy metal pollution index (HPI) is one of them. Antalya is, one of the big cities in Turkey, also growing fast; population and settlement in this city are increasing quickly. That is why two streams, Duden and Goksu, are selected to measure the heavy metal pollution. The 24 water samples from Duden Stream and 18 water samples from Goksu Stream were taken systematically in June 2018 and were analyzed by using HPI. The heavy metals, from the highest value to the lowest one, were Sr > Fe > Al > Mn > As > Ni > Cu > Pb > Cr > Se in Duden Stream; and Sr > Fe > Al > Ni > As > Cu > Mn > Pb > Cr > Se in Goksu Stream. The Sr and Al values have exceeded the standard permissible values in both of the streams. The heavy metal pollution index (HPI) was used to evaluate the potential risk. Regarding quality the water samples have been classified as “good” and “poor”, generally. On the other hand, some water samples had heavy metal pollution above standards. Especially, the water of D15 - D19 - D20 and G13 samples were determined as “very poor” according to the standards. As a conclusion, the anthropogenic factors and urbanization may be the cause of the pollution. Living creatures that use water may have health problems and ecological equilibrium may be hampered in these stations. Groundwater may be contaminated in these areas. So, the urbanization needs to be done more carefully.
Keywords: *heavy metal pollution index (HPI), urbanization, statistics, surface water, environment*

Introduction

The urbanization may cause pollution. This question becomes much more important, especially in industrial areas. The quality of environment has been influenced by the diffusion of pollutants in air, water and soil in large areas. The accumulation of pollutants has disturbed the ecological equilibrium, in time (Stoica and Baiulescu, 2008). The reason of the heavy metal pollution in water could be natural or anthropogenic (Zarazua et al., 2006; Mehrabi et al., 2015; Sobhanardakani, 2016). Bhuiyan et al. (2015) reported “The heavy metal evaluation index (HEI) showed strong correlations and provided better assessment of pollution levels with HPI.”

Duden Stream flows in the Lara Region of Antalya Bay and it is an example for pollution (Erdem and Topkaya, 2004). According to Yardimci et al. (2005), the other example is Goksu Stream which is the main source of Bogacay River. It feeds Bogacay River. So, Goksu Stream affects the water quality of the Bogacay River and the water quality changes temporally; the critical level of water quality was determined in summer because of the recreational activities. Cengiz et al. (2017) also evaluated the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

risk potential of metallic pollution in Bogacay River. According to this study, the samples from upstream showed lower risk potentials than those from downstream. Yalcin et al. (2016) also has investigated the contamination of the sediments by heavy metals along the banks of the Bogacay River. Demarco et al. (2018) and Uncumusaoglu et al. (2016) emphasized the importance of heavy metal pollution in streams in terms of environmental contamination and anthropogenic pollution. Besides, the heavy metal pollution in surface sediments has been also studied in the most recent papers (Ye et al., 2019; Sodrzeieski et al., 2018; Omwene et al., 2018).

Antalya, which is at the forefront of Turkey's major tourism centers and called the "Turkish Riviera", is quite rich in terms of water resources. The water is provided from underground waters. However, Antalya is located on tufa which has a karstic structure. Because of that, water moves frequently from the underground to the surface or from the surface to the underground. On the other hand, the population growth is remarkable in the 5th largest city of the country. Parallel to this, settlement areas are also expanding. The western part of the city is the best example of fast construction. The construction and also population growth continues on both sides of the city. Therefore, the protection of the existing water has become more important and the quality and/or quantity of water should be investigated to prevent pollution.

Although some studies have been made on this subject, no data has been obtained on heavy metal pollution in Duden and Goksu streams after the built-up and population growth. The surface water, which is coming out from the Kepez Hydroelectric Power Plant, reaches via two channels to the Mediterranean; one of them is Duden Falls in Lara tourist area, the other one is Bogacay River in Konyaalti district. However, the second one passes Goksu Stream before Bogacay River (Leventeli et al., 2017). In this study, heavy metal pollution in the surface water was investigated and analyzed statistical between Kepez Hydroelectric Power Plant and Duden Falls in Lara tourist area, called Duden Stream and also between Kepez Hydroelectric Power Plant and Bogacay River with Goksu Stream in Konyaalti district. The location map of the study area, including the sampling stations, is given in *Figure 1.*

Experimental

Study area

The study area is located in the city center of Antalya district which is one of the big cities in Turkey. The water which is coming out from the Kepez Hydroelectric Power Plant reaches via two channels to Mediterranean; one of them is Duden Falls in Lara tourist area, the other one is Goksu Stream with a part of the Bogacay River in Konyaalti district. There are agricultural, industrial and residential areas in the region.

Field sampling

The samples were collected from 42 locations in June 2018 based on morphological and land use properties of the study area. 1 L polythene containers are used for the collection according to standard procedures. Because of the dry season, surface water samples have been taken at this time. D in numbers of samples means "taken from Duden Stream", G means "taken from Goksu Stream". Goksu Stream has a 10.5 km length and Duden Stream has 28.5 km length. So, 24 samples were taken from Duden Stream and 18 samples from Goksu Stream. There are natural barriers like barbed wire,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

morass, prohibited agricultural areas and so on. The long symbols show the stations of Duden Stream and the round symbols show the stations of Goksu Stream (see *Fig. 1*).

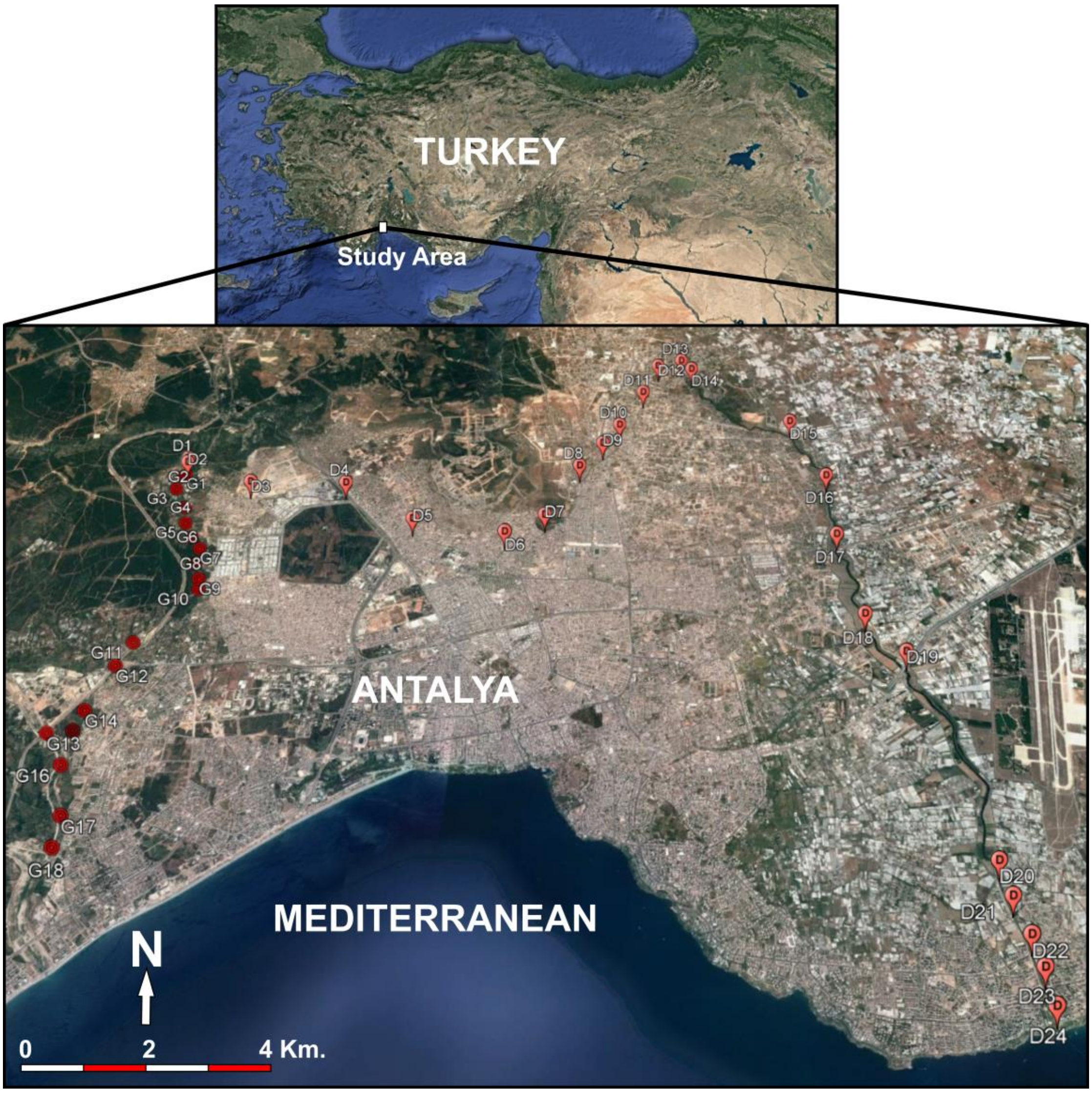

Figure 1. *The location map of the study area shows the sampling stations*

Laboratory methods

Preparation of samples

The experimental studies have been done in the Research Center Laboratory of Akdeniz University. Water samples acidified by adding 5 ml of nitric acid to 1 L polyethylene bottle ISOLAB mark tubes. The biological activities of microorganisms and bacteria that may be in the environment have been terminated in order to prevent the transformation of metals into other forms. The samples were kept in the refrigerator until analysis, and then they were filtered through the filter paper. 2 ml of nitric acid was added after pre-treatment to acidify the samples according to EPA 3005A (1992) method (Rohrbough et al., 1986; ASTM 1985). *Table 1* shows the device parameters of

Inductively Coupled Plasma – Mass Spectrometer (ICP-MS). A certified standard (SPS-SW2 surface water) was used to verify method trueness.

Table 1. *Device parameters of ICP-MS (Perkin Elmer Elan DRC-e)*

Spectrometer	Elan DRC-e (Perkin Elmer SCIEX, Norwalk, CT, USA)
Sample Introduction	Scott Spray Chamber
RF Power	1000
Skimmer Cone	Nickel
Sampler Cone	Nickel
Gas flow rates (L min^{-1})	Nebulizer gas flow:0.81 Auxillary gas flow:1.20 Plasma gas flow:19
Scanning mode	Peak hopping
Reaction gas, CH_4(ml min^{-1})	Cell gas A 0.2; RPq 0.8 BEC (ppb) 0.1463 (As 74.9216)
Number of sweeps/reading	20
Number of readings/replicate	1
Number of replicates	3
Auto sampler	CETAX ASX-520
Dwell time per AMU (ms)	50
Sample flush	Time (50), speed (+/- rpm)-48
Read delay	Time (15), speed (+/- rpm)-20

Statistical methods and heavy metal pollution index (HPI)

The results of the analyses have been given below with calculations and also with assessments have been made using the Descriptive Analysis, Multivariate Statistics Analysis (Correlations, Principal Component, Component Matrix, Hierarchical Analysis) and Heavy Metal Pollution Index (HPI). Multivariate Statistics Analysis has been made using SPSS-23 software. Besides that, EXCEL 2016 software has been used for HPI calculations, according to similar works.

Results and discussions

Chemical data, statistical surveys

The water samples were taken at the same time from the streams to investigate the existence of iron (Fe), lead (Pb), arsenic (As), copper (Cu), manganese (Mn), nickel (Ni), aluminum (Al), cobalt (Co), strontium (Sr), chromium (Cr), selenium (Se), antimony (Sb), mercury (Hg) and cadmium (Cd) elements. The amount of heavy metal elements obtained is given in *Table 2* in ppb (µg / L). However; antimony (Sb), mercury (Hg) and cadmium (Cd) were not detected in any samples. Cobalt (Co) values were below reporting limit. Chromium (Cr) and selenium (Se) values were below reporting limit generally, excepting some samples; Cr values are 1.7 in G1, 2.5 in G2, 1.2 in G3, 4.7 in G9, 4.3 in G10, 2.1 in D12 and 2.9 in D13; Se values are 0.4 in G7, 0.5 G9, 0.3 G10, 2.1 in D12 and 2.9 in D13.

Descriptive statistics of metal concentration are presented in *Table 3*. The heavy metals were arranged from the highest value to the smallest one as Sr > Fe > Al > Mn > As > Ni > Cu > Pb > Cr > Se in Duden Stream. On the other hand, the order of the metal values from the highest value to the smallest one for Goksu Stream was as Sr > Fe

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

> Al > Ni > As > Cu > Mn > Pb > Cr > Se. Among these metals, the Sr and Al values have exceeded the standard permissible values in both of tributaries. The values of the other metals were far below standard values.

Table 2. *The heavy metal values (ppb)*

NO	Coordinates		Pb	As	Cu	Fe	Mn	Al	Ni	Sr
D1	36S 0288246	4090629	N.D.	10	4.5	185	2.6	9	5.4	202
D2	36S 0288254	4090520	N.D.	9.9	3.4	183	1.3	5.9	4.3	317
D3	36S 0289513	4090059	2.2	9.6	4.9	212	1.8	7.5	23.5	421
D4	36S 0291260	4090070	8.1	8.9	30.7	254	7.9	13.3	9	298
D5	36S 0292577	4089230	5.5	9.1	4.7	189	1.3	6.8	24.6	309
D6	36S 0294242	4088960	1.5	9.2	6.3	198	1.6	16	18	305
D7	36S 0294935	4089369	2.9	9.6	4.9	248	3.2	9.3	5.2	336
D8	36S 0295524	4090582	N.D.	10.6	3.2	234	1.6	8.8	5	331
D9	36S 0295941	4091168	5.1	8.3	43.6	238	9.2	26.5	5.6	329
D10	36S 0296251	4091657	1.1	9.4	5.6	232	4.4	11.3	4.9	311
D11	36S 0296696	4092554	1.5	9.5	5.8	241	4.2	6	5.9	305
D12	36S 0296991	4093304	4.5	12.3	5.1	176	7.7	3.6	7.1	298
D13	36S 0297453	4093464	N.D.	12.1	30.6	156	9	3.7	5.1	285
D14	36S 0297650	4093229	N.D.	11.8	3.2	195	3.1	2.9	4	328
D15	36S 0299490	4091819	1.9	10.6	6.5	218	84.9	52.2	9.9	478
D16	36S 0300061	4090432	N.D.	11	5.1	185	30.8	9.9	4.5	425
D17	36S 0300127	4089027	N.D.	10.7	5	174	12.5	12.1	4.7	319
D18	36S 0300445	4087284	N.D.	11.6	3.5	151	15.5	6	4.1	357
D19	36S 0301044	4086519	2.3	7.8	3.8	221	85.4	81.3	18.8	365
D20	36S 0301940	4082808	4.3	9.7	4.8	213	82.5	78.4	19.4	325
D21	36S 0302046	4082257	N.D.	8.9	4.1	142	13.4	7.1	3.8	302
D22	36S 0302210	4081680	N.D.	10.2	3.1	168	12.9	16.7	3.2	298
D23	36S 0302310	4081209	N.D.	8.4	3.7	161	13.6	15.2	2.4	256
D24	36S 0302363	4080662	1.5	9.6	3.2	159	19	24.9	3.07	201
G1	36S 0288231	4090522	N.D.	9	3.8	238	1.2	3.5	11.2	299
G2	36S 0288179	4090460	1.9	8.3	3.7	227	1.4	5.8	4.5	301
G3	36S 0288131	4090151	0.7	9.4	4.1	239	1.4	3.5	12.8	322
G4	36S 0288388	4089712	N.D.	9.4	3.5	265	1.1	7.8	6.4	500
G5	36S 0288477	4089340	1	8.4	3.8	260	1	5.3	8	511
G6	36S 0288663	4089014	4.5	6.3	2.7	167	N.D.	1.2	8.1	455
G7	36S 0288862	4088752	N.D.	6.5	3.1	198	1	N.D.	5.2	361
G8	36S 0288854	4088410	N.D.	6.6	2.9	190	N.D.	2.6	6	358
G9	36S 0288992	4088071	N.D.	6.4	5.9	205	1	4.8	12.4	363
G10	36S 0289033	4087849	N.D.	6.3	4	185	1.1	6.2	7.3	353
G11	36S 0288180	4086714	N.D.	6.2	2.9	166	N.D.	2.6	4.9	357
G12	36S 0288003	4086239	3.7	6.4	3.6	157	N.D.	1.2	3.6	401
G13	36S 0287235	4084938	4.2	1.1	7.3	148	24	54	15	399
G14	36S 0287727	4085368	N.D.	6.7	3.5	182	N.D.	N.D.	2.9	420
G15	36S 0287639	4084992	2.4	7	17.4	174	N.D.	2.4	6.4	398
G16	36S 0287624	4084370	N.D.	6.9	5	164	6	14.4	8	366
G17	36S 0287869	4083497	N.D.	6.5	3.6	165	2.2	1.1	3.7	356
G18	36S 0287884	4082971	2.8	6.2	3.7	155	3.5	17.1	3.3	302

N.D. Not detected

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

Table 3. Descriptive statistics of water sample

Parameter µg/l	Duden Stream n = 24				Goksu Stream n = 18				Std. permissible value (Nasrabadi, 2015; Prasad and Bose, 2001)
	Min	Max	Mean	Std. dev.	Min	Max	Mean	Std. dev	
Pb	0	8.1	1.766667	2.243961	0	4.5	1.177778	1.632593	50
As	7.8	12.3	9.95	1.19855	1.1	9.4	6.866667	1.837518	50
Cu	3.1	43.6	8.304167	10.5732	2.7	17.4	4.694444	3.364079	1000
Fe	142	254	197.2083	33.28661	148	265	193.6111	37.16599	1000
Mn	1.3	85.4	17.89167	26.55961	0	24	2.494444	5.572355	300
Al	2.9	81.3	18.1	21.66478	0	54	7.416667	12.50309	10
Ni	2.4	24.6	8.394583	6.853334	2.9	15	7.205556	3.560812	20
Sr	201	478	320.875	61.13301	299	511	379	61.98102	50
Cr	0	2.9	.2083333	.7156126	0	4.7	.8	1.527012	10
Se	0	2.9	.2083333	.7156126	0	.5	.0666667	.157181	10

Significant correlation coefficients between 10 heavy metal behaviours in river water in 42 different stations are shown in *Table 4*. Positive correlation was determined between Pb and Fe, Cu, Ni; between As and Cr, Se; between Mn and Al; between Cr and Se in Duden Stream. The highest correlation between these metals was determined between Mn and Al (0.923 *). The positive high correlation between Mn and Al showed that their potential sources may be the same in Duden Stream. There is a positive correlation between As and Fe; between Mn and Al, Ni; between Cr and Se, between Al and Ni in Goksu Stream. High positive correlation between Mn and Al is determined (0.974 *). Besides, there is a high negative correlation relationship between As and Mn (-0.748*), As and Al (-0.715*) in Goksu Stream. These correlations showed that Mn and Al may be from the same source. However, it can be said that As has a different source than Mn and Al in Goksu Stream.

According to the total variance of heavy metal content, the statistical evaluation of the chemical analysis results was high and calculated as 79.460 % (*Table 5*). The explained total variance analysis is evaluated in two parts. One of them is “Initial Eigenvalues”; the other one is “Extraction Sums of Squared Loadings”. The percentage of explanations of each factor is given in order; the cumulatively highest value gives percentage of explanations of the used data. The numerical data of this ratio, which reaches 100, explain its explanatory rate. Therefore, the cumulative value obtained as 79.460% was stated to be high in the factor analyzes of the article. A total of 4 factors were determined by factor analysis. The first factor was explained by Al, Mn, Ni, Pb. The second, third and fourth factors were respectively represented by Cu, As and Fe (*Table 6*). Factors indicate possible sources. Metals within the same factor can be of similar origin in the first factor except other factors.

From the results of the hierarchical analysis, it is understood that three groups were formed among locations (*Fig. 2*). The first group of locations includes 28, 29 and 15 numbers. The second group of locations includes 1, 24 and 23 numbers. The third one includes total of the all numbers. These groups show similarities between the locations Excessive differences between locations show the excess of natural and anthropogenic factors acting on the streams. The analysis results are consistent with factor analysis and with the Principal Component Analysis.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

Table 4. *Relationships between behaviour of heavy metals*

		Pb	As	Cu	Fe	Mn	Al	Ni	Sr	Cr	Se
DUDEN	**Pb**	1									
	As	-.331	1								
	Cu	.483*	-.126	1							
	Fe	.533*	-.358	.282	1						
	Mn	.118	-.147	-.113	.107	1					
	Al	.249	-.383	-.024	.248	.923*	1				
	Ni	.483*	-.327	-.076	.262	.314	.425	1			
	Sr	.041	.129	-.047	.305	.446	.259	.314	1		
	Cr	.016	.566*	.332	-.299	-.107	-.202	-.108	-.151	1	
	Se	.016	.566*	.332	-.299	-.107	-.202	-.108	-.151	1*	1
GOKSU	**Pb**	1									
	As	-.470	1								
	Cu	.240	-.167	1							
	Fe	-.426	.764*	-.177	1						
	Mn	.403	-.748*	.162	-.328	1					
	Al	.437	-.715*	.168	-.299	.974*	1				
	Ni	.102	-.190	.182	.222	.541*	.513*	1			
	Sr	.163	.009	.054	.223	-.000	.005	-.018	1		
	Cr	-.268	.117	-.023	.238	-.133	-.101	.341	-.364	1	
	Se	-.324	-.112	-.027	.049	-.118	-.138	.191	-.138	.656*	1

*Correlation is significant at 0.01 level (2-tailed)

Table 5. *Total variance of heavy metal content*

Component	Initial eigenvalues			Extraction sums of squared loadings		
	Total	% of variance	Cumulative %	Total	% of variance	Cumulative %
1	2.452	30.649	30.649	2.452	30.649	30.649
2	1.441	18.007	48.656	1.441	18.007	48.656
3	1.305	16.311	64.967	1.305	16.311	64.967
4	1.159	14.493	79.460	1.159	14.493	**79.460**
5	.763	9.540	89.000			
6	.474	5.920	94.920			
7	.365	4.568	99.488			
8	.041	.512	100.000			

Extraction method: principal component analysis

Table 6. *Component matrix[a]; extraction method: principal component analysis*

	Component			
	1	2	3	4
Pb	**.600**	.483	-.317	-.316
As	-.073	.379	**.706**	.469
Cu	.217	**.788**	-.089	-.254
Fe	.310	.414	-.170	**.734**
Mn	**.800**	-.304	.421	.023
Al	**.880**	-.246	.268	-.150
Ni	**.698**	-.046	-.191	.103
Sr	.204	-.341	-.619	.450

[a]4 components extracted

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

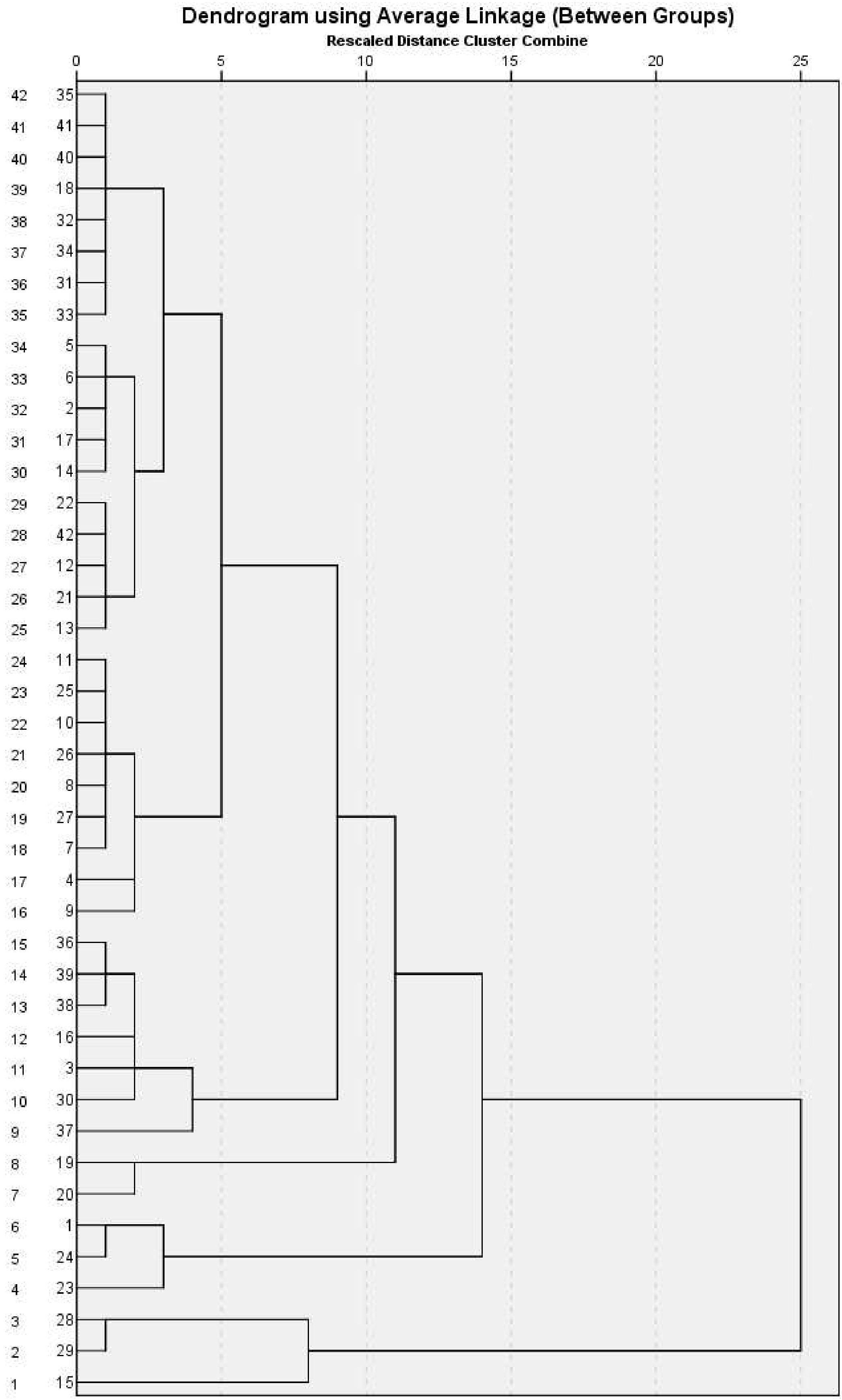

Figure 2. *Dendrogram of the results*

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DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

Heavy metal pollution index (HPI)

The pollution indexes are used to estimate the pollution of the water. Generally, heavy metal pollution index (HPI), heavy metal evaluation index (HEI) and degree of contamination (*Cd*) are used to evaluate water for drinking as well as irrigation purposes (Brraich and Jangu, 2015). Mehrabi et al. (2015) have also used heavy metal pollution index (HPI) to evaluate the level of contamination both in groundwater and in soil in Ahangaran mining area, Iran. They reported that HPI values were less than the critical index limit. Bhuiyan et al. (2015) have studied in Buriganga River, Bangladesh. The results showed that most of the samples exceeded the critical limit and "the intensity of pollution gradually decreased from the source to the downstream part of the river". Yang et al. (2015) have studied during the period 2008-2012 to understand the contamination in terms of heavy metal pollution and also used HPI for that. They had the following result: "The general trend of reduction in HPI appears not to have a seasonal variation and most likely resulted from the continued improvement in heavy metal pollution control strategies implemented by local environmental, agencies combined with a significant improvement in wastewater treatment capacities."

Prasad and Bose (2001) proposed the heavy metal pollution index (HPI) (Nasrabadi, 2015). The analysis results were interpreted based on these papers, in this study. Based on the weighted arithmetic average method, HPI shows the total quality of water compared to heavy metals (Horton, 1965; Mohan et al., 1996). The Heavy Metal Pollution Index (HPI) and the sub-index of each parameter (Qi) are calculated using the following correlations (*Eq. 1*).

$$Q_i = \sum_{i=1}^{n} \frac{(M_i - I_i)}{(S_i - I_i)} \text{x}100 \qquad HPI = \frac{\sum_{i=1}^{n} W_i Q_i}{\sum_{i=1}^{n} W_i} \tag{Eq.1}$$

Wi is the unit weight of the i-th parameter, and Qi is the sub-index of the i-th parameter. n is the number of parameters considered. Mi is the measured value of the parameter i. Ii and Si give the ideal and standard values of the i-th parameter. The HPI calculations are shown in *Table 7*. The average of HPI is 80.38833 for Duden Stream, 60.13997 for Goksu Stream. The graphical representations of the results are presented in *Table 8* with heavy metal pollution index (HPI) values. All values, except values of D15 - D19 - D20 - G13 samples, are below the 100.

Figure 3 shows the distribution of the heavy metal pollution index (HPI) values at the stations in the study area. The critical pollution index value is 100; this and above this value should be considered unacceptable (Prasad and Kumari, 2008; Prasad and Mondal, 2008; Reza and Singh, 2010; Ojekunle et al., 2016). According to Sirajudeen et al. (2014) the status categories of HPI are given in *Table 9*. According to the table there is no water in "very good" category. 14 water samples have been classified as "good" (D1 - D2 - D11 - D14 - D21 - G1 - G3 - G7 - G8 - G11 - G12 - G14 - G15 - G17); 20 water samples as "poor" (D3 - D4 - D5 - D7 - D8 - D10 - D12 - D13 - D16 - D18 - D22 - D23 - G2 - G4 - G5 - G6 - G9 - G10 - G16 - G18) and 8 water samples as "very poor (unsuitable for drinking)" (D6 - D9 - **D15 -** D17 - **D19** - **D20 -** D24 - **G13**). The HPI values of D15 - D19 - D20 and G13 samples were well above the limit.

The high HPI values were due to industrial waste waters, domestic sewage and landfill leachate (Milivojevic et al., 2016).

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DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

Table 7. *HPI calculations for both of the streams*

				DUDEN n = 24			GOKSU n = 18		
Heavy metals	S_i	I_i	W_i	M_i	Q_i	$W_i x Q_i$	M_i	Q_i	$W_i x Q_i$
As	50	10	0.02	9.95	-0.125	-0.0025	6.8666	-7.8335	-0.15667
Cr	10	-	0.1	0.2083	2.083	0.2083	0.8	8	0.8
Mn	300	100	0.003333	17.8916	-41.0542	-0.13685	2.4944	-48.7528	-0.16251
Fe	1000	100	0.001	197.2083	10.80092	0.010801	193.6111	10.40123	0.010401
Ni	20	-	0.05	8.3945	41.9725	2.098625	7.2055	36.0275	1.801375
Cu	1000	50	0.001	8.3041	-4.38904	-0.00439	4.6944	-4.76901	-0.00477
Se	10	-	0.1	0.2083	2.083	0.2083	0.0666	0.666	0.0666
Al	10	-	0.1	18.1	181	18.1	7.4166	74.166	7.4166
Pb	50	-	0.02	1.7666	3.5332	0.070664	1.1777	2.3554	0.047108
Sr	50	-	0.02	320.875	641.75	12.835	379	758	15.16
Total			0.415333	Total		33.38795	Total		24.97814
HPI values				80.38833			60.13997		

Table 8. *The values of heavy metal pollution index (HPI)*

DUDEN		GOKSU	
Station	**HPI**	**Station**	**HPI**
D1	43.99435	G1	47.56614
D2	46.92572	G2	51.28629
D3	72.53699	G3	49.65717
D4	66.4472	G4	70.34977
D5	60.97661	G5	66.32724
D6	78.40037	G6	51.17769
D7	57.75208	G7	46.7238
D8	55.7768	G8	43.55147
D9	98.8178	G9	65.68706
D10	59.78249	G10	63.56285
D11	47.09795	G11	42.73846
D12	52.10245	G12	43.20092
D13	53.28856	G13	**176.502**
D14	40.82054	G14	41.40662
D15	**177.8917**	G15	47.44171
D16	67.32998	G16	73.99047
D17	93.24475	G17	38.35314
D18	51.15154	G18	71.67112
D19	**242.1338**		
D20	**232.0681**		
D21	47.98676		
D22	70.51553		
D23	62.16189		
D24	80.9329		

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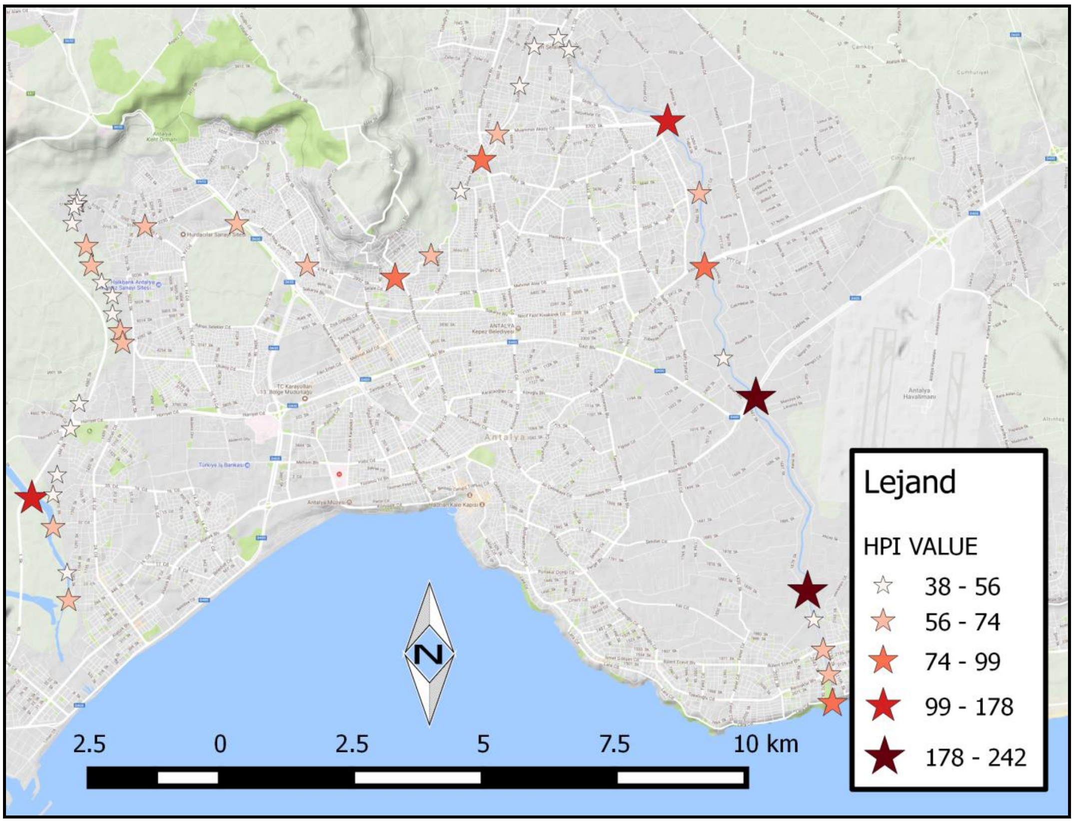

***Figure 3.** The distribution of the heavy metal pollution index (HPI) values at the stations in the study area*

***Table 9.** Status categories of HPI*

HPI	Quality of water (Sirajudeen et al., 2014)	Stations of study area (Duden and Goksu Streams)
0-25	Very good	---
26-50	Good	D1, D2, D11, D14, D21, G1, G3, G7, G8, G11, G12, G14, G15, G17
51-75	Poor	D3, D4, D5, D7, D8, D10, D12, D13, D16, D18, D22, D23, G2, G4, G5, G6, G9, G10, G16, G18
Above 75	Very poor (unsuitable for drinking)	D6, D9, **D15**, D17, **D19**, **D20**, D24, **G13**

Conclusions

The metal pollution ratio between the Kepez Hydroelectric Power Plant and the Karpuzkaldiran waterfall, which is defined as the “Lower Duden of the Duden Stream” in Lara and also surface water between Kepez Hydroelectric Power Plant and Bogacay River in Konyaaltı district, named Goksu Stream, were investigated using the HPI method. Firstly, aluminum (Al), lead (Pb), arsenic (As), copper (Cu), manganese (Mn), iron (Fe), nickel (Ni), cobalt (Co), strontium (Sr), chromium (Cr), selenium (Se), antimony (Sb), mercury (Hg) and cadmium (Cd) elements were investigated. Antimony (Sb), cadmium (Cd) and mercury (Hg) were undetectable and cobalt (Co) remained below the reporting limit.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

The mean values of aluminum (Al) and strontium (Sr) concentrations exceeded the permitted standard values, while the concentration of other metals remained well below the permissible standard, according to the descriptive statistics of the metal concentration of Duden Stream. When the relations between 10 heavy metal behaviours in stream water in 24 different stations were examined by multivariate analyses; the correlation between Cu and Pb, Fe and Pb, Ni and Pb, As and Cr, As and Se, Mn and metals were positively correlated, respectively, Cr and Sr have the same values and are highly correlated. The HPI critical value is set at 100 according to the literature. Accordingly, it has been determined that the calculated HPI value in water samples D15, D19 and D20 is well above the predicted critical value. The HPI value was consistent with multivariate statistical analyses.

4 factors influenced to the streams at high level with 79.460%. This value shows that the data used in the statistical analyzes are sufficient to explain the factors. The cumulative value calculated by SPSS shows that the percentage of explanations is high and adequate for 4 different factors.

The chemical similarities between the sampling stations show that there are 3 different locations in the region. According to the hierarchical analysis; 28, 29 and 15 samples have formed their dendrogram among themselves; 1, 24 and 23 samples have formed their dendrogram among themselves and all remaining samples have formed their dendrogram among themselves. The main reason of this grouping is the natural and anthropogenic effects.

The Goksu samples, according to the descriptive statistics of the metal concentration of the surface water, only the mean values of the concentration of strontium (Sr) exceeded the allowable standard values, while the concentration of other metals was far below the permissible standard. When the relationship between 10 heavy metal behaviours in stream water at 18 different stations was examined, it was found that there was a positive correlation between As and Fe, As and Mn, As and Al, Mn and Al, Mn and Ni, Al and Ni, Cr and Se metals respectively. It has been determined that the calculated HPI value in the case of water number G13 is above the predicted critical value.

There is no water in “very good” category. 14 water samples have been classified as “good” 20 water samples as “poor” and 8 water samples as “very poor (unsuitable for drinking)”. The HPI values of D15, D19, D20 and G13 samples were well above the limit. So, it is understood that the main source of pollution is anthropogenic.

Acknowledgements. The financial support of the Scientific Research Projects Unit of Akdeniz University is gratefully acknowledged.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24232436

THE EFFECTS OF WOOD VINEGAR ON SOME SOIL MICROORGANISMS

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(Received 14th Oct 2018; accepted 2nd Jan 2019)

Abstract. This study was carried out in order to determine the enzyme activity of the wood vinegar obtained from the hazelnut shells with the potential of bio-pesticide in agro-ecosystem soil, and its effect on the microfungi and heterotrophic bacteria in total. The study was realized in the production seasons of 2014-2015 and 2015-2016, in the ecological conditions of the province Muş (in Turkey) on winter wheat field and with four repetitions according to Randomized Blocks Experimental Design. The treatments within the scope of the experiment were conducted as the six different doses of wood vinegar at 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% mL, and the control treatment which was only provided with tap water. Repeated Measurement ANOVA was used to determine the effect of wood vinegar at different doses, the production seasons and periods (pre- and post-treatment) on relevant features. As based on the statistical inferences, only the Treatment × Year and Period × Year interaction effects in terms of the number of heterotrophic bacteria ($P = 0.000$ and $P = 0.011$); and only the Period × Year interaction effects for the number of microfungi ($P = 0.000$) were found statistically significant. Therefore, the effect of the treatments made on the number of heterotrophic bacteria varied by the production seasons. In a similar way, the effect of the production season and periods on the number of microfungi also changed as based on the term. While the effect of period ($P = 0.000$) and the Treatment × Period interaction ($P = 0.014$) effect were significant for Alkaline Phosphatase activity, Year × Period ($P = 0.001$) and Treatment × Period interactions ($P = 0.000$) were found significant as related to Beta Glucosidase activity. In conclusion, it may be stated that the wood vinegar used at different doses with the purpose of protecting plant and/or crop in wheat agro-ecosystem does not have a negative effect on microbial factors determined in the soil, and especially, the treatment of 3% mL has a positive effect on bacteria number and Beta Glucosidase enzyme activity.
Keywords: *agroecosystem, heterotrophic bacteria, microfungi, soil enzyme activities, wood vinegar*

Introduction

The primary objective of agricultural practices is to obtain abundant and high-quality crops without disturbing ecological balances (Topal, 2011). Within the scope of

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DOI: http://dx.doi.org/10.15666/aeer/1702_24372447

sustainable plant protection studies, herbal products may have a considerable part as stabilizer for synthetic pesticides or effecting the actions of synthetic pesticides in soil (Hagner, 2013). In the production of herbal pesticides, a variety of plants and various technologies have been used (Tiilikkala et al., 2011). Wood vinegar (WV) and other weak pyrolysis liquids are produced as a by-product of carbonization processes, and in the archeological studies carried out so far, it has already been detected that they were used in the Neanderthal's era (Tiilikkala et al., 2010). WV consists of water by 80-90% and the rest amount is of more than 200 organic compounds (Kim et al., 2008). The main components of WV are acetic acid and also organic acids, phenolic, alkane, alcohol and ester compounds (Jothityangkoon et al., 2008). WV is a substance that is toxic or slightly toxic to the non-target organisms in water and soil (Hagner, 2013). It has been detected that WV obtained from *Vitex pubescens* displayed antifungal effect (Orahami and Yoshimura, 2013). Baimark and Niamsa (2009) stated that WV could restrain the growth of fungus, as its involving strong phenolic compounds. Velmurugan et al. (2009) found out that the neutralized WV showed a strong antifungal effect. Jothityangkoon et al. (2008) detected that the contamination decreased, in their studies for the effect of WV on fungi producing aflatoxine. Namlı et al. (2014) stated that the case in which WV is treated as biocide in in-vivo conditions would provide beneficial outcomes, in accordance with the data they have already obtained in in-vitro conditions. Orahami et al. (2018) indicated in their studies that WV has had antifungal effect. Eric et al. (2012) stated that distilled and non-distilled WV had inhibited the bacteria. WV can activate the edaphon number in a short time and at maximum, and also has a promoting role in increasing the number of bacteria especially in the root parts of vegetables (Shi, 2003). Duan et al. (2016) put forth that the four types of WV, which they had involved in their studies, had a strong blocking effect on three sorts of bacteria. In plant protection activities, WV stands for a promising solution in terms of preventing the growth of pathogenic bacteria and fungi (Chalermsan and Peerapan, 2009). Lee et al. (2010), in their studies, pointed out that WV had a strong antimicrobial effect. Additionally, it was stated that the applied WV treatments had increased the phosphatase, protease, urease and invertase enzyme activities (Aleandri et al., 2012). It was also observed that the WV application at different concentration in sandy soil created an effect on microbial biomass and enzyme activity (β-Glucosidase, alkaline phosphatase and dehydrogenase) (Du et al., 2016). Koç et al. (2018) detected that WV was promising in increasing the enzyme activities in some of its applications. We consider that the determination of the most effective and appropriate WV dose is a highly important issue for plant protection activities. In this way, to maintain a pest control process without using any chemical substance will become possible. The objective in this research is to determine the effect of the wood vinegar obtained by means of the carbonization of hazelnut shells in the potential of bio-pesticide on alkaline phosphatase and beta glucosidase enzyme activities, bacteria and microfungi populations in agro-ecosystem soil.

Materials and methods

This study has been carried out in Krasunia odeska wheat field that belongs to BERCE Alparslan Agricultural Administration (height: 1276, lateral: 380 47' 33. 1815", long: 410 32' 45. 700") located at a distance of 12 km to the central part of the province Muş (in Turkey) (*Fig. 1*). The study was made in accordance with the

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DOI: http://dx.doi.org/10.15666/aeer/1702_24372447

Randomized Block Design and with four repetitions. Each parcel in experiment has the size of 25 m^2 and at least a 2-m-gap was spaced out between blocks and parcels (Anonymous, 2016a). The treatments made in the experiment was realized in the way of six different doses of WV at 0.5%, 1.0%, 2.0%, 3.0%, 4.0% and 5.0% mL, and the control provided with tap water only. WV treatments were carried out as to the schedule for fertilizing and agricultural spraying by BERCE Alparslan Agricultural Administration. Treatments were made by means of 16 L backpack sprayer (AnadoluPower APW-16, mode of operation: mechanical/operating by side pressing, filling chamber with filter, having an internal tank stirrer, can spray the liquid up to a maximum distance of 4 m, with an adjustable nozzle at 4 different types, 2.70 kg in weight). The classification by the texture of soil in the experimental area is argillaceous soil with the rate of clay by 63.29%, silt ratio by 25.8% and sand ratio by 10.9% (Koç, 2017). The climatic data of the research area, respectively, the 1st year (2014-2015), the 2nd year (2015-2016) and areal precipitation, average temperature and average relative humidity values of the last decade (long period average) were presented in *Table 1* (Anonymous, 2016b).

***Figure 1.** Experimental area*

***Table 1.** Some climate data for 2014-15, 2015-16 years and last ten years (LTY) in Muş province*

Total rainfall (mm)			**Mean temperature (°C)**			**Mean relative humidity (%)**		
2014-15	2015-16	LTY	2014-15	2015-16	LTY	2014-15	2015-16	LTY
740.4	790.1	740.5	11.55	11.48	10.62	55.02	54.00	60.79

Wood vinegar (WV) used in this study has been supplied from an establishment developing bio-coal and wood vinegar products by the gasifier of hazelnut shells (Namlı et al., 2014). Wheat seeds were sown by No-Till method. Inputs used in the treatment: bottom fertilizer (NP 20-20-0, 13.700 g/da) upon sowing on the 16th October of 2014 and height fertilizer in bolting period (46% urea, 10 kg/ha^{-1}) were applied. Furthermore, on the 26th May of 2015, to the parcels for which WV was applied, WV at different doses and to the control parcels only tap water was applied. Upon sowing, on the 12th September of 2015, bottom fertilizer (NP 20-20-0, 13.7 kg/ha^{-1}) and height fertilizer in bolting period (46% urea, 10 kg/ha^{-1}) were applied. Moreover, to the parcels which WV was applied on the dates of April 24th, April 30th, May 8th and June 6th, 2016, WV application at different doses was performed and the control parcels were provided with only tap water. The average humidity (%) content of soil samples in the experimental area was detected (*Table 2*).

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Table 2. *Average amount of moisture (%), according to production seasons in the experimental area*

Years	Sample time	Average moisture (%)
2014-2015	19.05.2015	24.90
	25.06.2015	19.60
2015-2016	21.04.2016	27.97
	25.06.2016	16.27

Soil sampling process involved the soil samples obtained from 8 different parts of each parcel (Yardım, 1996) by means of nematode sampling instrument (from a depth of 10 to 30 cm), and those samples taken were blended well to be aggregated. Samples were collected in sterile polyethylene nylon bags and preserved at +4 °C in the laboratory for further analysis. Treatments were realized once in 2014-2015 and four times in 2015-2016. The determination of humidity percentage in soil samples according to Craze (1990), the detection of total number of microfungi and bacteria according to Benson (2001), and the determination of Beta-glucosidase and Alkaline phosphatase enzyme activities were done according to Naseby et al. (1997). Repeated Measurement ANOVA has been used in analyzing data sets. Analysis results have presented as graphically (*Figs. 2–8*). All statistical analyses have been performed by using IBM SPSS (Ver. 24).

Statistical model used for analyzing data set was:

$$Yijkl = \mu + \alpha i + \beta j + \alpha\beta ij + \pi l(ij) + \gamma k + \alpha\gamma ik + \beta\gamma jk + \alpha\beta\gamma ijk + \gamma\pi kl(ij) + \varepsilon m(ijkl)$$

Yijkl: observed value for the number of heterotrophic bacteria, the number of microfungi and enzyme activity in k period of lth experimental unit of jth year in the ith treatment,
μ: overall population mean,
αi: effects of ith treatment (i = 1, 2, 3, …,7),
βj: effects of jth year (j = 1, 2),
αβij: treatment by year interaction,
π l(ij): random effect of the experimental unit l in ith treatment and jth year,
γk: effect of k th period (k = 1 = before, k = 2 after),
αγik: treatment by period interaction,
βγjk: year by period interaction,
αβγijk: treatment × year × period interaction,
γπkl(ij): experimental unit l by period interaction in ith treatment and jth year,
ε m(ijkl): random error term (Mendeş, 2013).

Results and discussion

Repeated Measurement ANOVA has been used for investigating effect of WV applications on the number of heterotrophic bacteria, the number of microfungi and enzyme activity (Winer et al., 1971; Mendeş, 2013). And the results of the Repeated Measurement ANOVA have been presented below:

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DOI: http://dx.doi.org/10.15666/aeer/1702_24372447

Effect of wood vinegar treatments on the number of heterotrophic bacteria

As based on the repeated measurement ANOVA results, only Treatment × Year and Period × Year interaction effects have been found statistically significant (P = 0.000 and P = 0.011). Therefore, the effects of treatments on heterotrophic bacteria have varied by years (2014-2015 and 2015-2016). Similarly, on the basis of period (pre- and post-treatment), the effect of year on heterotrophic bacteria have also differed. Treatment × Year and Period × Year interaction have been presented in *Figures 2* and *3,* respectively. As is seen in *Figure 2,* it has been determined that WV is effective in the treatments for 2014-2015 in terms of the decrease in heterotrophic bacteria, and however, there have been great differences in the case for 2015-2016.

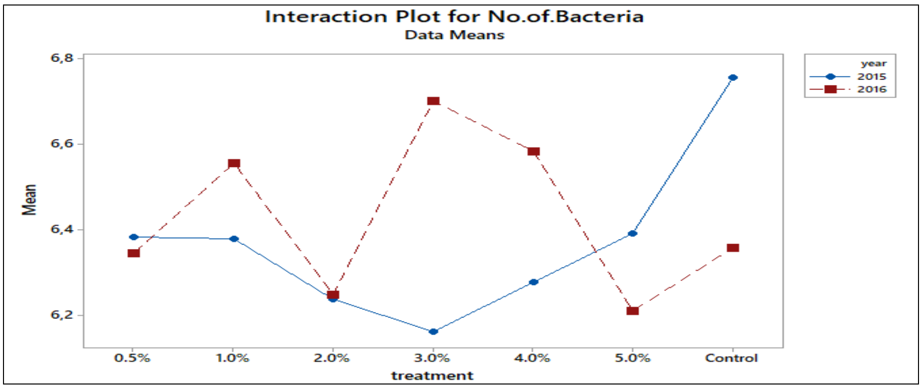

Figure 2. *Interaction plot for treatment by year in terms of number of heterotrophic bacteria*

When the Period × Year interaction plot is analyzed (*Fig. 3*), it is seen that the number of bacteria before the treatment in 2014-2015 is a bit more than the number of bacteria after the treatment. On the other hand, it can be observed that the post-treatment bacteria number in 2015-2016 is considerably more than the number for pre-treatment.

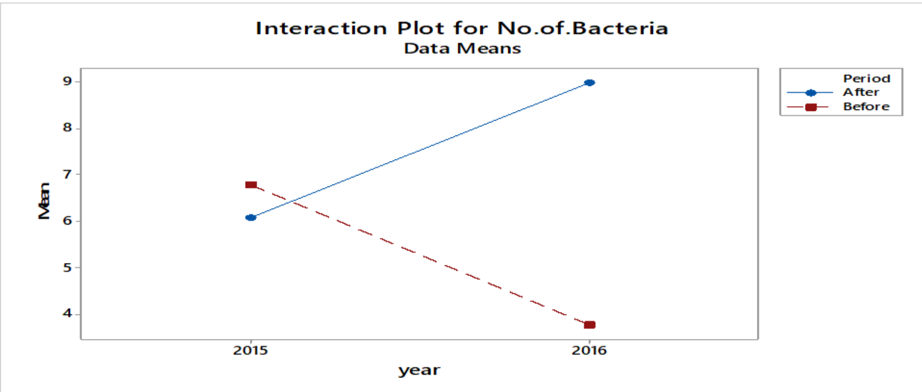

Figure 3. *Interaction plot for period by year in terms of number of heterotrophic bacteria*

Effect of wood vinegar treatments on the number of microfungi

Based on the Repeated Measurement ANOVA results, it has been seen that the only Period × Year interaction effect is statistically significant (P = 0.000). For this reason,

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the effect of year on the number of microfungi has varied by the period (before and after treatment). The Period × Year interaction plot is given in *Figure 4*. As it is seen in this plot, microfungi number in 2014-2015 is rather more, when compared to the period by 2015-2016, independently of treatment number. It is realized that the number of microfungi after treatment in 2015-2016 is greater than it is in 2014-2015.

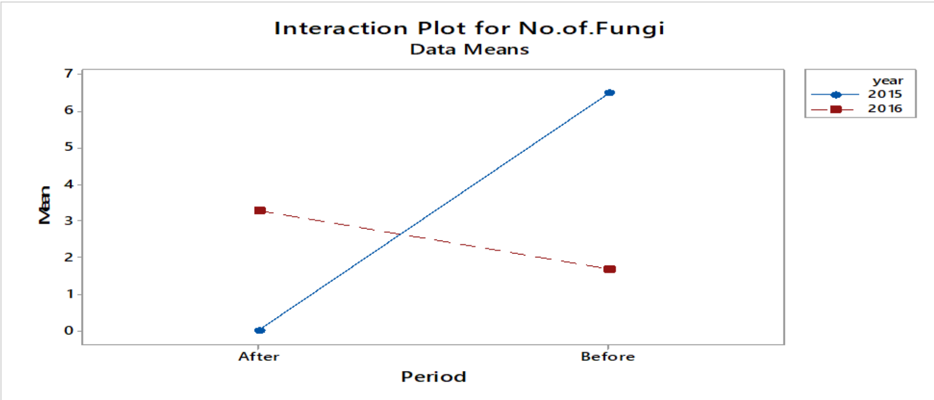

Figure 4. *Interaction plot for period by year in terms of number of microfungi*

Effect of wood vinegar treatments on enzyme activity

Considering the results of statistical analysis, it has been seen that the period effect (P = 0.000) and Treatment × Period interaction (P = 0.014) is significant. Therefore, the effects of treatments on Alkaline Phosphatase activity have varied by period (before and after treatment). Main effect plot for period and Treatment × Period Interaction plot are provided in *Figures 5* and *6,* respectively.

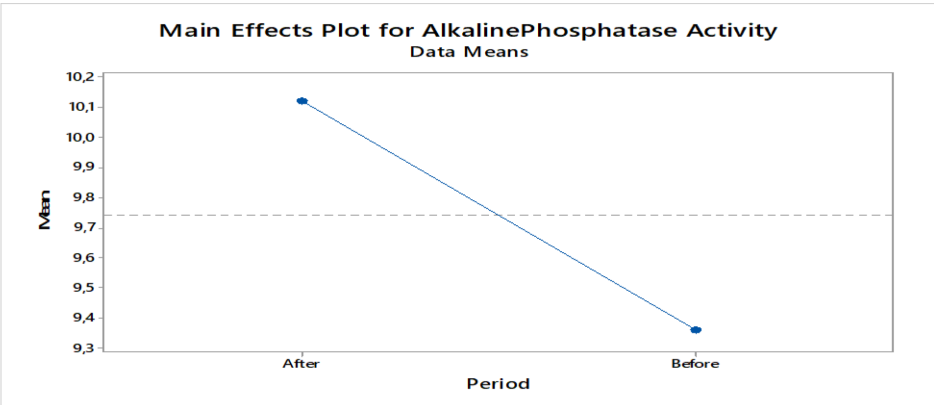

Figure 5. *Main effect plot for period in terms of alkaline phosphatase activity*

As it can be seen in *Figure 5,* the value of Alkaline Phosphatase activity after treatment is significantly higher than before treatment, independently of year and treatments. Treatment × Year interaction effect plot is given in *Figure 6*. When *Figure 6* is considered, the value of Alkaline Phosphatase activity has varied by treatments and years. However, the Alkaline Phosphatase activity at maximum is gained from the WV treatment of 1.0% mL, in 2014-2015. For 2015-2016, the highest value was obtained

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from the control treatment. As the reason for these results, it has been estimated that the case may be based on the extent, namely the highest, of WV doses used.

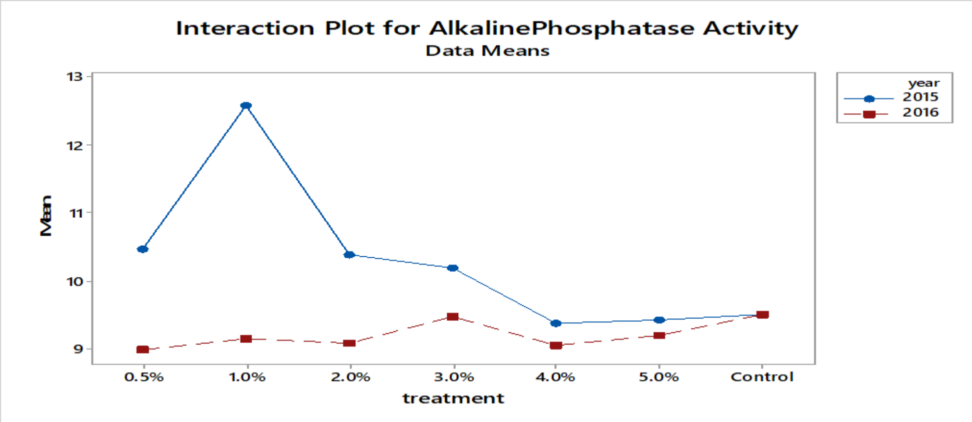

Figure 6. *Interaction plot for treatment by year in terms of alkaline phosphatase*

ANOVA results as related to Beta Glucosidase activity have revealed that Year × Period (P = 0.001) and Treatment × Period interactions (P = 0.000) are statistically significant. Therefore, the effect of year and treatments on Beta Glucosidase activity has varied by period (before and after treatment). Interaction plots for Year × Period and Treatment × Period are presented, respectively, in *Figures 7* and *8*. As it can be seen in *Figure 7,* the difference between pre-treatment and post-treatment is rather obvious, especially in 2015-2016.

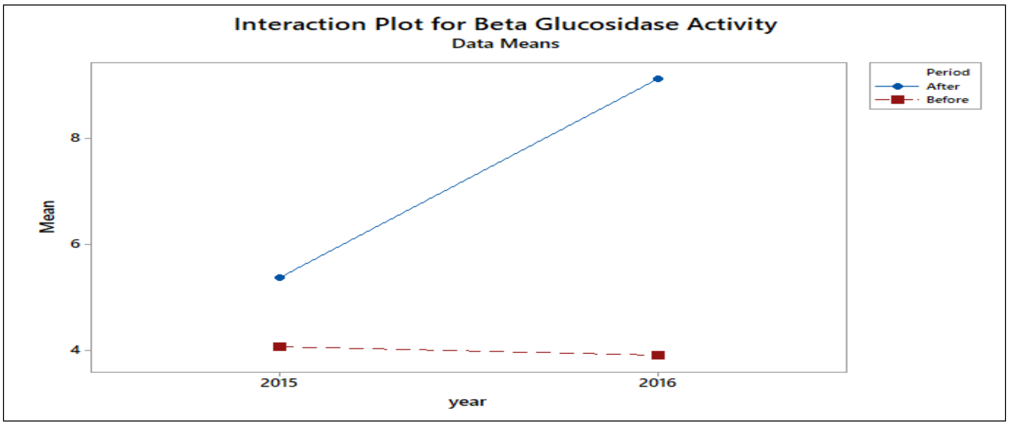

Figure 7. *Interaction plot for period by year in terms of beta glucosidase activity*

When the effect of treatments on Beta Glucosidase activity is considered (*Fig. 8*), it is seen that Beta Glucosidase activity has varied by treatment and years. In addition to this, while the highest value of Beta Glucosidase activity has been gained from the 3.0% mL treatment for the year 2014-2015, the maximum value for Beta Glucosidase activity has been received in the control treatment for 2015-2016. This result, as is in Alkaline Phosphatase activity, is thought to be arisen from that WV doses have been applied at higher levels, as well.

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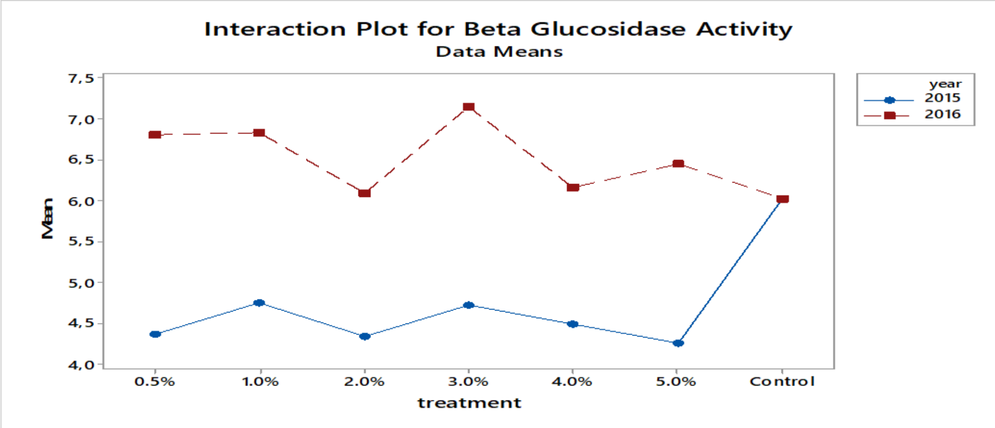

Figure 8. *Interaction plot for treatment by year in terms of beta glucosidase*

The utilization of bio-pesticides in agricultural practices as an alternative to the common pesticides is essential with regard to secure food, preservation of biological diversity and prevention of environmental pollution. In this study, the probable effect of wood vinegar obtained from the carbonization of hazelnut shells having the bio-pesticide potential on some microbial factors in agro-ecosystem soil has been investigated. It is realized that the findings reached at within the scope of this study are supported by the results of some similar studies that have been carried out before. For instance, the obtained findings as being relevant to the number of heterotrophic bacteria share similarity with the results in Shi (2003); however, it has been detected that they indicate some differences compared to the studies carried out by Nurhayati et al. (2005), Chalermsan and Peerapan (2009), Mao et al. (2010), Eric et al. (2012) and Duan et al. (2016). It has been thought that these differences may result from the changes related to the specific conditions, like climatic conditions, in the experimental area. When considered in terms of microfungi, especially in the production season of 2014-2015, it has been determined that WV treatments have effect on the decrease in the number of microfungi. Also, it has been noticed that the results similar to these findings have already been reached in the studies by Jothityangkoon et al. (2008), Baimark et al. (2008), Baimark and Niamsa (2009), Chalermsan and Peerapan (2009), Velmurugan et al. (2009), Lee et al. (2010), Ibrahim et al. (2013), Oramahi and Yoshimura (2013), Saberi et al. (2013), Namlı et al. (2014), Chuaboon et al. (2016), Ahadiyat et al. (2018) and Oramahi et al. (2018). It has been observed that the effect of WV treatments applied in this study on the enzyme activities (Alkaline phosphatase and Beta-glucosidase) varies according to before and after treatment. However, it is understood that the enzyme activities generally increase after WV treatments, and similar results have also revealed in the studies by Aleandri et al. (2012) and Koç et al. (2018). It can be said that WV treatments will be able to affect microbial biomass, as based on the findings obtained from this study, as Du et al. (2016) and Rui et al. (2014) stated before in their researches.

Conclusion

Pesticides which are widely used in agricultural practices harm the food safety and/or security, environment, biological diversity and biological chain. This issue affects soil

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DOI: http://dx.doi.org/10.15666/aeer/1702_24372447

productivity and human health in a serious and negative way, and these negative effects become more prominent in time. In accordance with the 2009/128/EC Framework Directives, the usage of pesticides must be minimized, and primarily the low-risk pesticides like bio-pesticides must be taken into consideration. Wood vinegar is a product which has a very low environmental risk and can quickly decay in the soil as a result of microbial activities. At this point, we consider that wood vinegar can be used as a bio-pesticide, i.e. as an alternative to the pesticides. In this study, it has been detected whether wood vinegar used in wheat agricultural eco-system has a negative effect on microbial factors determined in the soil, or not. As based on this, it can be put forth that wood vinegar will be able to be used securely as a pesticide. Additionally, the increase in enzyme activities and productivity may indicate that using WV practically is able to create a positive effect in crop production. Considering wood vinegar treatments, it can be stated that especially its doses and use frequencies have an effect on the microbial factors in soil. It is estimated that the dose of 3.0% mL will especially create a positive effect on the number and activity of biological factors in soil, in general.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24372447

FUZZY LOGIC APPLICATIONS IN HORTICULTURE AND A SAMPLE DESIGN FOR JUICE VOLUME PREDICTION IN POMEGRANATE (*PUNICA GRANATUM* L.)

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(Received 19th Oct 2018; accepted 28th Jan 2019)

Abstract. Fuzzy expert systems search for a solution based on the expertise of people who are experts in a particular field. This could be described as a kind of advisory system edited on computer. The use of natural language on the basis of fuzzy logic and easier understanding of system logs provide this technique to resolve many daily and current problems. In this study, a sample expert system to estimate juice volume in pomegranate was designed, using the fuzzy logic method, which closest to the logic of the human mindset. Recording of data was performed on the private farm of the province of Siirt, Turkey. The Fuzzy Logic Interface of MATLAB Program was used in the designing phase of the system. The evaluation of the model was carried out according to coefficient of determination and coefficient of correlation. The model revealed R^2 = 80% coefficient of determination, and r = 0.89 coefficient of correlation. With more informative parameters, the error rate can be decreased. Fuzzy logic seems one of the useful tools with prediction purposes in horticulture.
Keywords: *fuzzy expert system, Punica granatum L. cv. Zivzik, juice volume, fruit size, irrigation*

Introduction

Pomegranate (*Punica granatum* L.) is a tropical and subtropical fruit species, in the cultivation of which India, Iran and China are the top ranking in the world, followed by Turkey and USA. The annual production of pomegranate cultivation is over 100,000 tons in Turkey, which is available in 48 provinces of the country. Pomegranate is a perennial and drought-tolerant plant. Arid and semiarid zones are popular for growing pomegranate trees (Asgary et al., 2014). The flowering and fruit setting period varies according to low or high altitude conditions. This particularly affects the early or late opening of flowers. It is indicated that the colors and sizes are not normal due to the inadequate temperatures (Onur, 1988; El-Sese, 1988). Pomegranate juice is a good source of fructose, sucrose, and glucose. It also has some of the simple organic acids such as ascorbic acid, citric acid, fumaric acid, and malic acid (Asgary et al., 2014). Morever it is a polyphenol-rich fruit juice with a high antioxidant capacity. This fruit can help preventing or treating several cardiovascular risk factors including hypertension, hypercholesterolemia, oxidative stress, hyperglycemia, and inflammation (Chong et al., 2010; Wang et al., 2014; Sahebkar et al., 2017).

Agricultural production is a complex system requiring knowledge and information from many diverse sources. Agricultural specialist assistance is not always available for the farmer who needs it. In order to support the farmer expert systems were identified as

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a powerful tool with extensive potential in agriculture (Prasad and Vinaya Babu, 2006; Dath and Balakrishnan, 2013; Kolhe and Gupta, 2014).

During the last five decades, the potential of electronic data processing has been used to an increasing degree to support human decision making in different ways. Since the late 1970s and early 1980s decision support systems found their way into management and engineering. Evaluation, diagnosis, prediction could be classified as decision support systems. Even though fuzzy set theory can be used in all three "prototypes" we shall concentrate on "expert systems" only because the need and problem of managing uncertainty of many kinds is most apparent there; hence, the application of fuzzy set theory is most promising and advanced. In operations research the modeling of problems is normally being done by the OR-specialist. The user then provides input data and the mathematical model provides the solution to the problem by means of algorithms selected by the OR-specialist (Zimmermann, 2001). While the typical OR-model or software package normally supports the expert, an expert system is supposed to model an expert and make his expert knowledge available to non-experts for purposes of decision making, consulting, diagnosis, learning, or research (Konopasek and Jayaraman, 1984).

The most relevant approaches of fuzzy expert system (FES) in horticulture is fruit sorting and grading system (May and Amaran, 2011; Nandi et al., 2012; Razak et al., 2012; Teoh et al., 2013; Hasan et al., 2014; Suksawat and Komkum, 2015; Nandi et al., 2016); fruit counting (Kumar et al., 2017; Qureshi et al., 2017), plant watering management (Ge et al., 2013; Ying et al., 2017), plant yield prediction (Srinivasan et al., 2009; Papageorgiou et al., 2013). Brotons et al. (2017) in their study tried to find relation between pomegranate maturity index with to solar net radiation by means of fuzzy approach. As a result of the study, they reported the possibility of prediction the most likely date from which the fruit will be ready for harvest, for a given latitude.

The main purpose of this study was to introduce fuzzy expert systems in estimating the juice volume without requiring any laboratory work by using the pomegranate features such as different harvest time, irrigation amounts, and fruit size.

Materials and methods

Plant material

The plant material of the study is pomegranate (*Punica granatum* L.) cv. Zivzik, obtained from an 8 year-old orchard located at 37° 57’ 17” Northern and 41° 51’ 07” Eastern coordinates, in Kezer region of the Central district of Siirt, Turkey (*Fig. 1*). Experiment was settled with 3 replications according to the design of random blocks.

The irrigation in pomegranate cultivation is one of the important parameters and therefore we have considered it as one of the input parameters. The amount of irrigation water was designed based on the fact that 50%, 75% and 100% of the open water surface evaporation values, obtained from the experimental area, were applied as irrigation water when they reached 80 mm and 120 mm (Aydın et al., 2017). The amounts of irrigation water applied to the treatments were between 292.6 mm (50%), 438.8 mm (75%) and 585.2 mm (100%). In the experiment, during the 4 weeks from the middle of October to the beginning of November, five fruit samples from each Zivzik pomegranate tree were taken weekly and their physical and chemical properties were examined.

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***Figure 1.** Zivzik variety of pomegranate: fruit and orchard*

Much of human knowledge is a collection of rules and facts which, for the most part are neither totally certain, nor totally consistent, the storage of these vague and uncertain portions of knowledge by using fuzzy sets seems much more appropriate than the use of crisp concepts and symbolism, "management of uncertainty" of human thinking, when modeled in expert systems, might also increase efficiency, that is, decrease answering time (Zadeh, 1983; Zimmermann, 2001).

The fruit samples used were harvested respectively on October 16, October 23, October 31, and November 6, 2015. The fruits were determined in terms of fruit weight, fruit length, fruit width, aril yield (%), fruit juice ratio, total soluble solids (%), titrable acidity (%), TSS / TA and aril color. Pomological analyzes of these samples were carried out in the laboratory of Siirt University. Fruit size (FS) trait from the analyzed pomological characteristics was used in the prediction.

Fuzzy logic based expert system

An expert system is a computer program that solves problems that heretofore required significant human expertise by using explicitly represented domain knowledge and computational decision procedures (Kastner and Hong, 1984). The general structure of an expert system is shown in *Figure 2*. The Knowledge Acquisition Module supports the building of an expert system's knowledge base. The subject of knowledge acquisition for knowledge-based systems falls conveniently into two parts depending on whether the knowledge is elicited from the experts by knowledge engineers or whether that knowledge is acquired automatically by the computer using some form of automatic learning strategy and algorithms (Graham and Jones, 1988; Zimmermann, 2001).

General structure of FES for juice volume prediction in pomegranate is given in *Figure 3*.

Input and output values and their intervals in established model for the prediction of fruit juice volume (FJL) by means of FL are shown in *Table 1*. As the table shows, harvesting period (HP), amount of irrigation water (AIW) and FS were used as input data, and the output data of fruit juice volume were obtained.

Verbal expressions and fuzzy sets for HP, AIW, FS and FJV are given in *Table 2*. As *Table 2* shows, the verbal expressions established for HP were 1st period, 2nd period, 3rd

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period and 4th period; for AIW deficit, medium and enough; for FS very small, small, normal, large and very large; and for FJV very low, low, normal, high and very high.

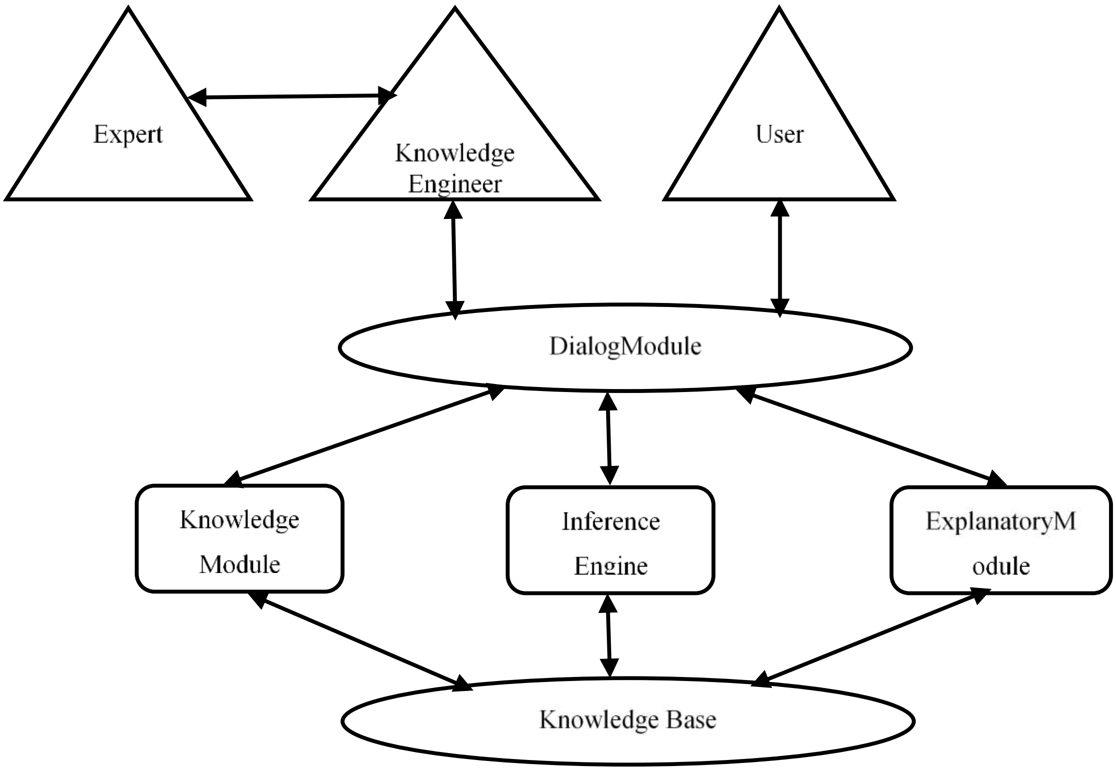

Figure 2. *Structure of an expert system*

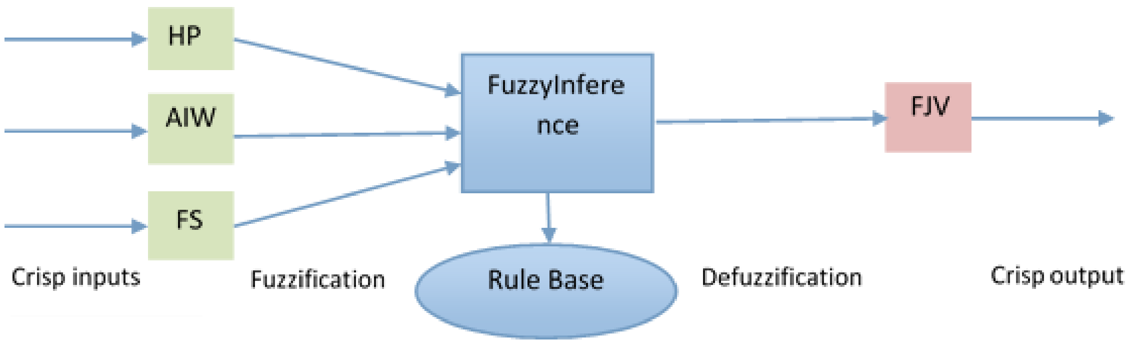

Figure 3. *Scheme of the developed FES*

Table 1. *Input–output values and their intervals*

Input/Output	Trait	Intervals
Input data	HP	0–5 (integer)
	AIW	0–4 (integer)
	FS	76–270 (gr)
Output data	FJV	13–73 (ml)

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Table 2. *Verbal expressions for HP, AIW, FS and FJV*

Trait	Verbal expression	Value intervals
HP	1st period	$0 < x < 2$
	2nd period	$1 < x < 3$
	3rd period	$2 < x < 4$
	4th period	$3 < x < 5$
AIW	Deficit	$0 < x < 2$
	Medium	$1 < x < 3$
	Enough	$2 < x \leq 4$
FS	Very small	$76 \leq x < 110$
	Small	$100 < x < 180$
	Normal	$170 < x < 200$
	Large	$190 < x < 220$
	Very large	$210 < x \leq 270$
FJV	Very low	$13 \leq x < 23$
	Low	$20 < x < 38$
	Normal	$37 < x < 47$
	High	$45 < x < 53$
	Very high	$52 < x \leq 73$

Various functions were used to graph input and output membership. Here, triangular (*Eq. 1*) and trapezoidal (*Eq. 2*) membership functions were used to calculate membership degrees of the input and output values generated (Baykal, 2004).

$$\mu_A(x;a_1,a_2,a_3) = \begin{cases} a_1 \leq x \leq a_2 & then \quad (x-a_1)/(a_2-a_1) \\ a_2 \leq x \leq a_3 & then \quad (a_3-x)/(a_3-a_2) \\ x > a_3 \ \text{ veya } \ x < a_1 & then \quad 0 \end{cases} \tag{Eq.1}$$

$$\mu_A(x;a_1,a_2,a_3,a_4) = \begin{cases} a_1 \leq x \leq a_2 & then \quad (x-a_1)/(a_2-a_1) \\ a_2 \leq x \leq a_3 & then \quad 1 \\ a_3 \leq x \leq a_4 & then \quad (a_4-x)/(a_4-a_3) \\ x > a_4 \ \text{ veya } \ \mathrm{x} < \mathrm{a}_1 & then \quad 0 \end{cases} \tag{Eq.2}$$

Mamdani inference technique was used as fuzzy inference method (Mamdani and Assilian, 1975). A centroid technique method (Cox, 1999) was used for defuzzification of fuzzy data. It finds the point where a vertical line would slice the aggregate set into two equal masses. Mathematically this centre of gravity (COG) can be expressed as:

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$$COG = \frac{\int_a^b \mu_A(x)x\,dx}{\int_a^b \mu_A(x)\,dx} \quad \text{(Eq.3)}$$

A centroid defuzzification method (*Eq. 3*) finds a point representing the centre of gravity of the fuzzy set A, on the interval ab.

Evaluation

In this study, coefficient of correlation and coefficient of determination (*Eq. 4*) (Spiegel et al., 2009) between real estimated and predicted by means of FES were calculated in term of accuracy and efficiency of the system.

$$R^2 = \frac{\sum_{i=1}^{n}(\hat{Y}_i - \bar{Y})^2}{\sum_{i=1}^{n}(Y_i - \bar{Y})^2} \quad \text{(Eq.4)}$$

where: Y_i – observed value, ($\hat{Y}_i$) – predicted value, $\bar{Y}$ – arithmetic mean, n – the total number of observations.

Results and discussion

As a result of analysis of variance, it was determined that there was a significant difference between harvesting periods according to juice volume ($p < 0.05$). A significant positive correlation was found between fruit weight and fruit juice volume ($p < 0.01$).

Descriptive analysis of the data revealed that the average fruit juice volume (FJV) of pomegranate in 4 HP and 3 different irrigation amount (deficit, medium, enough) varied from 18.39 ± 2.73 to 51.33 ± 9.20 ml; fruit weight of pomegranate (FS) from 91.18 ± 10.80 to 226.52 ± 25.80 g respectively.

For the FL realized via the MATLAB program (Mathworks, 2009), 3 inputs (HP, AIW, FS) and 1 output (FJV) were available. The HP graph formed in MATLAB, as illustrated in *Figure 4,* uses the four harvesting period as 4 the conditional fuzzy sets. In FES, however, this input is entered as discrete. The AIW graph formed in MATLAB and containing 3 verbal expressions is illustrated in *Figure 5*. It consists of 3 verbal variables. Since the amount of irrigation water generally changes according to the number of factors such as the amount of irrigation made by the farmer, the amount of rainfall, the amount of evaporation associated with the seasonal temperature, and the soil structure. Just farmer's decision concerning plant irrigation basically can be used. We used this input as the crisp input in our model. For deficit irrigation 1 will be entered, 2 for medium, 3 for enough irrigation. In the FS graph, illustrated in *Figure 6,* for the very small and very large fuzzy sets the trapezoidal membership functions was selected, and for the small, medium and large fuzzy set the triangular function was used. *Figure 7* shows the output graphic FJV. The FJV graph, divides the output data into five ranges as very low, low, normal, high and very high, and shows the verbal expression memberships. For the very low and very high fuzzy sets the trapezoidal membership function was selected; for the normal and high fuzzy sets the triangular function was used.

Rule base of the FES was contained of 25 rules. Rules were formed from the data (HP, AIW, FS) and taking into account the FJV as measured in the laboratory. *Table 3* shows some rules given in our FES.

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Table 3. *Some rules from developed rulebase*

Rule 1:	If HP is	'1st Period'	AIW is	'Medium'	FS is	'Small'	Then FJV is	'Normal'
Rule 2:	If HP is	'2nd Period'	AIW is	'Medium'	FS is	'Normal'	Then FJV is	'Low'
Rule 3:	If HP is	'3rd Period'	AIW is	'Enough'	FS is	'Very large'	Then FJV is	'Very high'
Rule 4:	If HP is	'4th Period'	AIW is	'Enough'	FS is	'Very small'	Then FJV is	'Very low'
Rule 5:	If HP is	'2nd Period'	AIW is	'Deficit'	FS is	'Normal'	Then FJV is	'Normal'

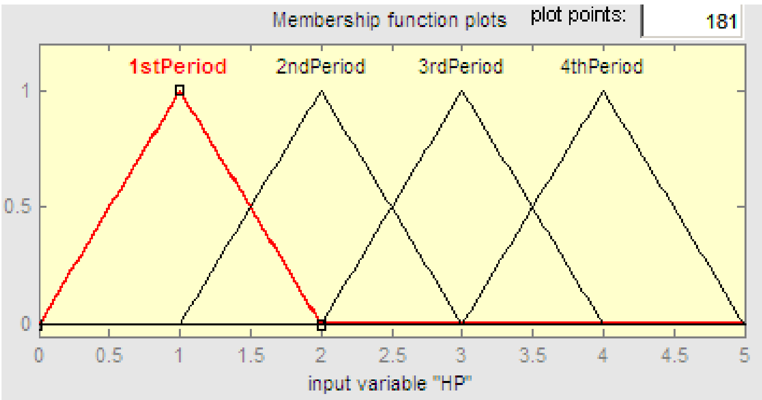

Figure 4. *HP membership function graph*

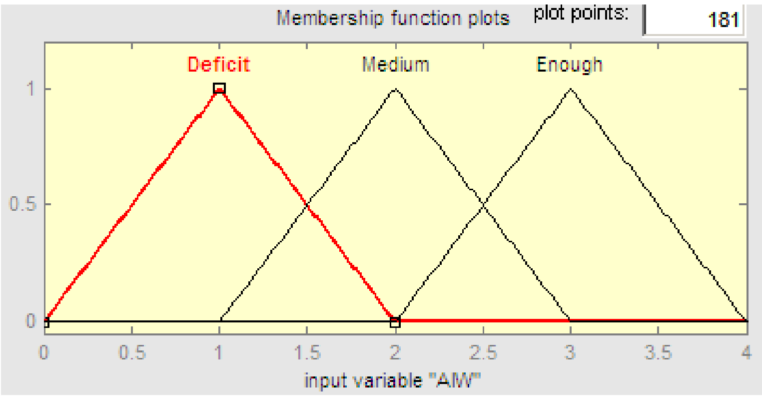

Figure 5. *AIW membership function graph*

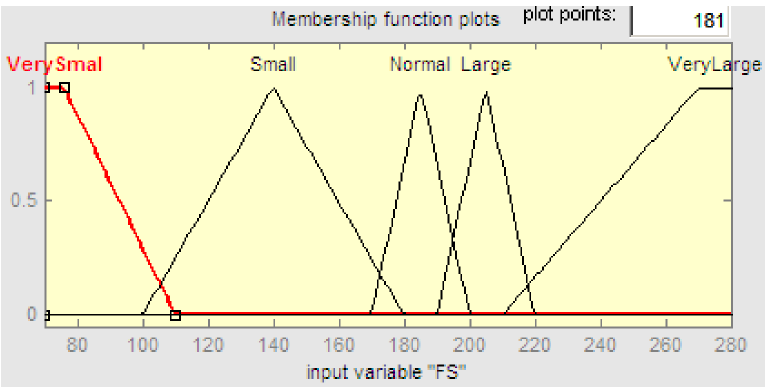

Figure 6. *FS membership function graph*

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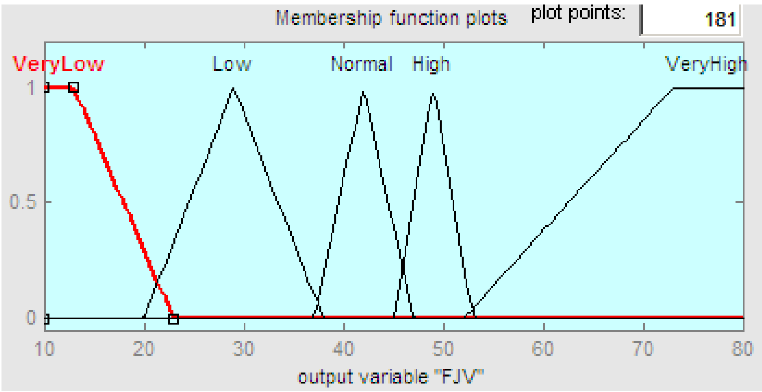

***Figure 7.** FJV membership function graph*

Figures 8 and *9* show the 3-dimensional relationships between the HP, AIW, FS and FS.

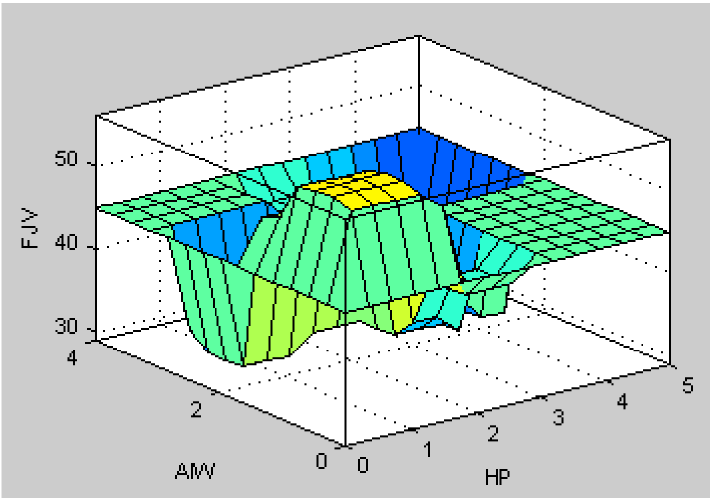

***Figure 8.** 3-D relation between AIW, HP and FJV*

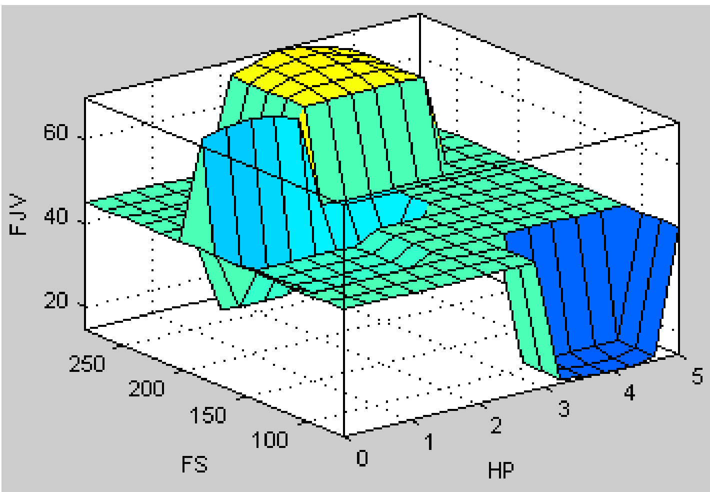

***Figure 9.** Relation between FS, HP and FJV*

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For example, given 1st Period for HP, 2-medium for AIW, and 165 mg for FS, the observed output value appears next to the statement 42 ml for FJV (*Fig. 10a*). Other results can be found by supplying different input data, for example, given HP = 3rd Period, AIW=enough, and FS = 250 mg, the appropriate response for these inputs is FJV = 69.3 ml (*Fig. 10b*).

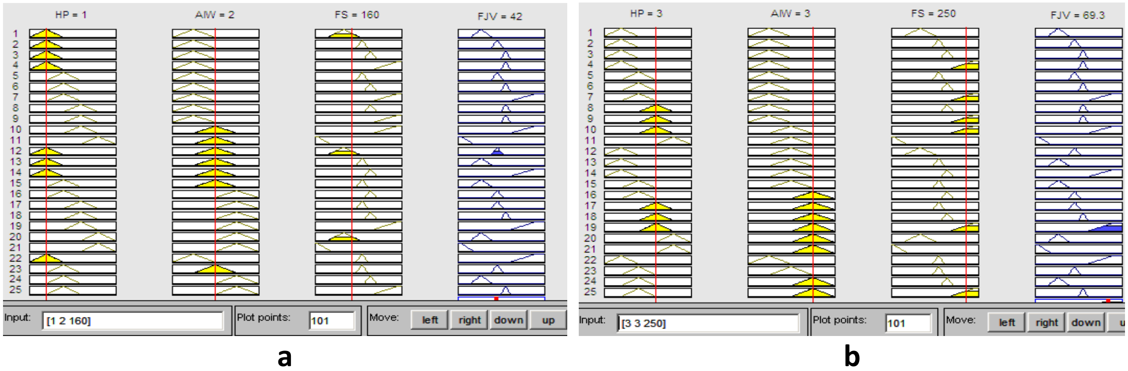

Figure 10. *Simulation results for the given inputs*

As shown in *Figure 10a* and *b,* different rules were fired for the different inputs. Thereafter, all data were run through the FL model, with the results of FJV. The coefficient of correlation between real estimated and predicted by means of FL was 0.89 (*Fig. 11*).

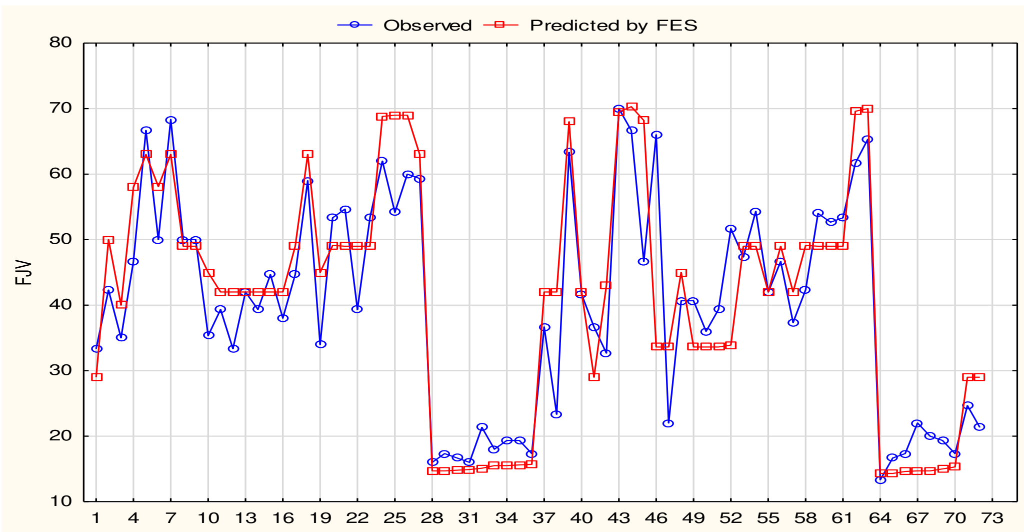

Figure 11. *Relation between FJV measured in laboratory and predicted via FES*

The coefficient of determination was found to be 80%. With the aim of fast, but accurate and easy way of finding volume, weight, and surface area of a crop in the study of Dorvlo et al. (2012) the prediction equation for weight can be used with an appreciable coefficient of determination of 68.89%. Prediction accuracy of the current study was found higher than the study of Dorvlo et al. (2012).

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Conclusion

This paper assayed pomegranate juice volume estimation by means of the FL. Using the input data of HP, AIW and FS model was able to predict fruit juice volume at a level of success. In the end, the study's FL model revealed coefficient of determination rate of 80%. It can be said that a fuzzy expert system can be applied in areas where mathematical modeling is difficult. In addition, the establishment of such expert systems in agriculture using fuzzy logic will help real-time, appropriate and rapid decision-making in land conditions, taking advantage of analytical decision-making capabilities of experts. In the model we build, we can develop an expert system, which gives more definite results in future by further developing and fuzzifiing the other inputs as much as possible, that can guide the producers of pomegranate breeding. Given these results, it is recommended that a prediction system be devised by adding more improved FL model with high sensitivity. Such a system will eliminate the need for intensive laboratory work in lieu of data obtained from farm. Considering all the factors, a more punctual and efficient system can be developed in the future.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24492460

BREEDING STATUS AND NEST CHARACTERISTICS OF ROSE-RINGED (*PSITTACULA KRAMERI*) AND ALEXANDRINE PARAKEETS (*PSITTACULA EUPATRIA*) IN ISTANBUL'S CITY PARKS

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(Received 25th Oct 2018; accepted 28th Jan 2019)

Abstract. Invasive non-native parakeet populations are increasing throughout Europe with proved negative impact on native fauna and Turkey is no exception. Rose-ringed and Alexandrine parakeets have established populations in Turkey's large cities but even basic distribution, abundance and breeding behaviour information is missing. This study aims at determining the breeding status and identifying the nest characteristics of Rose-ringed and Alexandrine parakeets in Istanbul's city parks. The study is carried out in 9 city parks during one breeding season. Data on the presence of breeding parakeet species, their nest characteristics and characteristic of suitable cavities on non-nesting trees were collected. Both species were recorded breeding sympatrically in 5 out of 9 city parks with probable breeding in one more park. When nesting, both species preferred high trees with high diameter but Rose-ringed parakeet nests were placed lower than those of Alexandrine parakeet's. Plane (*Platanus* sp.) species were the most used as a nesting tree for both species. This study reveals the first systematic observations on the breeding Rose-ringed and Alexandrine parakeet populations in Istanbul and serves as a basis for further detailed research on both species in Turkey.
Keywords: *invasive species, non-native fauna, nest selection, nest site competition, cavity nesters, environmental change*

Introduction

Invasive non-native species, a human-caused problem, are considered to be amongst the major problems for biodiversity in the Anthropocene (Pievani, 2014). Although there has been a debate on whether invasive species are themselves a cause for ecosystem change or they benefit from already disturbed ecosystems (Didham et al., 2005; Gurevitch and Padilla, 2004; Clavero and García-Berthou, 2005; Davis, 2003) studies show a correlation between invasive species presence and altered local biodiversity. Invasive non-native species can alter the local biodiversity by excluding native species through competition for resources (Mazzamuto et al., 2017), by predation (Wanless et al., 2007), by parasitism (Douda et al., 2017), by disease (Morand, 2017), by hybridization (Kovach et al. 2016) or by causing evolutionary changes (Mooney and Cleland, 2001). All these changes impact ecosystem functioning and therefore creates direct or indirect economic loss (Kaiser, 2006; Dehnen-Schmutz et al., 2004; Schwoerer et al., 2014).

Parakeets, especially the Rose-ringed parakeet (*Psittacula krameri*) are one of the widespread and common invasive bird species with well-established populations in Europe (Pârâu et al., 2016). Rose-ringed parakeets originate from Asia and Africa,

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mostly found around human settlements in Europe and is known to compete for nest-sites (Strubbe and Matthysen, 2009), or for food (Peck et al., 2014) with local species. Alexandrine parakeet (*Psittacula eupatria*), has its native distribution from Afghanistan to Vietnam and is not as widespread and as common as the Rose-ringed parakeet in Europe. Major Alexandrine parakeet populations were reported from Belgium, Germany and Netherlands, all with a couple of hundred individuals (Ancillotto et al., 2016) but Ancillotto et al., (2016) suggested that the number of Alexandrine parakeets is increasing and according to their model its establishment success might be benefiting from the past invasion of Rose-ringed parakeet in Europe.

Rose-ringed parakeet is smaller (40 cm) than Alexandrine parakeet (58 cm) (Juniper and Parr, 2010). In their native ranges both species occur in wide variety of habitats but mainly in woodlands; prefer tree cavities as nest; feed on wild and cultivated seeds, flowers and fruits and considered as serious crop pests (Parr and Juniper, 2010). In its native range, the clutch size of rose-ringed parakeet ranges from two to six (Lamba, 1966).

Two species of invasive non-native parakeets are common in Turkey, Rose-ringed and Alexandrine parakeet (*Psittacula eupatria*). The first observations on the established Rose-ringed parakeet populations in large cities (Istanbul, Ankara and Izmir) were recorded in the 1990s (Boyla et al., 1998). Alexandrine parakeet records, on the other hand, started to increase during the 2000s (eBird, 2012) and probably outnumbered Rose-ringed parakeets in some parts of Istanbul (Sahin, personal observation). Although both species were quickly increased in number in large cities, basic information on their distribution, abundance and breeding status are missing in Turkey.

It is for sure that in order to have a comprehensive understanding of the impact of invasive parakeet species on native biodiversity and to create an effective management plan we first need to understand their distribution, abundance and invasion ecology. Introduction of parakeet species is under strict control by legislation in European Union (European Commission, 2007), however, it is still uncontrolled in Turkey (Per, 2018). Turkey's rich bird diversity is already under various threats due to speed-up in development and urbanisation projects (Şekercioğlu et al., 2011) and the impact of invasive non-native species on native species such as Common starling (*Sturnus vulgaris*, Eurasian nuthatch (*Sitta europaea*), and Syrian woodpecker (*Dendrocopos syriacus*), is probably making it worse in urbanised areas. An immediate research on the topic is needed. This study attempts for the first time to use systematic observations to reveal information about breeding Rose-ringed and Alexandrine parakeets in one of the largest cities of Turkey. The aim of this study is to provide baseline information on the distribution, breeding status and nest preferences of two invasive parakeet species in Istanbul's city parks. Although this study was conducted in 2012, the results are still relevant as both species are present, breeding and moreover, likely to increasing in distribution and abundance in Istanbul according to the number of sightings in global bird-sightings database, e-bird (eBird, 2012). We expect that the baseline information revealed by this study will trigger further studies on the two invasive parakeet species in Turkey.

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Material and Methods

Study Area

This study is conducted in Istanbul, one of the largest cities in Turkey. Although two parakeet species can be seen in most parts of the city, especially in densely populated areas, the breeding occurs in parks where a high amount of old trees can be found together (Sahin, personal observation, see “Discussion”). Therefore this study was carried out in the historic parks of Istanbul that are managed by the Istanbul Metropolitan Municipality. Five of these parks (Beykoz (27.9 ha), Buyuk Camlica (12.9 ha), Kucuk Camlica (27.14 ha), Fethipasa (13.4 ha) and Cubuklu (16.3 ha)) are located in Asian and four of them (Emirgan (42.7 ha), Florya (67.5 ha), Gulhane (12.1 ha), Yildiz (39.2 ha)) are located in European side of Istanbul. The location and size of the parks are given in *Figure 1*.

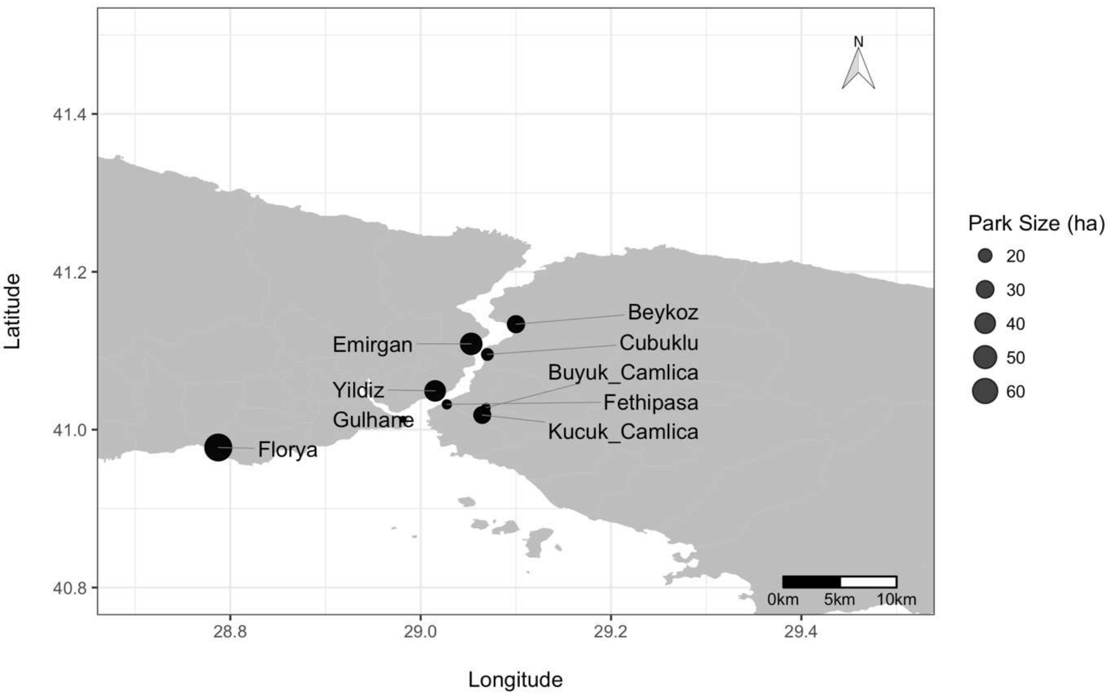

Figure 1. *Location and size of the studied parks in Istanbul. Size of the black circles represents the size of the parks in hectare*

The whole area is a transition zone between the dry Mediterranean and wet Black Sea climate. This impacts the climatic conditions between the northern and the southern part of the city; northern parts get more rain and are colder on average than southern parts.

As all the studied parks are managed, both native and non-native tree species with different ages can be found in these areas. The three most common Gymnosperm genera are *Pinus*, *Cedrus* and *Cupressus* whereas the three most common Angiosperm genera are *Quercus*, *Fraxinus* and *Platanus* for all parks. The total volume of trees belong to these two major classes are given in *Figure 2* and volumes of the most common Angiosperm genera were given in *Figure 3*.

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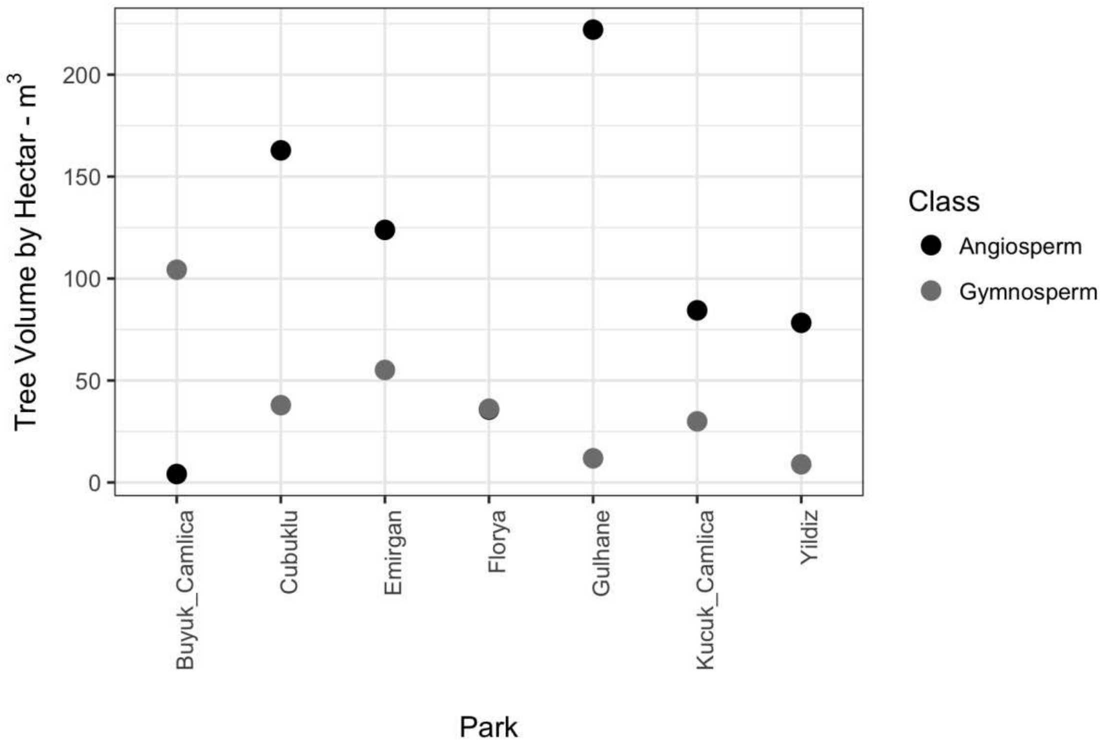

Figure 2. *Volume of Gymnosperm and Angiosperm trees in studied parks. Tree volumes are normalised by hectare for each park*

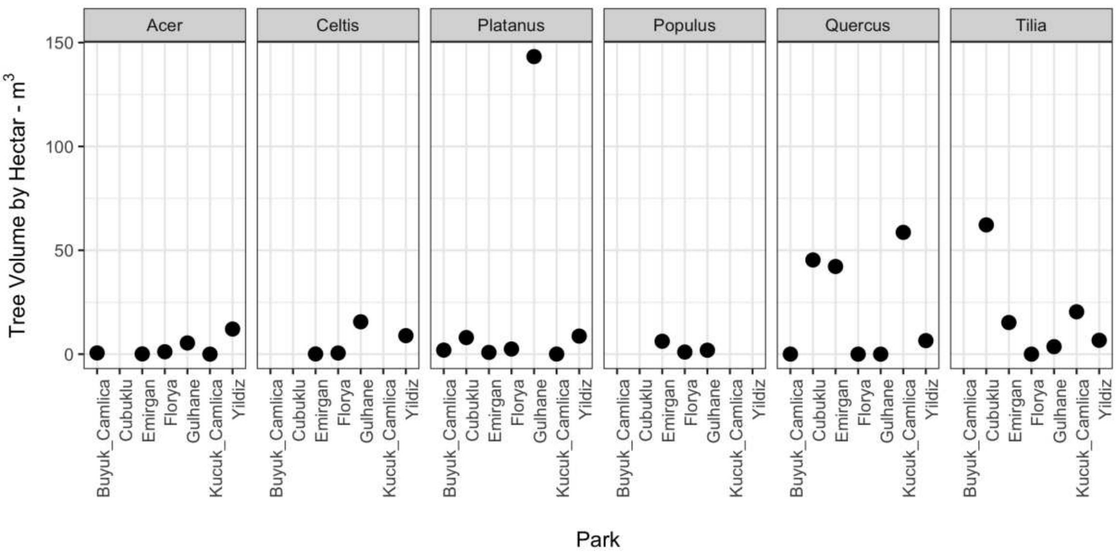

Figure 3. *Volume of the most common Angiosperm trees in studied parks. Tree volumes are normalised by hectare for each park*

The most common passerine species found in almost all parks includes Robin (*Erithacus rubecula*), Blackbird (*Turdus merula*), Great tit (*Parus major*), Blue tit (*Cyanistes caeruleus*), House sparrow (*Passer domesticus*), Chaffinch (*Fringilla coelebs*) among others.

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Data Collection

Two visits were made in breeding season between March-May in 2012. In the first (early) visit breeding status and the distribution of two parakeet species were recorded together with the number of adult individuals (recorded as the adult male and adult female / young male) in each park. In this first visit behaviour that indicates breeding (e.g. courtship, nest building etc.) and the location of detected nests were also recorded. In all parks, birds were counted by walking pedestrian paths.

In the second visit, data on nest characteristics were collected for all the nests determined in the first visit. Data on tree species, tree height and diameter, nest height, nest type (natural cavity, old nest for other species or nest box) and the nest orientation were collected. Tree height was measured with the clinometer, the orientation of nests was measured with a compass and the tree circumference was measured with a tape measure from 1.30 m height. Diameter at breast height (dbh) was calculated by dividing tree circumference with Pi number. To identify important factors shaping the nest selection data were collected on trees that have suitable cavities but no parakeet nests. These trees were randomly selected from maps created for forest management plans (Asan et al., 2002).

Statistical Analyses

To determine the factors that have an impact on nest selection, we compared data from nests and non-nest trees. To test within and between species differences among nest height, tree height, nest orientation and tree diameter we used one-way ANOVA tests on SPSS Version 21.0 (IBM Corporations Released 2012).

Results

Breeding Distribution and Abundance

Two species were found sympatrically breeding in 5 out of 9 city parks (*Table 1*). In Beykoz Park, birds were observed in suitable habitat during the breeding season, however, no breeding behaviour or nest were recorded, this area is marked as potential breeding area.

Table 1. *Breeding status of Rose-ringed parakeet (Psittacula krameri) and Alexandrine parakeet (Psittacula eupatria) in studied parks. Possible: species were seen during the breeding season, but no proof for breeding, Certain: active nest(s) were found in the park, No: no proof for breeding or no suitable habitat*

Park	**Breeding Status**	
	P. eupatria	P. krameri
Beykoz Park	Possible	Possible
Büyük Çamlıca Park	No	No
Emirgan Park	Certain	Certain
Fethipaşa Park	No	No
Florya Park	Certain	Certain
Gülhane Park	Certain	Certain
Çubuklu Park	No	No
Küçük Çamlıca Park	Certain	Certain
Yıldız Park	Certain	Certain

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DOI: http://dx.doi.org/10.15666/aeer/1702_24612471

In all parks visited and in all visits, Alexandrine parakeet was present during the breeding season where Rose-ringed parakeet was absent in two parks; Büyük Çamlıca and Çubuklu Parks. During the breeding season, the maximum number of individuals recorded for each species are 57 for Alexandrine parakeet in Gülhane Park and 37 for Rose-ringed parakeet in Küçük Çamlıca Park.

A total of 38 nests were found in studied parks during one breeding season, of which 15 belong to Rose-ringed parakeet and 23 belong to Alexandrine parakeet. In parks where both species breed sympatrically, nests of two species found congregated in specific parts of the park rather than scattered away from each other species' nesting area.

Nest Characteristics

The majority of Alexandrine parakeet nests were found on *Platanus* spp trees (especially *Platanus acerifolia* Wild.) followed by *Pinus pinea* and *Celtis australis*. All nests found to belong to Alexandrine parakeet were natural cavities. Similarly, the majority of Rose-ringed parakeet nests were found on *Platanus* spp trees (especially *Platanus acerifolia* Wild.) followed by *Pinus pinea*, *Tilia argantea*, *Populus tremula*, and *Sophora japonica*. Rose-ringed parakeets were observed using cavities that are used by *Dendrocopus* or *Sitta* species in some areas. These cavities were found on *Quercus robur* trees. The relative numbers of nesting trees for both species were given in *Figure 4*.

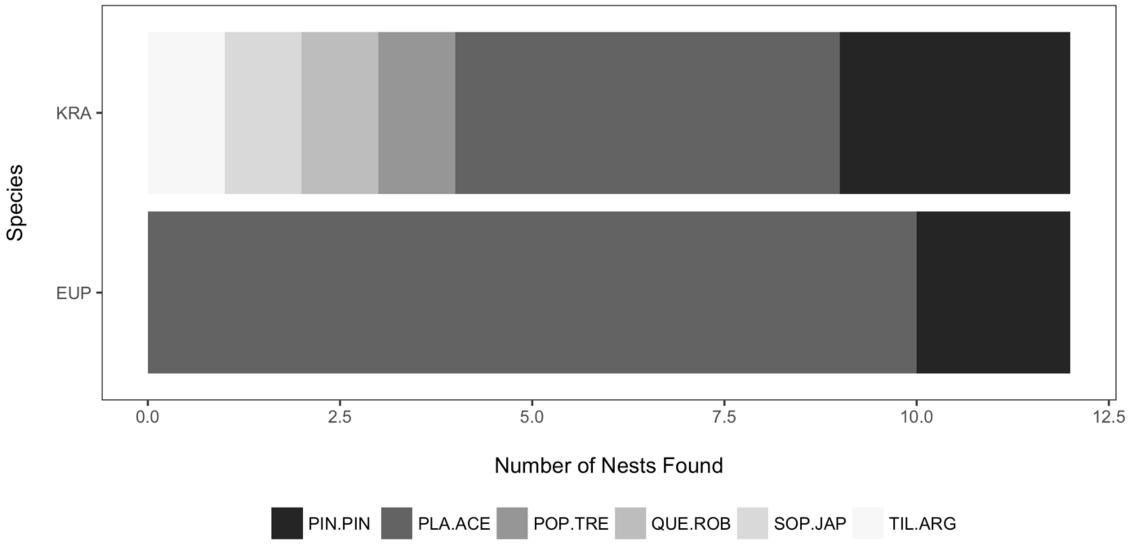

Figure 4. *Number and species of nesting trees for both parakeet species. KRA: Rose-ringed parakeet, EUP= Alexandrine parakeet, PIN.PIN: Pinus pinea, PLA.ACE: Platanus acerifolia, POP.TRE: Populus tremula, QUE.ROB: Quercus robur, SOP.JAP: Sophora japonica, TIL.ARG: Tilia argantea*

12 nests from each species were used in the analysis of comparing nest vs non-nest trees. Data from 24 non-nesting trees were used. For the 12 nests on which measurements were made, Alexandrine parakeet selected cavities placed higher on trees and trees with a wider diameter than Rose-ringed parakeet. The average height of Alexandrine parakeet nests was 8,6±3,6 m and the average tree diameter was 98,8 cm.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24612471

These values were 6,8±1,6 m and 64,58 cm respectively for Rose-ringed parakeet. According to test results, nests were higher and nesting trees had wider diameter than randomly selected non-nesting trees. These measurements were 4,5±2,1 m and 57,92 cm in non-nesting trees.

Comparing nest and non-nest tree characteristics for each species individually revealed that nest orientation (p=0,000; F= 59,668, df= 34) and breast height diameter (p= 0,001; F= 14,693; df= 34) is the important factors in selection of nests for Alexandrine parakeet whereas it is tree height (p= 0,000; F= 20,120; df= 34) and breast height diameter (p= 0,000; F= 99,965; df= 34) for Rose-ringed parakeet.

When both species' nest characteristics evaluated together against non-nesting trees, tree height is not a significant factor (*Figure 5A*) but nest heights were found significantly higher than cavities measured on non-nesting trees (p= 0,000; F= 24,528; df=47, *Figure 5B*). Nesting trees were significantly wider in breast height diameter than non-nesting trees (p= 0,000; F= 15,006; df=47, *Figure 5D*).

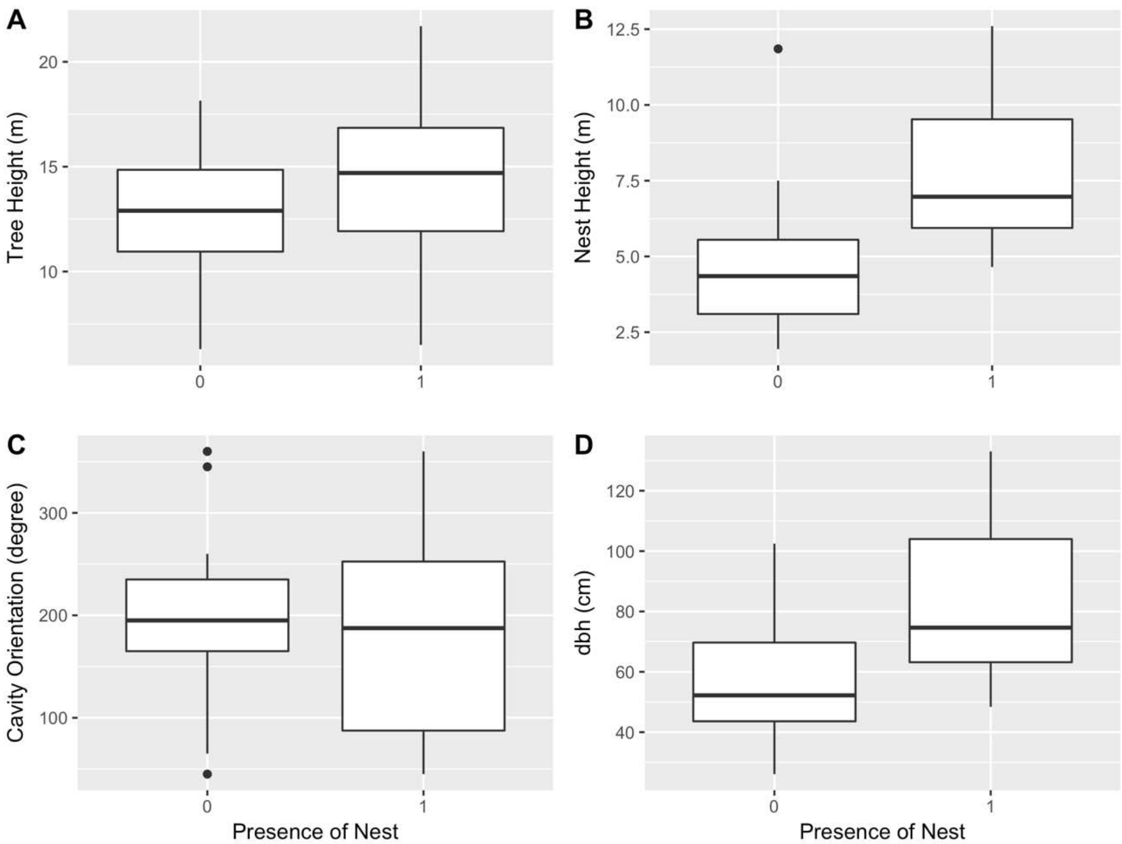

Figure 5. *Comparison of nesting (Presence of Nest =1, n=24) and non-nesting (Presence of Nest= 0, n=24) trees (95% CI). A: height of the tree, B: height of the cavity, C: orientation of the cavity, and D: diameter of the tree at breast height. Both species are evaluated together*

Discussion

This is the first systematic study on the two breeding parakeet species in Istanbul's city parks. The study covered large city parks. A preliminary work has been carried out to check the prevalence of the parakeet breeding sites in İstanbul. Accordingly, sighting data from an online database, e-bird (eBird, 2012), are mapped for both species and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24612471

stratified random selection was applied to the areas outside of large city parks. 6 randomly selected 2 x 2 km squares with different habitats were visited to record the breeding. Although parakeets were observed on old trees in alleys and in open areas no breeding has recorded in these squares. As Pithon (1998); Butler (2003); Strubbe and Matthysen (2011) and Czajka et al. (2011) suggests, European breeding populations of parakeets are concentrated in city parks where there is a high concentration of trees and at the same time anthropogenic activities. According to our preliminary study, breeding parakeets seem to show a similar selection for breeding habitat in Istanbul: both species were recorded breeding in 5 out of 9 city parks investigated in this study. The reason that parakeets are choosing city parks for breeding is probably related with the wide variety of tree species, including exotic ones, which serves as a rich source of food but also a safe breeding habitat relatively isolated from constant human disturbance. In a study carried out in a botanical garden, Claes and Matthysen (2005) found that exotic plant species constitute 36% of the diet of Rose-ringed parakeets. Strubbe and Matthysen (2011) found that male Rose-ringed parakeets, which are responsible for feeding breeding females have a foraging distance a few hundred meters away from the nests. City parks probably provide plenty of suitable nests and food source to both parakeet species during the breeding season.

Strubbe and Matthysen (2007) also found a positive correlation between breeding Rose-ringed parakeet numbers and cavity availability in its breeding areas. In Büyük Çamlıca Park where both species were absent, the majority of the area is covered with pine plantations and therefore does not provide a suitable habitat with many cavities and a wide variety of food resources. In Fethipaşa and Çubuklu Parks, there are less available cavities in comparison to other parks, which might be a reason for the absence of breeding.

In parks where breeding occurred *Platanus acerifolia* were the most preferred nesting tree by both species. Studies on Rose-ringed parakeets found that they most preferred *Platanus* spp. trees in the UK (Pithon, 1998) and Germany (Czajka et al., 2011). In Germany, Czajka et al., (2011) reported that for the Rose-ringed parakeet *Acer* and *Fraxinus* spp. are the most preferred nesting trees, following *Platanus* spp. Strubbe and Matthysen (2007) found that Rose-ringed parakeets prefer habitats that are dominated by *Fraxinus* spp. and that include *Populus* and *Ulmus* spp. in Belgium. In this study, no Rose-ringed parakeet nests were found on *Fraxinus* or *Acer* trees.

To the best of our knowledge, there is no study reporting nest characteristics of Alexandrine parakeet in its non-native range. In this study, we found that the average height of the studied Alexandrine parakeet nests was 8.6 m and average dbh was 98.8 cm. For Rose-ringed parakeet these values were 6.8 m and 64.6 cm respectively. According to Pithon (1998) the average height for Rose-ringed parakeet nests in the UK was 11.1 m and according to Butler (2003), it was 8.1 m. Choosing higher nests probably provides protection from potential predators by making it more difficult to access. In our study, we found that both species prefer higher cavities compared to suitable cavities on non-nesting trees. However, an explanation for the lower average height for Rose-ringed parakeet nests might be the competition between two species for nest cavities. As Alexandrine parakeet is larger than the Rose-ringed parakeet it might be possible that it is taking better cavities and pushing Rose-ringed parakeet to cavities with poorer conditions. This might also have revealed by the nest orientation data we collected. In our study, Alexandrine parakeet nest were mostly south oriented. Given the fact that prevailing winds in Istanbul are northerly, it is possible that Alexandrine

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24612471

parakeets are choosing nests with the south orientation to have a protection from the impact of northerly winds. This competition hypothesis requires a more detailed study.

Recent studies show that increasing parakeet populations are negatively impacting local species (Strubbe and Matthysen, 2009; Mori et al., 2017; Menchetti and Mori, 2014; Yosef et al., 2016). In our study, we observed that the Rose-ringed parakeets were interacting with Eurasian nuthatches *Sitta europaea*, Common starlings *Sturnus vulgaris*, Syrian woodpeckers *Dendrocopos syriacus*; and Alexandrine parakeets were interacting with Western jackdaws *Coloeus monedula*. Although these were only superficial observations, they still have a value in suggesting that competition for resources is probably occurring among invasive non-native parakeets and native species in Istanbul's large city parks. Furthermore, a third invasive bird species, Common myna *Acridotheres tristis* has established breeding grounds in a few of the parks investigated in this study (eBird, 2012). The presence of three invasive species might create complex interactions among invasive and native species (Orchan et al., 2013). This subject definitely requires further investigation. Furthermore, competition between two parakeet species might be affecting these interactions and creates a complex interaction among non-native and native species. These areas require detailed studies.

Conclusion

This is the first study that reports breeding status and nest-characteristics of Rose-ringed and Alexandrine parakeets, two invasive species in Istanbul's city parks and we found that both species breed sympatrically in all parks investigated. Our analyses demonstrated that both species prefer cavities placed higher and on wider trees in comparison to available but not occupied cavities. We could not detect a statistically significant difference between the nest characteristics of two species (not reported here), probably because of small sample size. Although this study was conducted in 2012, the presence and breeding status of both species has not changed in studied parks and moreover two species are seemingly increasing in abundance (eBird, 2012) with a third invasive bird species, Common myna, added to some of the parks. More detailed studies are required on the nest and habitat preferences with higher sample size, potential competition between two parakeet species and their impact on native bird fauna to better inform wildlife managers and protect urban native bird fauna.

Acknowledgements. This study was produced by Dilek Sahin's Master of Science Thesis.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24612471

COMPARATIVE STUDY OF METALS AND PHYTOCHEMICAL SCREENING IN AQUEOUS AND ACETONE EXTRACTS OF *CALOTROPIS PROCERA* AND *AJUGA BRACTEOSA*

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(Received 4th Oct 2018; accepted 16th Jan 2019)

Abstract. The main purpose of this study was to evaluate the antioxidant and phytochemical activities and metal contents from leaves, flowers and roots of regional Aak (*Calotropis procera* Ait.) and Hari Boti (*Ajuga Bracteosa* Wall.). Distilled water and acetone solvents were used to extract the antioxidants and phytochemical constituents. Diverse antioxidant and phytochemical activities such as flavonoid, phenolic, and flavonol contents, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), OH°, and H_2O_2 scavenging activities, Fe^{+2} chelating action, ferric reducing antioxidant power (FRAP), ascorbic acid and phosphomolybdenium complex essay were performed by using reported methods. Atomic absorption spectroscopy was used to evaluate the different metals such as Pb, Cd, Zn, Fe, Cu, Mg and K. The flavonoid contents (expressed as rutin equivalents) stated as 12.01-82.56 mg/g dry weight were higher than phenolic contents (expressed as gallic acid equivalents) quantified as 26.60-66.60 mg/g dry weight. *Ajuga bracteosa* extracts exhibited significant DPPH and OH radical scavenging activities. In all the analyzed samples, the highest concentration of iron (27.97 mg/L) was displayed by roots of *Ajuga Bracteosa.* Health risk index (HRI) revealed that level of Cd in roots and flowers of *Calotropis procera* and in flowers of *Ajuga bracteosa* was not safe for the consumption of humans as its concentration exceeded permissible limit.
Keywords: *ascorbic acid, Fe^{+2} chelating action, acetone, ferric reducing antioxidant power*

Introduction

Since old times, people are relying onto medicinal plants for their sicknesses. History for medicinal plants may be longstanding concerning illustration of mankind's historical backdrop. Since the mid of the 19th century diverse categories regarding bioactive compounds have been separated, characterized and identified. Among these, a number of compounds played a prime role in the discovery of new drugs. Many medicines manufactured using plant ingredients possess significant amount of alkaloids, tannins, flavonoids, and phenolic compounds, which play effective role in the cure of a variety of degenerative diseases (Ghias et al., 2012; Ali et al., 2015). Antioxidants help on chain softening responses and free radical searching action as well. All radicals have unpaired electrons and are profoundly breakable, also could extricate electrons starting with other particles to accomplish strength bringing on them harm. An over the top processing about reactive oxygen species (ROS) induces the oxidative stress thereby generating harm to DNA, proteins or lipids, and hampering their normal working (Pham-Huy et al., 2008). These biochemical alterations would involve in the aging process, and additionally in a developing rundown for mankind's diseases, for example, cancerous (Ziech et al., 2011), furthermore Alzheimer's disease (Galindo et al., 2010).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24732487

Cancer and cardiovascular diseases are considered main causes of death in United States of America (USA). Approximately, 32% deaths due to cancer could be evaded by nutritional adaptations. Extracts from curative herbs, vegetables, and fruits possess anticancer activity, akin to hormonal cure and chemotherapy (Wang et al., 2018). Oral doses of *Catharanthus roseus* flowers' aqueous extract to rats (diabetic) caused remarkable decrease in blood glucose, and decline in lipid profile. This oral administration also controlled the drop off in body weight (Suja, 2018). Fruits, vegetables and grains hold numerous widespread mixtures of phytochemicals. Antioxidants inferred from plants go about as diminishing operators, metal molecule chelators and also free radical scavengers. In the coming years, exceptional consideration are required to be paid towards exploring natural-based cell reinforcement extraction starting with plants such as, phenols, flavonoids and tocopherols (Wojdyłoa et al. 2007; Katalinic et al. 2006). Toxic metals accumulated in medicinal plants grown on contaminated soil might represent a danger to human wellbeing. Indeed purpose of ongoing research should generally be to lower the levels of contaminates. In national priorities list (NPL) of the substances which are established to cause the potential threat to human health because of recognized or alleged toxicity, arsenic ranks at first. Agency for toxic substances and disease registry (ATSDR) prioritizes substances in light of a mix about their toxicity, possibility of mankind's purposes of presentation and also recurrence. Lead, mercury, and cadmium rank at 2^{nd}, 3^{rd} and 7^{th} positions in the list of toxicants, separately (ATSDR, 2017). Mankind's interaction with metals might happen throughout world related activities principally through inward breath, dermal routes, mining and from water. Furthermore, contaminated food utilization and exposure to polluted air, soil and dust also enhance metal level in humans (Carlin et al. 2016). A thorough survey from claiming phytochemistry, medicinal and universal utilization of *Calotropis procera* (Aak) and *Ajuga bracteosa* (Kauri Boti) may be distributed by Ahmed et al. and Mubashir Hussain et al. separately (Ahmed et al. 2005; Mubashir Hussain et al. 2016). *Calotropis procera* had been used to treat large portions of infections such as ulcers, spoiling about skin, leprosy, piles, bronchial asthma, and illnesses of the liver, abdomen and spleen as well (Kartikar and Basu, 1994). *Ajuga bracteosa* is over 10-30 cm in stature also a prised, medicinal, soft, fragrant and villous herb. Furthermore, goes about similarly as antibacterial, anti-inflammatory, astringent, anthelmintic and antifungal. It can be used to cure intestinal infections, fever, phlegm, gout, palsy, amenorrhea and stiffness (Shen et al., 1993; Kaithwas et al., 2012). Different metals play a vital role in human body. Metals are characterized into two categories; fundamental or key metals and unnecessary or harmful metals. Key metals are copper, iron and Zinc, whereas nonessential or lethal metals are cadmium, chromium, lead and mercury. Key metals also become toxic when taken in high amount (Angelova et al., 2004). Intake of metals through intake of polluted therapeutic plants is related to human wellbeing dangers (Khan et al., 2007). Appraisals of wellbeing dangers to metals are as per the following; Fe is a basic element which limits many body capacities including supply of O_2 in the blood. Fe is also fundamental for giving vitality to the body. Praline hydrolase, ribonucleotide reductase, pyruvate oxidase, mitochondrial cytochrome, and tyrosine are the vital catalysts that entail the iron as a cofactor in the human body (Manore et al., 2009).

Copper assumes a central part in metabolism of energy, amalgamation and assurance of collagen protein. Proteins that oblige the Copper (II) particle as a co-variable in the body are superoxide dismutase and cytochrome-c-oxidase. Raised convergence of Cu in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24732487

human bodies causes many infections like gastrointestinal infection (Turkdogan et al., 2002). High accumulation of lead has troublesome health impacts such as respiratory and dermatogenic issues brought on by ingestion and dermal contacts of polluted soil (Cao et al., 2010; Wang et al., 2009). Abnormal amounts of lead cause hypertension, stomach related and apprehensive clutters, memory and focus issues, muscle and joint torment. Zinc is a critical mineral required for the body. Zinc controls many body functions, similar to resistant and stomach related framework, lessening of stress levels, vitality digestion and curing of wounds. Deficiency of zinc causes numerous infections such as low circulatory strain, development impediment and hindered bone improvement. Cadmium is not viewed as fundamental to human life. Unfriendly wellbeing impacts on individuals are displayed by overabundance cadmium introduction. From 20 to 30 years, cadmium is aggregated in the kidneys for a moderately extensive period and, at high measurements, harm respiratory framework and causes bone sickness. In the study zone these natural plants have been used to cure skin infections, fever, gout and asthma. The point of the available assessment may be to examine the cell reinforcement exercises, furthermore lethal levels for follow metals, additionally assess supplementary metals starting with chosen parts of *Ajuga bracteosa* and *Calotropis procera* developed in the region Bhimber AJK, Pakistan, eventually Tom's perusing utilizing different extraction solvents.

Methods and materials

Instruments/devices used

Double-beam UV-Visible Spectrophotometer, Model UV-1900, Shimadzo, AA-7000 Atomic Absorption Spectrophotometers, Shimadzo, and diamond saw blades (Cutter Diamond) were used in this study.

Reagents and chemicals

Folin-ciocalteu, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, hydrogen peroxide, L-ascorbic acid, and Rutin were purchased from Sigma Chemical Co. whereas H_2SO_4, H_3PO_4, Na_3PO_4, $HClO_4$, HNO_3, $(CH_3)_2CO$, Na_2CO_3, $NaNO_2$, $AlCl_3$, NaOH, CH_3COONa,), CH_3OH, $FeSO_4$, $C_6N_6FeK_3$, $C_2HCl_3O_2$, $FeCl_3$, HO_3P, $C_{12}H_7NCl_2O_2$, $(NH_4)_6Mo_7O_{24}$ were obtained from Merck (Germany).

Sampling area

The district Bhimber is situated at an altitude of 313 meters above sea level. It is located between latitude: 32-48 to 33-34 and longitude: 73-55 to 74-45, and has an area of 1516 km^2. People living in the rural and urban areas of district Bhimbr frequently use extracts of various parts of medicinal plants such as roots, leaves and flowers to cure ailments. Hence, the district was focused for this study (*Fig. 1*).

Sampling of diverse parts of plants

The study was aimed to detect phytochemicals and metals in medicinal plants collected from hilly areas of Bhimber Azad Jammu and Kashmir, Pakistan. Initially, a physical survey was carried out to select the sampling stations. Fresh flower, leaf and root samples (1-1.5 kg wet weight) of the targeted plants were collected separately in

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polythene bags from pre-decided locations during spring season of the year 2018. The samples were authenticated at the Department of Botany, Mirpur University of Science and Technology (MUST), Mirpur-10250 (AJK), Pakistan. The flowers and leafs were plucked with gloved hands whereas the diamond saw blades purchased from local market were used to cut and collect the root samples. Then, to remove soil particles, the collected plants' parts were washed carefully with tap water and distilled water respectively. The plants' parts were cut into small pieces and left to dry them out in mild sunlight for a number of days. The commercial beater was used to convert dried parts of the plants into coarse powder. The dried and powdered samples packed in pre-washed polythene bags were shifted to the lab for wet digestion and metal analysis.

***Figure 1.** Map of district Bhimber, the sampling area*

Digestion of plants' parts for metal analysis

Digestion protocol explained by Sharma et al. (2008) was followed. Various parts of the plant samples (powdered) were digested using the fixed ratio of concentrated H_2SO_4, HNO_3 and $HClO_4$. For this purpose, mixture of conc. sulphuric acid, perchloric acid and nitric acid (1:1:5) was utilised. One g of powdered plant sample taken in beaker was added 15 mL of acid mixture. The sample in beaker was heated for digestion at

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DOI: http://dx.doi.org/10.15666/aeer/1702_24732487

70 °C on hot plate in a fume hood. In order to avoid the evaporation of metal contents, the digestion process was carried out at slow rate. Near to dryness, 10 mL of acid mixture were added again and the temperature was increased slowly upto 90 °C to get around 5-8 mL of clear digested solution. Then, the cooled digested solution in the beaker was added 15 mL of nitric acid (0.05 N) and filtered by using Whatsman filter paper no. 41. The volume of the filtered digest was made upto 30 mL with 0.05 N nitric acid solution and stored at 4 °C in pre-washed, dried polythene bottles for metal analysis (Sharma et al., 2008).

Extraction of the plant samples for analysis of phytochemicals

Two-step extraction procedure was adopted for the investigation of antioxidant activity and phytochemical components. Water and acetone solvents were used in first and second steps for the extraction of hydrophilic and hydrophobic contents respectively.

For water extraction, 1 g powdered plant sample in 10 mL distilled water was centrifuged for 20 min at 6000 rpm and supernatant was shifted to a test tube. The same extraction practice was repeated thrice and each time upper layer was collected in the test tube. The solid residue left after the water extraction was further extracted three times using acetone (1:10 w/v) and the supernatant layer was saved in another test tube. Both the extracts containing phytochemical ingredients were stored at -10 °C to carry out further analysis in the department of Chemistr, Mirpur University of Science and Technology, Mirpur, which is situated at Pakistan country in the cities place category with the GPS coordinates of 73° 45’ 6.3720” E and 33° 8’ 54.2112” N.

Investigation of phenolic contents

The investigation of phenolic contents in water and $(CH_3)_2CO$ concentrates was finished with the assistance of the strategy depicted by Jing et al. (2015). Above all, arranged the ten time’s diluted Folin-ciocalteu by including one mL Folin-ciocalteu, finished the level up to ten mL with distilled H_2O. Around 1 mL of H_2O and $(CH_3)_2CO$ concentrate was mixed with 5 mL of diluted Folin-ciocalteu and 4 mL of 7.5% Na_2CO_3. Before measuring spectrophotometrically the absorbance at 760 nm, left the blend at room temperature to 90 min.

Investigation of flavonoids contents

Recognition of flavonoids was finished with the assistance of strategy altered by Jing et al. (2015). Around 5 mL of H_2O or $(CH_3)_2CO$ concentrates were added into 0.3 mL of 5% $NaNO_2$ for 5 min, and then included 0.3 mL of 10% $AlCl_3$ in the certain blend. Two mL of 1 M NaOH was included the blend to discontinue the response after six min. The surrendered blend was diluted to 10 mL and calculated the absorbance quickly at 510 nm. Rutin was utilized as standard and flavonoids were explained as rutin counterparts.

Investigation of flavonols

Flavonols in the samples were recognized by utilizing the strategy set by Kumaran and Karunakaran (2006). Around 2 mL of H_2O or $(CH_3)_2CO$ concentrate mixed with 2 mL of (50 g/L) CH_3COONa and 2mL of 2% aluminium chloride in the concentrate.

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The blend was permitted to left for 2.5 h and then measured the absorbance at 440 nm. Rutin was utilized as a standard for the detection of flavonols using calibration curve equation y = 0.0007x + 0.3591 (x = concentration of rutin, y = absorbance).

Scavenging activity of DPPH

Scavenging action of DPPH (2, 2-diphenyl-1-picrylhydrazyl) was documented by the strategy portrayed by Yu et al. (2002) and Aoshima et al. (2004). Around 1 mL of the specimen concentrate was added in 2.5 mL of DPPH (0.1 mM in CH_3OH) and held the blend in darkness for 30 min. At 517 nm, vanishing of the shade of DPPH was measured against the clear. The outcomes were measured as *Equation 1:*

$$\% \text{ inhibition} = \frac{\text{A Blank} - \text{A Sample}}{\text{A Blank}} \times 100 \quad \text{(Eq.1)}$$

Scavenging activity of OH°

The scavenging activity of OH° of concentrates of plants in H_2O and $(CH_3)_2CO$ was portrayed by Yu et al. (2004). Around 3 mL of H_2O or $(CH_3)_2CO$ concentrate was mixed in 1 mL of $C_{12}H_8N_2$ (0.04 M), 2 mL phosphate buffer (0.2 M), and 0.04 L of $FeSO_4$ (0.02 M). The reaction was begun by the addition of 0.1 mL of 7.0 mM hydrogen peroxide in the blend, and then measured absorbance at 560 nm. The scavenging activity was evaluated as expressed in *Equation 2:*

$$\text{Scavenging activity} = \frac{\text{A Blank} - \text{A Sample}}{\text{A Blank}} \times 100 \quad \text{(Eq.2)}$$

Scavenging activity of hydrogen peroxide

Aiyegoro and Okoh (2010) reported the method for the measurement of scavenging activity of H_2O_2. The 2.4 mL of hydrogen peroxide solution (4 mM) prepared in 0.1 M phosphate buffer (pH 7.4) was blended with 4 mL extract and incubated for 10 min at room temperature, then measured absorbance at 230 nm against the blank (Reimann and de Caritat, 2005)**.** Scavenging activity was calculated as *Equation 3:*

$$\text{Scavenging activity} = \frac{\text{A Blank} - \text{A Sample}}{\text{A Blank}} \times 100 \quad \text{(Eq.3)}$$

Chelating action of Fe^{+2}

The chelating capacity of Fe^{+2} ions was evaluated via the technique for Dinis et al. (1994). Around 2 mL $FeSO_4$ (0.125 mM) was mixed with 2 mL of water or $CH_3)_2CO$ extracts. Two mL ferrozine (0.3125 mM) was added into the blend to start the reaction and left the blend for ten min. The absorbance was measured at 562 nm against the blank. The accompanying equation was utilized to ascertain chelating action (*Eq. 4*):

$$\text{Chelating Activity} = \frac{(\text{A control} - \text{A Sample})}{(\text{A control})} \times 100 \quad \text{(Eq.4)}$$

Ferric reducing antioxidant power (FRAP)

Hazara et al. described the method for the detection of ferric lessening cancer prevention agent control (Hazara et al., 2008). Around 2 mL of concentrate was blended

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with the two mL phosphate buffer (0.2 M, pH 6.6) and two mL of 0.1% $C_6N_6FeK_3$ and held the mixture for twenty min at 50 °C. Subsequent to twenty min, the reaction was clogged by two mL $C_2HCl_3O_2$ (10%). The supernants were blended with 2 mL of $FeCl_3$ and 2 mL of H_2O and permitted to remain for 20 min and afterward absorbance was measured at 700 nm aligned with the clear. Ascorbic corrosive was utilized as positive control.

Ascorbic acid determination

Ascorbic corrosive substances were controlled by the strategy as expressed by Klein and Perry (1982). Dry water extract was re-extricated for HO_3P (1%, 10 mL) to 45 min and then filtered. 1 mL filtrate was blended in 9 mL $C_{12}H_7NCl_2O_2$ (0.8 g/1000 mL) and then measured absorbance inside thirty min at 515 nm. Ascorbic substances computed from L-ascorbic acid bend (0. 006-0.1 mg/mL; y = 3. 006x + 0. 007; R^2 = 0. 999), and results were communicated as ascorbic corrosive equivalents.

Phosomolybdenium complex assay

Prieto et al. described the method to measure Phosomolybdenium complex of the extracts spectrophotometrically (Prieto et al., 2006). Quickly 2 mL of sample in H_2O or $CH_3)_2CO$ was mixed in 6.6 mL claiming reagent (28 mol/L Na_3PO_4, 0.6 $molL^{-1}$ sulphuric acid and 4 $molL^{-1}$ $(NH_4)_6Mo_7O_{24}$ topped the mixture and incubated at 95 °C for 90 min. After cooling, the absorbance was measured at 695 nm alongside reasonable holding one mL of reagent and also one mL H_2O rather than extracts and after that subjected to the same exploratory conditions. The outcomes from three examinations, each kept running in triplicate, were communicated as the mean of relative antioxidant action (RAA) compared with that of vitamin-C.

Daily intake of metals (DIM)

Information regarding the daily ingestion of therapeutic plants was gathered during the study. DIM was computed as *Equation 5:*

$$\text{DIM} = \frac{\text{Cmetal} \times \text{CFactor} \times \text{Cfood intake}}{\text{Baverage weight}} \quad \text{(Eq.5)}$$

C_{metal} stands for metal contents in selected samples, C_{Factor} depicts the conversion factor (value is 0.083), $C_{food\ intake}$ utilization of therapeutic plants which is 100 mg per individual per day and $B_{average\ weight}$ corresponds to the normal body weight within review range which is 50 kg.

Health risk index (HRI)

$$\text{HRI} = \text{DIM} / \text{RfD} \quad \text{(Eq.6)}$$

RfD characterizes the reference oral measurement. The value of health risk index less than one represents the protected mode for nearby populace through intake of plants and the other way around (*Eq. 6*).

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Results and discussion

Flavonoids, flavonols and total phenol contents of the extracts

It has been identified that flavonoids show cell reinforcement endeavour and their outcomes on ethnical nourishment then wellbeing are impressive. Several recent studies have confirmed that phytochemicals including alkaloids, flavonoids, flavonols, phenols, terpenoids and steroids have colossal antioxidant and free radical scavenging activities. Plant extracts rich in polyphenols and essential phytoconstituents have been shown to display persuasive antioxidant and free radical scavenging activities in diverse antioxidant models (Farhan et al., 2012; Amari et al., 2014).

The flavonoids, flavonols and phenols are important as their hydroxyl groups are accountable for antioxidant effects in plants. Highest flavonoid contents were shown by water extract of leaves of *Ajuga bracteosa* (81.56 mg/g) and least contents (12.01 mg/g) were present in roots of *Calotropis procera* (*Table 1*). Leaves and flowers of *Calotropis procera* showed highest flavonol contents in water and acetone extracts respectively (98.98 mg/g, 60.27 mg/g). Least flavonol contents were present in water and acetone extracts of roots of *Ajuga bracteosa* (82.71 mg/g). Phenolic substance are measured as far as gallic equivalents having highest contents in leaves of *Ajuga bracteosa* (66.60 mg/g) followed by leaves of *Calotropis procera* (*Table 1*).

Table 1. *Phytochemical constituents in leaves, flowers and roots of Ajuga bracteosa and Calotropis procera*

Botanical name of therapeutic plants	Part of the plant taken	Local name	Total phenolic contents (mg/g)		Flavonoid contents (mg/g)		Flavonol contents (mg/g)	
			Water	Acetone	Water	Acetone	Water	Acetone
Ajuga bracteosa	Leaves	Hari Boti	66.60	56.10	81.56	40.77	20.48	15.44
Ajuga bracteosa	Flowers	Hari Boti	26.60	24.95	30.21	28.84	47.81	25.74
Ajuga bracteosa	Roots	Hari Boti	60.43	53.63	43.54	31.41	82.71	24.75
Calotropis procera	Flowers	Aak	59.67	21.26	32.62	26.72	60.27	16.83
Calotropis procera	Leaves	Aak	60.6 7	41.20	82.56	14.10	98.98	35.73
Calotropis procera	Roots	Aak	43.17	11.25	12.01	12.54	42.62	23.61

Antioxidant activity

The antioxidants from the leaves, roots and flowers of *Ajuga bracteosa* and *Calotropis procera extracted* in water and acetone are shown in *Table 2*.

The free radical scavenging activity of both hydrophilic and lipophilic antioxidants is estimated by the DPPH radical that is a stable free radical, and has been widely used as a sensitive and rapid tool. DPPH interact with antioxidants that neutralize the free radicals by transferring electrons or hydrogen atoms to DPPH (Archana et al., 2005). Watery extract of flowers of *Ajuga bracteosa* showed highest DPPH scavenging activity (97%) over roots and leaves (96%, 94%). The OH radical has been considered as a highly damaging ROS in free radical pathology, has ability to damage almost every molecule in living cells. The hydroxyl radical scavenging capacity of therapeutic plants is directly related to their antioxidant activity (Uttara et al., 2009). Roots of *Ajuga bracteosa* and leaves of *Calotropis procera* gave the same OH radical and hydrogen peroxide scavenging activity respectively in aqueous extracts (77%), while acetone

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extracts gave the poor OH radical and hydrogen peroxide scavenging activities. The higher antioxidant yield from leaves and blooms of Ajuga bracteosa and Calotropis procera with water solvent prominently indicate viability of this solvent towards antioxidant components from these materials.

Table 2. *Antioxidant activities in leaves, flowers and roots of Ajuga bracteosa and Calotropis procera*

Botanical name of therapeutic plants	Part of the plant taken	Local name	DPPH scavenging activity (%)		OH radical scavenging activity (%)		Hydrogen peroxide radical scavenging activity (%)	
			Water	Acetone	Water	Acetone	Water	Acetone
Ajuga bracteosa	Leaves	Hari Boti	94	81	30	48	23	02
Ajuga bracteosa	Flowers	Hari Boti	57	47	57	15	21	13
Ajuga bracteosa	Roots	Hari Boti	96	88	77	16	40	04
Calotropis procera	Flowers	Aak	94	77	75	14	78	16
Calotropis procera	Leaves	Aak	89	87	59	8	77	51
Calotropis procera	Roots	Aak	73	55	42	06	76	23

Metal chelating action

The chelating of Fe^{+2} by different plant extracts is evaluated via the method utilized by Dinis et al. (1994). Different studies revealed that the reducing power of biologically active compounds relative to their antioxidant action is reflected by the electron donation capability. Antioxidants are reducing agents, and inactivation of oxidants by reductants can be described as a reduction–oxidation (redox) reaction, in which one reaction species is reduced at the expense of the oxidation of the other (Gulcin et al., 2010). In the reducing power assay, the presence of antioxidants in the therapeutic plants would cause the reduction of Fe_3^+ to Fe_2^+ by donating the electron. Aqueous extract of leaves of *Ajuga bracteosa* was the most active extract interfered with the formation of ferrous and ferrozine complex, that is connected with redox metal catalysis incorporates chelating in regards to the metal particles before ferrozine followed by chelating activity of roots and flowers of *Ajuga bracteosa,* then, leaves of *Calotropis procera*. Acetone extracts showed less chelating activity towards Fe^{+2} (*Table 3*).

Table 3. *Fe^{+2} chelating activity and FRAP activity in leaves, flowers and roots of Ajuga bracteosa and Calotropis procera*

Botanical name of therapeutic plants	Part of the plant taken	Local name	Fe^{+2} chelating activity (%)		FRAP activity (%)	
			Water	Acetone	Water	Acetone
Ajuga bracteosa	Leaves	Hari Boti	67	10	68	43
Ajuga bracteosa	Flowers	Hari Boti	33	10	71	50
Ajuga bracteosa	Roots	Hari Boti	56	68	82	62
Calotropis procera	Flowers	Aak	17	21	78	82
Calotropis procera	Leaves	Aak	46	34	69	75
Calotropis procera	Roots	Aak	43	11	32	26

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DOI: http://dx.doi.org/10.15666/aeer/1702_24732487

Table 3 showed the ferric reducing antioxidant power (FRAP) activity in different parts of *Ajuga bracteosa* and *Calotropis procera* in aqueous and acetone extracts. Acetone extracts of *Calotropis procera* showed highest FRAP activity followed by water extracts of *Ajuga bracteosa*.

Phosomolybdenium complex assay and ascorbic acid determination

Phosphomolybdenium assay was expressed as mg AAE/100 g (*Table 4*).

Roots of *Ajuga bracteosa* and flowers of *Calotropis procera* showed significantly higher values in aqueous extract (212.9 mg/g, 207.65 mg/g) respectively. Aqueous extract of flowers of *Ajuga bracteosa* showed the second higher contents (100 mg/g). Lower contents were observed in acetone extracts of flowers of *Ajuga bracteosa* and leaves of *Calotropis procera* (6.0 mg/g, 6.65 mg/g). Significantly differences in ascorbic acid contents among different parts of two plants were recorded (*Table 4*). Flowers of *Ajuga bracteosa* and *Calotropis procera* had the highest and same ascorbic acid contents (0.469 mg/g) followed by roots of *Ajuga bracteosa* (0.379 mg/g). Lower contents were observed in roots of *Calotropis procera* (0.121 mg/g).

Table 4. *Phosomolybdenium complex assay and ascorbic acid contents in leaves, flowers and roots of Ajuga bracteosa and Calotropis procera*

Botanical name of therapeutic plants	Part of the plant taken	Local name	Phosomolybdenium complex assay (mg/g)		Ascorbic acid determination (mg/g)
			Water	Acetone	
Ajuga bracteosa	Leaves	Hari Boti	64.4	38.65	0.261
Ajuga bracteosa	Flowers	Hari Boti	100.15	6.0	0.469
Ajuga bracteosa	Roots	Hari Boti	212.9	13.9	0.379
Calotropis procera	Flowers	Aak	207.65	15.90	0.469
Calotropis procera	Leaves	Aak	38.90	6.65	0.261
Calotropis procera	Roots	Aak	47.20	21.50	0.121

Distribution of metals

Sources of different metals in soil, plants and water are anthropogenic activities and furthermore from parent materials; however it is hard to assess the ordinary foundation groupings of metals in soil, water and plants (Reimann and de Caritat, 2005). Thus it is surveyed that centralization of metals contains both a trademark geochemical partition and anthropogenic exercises (Acosta et al., 2010). The distribution of metals among selected plants is represented in *Table 5*. Except lead, all metals are present in considerable concentrations. Highest mean level is shown by iron followed by zinc. The decreasing order of concentration of metals is as follows; Fe > Zn > Cu > Ni > Cd > Pb. The relationship study was likewise done to evaluate the shared varieties of chose metals in selected plants.

The correlation coefficients of selected metals in medicinal plants are shown in *Table 6*. Compact disc Fe (r = 0.756) and Fe-Cu (r = 0.633) depicted the positive association. Be that as it may, exceptionally solid positive connection was appeared by Pb-Ni (r = 0.839). Strongest connection was appeared by Zn-Fe (r = 0.869) and Zn-Cd (r = 0.851), showing their conceivable basic starting point.

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Table 5. *Concentration (mg/kg) of metal contents in extracts of leaves, flowers and roots of selected medicinal plants (n = 3)*

Samples	Pb	Ni	Zn	Cd	Fe	Cu
Ajuga bracteosa, leaves	BDL	0.092± 0.005	0.449± 0.010	0.104± 0.011	8.678± 0.001	0.084± 0.055
Ajuga bracteosa, flowers	BDL	0.177± 0.035	0.500± 0.013	0.100± 0.051	12.83± 0.025	0.106± 0.012
Ajuga bracteosa, roots	BDL	0.113± 0.031	0.608± 0.004	0.105± 0.005	27.97± 0. 105	0.279± 0.051
Calotropis procera, leaves	0.015± 0.001	0.171± 0. 105	0.277± 0. 501	0.096± 0.012	6.910± 0.001	0.122± 0.001
Calotropis procera flowers	BDL	0.122± 0.010	0.307± 0.050	0.094± 0.016	4.045± 0.018	0.164± 0.005
Calotropis procera roots	BDL	0.136± 0.016	0.421± 0.007	0.097± 0.009	7.281± 0. 105	0.214± 0.004

BDL stands for below detection limit

Table 6. *Correlation co-efficient of selected metals in therapeutic plants*

	Pb	Ni	Zn	Cd	Fe
Ni	**0.839**				
Zn	-0.308	-0.279			
Cd	-0.402	-0.471	**0.851**		
Fe	-0.208	-0.178	**0.869**	**0.756**	
Cu	-0.109	-0.243	0.459	0.160	**0.633**

P ≤ 0.05 Significant

Cluster investigation of metals was done so as to study the multivariate seizure as appeared as dandrogram in *Figure 2*. Cluster examination gives the huge information on the premise of comparable qualities. Three clusters were observed for selected metals. First cluster exhibited close association of metals (Zn, Fe, and Cd). Cluster of Cu showed mutual relationship with first cluster depicted that the metal concentrations vary because of basic components of the soil minerals and they have lithogenic cause. Third Cluster was appeared by Ni and Pb which also entangled with other clusters revealed that this association may be contributed by the horticultural exercises and additionally dry statement of the suspended particulates.

The quartile circulation of metals in therapeutic plants is shown in *Figure 2*. Symmetric distribution was shown by essential metal i.e. iron. Narrow distribution was shown by Ni, Pb, Cu and Cd. on the ability about dirt towards a multivariable-based cation profession get ready fundamentally liable to physicochemical conditions, for example, pH, temperature and the propinquity of different particles within the dirt structure. Description of health risk assessment is represented in *Table 7*. Health risk index revealed that some metals have safe level in selected plants so that some parts of selected therapeutic plants can be used as drug development and as a supplement to human body. Higher levels of cadmium and iron are investigated by consuming different parts of selected plants.

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DOI: http://dx.doi.org/10.15666/aeer/1702_24732487

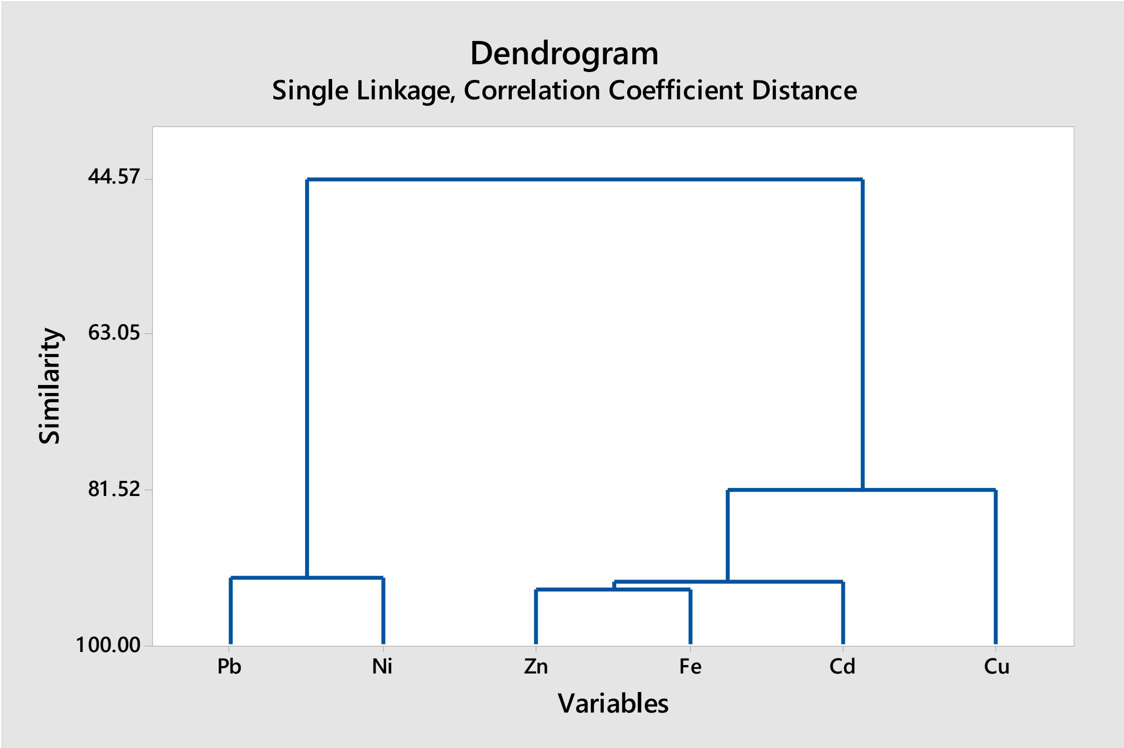

***Figure** 2. Cluster investigation of metals*

***Table** 7. Description of health risk assessment of metals in selected parts of the plants*

RfD	**DIM (mg/day)**						**HRI**					
Plants and their parts	***Ajuga bracteosa***			***Calotropis procera***			***Ajuga bracteosa***			***Calotropis procera***		
	L	**R**	**F**	**L**	**R**	**F**	**L**	**R**	**F**	**L**	**R**	**F**
Cu^b 0.040	0.014	0.046	0.018	0.020	0.055	0.027	0.35	1.15	0.43	0.50	0.88	0.68
Ni^b 0.020	0.015	0.018	0.029	0.028	0.023	0.202	0.75	0.90	1.47	1.40	1.13	1.01
Cd^b 0.001	0.012	0.017	0.017	0.016	0.016	0.016	12.4	1.74	**16.6**	0.002	**16**	**15.6**
Zn^b 0.300	0.075	0.100	0.075	0.046	0.070	0.051	0.25	0.33	0.25	0.153	0.94	0.17
Pb^b 0.0036	BDL	BDL	BDL	0.002	BDL	BDL	BDL	BDL	BDL	0.555	BDL	BDL
Fe^b 0.700	1.441	**4.64**	2.13	1.147	1.208	0.671	2.06	**6.63**	3.04	1.63	1.73	0.96

[b]USEPA, 2011, L = leaves, R = roots, F = flowers

Conclusion

This study gives an evaluation that chosen plants can be used as cancer prevention agents. Antioxidant properties and phytochemical contents varied among the water and acetone extracts of selected plants. Among these plants, *Ajuga bracteosa* extricate indicated extremely high antioxidant properties and elevated aggregate phenolic substance. It is demonstrated that flavonoid contents are the significant supporter of the cancer prevention agent properties of these plant extracts. The HRI indicates higher levels of cadmium present in roots and flowers of *Calotropis procera* and also in flowers of *Ajuga bracteosa.* The results also indicate that roots of *Calotropis procera* and flowers of both *Calotropis procera* and *Ajuga bracteosa* also exhibit less

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phytochemical constituents and antioxidant activity. Based upon the study outcomes, it is proposed that the users should avoid taking roots and flowers of *Calotropis procera* and flowers of *Ajuga bracteosa* to cure ailment. Further study is recommended to find and isolate the potential antioxidants to be used in the drug development

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ESTIMATION OF CARBON EMISSION IN THE EX-MEGA RICE PROJECT, INDONESIA BASED ON SAR SATELLITE IMAGES

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(Received 1st Nov 2018; accepted 28th Jan 2019)

Abstract. This paper aims to estimate the extent of the burned area in ex-Mega Rice Project (MRP) (Block A and Block E), Central Kalimantan and quantify the carbon emission as a result of peat fires in this area. A Synthetic Aperture Radar TerraSAR-X image was the main input in this study. Pre-processing included radiometric calibration, geometric reprojection and speckle filtering (Gamma MAP 7 × 7 kernel size). Maximum Likelihood (ML) classification was used for classifying burned areas. The classification result was validated based on data from the Kalimantan Forests and Climate Partnership report (KFCP). The burned area in 2010 as estimated by the TerraSAR-X satellite image HH/HV polarization was 47.6113 km^2 (4761.13 ha). Main pollutants (carbon dioxide (CO_2), carbon monoxide (CO), particulate matter (PM), black carbon (BC) and organic carbon (OC)) emissions were estimated based on an empirical equation. The average annual carbon releases into the atmosphere from this area were estimated to be 173,019.46 ton CO_2, 11,388.62 ton CO, 1,149.81 ton PM_{10}, 996.50 ton $PM_{2.5}$ 569.43 ton OC and 72.27 ton BC. This study suggests that the methodology proposed based on Earth Observation data can be used to locate burned areas in tropical environment and quantify carbon emissions.
Keywords: *burned area, emission factor, earth observation, forest fire, Ex-mega Rice Project, TerraSAR-X, dual polarization*

Introduction

Peatlands are wetland ecosystems frequently encountered in Southeast Asia. They play a vital role in the biosphere's biogeochemical processes (Immirzi, 1992). At a global scale, they hold an important ecological function as they represent a major store of soil carbon, sink for carbon dioxide and source for atmospheric methane (Strack, 2011); at a local scale they host habitats for several rare and endangered species (Posa, 2011). Page et al. (2011) estimated the volume of tropical peat to be 1758 Gm^3 (~18–25% of global peat volume) with 1359 Gm^3 located in Southeast Asia (77% of all tropical peat). During the last decades, the area of tropical peat swamps has been reduced and degraded due to deforestation, drainage, fires, clearing for agriculture and oil palm plantations and illegal timber harvest (Peace Report, 2018; Koh et al., 2009). From all these causes of environmental degradation, fires are a unique one that can be

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24892499

attributed partially to anthropogenic interference, however sometimes they can occur naturally; for instance, it has been demonstrated that fires have been observed during El Niño events as a direct consequence of the extensive drought incurred from the latter (e.g. in 1997, 2002, 2004, 2006 and 2009) (Ballhorn et al., 2009; Putra et al., 2008). Therefore, monitoring of tropical peatlands is crucially important for inventorying the magnitude of carbon stock and estimating the pollutants emitted to the atmosphere.

Deforestation has been a major cause of peatland disturbance; in 2005, 25% of all deforestation in SE Asia was on peatlands according to Rieley et al. (2008). Within Southeast Asia, Indonesia is the country with the largest share of tropical peat carbon stock estimated at 57.4 Gt (Page et al., 2011). In 1996, the Indonesian government initiated the Mega Rice Project (MRP) in the southern parts of Central Kalimantan. The aim of this project was to convert one million hectares of peat swamp forest to rice paddies. Consequently, forest cover decreased from 64.8% in 1991 to 45.7% in 2000, and clearance has continued since then. The MRP project led to frequently large fire occurrences during dry seasons. Approximately 4,740 km^2 in MRP have been damaged from severe fire episodes in 1997 and released 0.19 – 0.23 Gt of carbon to the atmosphere. This huge amount of carbon suggests that severe peat fires occurred in the MRP area (Putra et al., 2008).

Chemical species have long been detected and quantified directly from specialized satellite instruments such as the GOME instrument onboard ERS-2, the MODIS instruments onboard TERRA and AQUA satellites and the SCIAMACHY instrument onboard ENVISAT. Streets et al. (2013) provide a review on emission estimates from satellite retrievals. Another indirect approach to estimate gas emissions is to spatially quantify the phenomenon generating the emissions and then estimate the latter based on empirical relationships or models. This indirect approach is of special interest in land cover applications since Earth Observation has advanced significantly in estimating the spatial extent of deforestation activities and subsequently the calculation of environmental parameters (e.g. Kim et al., 2015; Zeng et al., 2018; Broich et al., 2017; Krasovskii et al., 2018; Joshi et al., 2015; Fawzi et al., 2018; Nugroho et al., 2018; Grinand et al., 2013). For instance, Carlson et al. (2013) estimated high-resolution carbon flux estimates from Kalimantan oil palm plantations based on land cover classification from Landsat data (at spatial resolution of 30 m). In the context of forest fires, an immediate consequence is forest loss and the associated outcome is the pollution emissions during the combustion (Page et al., 2002; Hooijer et al., 2010). Forest fires result to emission into the atmosphere of particulate matter (PM_{10}, $PM_{2.5}$), particulate black carbon (BC), organic carbon (OC) and gaseous emissions (carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides, non-methane volatile organic compounds (NMVOC), methane (CH_4), nitrous oxide (N_2O), sulfur dioxide (SO_2) and ammonia (NH_3)) (Shrestha et al., 2013). CH_4 and N_2O emissions from tropical peatlands are low (Strack, 2008). On a global scale, fire impacts the net carbon balance of global terrestrial ecosystems; this impact has been quantitatively assessed by Li et al. (2014). Recent research from Junpen et al. (2018) suggested that emissions from biomass open burning can be estimated based on burned area products derived from satellite data. In a similar context, Hawbaker et al. (2016) mapped the extent of burned area in the Southern Rocky Mountains, USA, subsequently use the LANDFIRE Fuel Characteristic Classification System to assign biomass loads and finally estimate biomass consumption and greenhouse gas emissions based on the CONSUME model. Several other studies have quantified burned area based on satellite data (e.g. Junpen et al.,

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2013; Permadi and Oanh; 2013). A critical aspect in such methodological approach is the spatial resolution of the satellite image which plays an important role in the accuracy of the burned area estimation and subsequently the estimation of pollutants emissions (Belenguer-Plomer et al., 2018; Yun and Pritchard, 2018). For a review of the strengths and limitations of remote sensing in estimating tropical forest biomass, the reader is directed to Mitchard (2015) and Saatchi (2015).

The aim of this paper is to assess the burned area based on fine spatial resolution Synthetic Aperture Radar (SAR) satellite images and field data, and consequently quantify the carbon emissions through empirical relationships that occurred from peat fires in blocks A and E of the MRP.

Materials and methods

Study area

Central Kalimantan, Indonesia is the second largest province of the island of Kalimantan and the third largest province of the country, covering 153,800 km^2. The climate is tropical with a significant amount of rainfall; the drier season usually begins in May and lasts until October while the wetter season covers the period between November and April. The temperature fluctuates between 25-27 °C and the rainfall ranges from 2,776 to 3,393 mm per year with an average of 145 rainy days annually (Moore et al., 2011). The central area of Central Kalimantan is covered by tropical forests while the southern area is swampy with an extensive river network. Peat swamp forest areas in Indonesia are primarily located at low altitudes in the coastal and sub-coastal lowlands of Papau (4.6 Mha), Sumatra (8.3 Mha) and Kalimantan (6.8 Mha) (Page et al., 1999); central Kalimantan contains one of the largest tropical peatland areas in the world. *Figure 1* presents the study area which is part of the MRP.

Satellite data

A SAR image was used in this study as the main input. TerraSAR-X is a high spatial resolution X-band SAR active system operating at 9.65 GHz centre frequency. The satellite was launched in 2007 and provides single, dual or full polarization data at a spatial resolution between 1 m and 16 m depending on the mode of acquisition. A TerraSAR-X Single Look Slant Range Complex (SSC) product in the DLR-defined COSAR binary format and the associated Multi Look Ground Range Detected (MGD) product in GeoTiff format were provided by ASTRIUM GEO-Information. The MGD product is a multi-look product with reduced speckle and approximately square ground resolution cells. The image was acquired in dual polarization (HH/HV) StripMap imaging mode on 05/04/2010 with a 6.6 m spatial resolution and 37.489 incidence angle. The image coordinates are oriented along the flight direction and the ground range. A simple polynomial projection was performed in range to an ellipsoid in order to achieve approximately quadratic pixels. The advantage of this product is the fact that no image rotation to a map coordinate system has been performed and interpolation artefacts are thus avoided.

Satellite image processing

The TerraSAR-X image was preprocessed by applying sequentially radiometric calibration, geometric reprojection and speckle filtering. An absolute radiometric calibration of Sigma Naught (σ0) was performed providing radar reflectivity per unit area at ground range. The simplified approach was followed where Noise Equivalent Beta Naught (NEBN) was ignored. The image was then reprojected onto the Geographic Lat/Lon (WGS 84) Coordinate Reference System (CRS) using the nearest neighbour resampling method. Finally the Gamma MAP filter with 7 × 7 kernel window was applied to reduce the speckle noise of the image as it has been shown to provide the best results in a tropical environment (Nuthammachot et al., 2017).

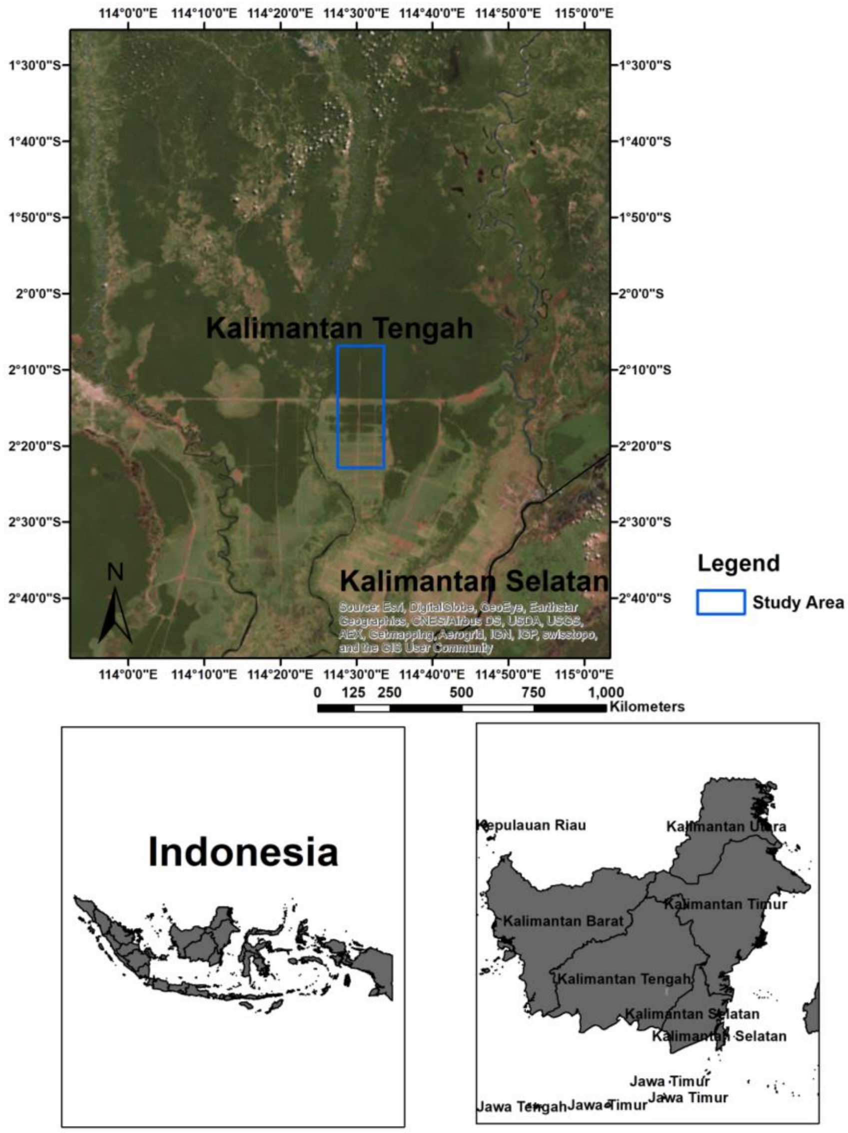

Figure 1. *A true-colour satellite image representation of the study area in Central Kalimantan, Indonesia*

For the detection of the burn areas the Maximum Likelihood (ML) algorithm was used to classify pixels corresponding to burned land as it is one of the most traditional classification algorithms developed, yet has been proven very powerful in a variety of remote sensing challenges. The thematic map produced indicating the burned area as estimated from TerraSAR-X dual polarization (HH/HV) was validated with the field

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24892499

data published in the Kalimantan Forests and Climate Partnership (KFCP) report (KFCP Vegetation Monitoring, 2014). The results of the validation were presented with an error matrix (contingency table) as suggested by Congalton (1991).

Emission estimation

The burned area calculated from the SAR data was used as an input to estimate the emission pollutants in this study area. Particulate matter and trace gas emissions from forest fires can be approximated based on the availability of field data in a given area. Hao and Liu (1994) present a methodology to estimate forest fire emissions (*Eq. 1*) based on which the emission of each pollutant can be quantified.

$$\mathrm{Em}_{\mathrm{i,j}} = \sum_{j} M_j \times \mathrm{EF}_{\mathrm{i,j}} \times 10^{-3} \qquad \text{(Eq.1)}$$

where:
$Em_{i,j}$ = Emission of pollutant i from land cover type j (tonne/yr)
M_j = Amount of burned biomass on land cover type j (tonne/yr)
$Ef_{i,j}$ = Emission factor of pollutant i from land cover type j (g/kg of dry matter)

The amount of biomass burned each year from forest fires was calculated by multiplying the factors burned area, fraction of total area burned annually, dry matter density and burning efficiency as presented in *Equation 2*. Dry matter density ρi and forest burned area fraction fj values were taken from the FAO Forest Resource Assessment (FAO, 2003).

$$M_j = \mathrm{A}_j \times f_j \times \rho_j \times \eta_j \qquad \text{(Eq.2)}$$

where:
A_j = Area of land cover type j (ha)
f_j = Fraction of total area burned annually of land cover type j
ρ_j = Dry matter density (tonne/ha)
η_j = Burning efficiency (oxidized in the combustion)

The emission factors of forest burning in a tropical forest environment were adapted from Andreae and Merlet (2013) and are provided in *Table 1*. The unit of emission factors (g/kg dry mass residue burning) was calculated by the division of the mass concentration of a pollutant (e.g. CO_2) {g} and the dry mass residue before burning {kg}. This parameter is reliable to use for pyrogenic emission factors for pollutants in tropical forest areas. In the literature, most of available CO_2 emission factors from biomass burning especially from forest fires are reported higher than 1,000 g/kg, e.g. Andreae et al. (2001), Chaiyo and Garivait (2014), Shrestha et al. (2013). In case of a complete combustion, the carbon released into the atmosphere would be equal to the carbon in the form of CO_2. As forest fire is frequently an incomplete combustion, the carbon discharged in the form of CO_2 represents more than 90% of the total carbon lose; CO emissions account for less than 5% of the total carbon release and minor carbonaceous compositions comprise of BC and OC (Chaiyo and Garivait, 2014). Consequently, the emission factor for CO_2 is higher comparing to other emission factors.

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Emission factors and other parameters i.e. dry matter density, burning efficiency for determining the biomass burned differ for each vegetation species, geographic location and environmental conditions. At global scale and national level, the estimation of emissions has been based on available guides such as the Intergovernmental Panel on Climate Change (IPCC) report 2006 for greenhouse gases emission inventory and research data or the approach proposed from Andreae and Merlet (2001). However, a country-specific value is needed to apply to Indonesia area for accurate data. In this study, we used primary data from Asian countries to estimate carbon emission. When the default parameters have been unavailable for Asia countries, the others parameters for a simlar climatic conditions and vegetation type have been the first choice (Junpen et al., 2013; Phairuang et al., 2017).

Table 1. *Pyrogenic emission factors for pollutants of interest for the tropical forest environment (adapted from Andreae and Merlet, 2013)*

Pollutant (abbreviation)	Pollutant	Tropical forest (g/kg)
CO_2	Carbon dioxide	1580
CO	Carbon monoxide	104
PM_{10}	Particulate matter less than 10 microns in diameter	6.5-10.5
$PM_{2.5}$	Particulate matter less than 2.5 microns in diameter	9.1
OC	Organic carbon	5.2
BC	Black carbon	0.66

Results and discussion

Burned areas enclosed in the MRP sites at Central Kalimantan, Indonesia were detected using a dual polarization TerraSAR-X image acquired in April 2010. *Figure 2* depicts the burned area as reported by the KFCP report (KFCP Vegetation Monitoring, 2014) and as estimated from the SAR image classification followed in this paper. It is apparent that the spatial distribution of the binary classification results from the classification of the SAR image correspond closely to the results reported by the KFCP. We estimated that the cumulative burned area calculated from the SAR image processing algorithm accounts for 47.6113 km^2 (4761.13 ha). The cumulative burned area reported from the reference map was 50.4128 km^2 (5041.28 ha). It has to be noted that these two datasets refer to two consecutive years. *Table 2* provides the accuracy assessment, with the reference data represented from the KFCP report (KFCP Vegetation Monitoring, 2014). The overall accuracy was 88.26%. The commission error for the burned area was 26.21%, which is attributed to the fact that several very small areas scattered around the image (salt and pepper effect) were classified as burned areas while they have been noise from the SAR image instead. A larger kernel in the speckle filter could have eliminated these pixels and reduce the commission error, however other artefacts would have been introduced in the image by selecting a kernel with a larger than 7 × 7 window size. The omission error was 33.80% and these are areas at the south-east side of the image and further away from the drainage canals; either the

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DOI: http://dx.doi.org/10.15666/aeer/1702_24892499

classifier has underestimated the burned effect in this part of the image, either this was a direct consequence of the difference in date acquisition of the two data sets.

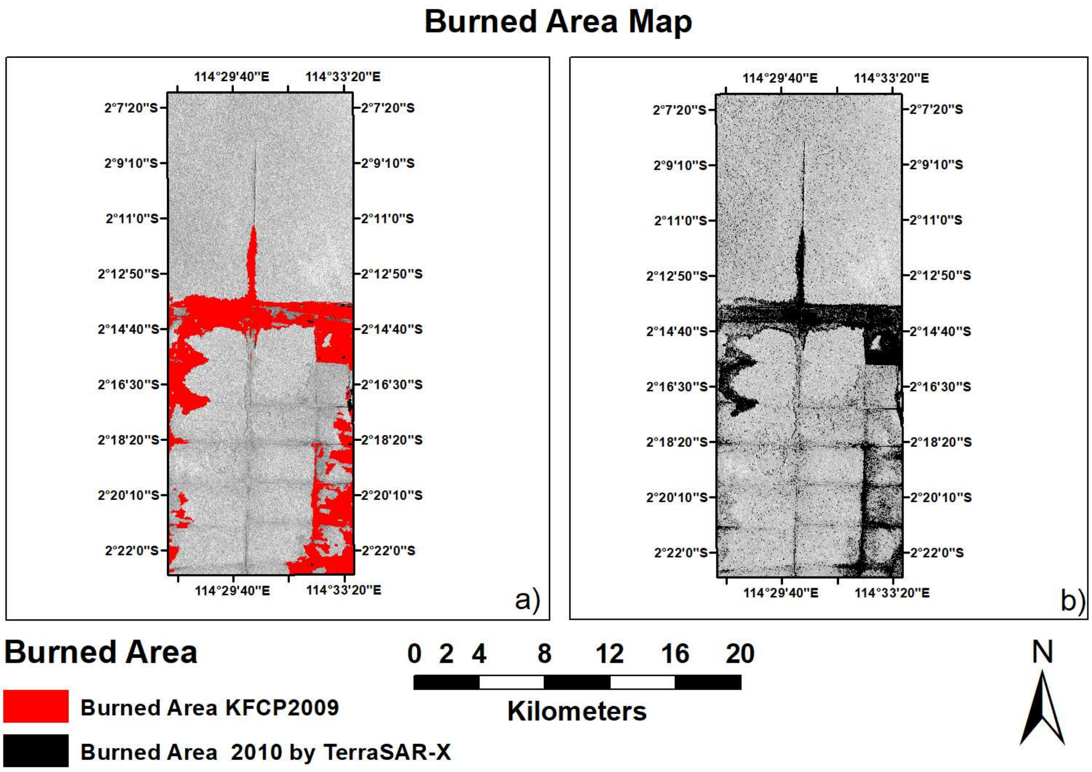

Figure 2. *Burned area map in the location of the MRP site Central Kalimantan, Indonesia: (a) burned area in 2009 by KFCP (KFCP Vegetation Monitoring, 2014); (b) burned area in 2010 by TerraSAR-X satellite image HH/HV polarization*

Table 2. *Confusion matrix illustrating the results of the estimation of the burned area based on the SAR data and using the KFCP as reference data (KFCP Vegetation Monitoring, 2014). The numbers indicate number of pixels (and percentage for omission/commission errors and overall accuracy)*

		Reference data			
		Unaffected	**Burned area**	**Total**	**Commission error (%)**
SAR data	**Unaffected**	22105909	2049716	24155625	8.48546
	Burned area	1425878	4014568	5440446	26.208844
	Total	23531787	6064284	29596071	
	Commission error (%)	6.05937	33.799802		
	Overall accuracy (%)	88.26			
	Kappa	0.63			

The estimation of pollutant emissions from burned areas in the area of interest based on the SAR data classification results (*Table 3*) showed that the emission pollutants of

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DOI: http://dx.doi.org/10.15666/aeer/1702_24892499

CO_2 was 173,019.46 ton, CO (11,388.62 ton), PM_{10} (1,149.81 ton), $PM_{2.5}$ (996.50), OC (569.43 ton) and BC (72.27 ton). These results indicate that CO_2 has the highest annual carbon releases into the atmosphere in this study area while BC are estimated to the lowest value comparably (*Table 3*).

Table 3. *Emission pollutants estimated based on the identified burned areas from the SAR satellite image classification*

Pollutant	Tropical burned forest (tons)
CO_2	173,019
CO	11,388
PM_{10}	1,149
$PM_{2.5}$	996
OC	569
BC	72

Conclusions

In this paper the burned area of a tropical peatland area in Central Kalimantan, Indonesia was estimated based on a very high spatial resolution SAR satellite image and evaluate against field collected data reported from KFCP (KFCP Vegetation Monitoring, 2014). The two results agree spatially in regard to the distribution of burned areas. The burned area estimated from the SAR image was subsequently input in an equation developed to estimate pollution emittance. We found that the average annual carbon release from the area of study into the atmosphere is 173,019.46 ton CO_2, 11,388.62 ton CO, 1,149.81 ton PM_{10}, 996.50 ton $PM_{2.5}$, 569.43 ton OC and 72.27 ton BC. The workflow presented, based on SAR satellite data, can be followed to map the burned area, estimate the cumulative land affected by fire and its corresponding biomass and quantitatively approximate the pollutants emission. The future research direction is a comparative analysis between SAR and other optical satellite images, such as Landsat and Sentinel-2, to improve the accuracy of the results.

Acknowledgements. The authors would like to express their profound appreciation and gratitude to GEO-Informatics Research Center for Natural Resource and Environment and Southern Regional Center of Geo-Informatics and Space Technology for making available facilities for the completion of this study. We would also like to thank the Faculty of Environmental Management, Prince of Songkla University for providing funding in support of this research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_24892499

BUTTERFLY COMMUNITY STRUCTURE AND DIVERSITY IN SANGIHE ISLANDS, NORTH SULAWESI, INDONESIA

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(Received 3rd Nov 2018; accepted 28th Jan 2019)

Abstract. Butterflies have an important role in the ecosystem of Sangihe Island, North Sulawesi, Indonesia. Currently, data on the diversity of butterflies on the island are still lacking and have not been published yet. Therefore, this study was aimed to analyze the structure of the butterfly community and its diversity in Sangihe Island, North Sulawesi, Indonesia. The research was conducted from March 2018 to May 2018 in Sangihe Island, North Sulawesi. Sampling was performed at three types of habitat: a farm, a forest edge, and bushes. Sampling method used was surveyed with purposive sampling. A collection of butterflies was gathered by the sweeping method using sweep net following the transect line randomly for 500 m long. At each habitat, four transects were set and the collection step was duplicated. Sampling was performed from 8.00 am to 15.00 pm. The collection comprised of 5 families, 39 species, and 944 individuals. The most commonly found family was Nymphalidae, while the most abundant species were *Junonia hedonia intermedia* and *Eurema tominia*. Species abundance, species richness index, species diversity index, and species evenness index were found to be the highest at the forest edge habitat, while the lowest ones were from the bushes. The diversity of the butterflies at the forest edge habitat was higher since host plants were located in the area, and the vegetation served as a food source and shelter for the animals.
Keywords: *forest edge, sweep net, Junonia hedonia, Nymphalidae, host plant*

Introduction

Sangihe Island is one of the most remote islands in the province of North Sulawesi, which directly borders with the Philippines. The Sangihe Island biodiversity involves a variety of plants and animals including butterflies (Shekelle et al., 2008; Central Agency on Statistics, 2015).

Butterflies are part of Lepidoptera, a type of insect with scaled wings. The scales provide beautiful colour patterns such as ribbon, nodes, and circles (Braby, 2004; Arya et al., 2014). The beautiful color patterns make several types of butterflies highly hunted and traded as collection, thus making them declared as endangered species (Coote, 2000). The diversity of butterflies in Indonesia is abundant, which consists of about 1,600 species out of 20,000 species among all species of butterflies worldwide (Alarape et al., 2015). The rich biodiversity in Indonesia could only be rivalled by the tropical countries in South America, such as Brazil and Peru that have about 3,000 species. The king butterfly, *Troides hypolitus*, is mostly found in the western part of Indonesia and Sulawesi, with some species spread in Maluku and Papua. Sulawesi is an island with the highest uniqueness of butterflies in Indonesia. From 557 species of butterflies spread in

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DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

Sulawesi, 239 of them (more than 40%) are endemic and can only be found on the island, such as *Papilio blumei*. Meanwhile, more than 600 species are spread in Java and Bali islands, 40% of which are endemic species (Amir and Kahono, 2008; Nidup et al., 2014).

Butterflies have an important role in maintaining the biodiversity with their function as pollinator that help pollination in plants, thus they help breeding of plants naturally (Plona, 2002; Perveen and Haroon, 2015). Moreover, butterflies are an important food for birds, reptiles, spiders and other predatory insects (Haroon et al., 2013). In terms of conservation of the ecosystem, butterflies are popular as bioindicators of the change of quality of the environment (Patil and Shende, 2014; Arya et al., 2014; Ghosh and Mukherjee, 2016).

Butterflies are often used as a model by scientists to estimate and predict biodiversity due to the effect of local and global climate change, environmental damage, distribution of plants and animals, forest management on the natural ecosystem (Axmacher et al., 2004; Vu et al., 2015). Several research studies have shown that deforestation in tropical countries due to illegal logging, over volume of logging, and clearing of forest area to provide space for farms, all could affect the distribution, structure, and composition of the community, diversity of the species of butterflies (Van Vul and Quang Vu, 2011; Nidup et al., 2014; Widhiono, 2015; Vu et al., 2015; Nkongolo and Bapeamoni, 2018). Butterflies are also affected by environmental variations and changes in the forest structure; moreover, they are also affected by the abundance of plants they feed on (Bora and Meitei, 2014; Castro and Espinosa, 2015). Butterflies are also highly sensitive to climate change (Parmesan and Yohe, 2003). Therefore this study was aimed to analyze the community structure and biodiversity of butterflies at Sangihe Island, North Sulawesi, Indonesia.

Materials and methods

Study area and types of land use

The study was performed from March to May 2018 at Sangihe Island, North Sulawesi, Indonesia (*Fig. 1*). Sampling was done on 3 types of habitat: forest edge, farming area, and bushes. In each habitat, 4 transect lines of 500 m were laid out. On each transect, air temperature, air humidity, canopy closure, altitude, and coordinates, were measured. Measurement of air temperature and humidity was carried out using Thermo-Hygrometer while taking of butterfly samples. The percentage of canopy coverage in each habitat was obtained by making a plot of 20 m x 20 m on each transect so that there were 4 plots in each habitat type. The percentage of canopy coverage in each plot was obtained by vertically projecting tree canopy coverage on the plot. Vertical projection on the flat area of the land was then converted into percentage, with the area of the plot as a denominator, so that the value of canopy coverage will be obtained for each plot in each transect. Altitude and coordinates were measured using a Global Positioning System (GPS) on each transect line.

The forest edge is a habitat that contains variety of plants such as *Ficus* sp (Moraceae), *Alstonia macrophylla* (Apocynaceae) and *Garcinia* sp. (Clusiaceae). At this habitat, there was a river with 8-10 m width. The area had a daily temperature ranging from 28.1-29.9 °C, the humidity of 82-87, canopy coverage of 76-86%, the altitude of 120-201 m above sea level, and it is located at the following coordinates 03°30’05.56” S and 125°31’43.31” E.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

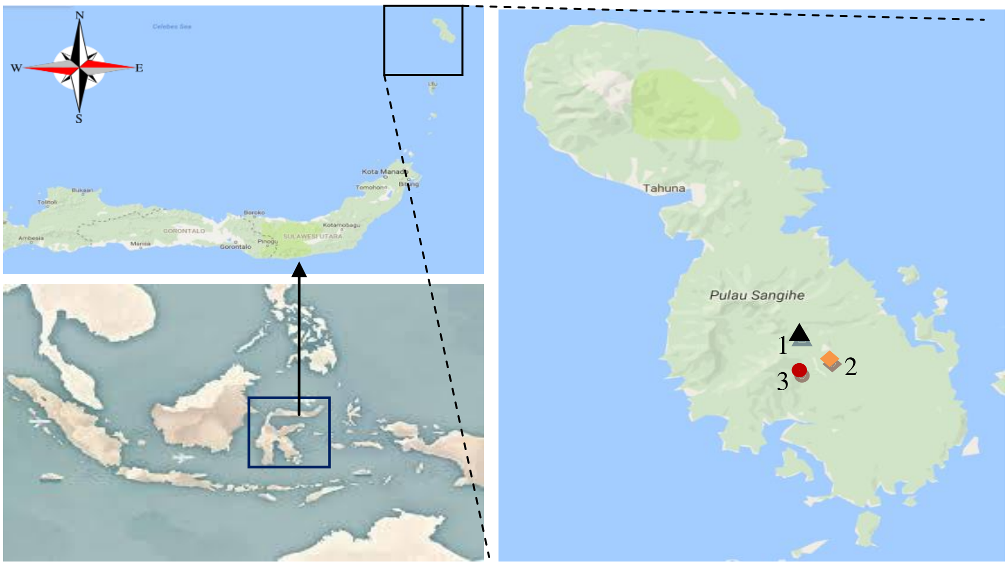

Figure 1. *Map of the study area in Sangihe Islands, North Sulawesi, Indonesia (1. edge forest, 2. farm area, 3. bushes)*

The farm area is an area managed by the local community and planted with a variety of crops such as cloves (*Eugenia aromatica*), coconut (*Cocos nucifera*), banana (*Musa* sp) and cassava (*Manihot utilisima*). The farming area selected in this study had a daily temperature of 29.2-30.3 °C, the humidity level of 79-82, canopy coverage of 70-80%, the altitude of 61-97 m above sea level, and it is located at the following coordinates 03°26’21.27” S and 125°36’01.77” E.

The bushes area selected in this study was formerly a farming area that has been abandoned by the local community and covered with plants such as ferns (Pteridophyta), grass (Poaceae), and bamboo (*Bambusa* sp). This habitat had a daily temperature of 33.0-35.3 °C, the humidity of 51-65, canopy coverage of 40-57%, the altitude of 120-137 m above sea level, and it is located at the coordinates of 03°26’52.73” S and 125°35’42.28” E (*Fig. 2*).

Sampling

Research method used was survey method through purposive sampling. Butterflies were collected with a sweeping method by using a net following transect lines that were set randomly for 500 m length and 5 m width to each side, left and right (Hamer et al., 2003). At each habitat, four transects were prepared and at each transect, sampling was duplicated. The total number of transects was 12. Sampling was performed at 8 am-3 pm (Peggie and Amir, 2006). From every species, only one butterfly was collected. When similar species was found, it was released back to nature. Sampling was performed every month for 3 months. Sampling was done monthly for three months. Butterfly sampling for 3 months was quite representative and can provide information about the diversity of butterflies in the Sangihe Islands. This is because those three months were during the dry season when the weather conditions were sunny. Those conditions were very suitable for butterflies to live. During this period, most nectar-rich plants began to flower (Rizal, 2007), thus providing butterflies food sources and supporting the life and breeding of butterflies.

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Figure 2. *Photographs of study sites (a) Sangihe Island, (b) edge forest, (c) farm area, (d) bushes*

Identification of butterfly samples

The process of identifying and classifying the specimens was based on the following identification books: Practical Guide to the Butterflies of Bogor Botanic Garden (Peggie and Amir, 2006), Agricultural entomology (Jumar, 2000), Butterflies of The South East Asian Island, Part I Papilionidae, Part II Pieridae-Danaidae, Part III Satyridae-Lybytheidae, Part IV Nymphalidae (I), Part V Nymphalidae (II) (Tsukada and Nishiyama, 1982, 1981,1982,1985, 1991), and Insect in Halimun Mountain National Park Bagian Barat (Amir et al., 2003).

Data analysis

Data analysis in this study was performed on several parameters: species abundance (n), species richness index (S), species diversity index (H) and species evenness index (E). Species abundance is the number of individual in each species found in each habitat. At all habitats, the indices used were Margalef species richness index (DMg), Shannon species diversity index (H) and Pielou species evenness index (J) (Magurran, 1988).

Species richness index (Dmg) was calculated using Margalefs index to show the number of species in a certain type of habitat (Magurran, 1988), using *Equation 1:*

$$\text{Species richness Indeks (Dmg)} = \frac{(S-1)}{\ln S} \qquad \text{(Eq.1)}$$

where: S = The number of species.; ln = Natural logarithm (natural number).

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Meanwhile, species diversity index was calculated using the Shannon-Weiner diversity index (H) in Magurran (1988) to describe the diversity of butterfly species, using *Equation 2:*

$$\text{Spesies diversity index } (H') = -\sum_{i=l}^{s} (Pi) \ln (Pi) \qquad \text{(Eq.2)}$$

where: Pi = the proportion of each species; ln = natural logarithm (natural number).

At the same time, to show the level of evenness of individual abundance in each butterfly species and as an indicator of species dominance in each habitat, species evenness index was calculated using Pielou's evenness index (Magurran, 1988), using *Equation 3:*

$$\text{Species evenness index (E)} = \frac{H'}{Ln\ S} \qquad \text{(Eq.3)}$$

where: H' = species diversity index; ln = natural logarithm (natural number); S = the number of species.

All indexes were calculated with the help of the program PAST 2.17 (Hammer et al., 2001). Statistical analysis to analyze the difference in species abundance, species richness index, and species evenness index among habitats was performed using the software Statistica version 6, with one-way ANOVA and Duncan test at a confidence level of 95% (StatSoft, 2001; Ohsawa, 2005). To check the relationship between species diversity with other physicochemical parameters in nature, the Spearman correlation was used using the software Statistica version 6 (StatSoft, 2001).

Analysis of similarity between butterfly communities was performed with Sorensen similarity index, by checking the existence and inexistence of certain species of butterfly (Magguran, 1988). The index was calculated using Biodiv 97, macro software in Excel (Shahabuddin et al., 2005). Index of dissimilarity (1 - Sorensen index) was used to do cluster analysis (Krebs, 1999; Ludwig and Reynold, 1988). Cluster analysis of every community was ordered hierarchal in the form of a dendrogram. The dendrogram was prepared using the program Statistica for Windows 6 (StatSoft, 2001). The clustering process was based on unweighted pair group with arithmetic mean and Euclidian distance (Lewis, 2001).

Results

Structure and composition of butterflies

The results found 5 families of butterflies in Sangihe Island, consisting of 39 species and 944 individuals. The families found were Nymphalidae, Papilionidae, Lycaenidae, Pieridae, and Hesperidae. The family with the highest number of species and individuals was Nymphalidae, with 19 species and 508 individuals (53.81%), followed by the family Papilionidae with 8 species and 214 individuals (22.67%). The family Nymphalidae was the most dominant family that was found in all sampling areas. Meanwhile, Hesperidae was the family with the least number of species, with only 2 species and 5 individuals (0.64%). The family Hesperidae was only found at the forest edge and farm and not in the bushes (*Table 1* and *Fig. 3*).

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***Table 1.** Number of families, species, and individuals found in three types of Habitat in Sangihe Island, North Sulawesi*

No	Family/Species	Number of Individuals			Grand	%
		Faram	Bushes	Edge Forest	Total	
I	**Nymphalidae**					
1	*Junonia hedonia intermedia*	58	73	64	195	20.66
2	*Cupha arias*	16	8	19	43	4.56
3	*Danaus affinitis fulgarata*	23	7	6	36	3.81
4	*Euploea leucostictos westwodi*	3	8	21	32	3.39
5	*Danaus genutia leucoglene*	12	0	11	23	2.44
6	*Hypolimnas bolina*	2	0	20	22	2.33
7	*Lasippa neriphus tawayana*	9	0	11	20	2.12
8	*Danaus ismare alba*	1	15	3	19	2.01
9	*Melanitis leda obsolete*	13	3	2	18	1.91
10	*Parthenos sylva salentia*	2	0	14	16	1.69
11	*Idea leuconoe*	4	3	8	15	1.59
12	*Mycalesis janardana*	3	7	5	15	1.59
13	*Orsotriaena medus*	14	0	1	15	1.59
14	*Ideopsis juventa tontoliensis*	8	1	4	13	1.38
15	*Lohara optalmica*	6	3	2	11	1.17
16	*Mycalesis horsfieldi*	5	0	2	7	0.74
17	*Terinos clarisssa*	0	0	5	5	0.53
18	*Faunis manado*	2	0	0	2	0.21
19	*Cyrestis acilia*	0	0	1	1	0.11
II	**Papilionidae**					
20	*Graphium meyeri*	28	8	36	72	7.63
21	*Papilio rumanzovia*	3	25	34	62	6.57
22	*Papilio polytes*	7	22	10	39	4.13
23	*Papilio sataspes*	0	0	14	14	1.48
24	*Graphium milon*	6	0	5	11	1.17
25	*Graphium agamemnon*	0	5	3	8	0.85
26	*Papilio gigon*	2	0	3	5	0.53
27	*Papilio helenus*	0	3	0	3	0.32
III	**Lycaenidae**					
28	*Pithecops phoenix*	20	0	9	29	3.07
29	*Jamides aratus*	4	0	22	26	2.75
30	*Jamides celeno*	2	1	3	6	0.64
31	*Lampides boeticus*	0	0	5	5	0.53
32	*Rapala ribbei*	3	0	0	3	0.32
IV	**Pieridae**					
33	*Eurema tomina*	21	19	38	78	8.26
34	*Eurema blanda*	8	11	12	31	3.28
35	*Eurema hecabe*	5	6	18	29	3.07
36	*Catopsilia pamona flava*	0	2	7	9	0.95
37	*Gandaca blanda*	0	0	9	0	0.00
V	**Hesperidae**					
38	*Borbo cinnara*	4	0	0	4	0.42
39	*Erionota thrak*	0	0	2	2	0.21
	Total	294	230	420	944	100.00

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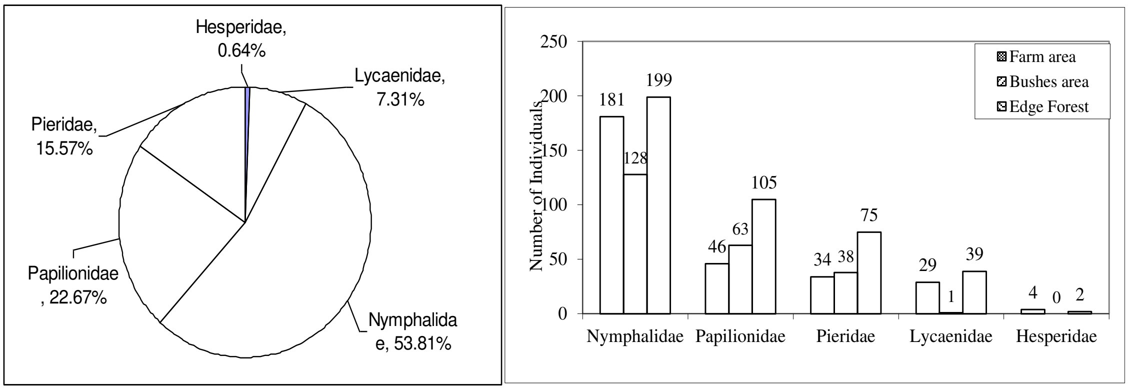

Figure 3. *The abundance of family butterflies found in three types of habitat at Sangihe Island, North Sulawesi*

Junonia hedonia intermedia is a butterfly species with the highest abundance (20.66%) compared to other species, followed by *Eurema tominia* with the abundance of 8.26%. Butterfly species with the lowest abundance with only 1 individual (0.11%) was *Cyrestis acilia* (*Table 1* and *Fig. 4*).

The species of butterflies found in all three habitats were not similar. There were species that were found in all 3 habitats, some were found only in 2 habitats, and some others were found only in 1 habitat. There were 17 species found in all types of habitat and 12 species found in only 2 habitats. Meanwhile, 3 species were found only in farm area while *Papilio helenus* was found only in the bushes. The species found only in the forest edge habitat were 6 species (*Table 1*).

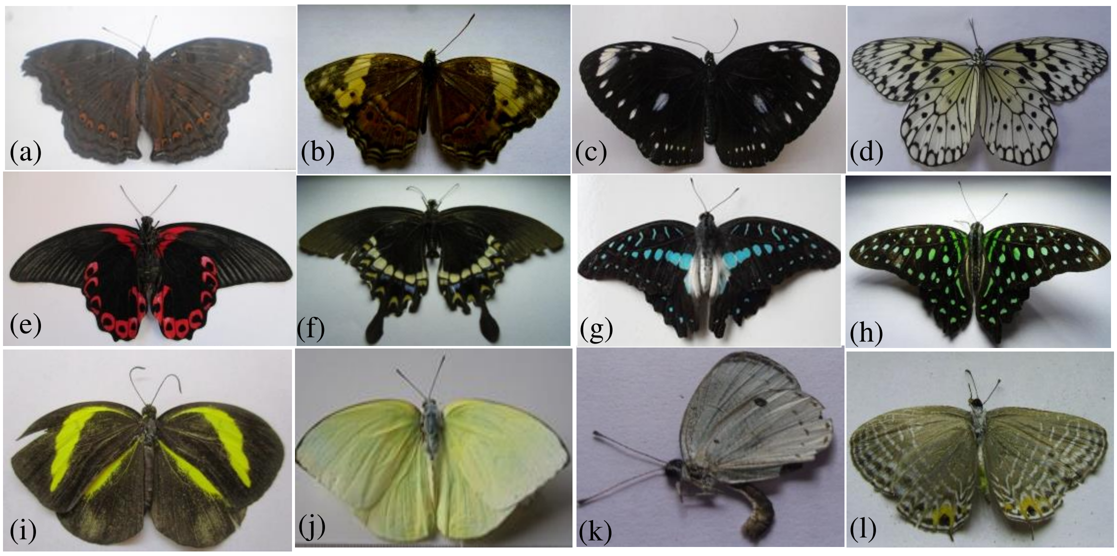

Figure 4. *Several species of butterflies found in the Sangihe Islands. Families Nymphalidae: (a) Brown pansy (J. hedonia intermedia), (b) Heliconians (C. arias), (c) Westwood's king crow (E. leucostictos westwoodi), (d) Paper kite (I. leuconoe); Papilionidae: (e) Red Mormon (P. rumanzovia), (f) Common Mormon (P. polytes), (g) Meyer's Triangle (G. meyeri), (h) Tailed jay (G. agamemnon); Pieridae: (i) Grass yellows (E. tominia), (j) Lemon Emigrant (C. pamona flava); Lycaenidae; (k) Sulawesi Quaker (P. phoenix), and (l) Common Cerulean (J. celeno)*

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):2501-2517.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

Butterfly species diversity

The average value of species abundance (118.00 ± 33.46), species richness index (4.52 ± 0.48), species diversity index (2.82 ± 0.14) and species evenness index (0.91 ± 0.02) were found to be the highest at forest edge habitat, while the lowest one was found in the bushes habitat (*Table 2; Fig. 5*). One-way ANOVA and Duncan tests showed that there were significant differences in terms of richness index (ANOVA: F = 15.72; P < 0.05), and diversity index of species (ANOVA: F = 32.96; P < 0.05), among habitats, while abundance (ANOVA: F = 4.05; P > 0.05) and evenness indices (ANOVA: F = 2.04; P > 0.05), were not significantly different. The species richness index and species diversity index of butterflies found in the farm were significantly different with those found in the forest edge and bushes habitats (*Table 2; Fig. 5*).

Table 2. *Species diversity of butterflies in three types of habitat at Sangihe Island, North Sulawesi (Average ± St dev)*

Habitat	Transect	Abundance species	Richness index	Diversity index	Evenness index
Farm area	1	106	4.50	2.72	0.88
Farm area	2	60	3.18	2.35	0.89
Farm area	3	44	3.44	2.35	0.89
Farm area	4	92	3.54	2.51	0.89
Average ± St dev		75.50±24,67	3.66±0.50	2.48±0.15	0.89±0.01
Forest edge	1	100	4.13	2.71	0.91
Forest edge	2	141	5.05	3.02	0.93
Forest edge	3	158	4.94	2.88	0.88
Forest edge	4	73	3.96	2.66	0.92
Average ± St dev		118.00±33.46	4.52±0.48	2.82±0.14	0.91±0.02
Bushes area	1	42	2.14	1.92	0.88
Bushes area	2	44	2.91	1.90	0.76
Bushes area	3	87	2.69	2.04	0.80
Bushes area	4	63	1.93	2.06	0.94
Average ± St dev		59.00±18.12	2.42±0.40	1.98±0.07	0.84±0.07

Correlation between species diversity and environmental factors

The results of temperature measurements in farm area habitat showed an average value and standard deviation of 29.68 °C ± 0.40, edge of forest showed an average value and standard deviation of 28.58 °C ± 0.77, and bushes habitat showed an average value and standard deviation of 34.20 °C ± 0.83. Average air humidity on farm area was 80.70% ± 1.09, on edge of the forest was 83.50% ± 1.12, and bushes habitat was 57.75% ± 5.26. Canopy coverage on farm area was averagely 74.75% ± 3.56, on edge of the forest was averagely 81.75% ± 4.02, and on bushes habitat was averagely 48.00% ± 6.28 (*Table 3*).

Correlation between the diversity of butterfly species with the environmental factors showed that temperature was negatively correlated with species diversity index (-0.85) and species richness index (-0.77) (*Table 4*). This result shows that the lower the temperature, thus the higher the species diversity and species richness indices of butterflies. Species richness index and species diversity index of butterflies were

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DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

positively correlated with humidity and canopy coverage percentage. Meanwhile, the altitude did not give significant effect on species diversity of butterflies.

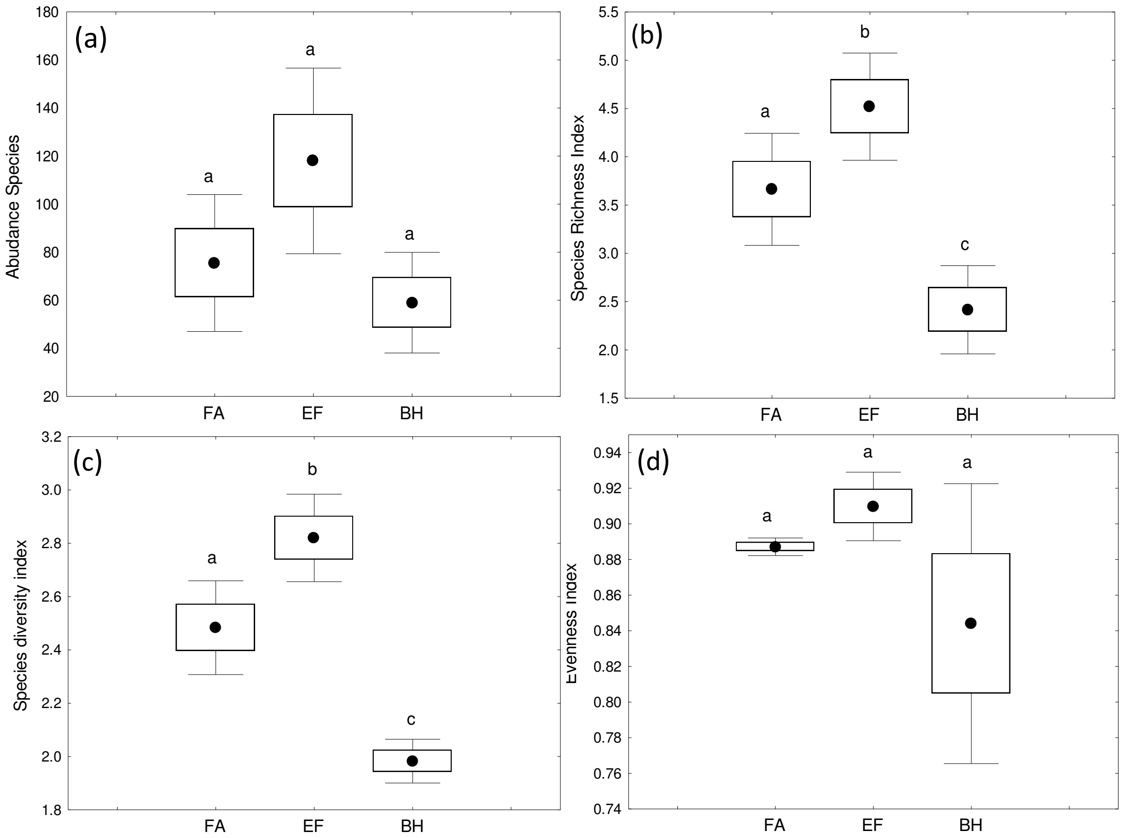

Figure 5. *The influence of habitat types on (a) abundance, (b) richness (c) diversity and (d) evenness species index in Sangihe Islands, North Sulawesi. (FA: farm area; EF: an edge of the forest, BH: bushes. (●): mean, (□): ± SE, (⌶): ± SD. The same letter in the same plot did not differ significantly according to Duncan's test at 95% confidence level)*

Table 3. *Species diversity butterflies and environmental factor in three types of habitat at Sangihe Island, North Sulawesi*

Habitat	Transect	Species diversity				Environmental factors			
		Abudance Species	Richness index	Diversity Index	Evennes index	Temperature (0C)	Humidity (%)	Canopy (%)	Altitude (M asl)
Farm area	1	106	4.50	2.72	0.88	29.7	79	70.00	77
Farm area	2	60	3.18	2.35	0.89	29.5	82	75.00	95
Farm area	3	44	3.44	2.35	0.89	29.2	81	80.00	61
Farm area	4	92	3.54	2.51	0.89	30.3	81	74.00	97
Average±Stdev		75.50±24,67	3.66±0.50	2.48±0.15	0.89±0.01	29.68±0.40	80.75±1.09	74.75±3.56	82.5±14.65
Forest edge	1	100	4.13	2.71	0.91	28.1	83	86.00	120
Forest edge	2	141	5.05	3.02	0.93	29.9	82	80.00	178
Forest edge	3	158	4.94	2.88	0.88	28.3	85	85.00	196
Forest edge	4	73	3.96	2.66	0.92	28.0	84	76.00	201
Average±Stdev		118.00±33.46	4.52±0.48	2.82±0.14	0.91±0.02	28.58±0.77	83.50±1.12	81.75±4.02	173.75±32.19
Bushes area	1	42	2.14	1.92	0.88	35.3	51	50.00	130
Bushes area	2	44	2.91	1.90	0.76	34.0	60	40.00	137
Bushes area	3	87	2.69	2.04	0.80	34.5	55	45.00	125
Bushes area	4	63	1.93	2.06	0.94	33.0	65	57.00	120
Average±Stdev		59.00±18.12	2.42±0.40	1.98±0.07	0.84±0.07	34.20±0.83	57.75±5.26	48.00±6.28	128.00±6.28

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DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

Table 4. *Correlation coefficient among butterflies diversity and environmental factor*

Variable	Temperature	Humidity	Canopy	Altitude
Abundance species	-0.49	0.53	0.55	0.47
Richness index	-0.77*	0.79*	0.75*	0.35
Diversity Index	-0.85*	0.86*	0.86*	0.34
Evenness index	-0.57	0.57	0.70*	0.12

*Marked correlations are significant at p < .05000, N = 12

Community similarity

Composition similarity of butterfly community in Sangihe Island showed that the highest similarity index was between the forest edge and farm habitats (0.85 or 85%). The lowest Sorensen similarity index was found between the forest edge and bushes habitats (0.67 or 67%) (*Table 5*).

A dendrogram was produced using *unweighted pair group method with arithmetic mean* (UPGMA) and it shows that there were 2 groups formed, where forest edge habitat was grouped together with the farm habitat (*Fig. 6*). This result shows that the butterfly community at forest edge habitat was highly similar to that found in the farm habitat.

Table 5. *Matrix about butterfly community similarity among habitats in Sangihe Island*

Habitat	Farm area	Edge forest	Bushes
Farm area	1.00	0.85	0.68
Edge forest	0.85	1.00	0.67
Bushes	0.68	0.67	1.00

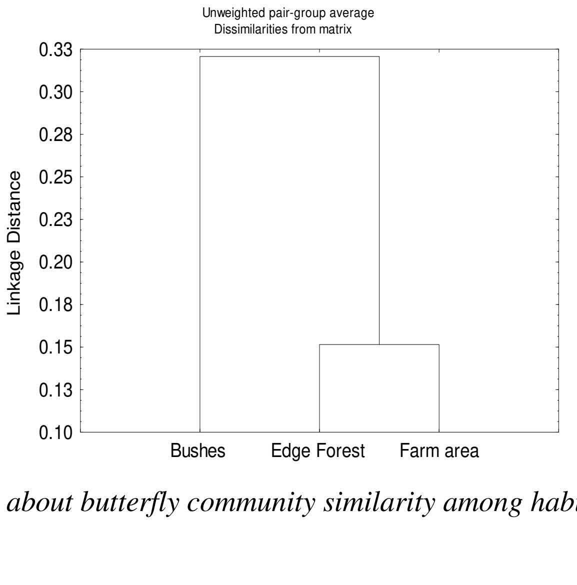

Figure 6. *Dendrogram about butterfly community similarity among habitats in Sangihe Island*

Discussion

The number of butterfly species found in the research was only 0.20% of all species worldwide, 2.23% of species found in Indonesia, and 6.96% of species found in Sulawesi. There are about 19,238 of butterfly species worldwide (Patil et al., 2017),

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1,750 species in Indonesia (Peggie, 2014), and 560 species in Sulawesi (Vane-Wright and de Jong, 2003). The number of butterfly species found in Sangihe Island in this study was lower than the results of several other studies carried out on the mainland of North Sulawesi. Studies in Manembo-Nembo Nature Reserve (Koneri and Maabuat, 2016) and Gunung Tumpa National Park (Tallei et al., 2015), North Sulawesi managed to find 44 and 50 species, respectively.

Several studies in other islands reported a higher number of butterfly species as compared to the current results found in Sangihe Island. Koneri and Saroyo (2012) reported 28 butterfly species in Manado Tua Island, Manado, North Sulawesi, while Lamatoa, et al. (2013) collected 19 species of butterfly in Mantehage Island, North Sulawesi. Moreover, Rosmidi et al. (2017) reported 27 butterfly species found in Perhentian Island and Bidong Island, Terengganu, Malaysia. The difference in the number of species found in each sampling location is affected by sampling area, sampling time, sampling method, habitat's condition and sampling period. The difference in the number of species between an island and the mainland is affected by the island area and distance between the island and the mainland. This result is in accordance with the biogeography theory of islands that mentions small and far islands support a lower number of species compared to bigger and nearer islands (Sumarto and Siahaan, 2012).

The family Nymphalidae was found abundantly in the study since it is a type of butterfly family that can adapt well to environmental conditions, has a large number of family members, and tend to be polyfag. The polyfag characteristic allows Nymphalidae to fulfill its need on host plant although the plant is not available. These reasons cause Nymphalidae exist in a relatively high number of species and individuals in Sangihe Island. According to Rosmidi et al. (2017), this phenomenon could be due to a large number of species in the family as well as their wide variety of selection of food, including flowers, fruits, honeydew, tree sap, rotten materials, and decomposing carcasses; moreover, they also have good adaptation on feeding on a wide range of host plants.

The host plants of Nymphalidae butterflies include Annonaceae, Asteraceae, Verbenaceae, Moraceae, Rubiaceae and Anacardiaceae (Lamatoa, et al., 2013). Plant species that are often found in Sangihe Island include *Ficus* sp (Moraceae), *Mussaenda pubescens* (Rubiaceae), *Garcinia* sp (Clusiaceae), *Lantana camara* (Verbenaceae), *Cananga odorata* (Annonaceae), *Mangifera indica* (Anacardiaceae), *Ficus benjamina* (Moraceae), Citrus sp (Rutaceae) and *Eupatorium inulifolium* (Asteraceae). A distinct difference in terms of the dominant family found in several places is due to the fact that distribution of butterflies is affected by the distribution of the host plant and the ecology (Amir et al., 2003; Panjaitan, 2008). The family Nymphalidae could be found easily all year since they are generalist and some types of this family do not depend only on the availability of flower nectar since they could get their food from rotten fruits or even animal urine (Sarma et al., 2012; Sari et al., 2013).

The species *Junonia hedonia intermedia* was found the most in the study. This species has wings with dark brown colour and orange eye pattern near the edge of the wings. It is able to fly fast and often found standing on the ground or leaves to get sunlight. Several studies reported finding this species standing on the ground or damp stones searching for minerals. This species was abundantly found in the study since it is part of the family Nymphalidae and has polyfag characteristic. Polyfag butterflies could multiply on many types of plants. Their larvae could survive in low diversity of host

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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plants (Helmann, 2002). The high existence of a butterfly species is supported by the availability of plants as sources of food (Yamamoto et al., 2007).

The existence of butterflies in a habitat highly depends on the condition of the habitat and the availability of sources of nectar as well as host plants. Several species of butterflies were found in low frequency and limited only to a type of habitat. Species of butterflies that were found in low frequency with limited distribution are those that are sensitive to the damage of the habitat (Sunduvu and Dumbuya, 2008). Damage to the habitat causes fragmentation and extinction of plants as sources of nectars and host to those butterflies (Hardy et al., 2007).

Species abundance, species richness index, species diversity index, and species evenness index were found to be the highest in the forest edge habitat since this habitat has a variety of plants that can act as a food source as well as host plants for the butterflies. The high species diversity of butterflies in the forest edge habitat could also be due to the existence of a river in the area that can support the life of the butterflies. Butterflies need minerals to survive, which are usually gained from the soil surface, rocks, or riverbank. Riverbank has a variety of vegetations, rocks, sand, mud, and water that attract more butterflies to get water and nutrient from wet rocks, sand, and mud along the riverbank (Van Vu and Quang Vu, 2011).

There was low diversity of butterflies in the bushes habitat since the habitat has a low number of flowery plants and mostly contains grass (Poaceae) and ferns (Pteridophyta). The bushes habitat was originally forest that was converted into farm area. When the farm was abandoned, it turned into bushes. The low species diversity index shows the low availability of food sources and adaptability of the butterflies towards limited environmental factors. Conversion of the forest into farms could reduce natural biodiversity. Several studies have shown that conversion of the natural habitat is the cause of the decline of butterfly species (Chowdhury et al., 2017). Species diversity could decline due to fragmentation of the habitat (Bayero, 1995).

A decrease in species diversity of butterflies was also reported due to deforestation in Kalimantan (Schulze and Fielder, 1998) and in Sulawesi (Fermont et al., 2005). Change of habitat due to change of function of parts of the forest could be a serious threat to the existence of specific species of butterflies in Indonesia. There is a correlation between the diversity of butterflies and their habitat. Larvae and adult butterflies depend on the diversity of host plants. Although butterflies could migrate to new areas, if the plant food is extinct, thus the butterflies also go to extinction. Hamer and Hill (2000) conducted a study on butterfly community in tropical areas and reported that abundance and diversity of butterfly species were higher in forests that have not been used for logging yet compared to forests with logging activity. Their study showed that damage to the forest highly affects species diversity of butterflies. The diversity of butterflies increases when the scale of the habitat and the complexity of the vegetation structure increase (Nkongolo and Bapeamon, 2018). Sharma and Joshi (2009) reported that there was a correlation between the complexity of the habitat structure, shape of vegetation, and diversity of butterflies. High vegetation diversity will increase the diversity of butterflies. The environmental support system such as the availability of habitat, water, mineral, food, temperature, and humidity affect the diversity of butterflies (Mas'ud et al., 2018).

Species evenness index was found the highest at the forest edge habitat. High evenness index shows the lack of dominant species of butterflies. The smaller the

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species evenness index, thus the less equal the species distribution is and there is a domination by certain species of butterfly (Magurran, 1988).

Species richness index and species diversity index of butterflies were found to be positively correlated with humidity and vegetative canopy, but negatively correlated with the air temperature. Temperature highly affects the activity of butterflies, their distribution, growth, and breeding. The temperature at the bushes habitat was higher than the forest edge habitat and the farm area. High air temperature causes a decrease of the volume of nectar secretion in flowers (Efendi, 2009), thus butterflies reduce their activity in the area and move to the forest area that is warm in order to save their energy and reduce evaporation of moisture from their body. This phenomenon causes low species diversity and number of individuals of butterflies found in the bushes habitat.

Rajagopal et al. (2011) reported that species richness index of butterflies is affected by air temperature and humidity. Moreover, Kunte (2001) and Pandhye et al. (2006) also reported that temperature and rainfall are two important factors that directly affect richness and population of butterflies. Tree canopy highly affects the diversity of butterfly species since they can provide protection and food source for the butterflies. There are several species of butterflies that live in a habitat with thick forest canopy, but on the other hand, there are also species that live in a habitat with less forest canopy.

Species similarity among habitats could be measured with similarity index or species similarity coefficient. Similarity index is also used as a basis for cluster analysis. The highest similarity index was found between the forest edge and farm habitats. This shows that there is a high similarity of butterfly species found in the forest edge and in the farming area. This phenomenon could be due to the fast movement of butterflies from one habitat to another and the variety of vegetation in habitats, thus affecting the existence of butterflies. Kendeigh (1980) reported factors that cause similarity of species between two habitats to include a short distance between the two habitats, a similar composition of vegetation, and others.

Conclusions

Butterfly species found in Sangihe Island consisted of 5 families, 39 species, and 944 individuals. Nymphalidae is the most abundant family with the highest number of species when compared to other families, since the family can adapt well to the environmental condition, has the most number of family members, and tend to be polyfag. The polyfag characteristic allows the Nymphalidae to fulfill its need for host plan although the host plant is not available. Based on the type of habitat, the highest species richness index, species diversity index, and species evenness index were found in the forest edge habitat, while the lowest ones were found in the bushes habitat. The difference in the diversity of butterflies among habitats was due to the different structure and complexity of the vegetation in each habitat. High vegetation diversity will increase the diversity of butterflies. Availability of resources such as habitat, water, mineral, food, temperature, and humidity affect the diversity of butterflies. Meanwhile, environmental factors such as temperature and humidity highly affect the activities, distribution, growth, and breeding of butterflies. The temperature at the bushes habitat was higher than the other two habitats. Therefore, the volume of nectar secretion in flowers decreases, so the butterflies reduce their activities in the area or move to the warm forest edge area.

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Based on the results of this study, it is recommended to conduct further research on plant species which have an ecological role and function as food sources and hosts for butterflies. The time period of research might be also extended so that the research can be conducted during the rainy season as well. The use of herbicide to kill grasses and weeds should be carefully monitored because it can kill butterflies too. Agroforestry system can be implemented in forest areas to increase vegetation diversity, and consequently to add more host plants and food sources for butterflies.

Acknowledgements. The authors would like to thank the Directorate of Research and Community Service and Directorate of Research and Development, the Ministry of Research, Technology, and Higher Education, for their financial support for the research through Competency-based Research 2018.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25012517

EVALUATION OF *LABEO CALBASU* FISHERY STATUS USING SURPLUS PRODUCTION MODELS IN KAPTAI RESERVOIR, BANGLADESH

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(Received 4th Nov 2018; accepted 11th Jan 2019)

Abstract. Surplus-production models were used for estimating the sustainable exploitation of *Labeo calbasu* fish stock in Kaptai reservoir, Bangladesh. The annual catch and effort data of 14 years (2001-2014) were analyzed to estimate the Maximum Sustainable Yield (MSY) using software packages CEDA and ASPIC. The average production was found 156 mt while the topmost and undermost catch was 263 mt (in 2009) and 108 mt (in 2003) respectively MSY and CV of Fox model for normal, log normal and gamma assumption were 87 mt (0.339), 65 mt (0.464) and 67 mt (0.551) respectively whereas values from Schaefer model were 139 mt (0.055), 130 mt (0.055) and 137 mt (0.064) and 139 mt (0.059), 130 mt (0.056) and 137 mt (0.064) in Pella-Tomlinson models. As regards, ASPIC estimated MSY and CV values for Fox and Logistic models were 127 mt (0.054) and 133 mt (0.057) respectively. Higher R2 values (above 0.879) in ASPIC in contrast with CEDA (0.71-0.818) represent its better fitting to data. To be conservative, we choose the MSY of 80-100 mt which indicates overexploited condition of *L. calbasu* in Kaptai reservoir and actions should be taken for sustainable management.
Keywords: *population dynamics, CEDA, ASPIC, maximum sustainable yield, exploitation, management*

Abbreviations: ASPIC - A Stock Production Model Incorporating Covariates; *B* - biomass; B/B_{MSY} - ratio of biomass to B_{MSY}; BDM - Biomass Production Model; B_{MSY} - biomass at giving MSY; CEDA - Catch and Effort Data Analysis; CPUE - Catch Per Unit Effort; *CV* - Coefficient of Variation; DoF - Department of Fisheries; *F* - fishing mortality; F/F_{MSY} - ratio of fishing mortality to F_{MSY}; F_{MSY} - fishing mortality rate at MSY; FAD - Fish Aggregating Device; FRSS - Fisheries Resource Survey System; IP - Initial Proportion; *K* - carrying capacity; MF - Minimization Failure; MSY - Maximum Sustainable Yield; *q* - catchability coefficient; *r* - intrinsic population growth rate; R^2 - coefficient of determination; SD - Standard Deviation; SE - Standard Error; SPM - Surplus Production Model

Introduction

Fishery is one of the lion's share consequential sources of revenue and socio-economic industry in Bangladesh as the country is glorified with hundreds of rivers and ditches, large coastal waterbody, a huge portion of wetlands, oxbow lakes, Bay of Bengal etc. Kaptai reservoir locally known as Kaptai Lake (latitude 22°22'-23°18' N; longitude 92°00'-92°26' E, *Fig. 1*) is the largest artificial freshwater resource of South-East Asia (Fernando, 1980; Haldar et al., 1991). It was constructed for hydro-electrical power generation damming the river Karnafuli situated in the Chittagong hill tracts in 1961. However fisheries, flood control, drainage and irrigation are considered as secondary option. The total outer area and average deepness of water is about 68,800 ha

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

and 9 m respectively containing the highest depth of 32 m (Alamgir and Ahmed, 1986; Haldar et al., 2003). The significant role of Kaptai reservoir in fishery production and socio-economic sectors has made this reservoir one of the most potential fisheries support of Bangladesh.

Kaptai reservoir constitutes a major portion of inland water resources. According to the report of Ahmed (1999) it comprises about 46.8% of the entire pond area of Bangladesh offering a huge potential for fish production. The major fish landing sites of the reservoir are shown in *Figure 1*. Ahmed et al. (2001) stated that during their study in this lake, fishery has been found to contribute around 6000 mt annually and the number of engaged fishers were estimated as 5560. Mesbahuddin (1966) documented that a small scale fishers group first started fishing here in 1963 using gill nets, seine nets, hooks and lines. About 74 freshwater species produce around 6000 mt of fish every year (Ahmed et al., 2001; Chakma, 2007). But according to the recent production records, a declining trend has been found in the productivity of high-value fish with the courses of time.

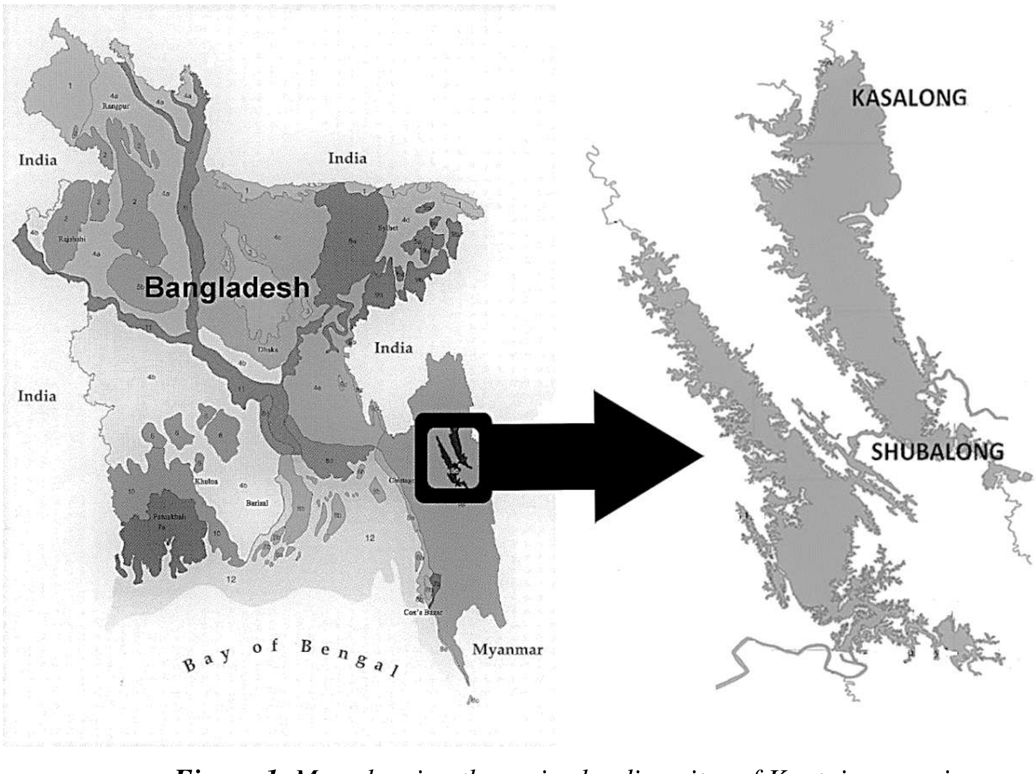

Figure 1. *Map showing the major landing sites of Kaptai reservoir*

Borre et al. (2001) stated that it could happen because the world's lakes suffer from some crucial threats for example quick eutrophication, invasive species, noxious pollution, excess fishing, alteration in water direction, acidic water and altered climate. Remarkably, Kaptai reservoir is also troubled by invasive species, overfishing of certain species and water diversion issue due to regulating electricity. Thus the performance of Kaptai reservoir has been suffering from a host of environmental, socioeconomic and management constraints as well affecting its potential.

Productivity record says that highly commercial important carps such as *Labeo rohita*, *Catla catla*, *Cirrhinus cirrhosis*, *L. calbasu* and *Tor tor* were found to show a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

dramatic decrease and it has been noted that the carp production was about 81% of the total production in 1965/66 while now it is only 5% (Alamgir and Ahmed, 1986). *L. calbasu* is an important profit-making omnivorous fish found in slow running rivers, ponds and lakes and normally attains a length of about 90 cm and weight of 5.5 kg. It is available throughout India except in Kerala (Jayaram, 1999), Bangladesh, Pakistan, Nepal, Myanmar, Burma, Thailand, Yunnan and also South China. Moreover, being a profitable species it is often cultured in South Western China (Jhingran, 1982). The productivity record of 2001-2014 reveals that the annual average yield of carp was about 415 mt while the average production of *L. calbasu* per annum comprises 156 mt (37.6% of total carp production) in this reservoir.

Hitherto, the orange fin *Labeo* along with other species have suffered from blind commercial fishing, dreadful conditions of ecosystem, destruction of habitat, lack of policy implementation, reduction of water level, toxic waste, using of unconstitutional fishing gears, deterioration of breeding ground, catching of juveniles etc. In this regard, the understanding of population dynamics of aquatic resources is essential for getting the maximum benefit and protecting water resources. Undoubtedly, the principal management approaches of a species are to assess its stock. The aim of this assessment includes the understanding of fish population dynamics (Jennings et al., 2001) predicting the fish population for alternative management approaches if needed (Hilborn and Walters, 1992).

In accordance with fish population ecology and economics, MSY (maximum sustainable yield MSY) is the largest harvested sustainable catch from a species' stock under the privileges of current ecological conditions where non-equilibrium biomass production models (BDMs) (also known as surplus production models, SPMs) could be used to assess the MSY value for a fish stock. The stereotyped SPMs which has been used to assess fishery resources have got universality as it is easier to run. MSY parameters can be determined directly from catch and effort (CPUE) data (Polacheck et al., 1993). In spite of being questioned the MSY, estimated from surplus production models has kept increasing its popularity as fishery management target biological reference points (Ricker, 1975; Pitcher and Hart, 1982; Hilborn and Walters, 1992; Prager, 1994; Quinn and Deriso, 1999; Maunder et al., 2006). Nevertheless, the MSY reference points B_{MSY} (biomass at giving MSY, B_{MSY}) and F_{MSY} (fishing mortality rate at MSY, F_{MSY}) are frequently considered as management benchmark (Jacobson et al., 2002).

However, past works have been done focusing on the biological and limnological aspects of Lake Fishery (Chowdhury and Mazumder, 1981; Azadi, 1985; Mahmood, 1986; Hye and Alamgir, 1992; Ahmed et al., 1994) and on its socioeconomic aspects (Ahmed, 1999; Hye, 1988). Although some studies have been done on conservation *L. calbasu* of Kaptai lake (Alam et al., 2000; Haroon et al., 2002; Nahiduzzaman et al., 2012; Hasan et al., 2013; Kabir and Quddus, 2015) but no work has been done yet neither to estimate the sustainable yields nor to assess the stock size of *L. calbasu* in Kaptai reservoir using surplus production models. Studies on population dynamics and assessment of any fishery are of great importance in management. Therefore, it is crucial that fisheries experts deliver a dependable diagram of stock dynamics and stock status to the authorities (Lynch et al., 2012). Thus, different production models have been used in this study aiming at the estimation of the size of mentioned fish production. The study has been performed to draw the required management accesses for its sustainable exploitation which may help fishery administrators and fishery

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

biologists to achieve the target. Therefore, the aim and scope of the article was to provide new perspective of surplus production models for the sustainable exploitation of the high value *L. calbasu* stock in Kaptai lake estimating its MSY.

Materials and methods

Data assemblage

The catch and effort data of Orange fin *Labeo* from 2001 to 2014 were obtained from the data documented by Fisheries Statistical Report of Bangladesh compiled by FRSS (Fisheries Resource Survey System, FRSS), DoF (Department of Fisheries, DoF). The catch data has been presented in weight (mt) while the CPUE in the form of catch captured per boat. The non-equilibrium models have been used to analyze the data. The reservoir is dominated by multi-species fishery and one gear can be used to catch different fishes at a time. Professional, seasonal professional and subsistence fishers use traditional fishing methods and artisanal gears such as seine net, cast net, gill net, lift net, hooks and lines and one type of fish aggregating device (FAD). Mostly Gill net and Sein Net are used with mechanized and non-mechanized vessels engaging for fishing. The mechanized crafts spend 8 to 12 h a day in each voyages, while non-mechanized are operated on daily basis where duration of operation is not fixed. The number of fishing boat fluctuated from 634 to 1702 in the study period. Use of fishing gears and its operating time vary depending on water depth, weather condition, fish abundance, type of fishing grounds, fisherman and vessels as well. Thus, the efforts were taken as fish captured by mechanize and non-mechanized boats per day. However, generally the fishing duration of seine net, gill net, cast net, lift net and FAD are 12 h, 13 h, 4 h, 12 h and 8 h per day, respectively (Roy et al., 2018).

Biomass production models

The available fishery census of *L. calbasu* were analyzed by CEDA (catch and effort data analysis) and ASPIC (a stock production model incorporating covariates) (Hoggarth et al., 2006; Prager, 2005). The Biomass Dynamics Models (BDMs) consist of Schaefer, Fox, and Pella-Tomlinson models.

The most frequently used Schaefer model (Schaefer, 1954) is established on a logistic population growth model (*Eq. 1*):

$$\frac{\mathrm{d}B}{\mathrm{d}t} = rB(B_\infty - B) \qquad \text{(Eq.1)}$$

Later on, considering the Gompertz growth equation Fox suggested the following analysis (*Eq. 2;* Fox, 1970):

$$\frac{dB}{dt} = rB(1nB_\infty - 1nB) \qquad \text{(Eq.2)}$$

However, the final generalized equation (*Eq. 3*) was stated by Pella and Tomlinson (1969):

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DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

$$\frac{dB}{dt} = rB(B_{\infty}^{n-1} - B^{n-1}) \quad \text{(Eq.3)}$$

where B = the biomass, t = time, B_{∞} = carrying capacity, r = intrinsic growth rate

CEDA (version 3.0.1)

The computer package CEDA version 3.0.1 has been built based on non-equilibrium surplus production models (Schaefer, 1954; Pella and Tomlinson, 1969; Fox, 1970). It follows the standard dynamics of the fish production models. Moreover, it also expresses three error assumptions named normal, log-normal and gamma. *CV* (coefficient of variation) of the estimated MSY could be calculated from the output confidence interval. CV of *Tables 2* and *3* were determined by bootstrapping method.

ASPIC (version 5.0)

The ASPIC package denotes the association of both Logistic (Schaefer) and Fox (a special case of GENFIT) production models. This package permits the bootstrap estimation of variability and is efficiently adaptable in case of handling different fishing patterns. Both the CEDA and ASPIC packages require an input value of initial proportion that is IP. Initial proportion defines the progressive fishery data series. IP equal to zero or near to zero implies that the fishery started from maiden state while the value of IP close to one refers to the fishery started from a heavily exploited population. The primary production was about 73% of the maximum catch so IP = 0.7 was used in this study for both CEDA and ASPIC computer packages. R^2 values determine the goodness-of-fit of the model to data.

Results

The observed highest and lowest catch were in 2009 (263 mt) and in 2003 (108 mt) respectively while the average yield was 156 mt in this period. The topmost and undermost values of CPUE were found as 0.255 mt/boat (in 2001) and 0.066 mt/boat (in 2014) respectively (*Table 1*) containing the average CPUE 0.148 mt/boat. It shows the increasing trend of effort causes decreasing rate of catch and CPUE with a slight fluctuation in courses of time.

CEDA result

CEDA has been found to be sensitive towards different IP values. Consequently, it has produced different outputs MSY estimations for different IP inputs (*Table 2*). In some cases, gamma error assumption has been found to exhibit minimization failure in different models (for IP = 0.1, 0.8 and 0.9). However, minimization failure was also observed in Fox model for IP = 0.8. *Table 3* shows the computed parameters for IP = 0.7. According to Fox model the R^2 values of normal, log normal and gamma assumption were 0.71, 0.75 and 0.734 respectively while IP = 0.7 (*Table 3*). On the other hand, the R^2 values calculated by Schaefer and Pella-Tomlinson for all error assumptions remained the same as 0.81, 0.818 and 0.815 orderly.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

Table 1. *Time series catch (mt) and CPUE (mt per boat) statistics (2001-2014) of Labeo calbasu of Kaptai reservoir, Bangladesh*

Year	Catch	Effort	CPUE
2001	192	754	0.255
2002	163	864	0.189
2003	108	670	0.161
2004	189	912	0.207
2005	121	634	0.191
2006	169	998	0.169
2007	149	956	0.156
2008	143	967	0.148
2009	263	1543	0.17
2010	173	1534	0.113
2011	148	1587	0.093
2012	125	1624	0.077
2013	123	1698	0.072
2014	113	1702	0.066
Sum	2179	16443	2.067
Max	263	1702	0.255
Min	108	634	0.066
Avg	156	1175	0.148
MEDIAN	148.5	982.5	0.158
SD	41.184	411.404	0.056
Variance	1696.093	169253.5	0.015
SE	11.007	109.952	0.015
CV	0.265	0.35	0.382

Note: Statistical analysis of base data sets are shown here

Table 2. *MSY (maximum sustainable yield) and CV (coefficient of variation) for Labeo calbasu in Kaptai reservoir, estimated by CEDA for IP = 0.1-0.9*

IP	Model								
	Fox			***Schaefer***			***Pella Tomlinson***		
	Normal	**Log normal**	**Gamma**	**Normal**	**Log normal**	**Gamma**	**Normal**	**Log normal**	**Gamma**
0.1	216	237	MF	357	312	MF	357	312	MF
	0.074	**0.033**	**MF**	**0.186**	**0.155**	**MF**	**0.1**	**0.165**	**MF**
0.2	157	142	142	202	186	189	202	186	189
	0.063	**0.097**	**0.099**	**0.089**	**0.133**	**0.122**	**0.097**	**0.134**	**0.125**
0.3	140	119	136	155	132	146	155	132	146
	0.059	**0.11**	**0.07**	**0.092**	**0.16**	**0.113**	**0.094**	**0.161**	**0.119**
0.4	135	136	132	137	114	130	137	114	130
	0.056	**0.035**	**0.064**	**0.078**	**0.154**	**0.104**	**0.084**	**0.149**	**0.107**
0.5	134	120	130	132	105	127	132	105	127
	0.061	**0.072**	**0.072**	**0.073**	**0.164**	**0.096**	**0.071**	**0.158**	**0.092**
0.6	133	128	126	134	135	130	134	135	130
	0.074	**0.044**	**0.082**	**0.063**	**0.043**	**0.075**	**0.068**	**0.045**	**0.074**
0.7	87	65	67	139	130	137	139	130	137
	0.34	**0.46**	**0.55**	**0.055**	**0.055**	**0.064**	**0.059**	**0.056**	**0.064**
0.8	MF	3	MF	144	131	142	144	131	142
	MF	**1.403**	**MF**	**0.052**	**0.044**	**0.053**	**0.051**	**0.05**	**0.046**
0.9	3.95E-04	3.50E-01	MF	148	145	147	148	145	147
	1.37E+05	**1.47E+02**	**MF**	**0.038**	**0.021**	**0.038**	**0.038**	**0.021**	**0.038**

CV is written below MSY values; MF represents minimization failure

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DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

Table 3. *Parameters computed by using CEDA computer package for Labeo calbasu in Kaptai lake (IP = 0.7) as the initial catch was about 73% of the maximum catch*

Model	R^2	*K*	*q*	*r*	MSY	*B*	R_{yield}	*CV*	B_{MSY}
Fox (Normal)	0.71	2087	1.74E-04	0.114	87	390	74.53	0.339	768
Fox (Log Normal)	0.75	2616	1.34E-04	0.067	65	471	54.45	0.464	962
Fox (Gamma)	0.734	2585	1.73E-04	0.07	67	474	56.332	0.551	951
Schaefer (Normal)	0.81	815	4.17E-04	0.684	139	143	80.734	0.055	407
Schaefer (Log Normal)	0.818	1048	3.19E-04	0.495	130	190	77.147	0.055	524
Schaefer (Gamma)	0.815	878	3.84E-04	0.623	137	158	80.844	0.064	439
Pella Tomlinson (Normal)	0.81	815	4.17E-04	0.684	139	143	80.734	0.059	407
Pella Tomlinson (Log Normal)	0.818	1048	3.19E-04	0.496	130	190	77.148	0.056	524

K: carrying capacity; *q*: catchability coefficient; *r*: intrinsic population growth rate; MSY: maximum sustainable yield; *CV*: coefficient of variation; R^2: coefficient of determination; *B*: current biomass; B_{MSY}: biomass at giving MSY

Fox model determined the values of MSY and CV for normal, log normal and gamma assumption as 87 mt (0.339), 65 mt (0.464) and 67 mt (0.551) respectively whereas values of these parameters were 139 mt (0.055), 130 mt (0.055) and 137 mt (0.064) in Schefer and 139 mt (0.059), 130 mt (0.056) and 137 mt (0.064) in Pella-Tomlinson models in that order. The estimated values of carrying capacity K were higher while the values of catchability coefficient q and intrinsic population growth rate r were lower in Fox model than Schaefer and Pella-Tomlinson models.

The graphical demonstration of observed and expected annual catch values has been shown in *Figure 2*. It illustrates an adjacent relationship between observed and estimated catch values for all the error assumptions used in every model.

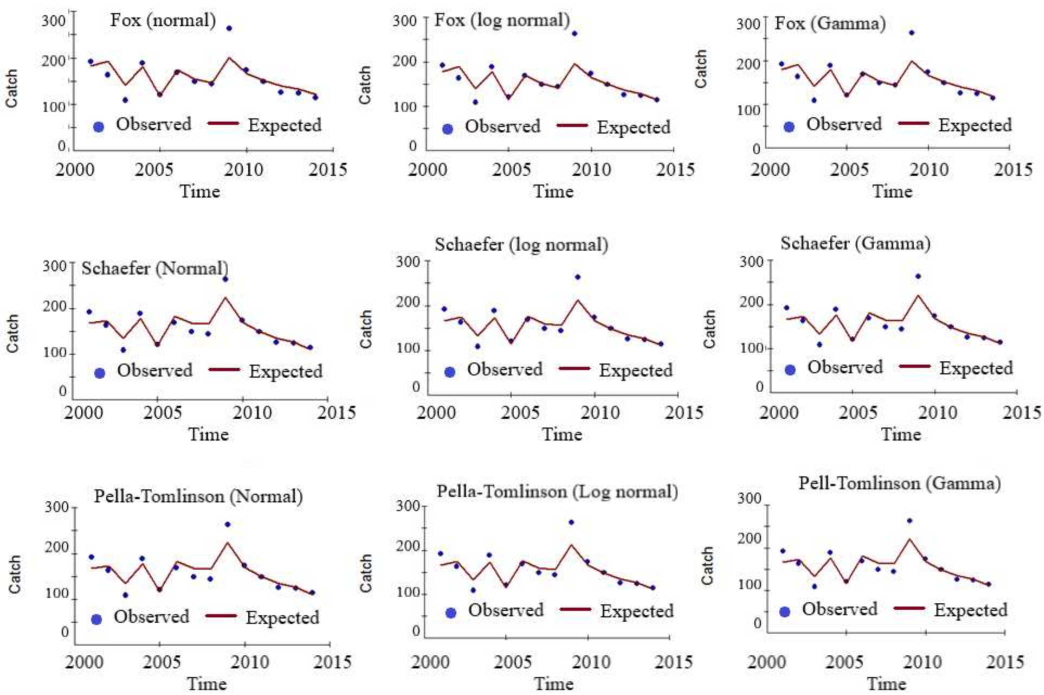

Figure 2. *Annual expected (lines) and observed (dots) catches (mt) using IP = 0.7 from three production models (Fox, Schaefer and Pella-Tomlinson models) and three error assumptions (normal, log normal and gamma) from the CEDA computer package for Labeo calbasu fishery of Kaptai reservoir*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

ASPIC results

Table 4 shows the calculated parameters for IP = 0.7 where MSY and CV values for Fox and Logistic models were 127 mt (0.054) and 133 mt (0.057) respectively. The R^2 value for Logistic model (0.887) was higher than the Fox model (0.879). This result discloses its better fit to data than Fox model. According to Fox model the estimated values of F_{MSY} (fishing mortality rate at MSY, F_{MSY}), B_{MSY} (stock biomass at giving MSY, B_{MSY}) and K were 0.152, 832 mt and 2260 mt respectively while those values for Logistic model have been found as 0.281, 472.7 mt and 945 mt respectively.

Table 4. *Parameters estimated by using ASPIC software for Labeo calbasu in Kaptai reservoir (IP = 0.7)*

Model	*IP*	*R-squared*	*K*	*q*	*MSY*	B_{MSY}	F_{MSY}	*CV*
Fox	0.7	0.879	2.26E+03	2.70E-04	127	832	0.152	0.054
Logistic	0.7	0.887	9.45E+02	3.35E-04	133	472.7	0.281	0.057

MSY (maximum sustainable yield), q (catchability coefficient) K (carrying capacity), F_{MSY} (fishing mortality rate at MSY), B_{MSY} (stock biomass giving MSY), CV (coefficient of variation) and R^2 (coefficient of determination)

Different parameters computed for IP = 0.1 to 0.9 have been shown in *Table 5*. The estimation of different output parameters for different IP input indicates the sensitivity of ASPIC to IP values.

Table 5. *Comparison of ASPIC parameters for Fox and Logistic models by changing the starting initial proportion, (IP 0.1` 0.9) level*

Model	*IP*	*R-squared*	*K*	*q*	*MSY*	B_{MSY}	F_{MSY}	*CV*
Fox	0.1	0.879	2319	2.67E-04	126.5	853.1	0.148	0.06
	0.2	0.879	2324	2.67E-04	126.7	855.1	0.148	0.065
	0.3	0.879	2325	2.68E-04	126.7	855.2	0.148	0.054
	0.4	0.878	2246	2.73E-04	127.1	826.2	0.154	0.063
	0.5	0.879	2333	2.68E-04	126.9	858.2	0.148	0.063
	0.6	0.879	2326	2.68E-04	126.7	855.9	0.148	0.046
	0.7	0.879	2262	2.70E-04	126.7	832	0.152	0.054
	0.8	0.879	2325	2.68E-04	126.7	855.3	0.148	0.075
	0.9	0.879	2328	2.68E-04	126.8	856.4	0.148	0.065
Logistic	0.1	0.887	944	3.35E-04	133.1	472.1	0.282	0.06
	0.2	0.877	13250	1.47E-04	277.4	6624	0.042	0.866
	0.3	0.887	943	3.35E-04	133.1	471.6	0.282	0.06
	0.4	0.887	950	3.33E-04	132.9	475.2	0.28	0.064
	0.5	0.887	938	3.37E-04	133.3	469.1	0.284	0.06
	0.6	0.887	942	3.36E-04	133.2	471.1	0.283	0.067
	0.7	0.887	945	3.35E-04	133	472.7	0.281	0.057
	0.8	0.887	950	3.33E-04	132.9	474.9	0.28	0.064
	0.9	0.887	952	3.33E-04	132.9	475.8	0.279	0.059

q (Catchability coefficient), K (carrying capacity), F_{MSY} (fishing mortality rate at MSY), B_{MSY} (stock biomass giving MSY), CV (coefficient of variation) and R^2 (coefficient of determination)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

The ranges of MSY was 126 mt– 277 mt in ASPIC whereas it was 3 mt – 39500 mt for CEDA which depicts the lesser sensitivity of ASPIC to IP values. Again, the higher value of R^2 using ASPIC software provides its evidence to be better fit than CEDA. ASPIC estimated values of fishing mortality (*F*) and biomass (*B*) of *L. calbasu* for IP = 0.7 have been shown in *Table 6*. It shows an upward trend of fishing mortality rate and descending rate of biomass which has ultimately caused the increasing of F/F_{MSY} and decreasing of B/B_{MSY} in the long run of time. However, the changes in F/F_{MSY} and B/B_{MSY} defines that the overexploitation was being done consistently for the last years.

Table 6. *Fishing mortality (F) and biomass (B) from ASPIC (IP = 0.7) from 2001 to 2014*

Year	Model							
	Fox				Logistic			
	F	***B***	F/F_{MSY}	B/B_{MSY}	***F***	***B***	F/F_{MSY}	B/B_{MSY}
2001	0.242	826	1.59	0.993	0.3	701	1.04	1.48
2002	0.22	761	1.45	0.914	0.272	621	0.965	1.31
2003	0.147	723	0.97	0.869	0.183	582	0.65	1.23
2004	0.267	741	1.75	0.891	0.334	598	1.19	1.27
2005	0.178	677	1.17	0.814	0.223	537	0.79	1.14
2006	0.257	681	1.69	0.818	0.321	546	1.14	1.16
2007	0.24	635	1.58	0.763	0.298	509	1.06	1.08
2008	0.24	608	1.57	0.731	0.294	492	1.04	1.04
2009	0.519	586	3.41	0.705	0.643	482	2.28	1.02
2010	0.429	439	2.82	0.527	0.54	349	1.92	0.739
2011	0.428	372	2.81	0.447	0.537	295	1.91	0.625
2012	0.408	323	2.68	0.388	0.509	257	1.81	0.545
2013	0.451	291	2.96	0.35	0.558	235	1.98	0.497
2014	0.472	256	3.10	0.308	0.583	207	2.07	0.439

F= fishing mortality, *B*= biomass, F/F_{MSY}= ratio of fishing mortality to F_{MSY}, B/B_{MSY}= ratio of biomass to B_{MSY}

Discussion

Study on population dynamics is of great importance since the sustainable management policy of capture fisheries depends on the stock assessment outcomes. The main objective of this study was to estimate the MSY of *L. calbasu* in Kaptai reservoir, Bangladesh through non-equilibrium surplus production models using CEDA and ASPIC software packages. MSY from surplus production models is the reflector of a fishery stock in a certain area and thought as a biological reference point for attaining the sustainable production target (Hilborn and Walters, 1992; Prager, 2002; Musick and Bonfil, 2004). Greater MSY than the recent catch projects mushrooming fishery stock which permits more fishing till the estimated MSY while MSY lesser than current catch determines the resource as overexploited where conservation is must for feasible production. Equal figures for both parameters define the equilibrium status of the fishery. Based on the analysis in *Tables 2–6,* the estimated MSY for Fox model ranged 65-87 mt while Schaefer and Pella- Tomlinson determined ranging 130-139 mt which supports Fox model being more conservative than others.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

On the other hand, the ASPIC estimated value of MSY (127-133 mt) is above than the recent catch (113 mt). Ewald and Wang (2010) has already reported about this uncertainty of MSY. Besides maximum sustainable yield, F_{MSY} and B_{MSY} also play role in sustainable fisheries management. The values of F/F_{MSY} had an upward trend meanwhile values of B/B_{MSY} decreased in the passage of time. Moreover, R^2 values from ASPIC (above 0.879) is higher than that of CEDA (0.71-0.818) which may pinpoint it is better fitting to the computed data. To be conservative we choose the MSY of 80-100 mt in this study, because the latest catch is higher than the MSY value, we found the overexploited condition of *L. calbasu* in Kaptai reservoir and managerial actions for its conservation are required.

Even though MSY is commonly used as biological reference point but when the unexplored CPUE data in indexing fish population abundance is used the interpretation, usage of derived population and management keys should be done carefully (Panhwar et al., 2012). The catch and effort data are required for surplus production models to appraise the status of stock (Mehanna and El-Gammal, 2007). In fact, in fish population and dynamics these models are the first conventional methodical tools of assessing an exploited fish stock when the data consists of year wise catches and some abundance index as well. Although these are not realistic as age-structured models but useful in representing yield policies (Jensen, 2002b). Not only that sometimes they could even produce better estimation of BRP (biological reference points) than age-structured models (Prager, 2002) and comparatively more suitable for management purposes at very practical cost (Haddon, 2011). Statistically the fluctuation in the data of catch, effort or CPUE indicates the changes of stock status. Contradictory relation between effort and catch suggest the swift downturn of fish stock. In some studies on marine fishery show the increasing rate of catch in spite of higher rate of effort (Balli et al., 2011; Kumar et al., 2012; Panhwar et al., 2012; Panhwar et al., 2013; Kalhoro et al., 2013; Mohsin et al., 2017; Karim et al., 2018). Remarkably, in our study greater rate of effort caused the catches to be reduced. The reason behind this consequence is the confined nature of reservoir. So, there is neither migration of fishes from other sources nor as a result lesser recruitment occurs there.

This study examined the current status of orange fin *Labeo* fishery stock of Kaptai reservoir using non-equilibrium SPMs which clearly projects that this fishery is in overexploited condition. In general, surplus production models comprise some unavoidable assumptions, many of which are not met in nature. These assumptions consist of interaction less species, independent r (intrinsic growth rate) over age composition, environmental factors free population, constant catchability coefficient, single stock unit, simultaneous fishing and natural mortality, accurate catch and effort statistics and consistent gear or vessel efficiency. In spite of having above mentioned assumptions its critical usage has made it as a powerful tool for fish stock assessment (Musick and Bonfil, 2005). Ahmed and Hambrey (1999) documented that the use of unlicensed fishing gears as well as non-permissible mesh size and brush shelters causes fingerlings reduction during the post-stocking period. Although every year, the authorities impose a ban on fishing from May 1 to July 31 considering the safety fish breeding and sound production in reservoir but subsistence fishermen and tribal people continue catching fish for home consumption and illegal marketing**.**

In light of the above, the following conservation schemes are suggested:

1. Addressing special legislative framework for Kaptai reservoir fisheries management as well as unbiased enforcement of this constitution

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DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

2. Restricting illegal gears and mesh size
3. Boycotting undersized fish harvest
4. Improving the ecological condition for providing the fishes a healthy home
5. Introducing sanctuary for better and safe growth

In brief, considering the excessive fishing pressure the existing lake fishery should be investigated further wisely for appropriate management.

Conclusion

This is the first attempt to use non-equilibrium SPMs for stock assessment in the reservoir fishery of Bangladesh. The estimated outputs of both CEDA and ASPIC computer packages exhibit that the influential money earning *Labeo calbasu* fishery in Kaptai reservoir is overfished. Uncertainty in determining MSY could happen because of commercial data collection drawback as well as for the apprehensive reliability of CPUE data set. Moreover, there is not enough independent information to test its reliability. Therefore, during the time of interpretation and usage of derived population as well as management parameters obvious tentative steps should be considered. In the light of uncertainties of fisheries science further studies could be done to assess the fish stock accurately along with the studies of the improvement of artificial breeding techniques, modification in genes to increase its adaptability in adverse environmental condition, implementation of different culture techniques etc. This study is to provide an initial concept of stock assessment of *L. calbasu* fishery through surplus production models.

Acknowledgements. The authors certify the support of Chinese Scholarship Council (CSC) and Ocean University of China with immense pleasure.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25192532

POTENTIAL IMPACTS OF WEATHER AND TRAFFIC CONDITIONS ON ROAD SURFACE PERFORMANCE IN TERMS OF FOREST OPERATIONS CONTINUITY

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(Received 6th Nov 2018; accepted 4th Feb 2019)

Abstract. The aim of this study was to evaluate the changes in forest road pavement bearing capacity (PBC) depending on meteorological conditions, traffic effects and horizontal curve parameters for a year on a monthly basis. Within this context, two different roads were investigated and measured with dynamic cone-penetrometer. The total number of the measurement points was 265 for traffic-restricted road (road no: 001-RN1) and 315 for open traffic road (road no: 005-RN2). In the study, three multiple regression models were developed to estimate PBC values on forest road. According to Model1, which was developed to estimate PBC values depending on vehicle traffic and on meteorological factors for alignment section of the RN2, the adjusted R^2 was found to be 0.635. In Model2 for the curve section of the RN2, the adjusted R^2 was found to be 0.711. Model3 for RN1 depending on meteorological factors demonstrated that the accuracy of PBC estimation had a high adjusted R^2, which was 0.952. In conclusion, PBC values can be estimated at high accuracy. Furthermore, traffic load has a strong effect on PBC. On the other hand, temperature has an important negative effect on the variation in PBC on RN1.
Keywords: *forest road, meteorological data, traffic volume, bearing capacity, pavement*

Introduction

Roads provide access for people to work, enjoy, or consider natural ecosystems (Demir, 2007). Forest road ecosystem includes both the paved and unpaved rights of way and adjacent structure, including other infrastructure, ditches, drainage features, and other components that provide the means for vegetation to establish and provide habitat for associated plants and animals (Lugo and Gucinski, 2000). Forest roads have several functions with respect to the management of forestry activities (Acar, 2016; Demir and Hasdemir, 2005). One of these main functions is the transport of timber from its point of felling to the mill. It accounts for a high proportion of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

costs to the industry (Dawson, 2001). For that reason, different heavy vehicles operate on forest roads to manage forestry activities and forest operations. On the other hand, therefore, so as to fulfil these functions, pavement is an important element on forest roads (Akay et al., 2018). However, land degradation and pavement deteriorates in time depending on climate factors, traffic load, maintenance works, slope degree, canopy closure and other factors (Haas, 2001; Tighe et al., 2003; Akgul et al, 2017; Akgul and Hasdemir, 2018; Gokbulak et al., 2018; Sheikh et al., 2017; Yurtseven et al., 2019). Also, pavement deterioration occurs depending on pavement surface compaction rate. Pavement deterioration is the most important factor for traffic safety and safe drive of vehicles. Pavement performance changes depending on deterioration (Akgul et al., 2016).

Pavement deterioration can be slowed down or stopped with proper maintenance. For this reason, it is essential to evaluate the structural condition of a pavement, for example its bearing capacity (Domitrovic and Rukavina, 2013). On the other hand, Kiss et al. (2016) emphasized that in order to prevent significant road deterioration, high bearing capacity is required for the roads which are intensively used by the vehicles. The bearing capacity of a pavement system is defined as the number of wheels passages that it can support before it reaches structural distress (O'mahony et al., 2000). Direct measurement of the bearing capacity is not possible. Instead, the deflection caused by a known load can be measured, and then the bearing capacity can be calculated (Primusz et al., 2015). Most of the devices used to measure the bearing capacity of forest roads express the measurement results with regard to elastic modulus (Kaakkurivaara et al., 2015)

The bearing capacity of a pavement mainly depends on its structure (Trzcinski and Kaczmarzyk, 2006). Besides, the diversity of traffic load and extreme meteorological conditions affect pavement structure, for example its bearing capacity (Bocz, 2009). The stresses caused by traffic load affect each pavement layer differently. For example, it causes deformations and structural changes in the pavement. Climate conditions significantly affect pavement stiffness and bearing capacity (Szentpeteri, 2013). In spring, the bearing capacity of pavement decreases because of the increase in the amount of moisture on subgrade (Charlier et al., 2009; Vestin et al., 2018). The bearing capacity of pavement is easily determined by a Dynamic Cone Penetrometer (DCP). On the other hand, DCP is a simple testing device for measuring in situ compaction, density, strength or stiffnes (Wu and Sargand, 2007; Puppala, 2008).

The objective of the study was to monitor the changes in forest road pavement bearing capacity (PBC) on two different forest roads depending on meteorological conditions, traffic effects (vehicle passages, traffic load, vehicle tonnage etc.) and road sections (horizontal curve and alignment).

Material and methods

Study area

The research area is located in the northern part of Istanbul University's Education Research and Practice Forest close to Sariyer, Istanbul. The research field is at Thracian side of the Marmara Region between 28° 59' 17" – 29° 32' 25" east longitudes and 41° 09' 15" – 41° 11' 01" north latitudes according to Greenwich. Within the scope of the study, two different forest roads [road no: 001 (RN1) and road no: 005 (RN2)] were selected as study areas (*Fig. 1*).

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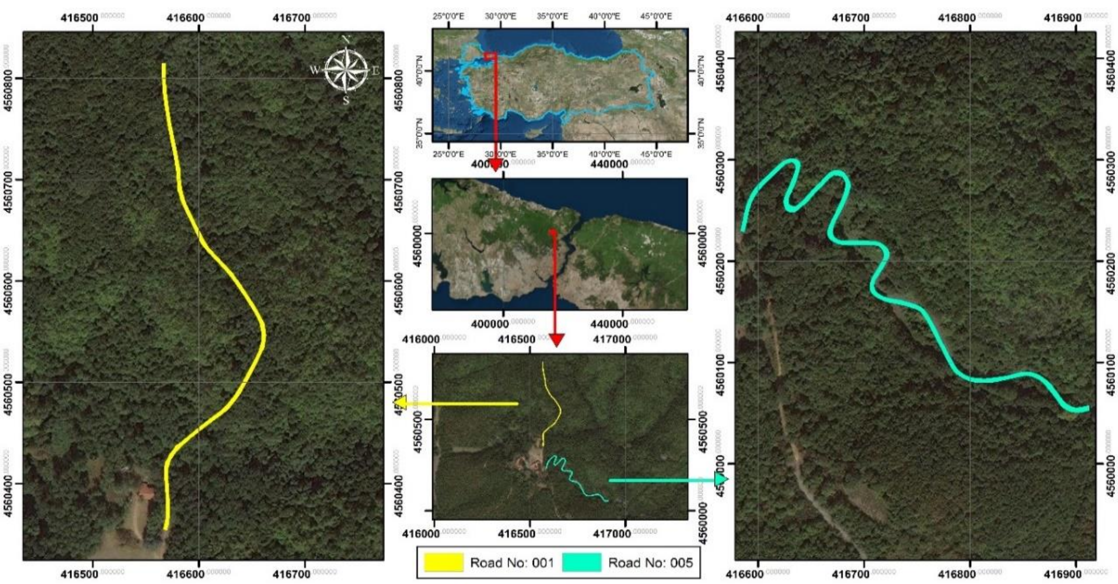

Figure 1. *Location map of the study area*

The RN1 and RN2 were classified as Normal Type-B forest road with 4-m platform width. The total length of the RN1 was 530 m while the total length of the RN2 was 684 m. The average slope of the RN1 was 8%, while the average slope of the RN2 was 12%. The RN1 was composed of nine horizontal curves while RN2 was composed of eleven horizontal curves. The minimum curve radius was 9.0 m while maximum curve radius was 200 m for RN1, and the minimum curve radius was 6.8 m while maximum curve radius was 50.3 m for RN2 (*Table 1*).

Table 1. *Geometrical specifications of roads*

	No	Type	Length (m)	Radius (m)	Direction (grad)	Start station (m)	End station (m)	Delta angle (grad)	Chord length (m)	Degree of curvature by arc (grad)
RN1	1	Alignment	8.521		15.064	0+000.00	0+008.52			
	2	Curve	11.285	39.602		0+008.52	0+019.81	18.140	11.246	48.226
	3	Alignment	31.272		396.924	0+019.81	0+051.08			
	4	Curve	38.758	42.441		0+051.08	0+089.84	58.136	37.425	44.999
	5	Alignment	25.245		55.060	0+089.84	0+115.08			
	6	Curve	78.910	200		0+115.08	0+193.99	25.117	78.399	9.549
	7	Alignment	19.991		29.9432	0+193.99	0+213.98			
	8	Curve	24.327	23.971		0+213.98	0+238.31	64.608	23.296	79.675
	9	Alignment	36.185		365.334	0+238.31	0+274.49			
	10	Curve	35.593	200		0+274.49	0+310.09	11.329	35.546	9.549
	11	Alignment	13.724		354.004	0+310.09	0+323.81			
	12	Curve	18.178	64.663		0+323.81	0+341.99	17.896	18.118	29.535
	13	Alignment	8.440		371.901	0+341.99	0+350.43			
	14	Curve	65.77	200		0+350.43	0+416.20	20.936	65.478	9.549
	15	Alignment	0.386		392.837	0+416.20	0+416.59			
	16	Curve	0.515	9.066		0+416.59	0+417.10	3.614	0.515	210.652
	17	Alignment	56.472		389.223	0+417.10	0+473.57			
	18	Curve	28.090	200.000		0+473.57	0+530.00	8.941	28.067	9.549
	19	Alignment	15.711		398.164	0+501.66	0+517.38			

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

RN2	1	Alignment	21.816		13.081	0+000.00	0+021.82			
	2	Curve	28.317	50.353		0+021.82	0+050.13	35.801	27.945	37.929
	3	Alignment	30.400		48.882	0+050.13	0+080.53			
	4	Curve	22.514	8.161		0+080.53	0+103.05	175.619	16.024	234.015
	5	Alignment	30.161		224.502	0+103.05	0+133.21			
	6	Curve	18.797	6.830		0+133.21	0+152.00	175.195	13.402	279.609
	7	Alignment	40.883		49.306	0+152.00	0+192.89			
	8	Curve	32.824	11.772		0+192.89	0+225.71	177.500	23.178	162.231
	9	Alignment	38.428		226.807	0+225.71	0+264.14			
	10	Curve	22.304	11.294		0+264.14	0+286.44	125.716	18.852	169.096
	11	Alignment	30.312		101.090	0+286.44	0+316.75			
	12	Curve	23.562	11.457		0+316.75	0+340.32	130.919	19.624	166.691
	13	Alignment	22.422		232.010	0+340.32	0+362.74			
	14	Curve	23.553	12.642		0+362.74	0+386.29	118.612	20.292	151.075
	15	Alignment	26.817		113.398	0+386.29	0+413.11			
	16	Curve	21.074	23.375		0+413.11	0+434.18	57.395	20.368	81.704
	17	Alignment	49.803		170.793	0+434.18	0+483.99			
	18	Curve	44.610	33.759		0+483.99	0+528.60	84.123	41.434	56.572
	19	Alignment	28.883		86.670	0+528.60	0+557.48			
	20	Curve	29.577	25.601		0+557.48	0+587.06	73.550	27.96	74.601
	21	Alignment	20.584		160.221	0+587.06	0+607.64			
	22	Curve	23.604	19.822		0+607.64	0+631.24	75.800	22.234	96.350
	23	Alignment	53.464		84.412	0+631.24	0+684.71			

Meteorological data (weather conditions)

Weather data was continuously recorded at the adjacent weather station at the Green Roof Research Site located in Istanbul University Faculty of Forestry. Weather data was measured by an automated weather station (DeltaOhm HD2003). Precipitation measurements (hourly basis) were performed using a rain gauge (DeltaOhm HD2003 tipping bucket, measurement accuracy ±1%). Also, weather data recorded during study period were compared to long term meteorological data between 1929 and 2017 for the research field which were listed in *Table 2* (General Directory of Meteorology-GDM, 2018).

Table 2. *Long term meteorological data from Istanbul (1929-2017) (GDM, 2018)*

	Months												Annual
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	
Mean temperature (°C)	6.0	6.1	7.7	12.0	16.7	21.4	23.8	23.8	20.1	15.7	11.7	8.3	14.4
Maximum temperature (°C)	8.4	9.0	10.9	15.4	20.0	24.6	26.6	26.8	23.7	19.1	14.8	10.8	17.5
Minimum temperature (°C)	3.1	3.1	4.2	7.6	12.1	16.5	19.4	20.1	16.8	12.9	8.9	5.5	10.8
Precipitation (mm)	106.0	77.7	71.4	45.9	34.4	36.0	33.3	39.9	61.7	88.0	100.9	122.2	817.4

Data collection for traffic characterization

Within the scope of the study, camera traps were installed to observe traffic characterization of the RN2. The RN1 was restricted to vehicle passages for one year. The camera traps (Bushnell Trophy Cam, 8MP) were positioned on tree trunks at both the start and end section of the RN2. The camera traps were programmed to take photo at 1 sec interval. The pictures taken by the camera traps were controlled on a monthly basis from September 2015 to September 2016. Vehicle tonnages were calculated in

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DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

four groups (automobile, crossover & SUV, minibus, pickup) according to vehicle types.

Measurement of PBC and data collection

In this study, in order to measure PBC, 53 measurement lines (30 lines in curve section; 23 lines in alignment section) at 10-m intervals were determined along the RN1 while 63 measurement lines (29 lines in curve section; 34 lines in alignment section) at 10-m intervals were determined along the RN2 (*Fig. 2*).

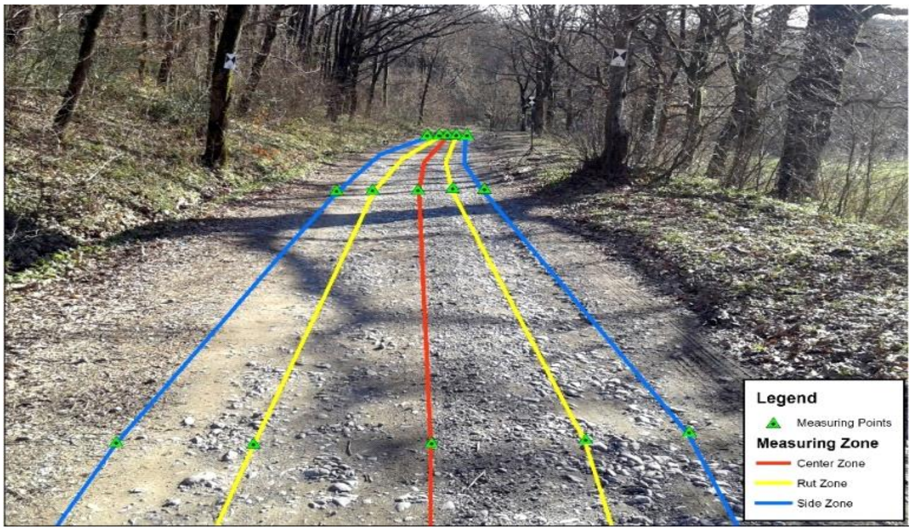

Figure 2. *Penetrometer measuring points*

Five penetrometer measurement points were set on each measurement line with a right angle to the road platform. In total, 265 measurement points (150 points in curve section; 115 point in alignment section) were established for the RN1 and 315 measurement points (145 points in curve section; 170 points in alignment section) were established for the RN2.

The measurement points were fixed with nails (20 cm length). The coordinates of each measurement point were measured by Pentax W800 total station in UTM ED1950 coordinate system (*Fig. 3*).

Figure 3. *Establishment of measurement points*

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PBC was measured using a penetrologger (Eijkelkamp Agrisearch Equipment, the Netherlands). Thirty-degree cones were used with a cone basal surface area of 1 cm^2 (nominal diameter 11.28 mm) (*Fig. 4*). Data collection was conducted from October 2015 to September 2016 on a monthly basis.

Figure 4. *Data collection*

Statistical analysis

All statistical analyses were performed using SPSS 23.0 statistical package. In order to estimate the effects of road geometrical properties on PBC, two different multiple regression models were developed for both road alignments and curves on the RN2. Moreover, multiple linear regression model was developed for the in RN1 to estimate the effects of meteorological parameters on PBC. To evaluate the accuracy of the mathematical model by regression analysis, 80% of the total number of the variables ($N = 1668$ for alignment section of RN2; $N = 1371$ for curve section of RN2; $N = 2540$ for RN1) were randomly selected and used as calibration data, while 20% of them ($N = 432$ for alignment section of RN2; $N = 369$ for curve section of RN2; $N = 640$ RN1) were also used as testing data. Furthermore, paired sample *t*-Test and correlation analysis were used to calculate the significance level of the models.

Results and discussion

Results of meteorological data

According to the meteorological data, the minimum average temperature was measured in February as 6.50 °C while the maximum was measured in August as 24.52 °C. The maximum total precipitation was measured in February as 130.1 mm while the minimum was measured in September as 9.6 mm (*Table 3*).

Results of traffic characteristics

According to the camera traps, 4598 vehicle passages were observed from September 2015 to September 2016 (*Table 4; Fig. 5*). The minimum total vehicle passages were in January as with 80 passages while the maximum total vehicle passages on monthly basis was in September 2016, which reached 1604 passages. Moreover, the average speed of vehicles was calculated as 30 km/h for this road.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

Table 3. *Meteorological data*

Year	Months	Mean temperature (°C)	Total precipitation (mm)	Rainfall duration (h)	Precipitation intensity (mm/h)	Mean pressure (Mbar)
2015	November	19.19	143.4	359	0.40	987.46
	October	13.75	75.5	155	0.48	992.48
	December	10.52	56	223	0.25	991.78
2016	January	6.66	87.3	365	0.240	991.48
	February	6.50	130.1	399	0.33	990.25
	March	10.70	63.4	177	0.36	985.77
	April	11.80	35.6	125	0.28	985.20
	May	15.36	40.6	154	0.26	984.20
	June	18.89	50.8	109	0.46	983.75
	July	23.72	45.1	73	0.62	985.03
	August	24.52	34.3	42	0.82	984.47
	September	23.47	9.6	36	0.27	986.18

Table 4. *Traffic characteristic of RN2*

Month	Year	Automobile (1250 kg)		SUV-Crossover (2000 kg)		Minibus (2000 kg)		Pickup (3300 kg+)		Total passes	Total Tonnage (ton)
		Passes	Tonnage	Passes	Tonnage	Passes	Tonnage	Passes	Tonnage		
September-October	2015	98	122500	28	56000	42	84000	46	151800	214	414300
October-November		106	132500	20	40000	50	100000	22	72600	198	345100
November-December		172	215000	88	176000	70	140000	78	257400	408	788400
December-January		26	32500	6	12000	20	40000	28	92400	80	176900
January-February	2016	90	112500	52	104000	108	216000	70	231000	320	663500
February-March		190	237500	46	92000	88	176000	54	178200	378	683700
March-April		204	255000	60	120000	56	112000	58	191400	378	678400
April-May		110	137500	62	124000	94	188000	66	217800	332	667300
May-June		144	180000	58	116000	100	200000	54	178200	356	674200
June-July		204	255000	16	32000	12	24000	16	52800	248	363800
July-August		140	175000	48	96000	8	16000	4	13200	200	300200
August-September		172	215000	48	96000	8	16000	32	105600	260	432600
September-November		852	1065000	116	232000	408	816000	228	752400	1604	2865400
Mean		**193**	**241154**	**50**	**99692**	**82**	**163692**	**58**	**191908**	**383**	**696446**

Results of PBC on monthly basis

PBC values measured monthly were calculated as mean values and are listed in *Table 5*. It was divided into two main columns as curve section and alignment section for monitoring PBC differences between the RN1 and RN2. The table demonstrates that the measured PBC values of the RN1 were lower than those of the RN2. Furthermore, the measurement results showed that PBC values of the curve sections were relatively lower than those of the alignment sections on the RN2. A comparison of PBC values between the curve section of the RN1 and that of the RN2 revealed that PBC values of the RN2 was almost two times higher than those of the RN1 (*Fig. 6*).

Figure 5. *Sample pictures which were taken from camera traps*

Table 5. *Mean PBC values on curves and alignment for RN1 and RN2*

Month	RN2						RN1					
	Curve section			Alignment section			Curve section			Alignment section		
	Side Zone (MPa)	Center Zone (MPa)	Rut Zone (MPa)	Side Zone (MPa)	Center Zone (MPa)	Rut Zone (MPa)	Side Zone (MPa)	Center Zone (MPa)	Rut Zone (MPa)	Side Zone (MPa)	Center Zone (MPa)	Rut Zone (MPa)
October 2015	1.985	2.000	1.995	2.036	2.037	2.045	0.582	0.594	0.589	0.570	0.563	0.569
November 2015	2.207	2.234	2.233	2.204	2.202	2.213	0.976	0.991	0.984	0.973	0.985	0.988
December 2015	2.080	2.094	2.097	2.321	2.335	2.340	0.860	0.842	0.867	0.872	0.894	0.887
January 2016	1.922	1.950	1.949	2.129	2.134	2.145	0.681	0.691	0.688	0.591	0.600	0.600
February 2016	1.894	1.919	1.919	2.179	2.190	2.197	0.871	0.852	0.864	0.722	0.730	0.722
March 2016	1.811	1.823	1.831	2.157	2.164	2.172	1.049	1.055	1.048	0.987	1.001	0.988
April 2016	1.632	1.650	1.653	1.886	1.905	1.899	0.785	0.779	0.791	0.557	0.570	0.562
May 2016	1.640	1.667	1.667	1.873	1.883	1.887	0.686	0.694	0.688	0.688	0.670	0.695
June 2016	1.915	1.943	1.939	2.030	2.038	2.045	0.664	0.664	0.671	0.868	0.875	0.876
July 2016	1.892	1.921	1.920	1.969	1.980	1.985	0.740	0.745	0.740	0.663	0.678	0.672
August 2016	1.889	1.918	1.917	1.940	1.945	1.959	0.738	0.729	0.735	0.634	0.644	0.639
September 2016	1.970	2.000	1.999	2.073	2.076	2.089	0.588	0.582	0.588	0.494	0.500	0.492
Mean	**1.903**	**1.927**	**1.927**	**2.066**	**2.074**	**2.081**	**0.768**	**0.768**	**0.771**	**0.718**	**0.726**	**0.724**

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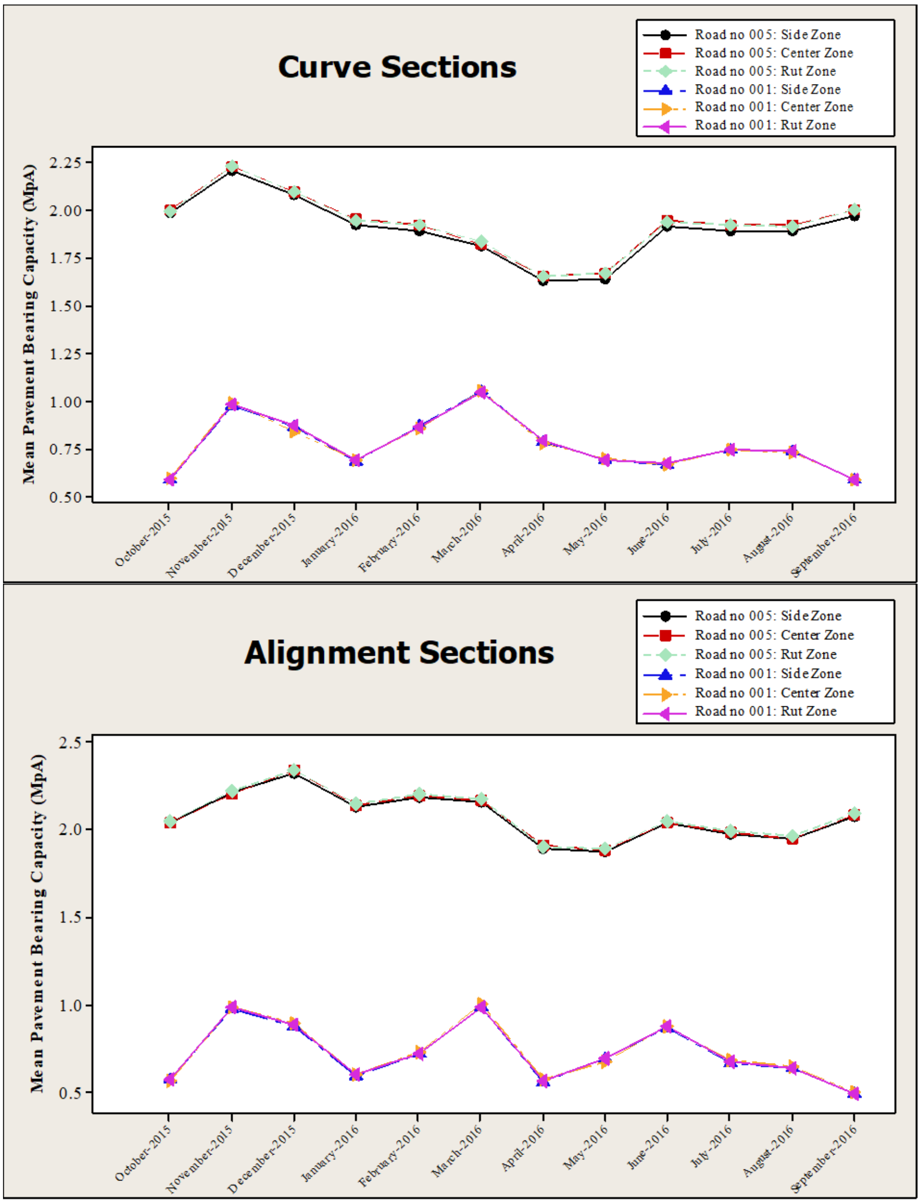

Figure 6. *Mean bearing capacity on curve sections and alignment sections of RN1 and RN2*

Besides, PBC values of the alignment section on the RN2 were found as three times higher than those on the RN1 (*Fig. 7*). PBC values were also observed to be lower in spring than in other seasons.

Results of statistical model

The descriptive statistics of the calibration data used in multiple regression model for the curve section ($N = 1371$) of the RN2 are listed in *Table 6,* while they are presented in *Table 7* for the alignment section ($N = 1668$) of the RN2. The descriptive statistics of the calibration data ($N = 2540$) for the RN1 are listed in *Table 8.*

In order to estimate the effects of road geometrical properties on PBC, two different multiple regression models were developed for both road alignments and curves on the RN2. Moreover, multiple linear regression model was developed to estimate the effects

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of meteorological parameters on PBC on the RN1. In all regression models, PBC was considered as the dependent variable. In the regression model for the alignment section of the RN2; road zone (Z), total precipitation (Tp), tonnage (Tn), passages (Ps) were considered as independent variables, in the regression model for the curve section of the RN2; road zone (Z), curve radius (CuR), total precipitation (Tp), precipitation intensity (Pi), passages (Ps), tonnage (Tn), curve length (CuL), while in regression model for RN1 road zone (Z), total precipitation (Tp), temperature (Tm), pressure (Prs) were considered as independent variables.

At the stage of testing the statistical relationship between the variables for the curve section of the RN2, the results of Pearson's correlation coefficients and their significance levels ($p < 0.01$) and correlation analysis are shown in *Table 9*. It was found that there was a strong positive relationship between PBC and ln(Z), (Z).

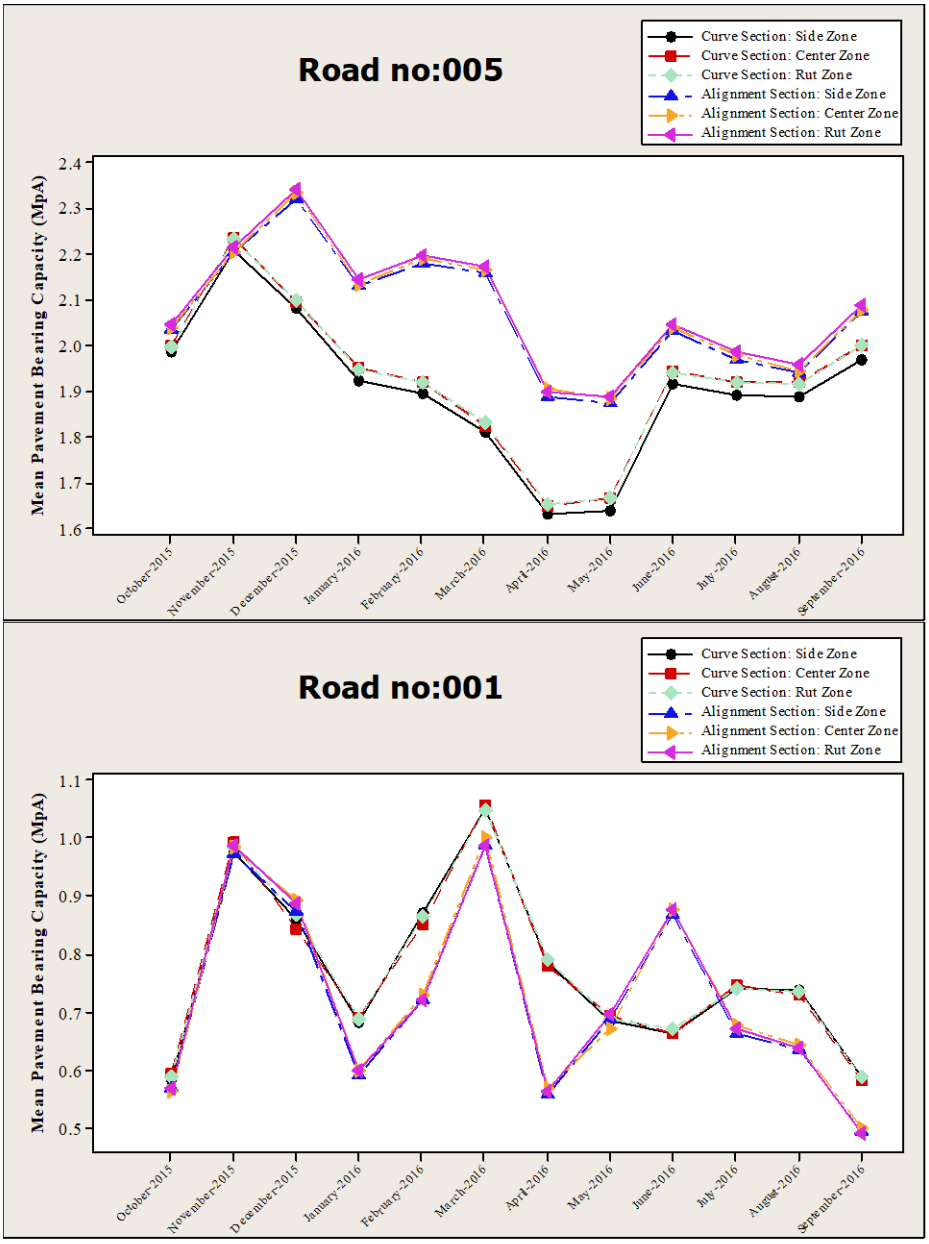

***Figure 7.** Mean bearing capacity on RN1 and RN2*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

Table 6. *Descriptive statistics for curve section of RN2*

RN2 Curve section	N	Minimum	Maximum	Mean		Std. deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
ln(Z) (road zone)	1371	0.693	1.386	0.994	0.006	0.267	0.071
Z (road zone)	1371	2.000	4.000	2.800	0.018	0.749	0.560
CuR (m)	1371	6.830	50.353	21.270	0.312	13.003	169.078
Tp (mm)	1371	9.600	143.400	64.308	0.906	37.803	1429.067
Pi (mm/h)	1371	0.239	0.817	0.398	0.004	0.168	0.028
Ps (passes)	1371	80.000	1604.000	383.170	9.079	378.728	143434.739
Tn (kg)	1371	300.200	2865.400	784.392	16.003	667.537	445605.726
CuL (m)	1371	18.797	44.610	27.882	0.185	1.220	1.488
PBC (MPa)	1371	0.010	3.900	1.918	0.029	7.721	59.612

Table 7. *Descriptive statistics for alignment section RN2*

RN2 Alignment section	N	Minimum	Maximum	Mean		Std. deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
ln(Z) (road zone)	1668	0.693	1.386	0.992	0.007	0.268	0.072
Z (road zone)	1668	2.000	4.000	2.800	0.018	0.752	0.566
Tp (mm)	1668	9.600	143.400	64.507	0.928	37.910	1437.178
Tn (kg)	1668	300.200	2865.400	781.825	16.300	665.697	443152.172
Ps (passes)	1668	80.000	1604.000	382.140	9.246	377.619	142596.100
PBC (MPa)	1668	0.020	3.996	2.080	0.030	1.244	1.547

Table 8. *Descriptive statistics for RN1*

RN1 All road sections	N	Minimum	Maximum	Mean		Std. deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
ln(Z) (road zone)	2540	0.693	1.386	0.995	0.005	0.268	0.072
ln(Tp) (mm)	2540	2.262	4.966	3.968	0.014	0.682	0.466
ln(Tm) (°C)	2540	-1.431	3.200	1.064	0.037	1.852	3.409
ln(Prs) (Mbar)	2540	4.238	6.900	5.785	0.025	1.266	1.602
ln(PBC) (MPa)	2540	-4.605	6.900	2.513	0.078	3.945	15.564

Table 9. *Correlations between variables for curve section of RN2*

	ln(Z)	*Z*	*CuR*	*Tp*	*Pi*	*Ps*	*Tn*	*CuL*	*PBC*
ln(Z)	1								
Z	0.994	1							
CuR	0.009	0.010	1						
Tp	-0.006	-0.007	-0.001	1					
Pi	-0.005	-0.006	-0.005	-0.143	1				
Ps	0.014	0.015	0.018	-0.479	-0.289	1			
Tn	0.015	0.016	0.013	-0.370	-0.329	0.919	1		
CuL	0.004	0.006	0.495	0.017	-0.002	0.004	-0.005	1	
PBC	**0.758***	**0.718***	0.024	0.031	0.029	0.007	0.035	-0.090	1

*Correlation is significant at the 0.01 level

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DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

Also, according to Pearson's correlation coefficients and their significance levels ($p < 0.01$), a strong positive correlation was found between PBC and ln(Z), (Z) (*Table 10*).

Table 10. *Correlations between variables for alignment section of RN2*

	ln(Z)	*Z*	*Tp*	*Tn*	*Ps*	*PBC*
ln(Z)	1					
Z	0.995	1				
Tp	-0.005	-0.005	1			
Tn	0.010	0.011	-0.367	1		
Ps	0.013	0.013	-0.477	0.917	1	
PBC	**0.713***	**0.673***	0.036	0.042	0.019	1

*Correlation is significant at the 0.01 level

Considering the correlation analysis results evaluated for the RN1, their significance levels ($p < 0.01$), there was a weak correlation between PBC and Z unlike the RN2 (*Table 11*). However, a strong negative correlation was found between PBC and Tm, Prs.

Table 11. *Correlations between variables for RN1*

	ln(Z)	*ln(Tp)*	*ln(Tm)*	*ln(Prs)*	*ln(PBC)*
ln(Z)	1				
ln(Tp)	-0.011	1			
ln(Tm)	0.000	-0.072	1		
ln(Prs)	-0.002	0.012	0.973	1	
ln(PBC)	**0.069***	0.003	**-0.951***	**-0.973***	1

*Correlation is significant at the 0.01 level

F test and adjusted R^2 statistics of the multiple linear regression models indicated that all three models were effective predictors of PBC. According to model 1, which was developed to estimate PBC values depending on vehicle traffic effects and meteorological factors for the alignment section of the RN2, adjusted R^2 was found as 0.635. Moreover, in Model 2 developed to estimate PBC values depending on vehicle traffic effects, curve parameters and meteorological factors for the curve section of the RN2, adjusted R^2 was found as 0.711. Model 3, which was developed to estimate PBC values for all sections of the RN1 depending on meteorological factors, adjusted R^2 was found as 0.952 (*Tables 12* and *13*).

Table 12. *Statistical summary of regression model RN1 and RN2*

Road No	Road section	N	Adjusted R^2	Std. error of the estimate	F	Sig.
RN2	Alignment	1668	0.635	0.750	582.219	0.000
RN2	Curve	1371	0.711	0.664	421.854	0.000
RN1	All road	2540	0.952	0.866	12519.627	0.000

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DOI: http://dx.doi.org/10.15666/aeer/1702_25332550

Table 13. Summary of regression model coefficient RN1 and RN2

Model no	Road no	Road section	Model	Unstandardized coefficients		Regression model
				B	Sig.	
Model 1	RN2	Alignment	Constant	-1.214	0.000	*Y = -1.214+18.684ln(Z)-5.519Z+0.002Tp+0.0002Tn+0.0003Ps*
			ln(Z)	18.684	0.000	
			Z	-5.519	0.000	
			Tp	0.002	0.007	
			Tn	0.0002	0.000	
			Ps	0.0003	0.019	
Model 2		Curve	Constant	-1.387	0.000	Y = -1.387+18.336ln(Z)-5.326Z+0.021CuL+0.008CuR +0.002Tp+0.0003Tn+0.427Pi-0.0004Ps
			ln(Z)	18.336	0.000	
			Z	-5.326	0.000	
			CuL	-0.021	0.000	
			CuR	0.008	0.000	
			Tp	0.002	0.005	
			Tn	0.0003	0.000	
			Pi	0.427	0.000	
			Ps	-0.0004	0.002	
Model 3	RN1	All Road	Constant	17.867	0.000	$Y = e^{17.867+1.001ln(Z)-2.849ln(Prs)-0.130ln(Tm)+0.065(Tp)}$
			ln(Z)	1.001	0.000	
			ln(Prs)	-2.849	0.000	
			ln(Tm)	-0.130	0.003	
			ln(Tp)	0.065	0.016	

As it is seen in *Table 12,* according to results of RN1's adjusted R^2 it was possible to closely estimate PBC without traffic effects on forest road.

Also, in the study, developed regression models validated with test data (observation data). Scatter plot for model 1 had a linear correlation with $R^2 = 0.57$ between the observed and predicted PBC ($N = 432$), scatter plot model 2 had a linear correlation with $R^2 = 0.59$ between the observed and predicted PBC ($N = 369$) while scatter plot for model 3 had a linear correlation with $R^2 = 0.96$ between the observed and predicted PBC ($N = 640$) (*Figs. 8, 9,* and *10*).

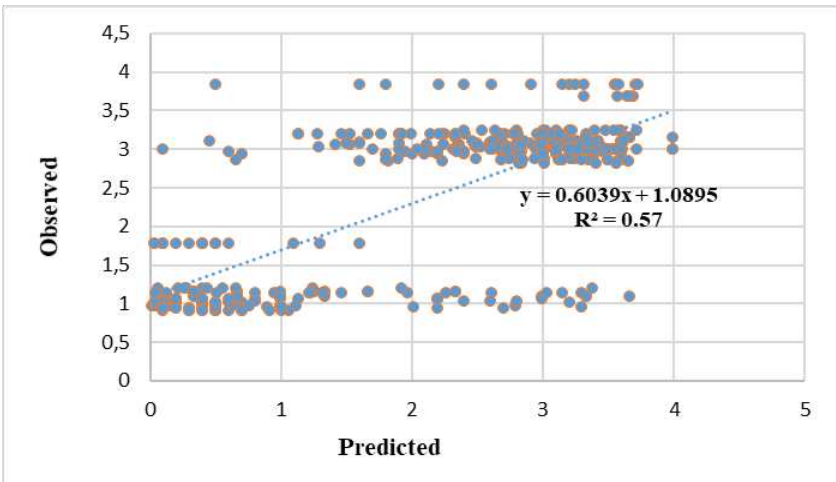

Figure 8. *Validation of predicted and observed PBC values for model 1*

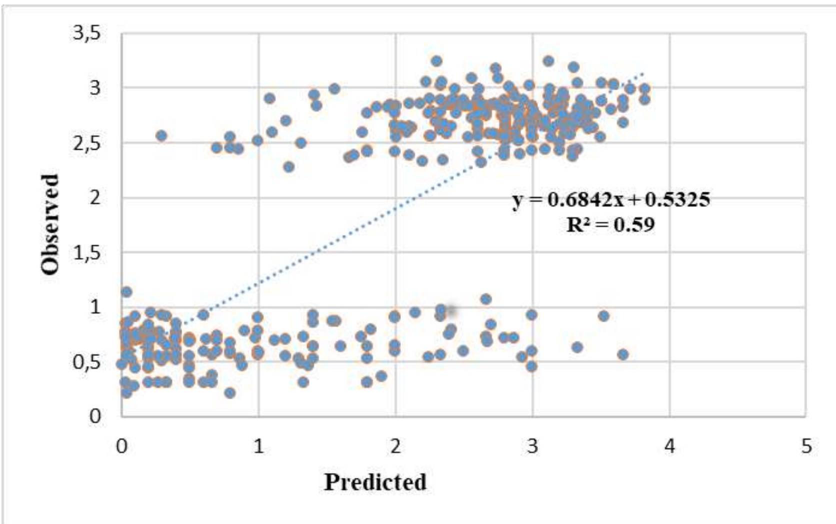

Figure 9. *Validation of predicted and observed PBC values for model 2*

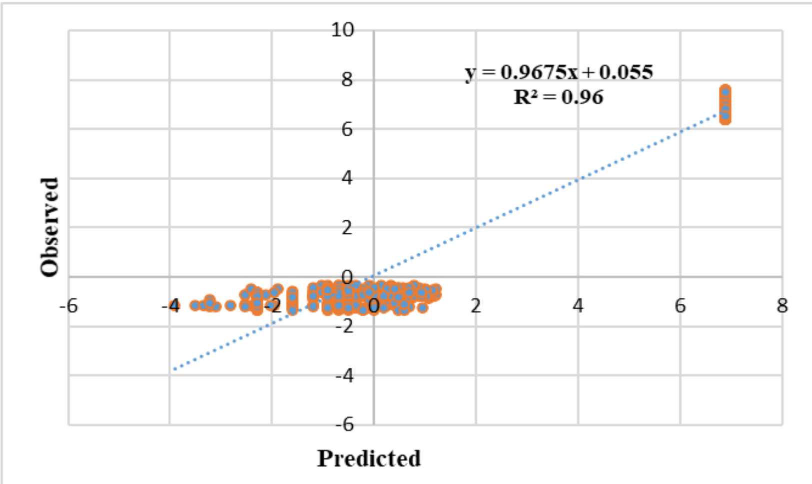

Figure 10. *Validation of predicted and observed PBC values for model 3*

Within the scope of the study, two different forest roads were investigated for monitoring the changes in PBC in one year. One of these roads was restricted to vehicle passages during the study. Hence, on this road (RN1); the effects of meteorological factors on PBC were investigated. Furthermore, on the other road (RN2), the changes in PBC were monitored depending on road sections, traffic effects and meteorological effects.

A comparison of the mean PBC between two roads, it was observed that PBC values on the RN2 were almost two or three times higher in the RN1. The most likely reason might be traffic load on the RN2. Similarly, Săceanu (2012) found a strong correlation between PBC and vehicle passages and tonnage. Our results showed that traffic load (vehicle passages and tonnage) was an important factor. In addition, during the measurement period on the RN2 which is open to traffic, there is a general decrease in the overall PBC values caused by the traffic load. In this regard, Salour (2015) stated that the combination of environmental factors and intensive vehicle traffic increased the

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deterioration of the road structure. The PBC values of the curve sections that we found were relatively lower than those of the alignment sections on the RN2. The possible reasons for that difference might include the changes (braking, accelerating) in the speed of the vehicles in the curve and alignment regions and lateral shifts of vehicles. Moreover, Das et al. (2016) reported that vehicle of placement in curve was different when compared to alignment, and the radius of curve also affected the lateral position of the vehicle.

PBC values were observed to be lower in spring than in other season. In order to explain the reason for this, Charlier et al. (2009) emphasized that the PBC decreases in spring because of the increase in the amount of moisture on subgrade. On the other hand, in connection with this, Yoshida et al. (2016) mentioned the need to have adequate drainage facilities to maintain the sustainability of the bearing capacity. Adlinge and Gupta (2013) also stated that moisture significantly reduced the strength of subgrade. Demir et al. (2012) and Erdem et al. (2018) stated that there is a parallel relationship to between monthly precipitation and sediment production. Monthly sediment production from unpaved forest road was significantly higher than that of paved forest road and undisturbed area. It clearly shows that a stabilizing cover on a forest road led to less sediment production and more soil protection (Demir et al., 2012; Erdem, 2018). The results of previous studies are similar to ours (Kaakkurivaara et al., 2015; Salour, 2015; Grajewski, 2016).

According to the correlation of the results, PBC values had a high negative correlation with Temperature (Tm) on RN1. Similar to these results, Pan et al. (2015) and Motiejūnas et al. (2010) reported that the increase in temperature on asphalt roads led to a decrease in the PBC. On the RN2, there was a strong relationship between PBC and road zone (Z) values both in the curves and alignments. On the RN1, however, there was a weak relationship between PBC and zone (Z). This might be probably because there was no traffic load on the RN1 while there was traffic load on the RN2. According to the correlation results, PBC values had a high correlation with road zone. We have the impression that it was possibly due to the wheel track.

Conclusions

Road surface stability is an important factor on sustainability of forest operations in all seasons. In this context, the PBC value is an indication of the continuity of forest operations. Also, these values and models can be useful to managers for decision making stage in forestry activities.

So, traffic load, especially vehicle tonnage had a strong effect on PBC. On the other hand, temperature had a significant effect on the variation in PBC on the RN1. However, three multiple regression models were developed to estimate PBC values. According to the regression models, PBC values could be estimated at high accuracy. These models will also provide useful results to managers for decision-making in the future. Moreover, fixed measurement points constituted in study can be used to generate pavement compaction maps, PBC changes map etc. for future studies.

Acknowledgements. This paper is supported by the Scientific and Technological Research Council of Turkey (TUBITAK) with the grant number 214O214. Also, authors thank the editor and anonymous reviewers for their constructive comments, which helped us to improve the manuscript.

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EFFECT OF SHADE TREATMENTS ON MORPHOLOGY, PHOTOSYNTHETIC AND CHLOROPHYLL FLUORESCENCE CHARACTERISTICS OF SOYBEANS (*GLYCINE MAX* L. MERR.)

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(Received 11th Nov 2018; accepted 16th Jan 2019)

Abstract. In maize soybean intercropping-system maize plants significantly increase the shade density at soybean canopy, and few people studied the threshold level of shade for sustainable production of soybean in this system. This experiment was started to determine the effect of four different shade treatments T_{75} (75%); T_{50} (50%); T_{25} (25%); T_0 (0%, control) on morphology, physiology and yield of soybean plants. Relative to T_{75}, treatments T_{25} and T_0 significantly increased the stem diameter and stem breaking strength while plant height was decreased. The chlorophyll a, chlorophyll b, and chlorophyll a + b, photosynthetic and chlorophyll fluorescence characteristics were improved as shade decreased, and maximum values were observed in T_{25} and T_0. Similarly, enzymatic activity of Rubisco was accelerated from T_{75} to T_0. However, genes related to sucrose synthesis (SS and SPS) were down-regulated by increasing shade (T_{75}). Importantly, non-significant differences were measured for seed-yield between T_{25} and T_0, and the plants of soybean under T_{25} produced 88% of T_0 yield. Overall, these results implied that agronomist should develop an appropriate intercropping planting pattern where the maximum shade density ranges from 20 to 30 % to obtain higher seed yield of soybean crop under intercropping-system.
Keywords: *Rubisco, intercropping, chlorophyll fluorescence characteristics, sucrose synthesis, seed-yield*

Introduction

Sun radiations are among the most important abiotic factors for agricultural production (Yang et al., 2014, 2018a). For most crop plants, even a slight increase or decrease in light intensity leads to considerable changes in photosynthetic characteristics (Wu et al., 2017). Light intensity affects the central processes of crops such as physiology, biochemistry and cell division (Kong et al., 2016; Yang et al., 2018b; Wu et al., 2018). Indeed, the numerous plant processes impair with decreasing light intensity which bring dramatic developmental and physiological changes, leading to a rapid decrease of these processes (Yang et al., 2015; Wu et al., 2016). Shading conditions could affect carbon balance of crop plant because the carbohydrate (sugars) demand increases while its production decreases: rates of physiological processes rise while the photosynthetic yield reduces (Yang et al., 2018a). Accordingly, tolerance to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

shade stress reduced at low photosynthetic rate in C_3 plants (Su et al., 2014). Moreover, the pattern of carbohydrates (Sugars) into expensive processes, like the biosynthesis of defense proteins (notably light-harvesting chlorophyll protein) raises with increasing shade (Yang et al., 2018a). The photosynthetic rate is the major driver of crop plant carbon balance, optimum and continuous availability of light should also be considered into account to study the plant responses to shade stress.

Crop growth as dry matter production is largely dependent on current photosynthesis and, therefore, one of the main important changes by shade stress in crop growth is ascribed to its huge reduction of net photosynthesis (Yang et al., 2018b). Reductions in photosynthesis could occur thanks to two main principle mechanisms (Yang et al., 2017): (i) decrease CO_2 diffusion into leaves, since the decrease internal and stomatal conductance (g_i and g_s, respectively), and (ii) metabolic potential inhibition for photosynthesis by inhibiting the leaf growth and enlargement by controlling the cell proliferation (Wu et al., 2017, 2018). However, further investigations are needed due to the relative significance of such mechanisms is debatable. The amount and activity of important enzymes involved in CO_2 fixation and regeneration of rubisco-1, 5-bisphosphate (RuBP) determined the metabolic potential of photosynthesis in plants under different conditions (Seemann and Sharkey, 1986; Delfine et al., 1999; Redondo-Gómez et al., 2007) as well as the activity and content of light capturing components, electron transport fragments, and energy transferring enzymes (Kao et al., 2003; Ranjbarfordoei et al., 2006; Stepien and Klbus, 2006). In photosynthesis Rubisco (RuBP carboxylase or oxygenase) catalyzes the process of CO_2 fixation (Mauser et al., 2001), which is directly involved in the first phase of Calvin Benson cycle and accounting for 12 to 35 percent of the leaf protein especially in C_3 crop plants (Evans and Seemann, 1989). In past reports, it has been revealed that the main biochemical restraint involved in shade-associated down regulation of net photosynthetic rate was reduction in the amount or activity of Rubisco (Evans and Seemann, 1989).

In past few years, chlorophyll fluorescence measurements have been known as an informative and useful indicator characterizing different light responses of photosynthesis. Considerable attention was paid to investigate and to determine the important characteristics of this technique (Schreiber et al., 1995). Chlorophyll fluorescence mainly and effectively used to measures the quantum yield of photosystem II and photo-inhibition by determining the potential quantum yield under prevailing light and shade conditions (Rascher et al., 2000). Shade significantly affected the performance and structure of the photosynthetic apparatus (Yao et al., 2017). It blocks the energy transport from PSII to PSI, reduces the leaf thickness, palisade and spongy tissues which results in low chlorophyll fluorescence (Wu et al., 2017; Yao et al., 2017). Thus, in this present study we aim to investigate the chlorophyll fluorescence parameters of soybean plants in response to different shading conditions because plant photosynthetic characteristics are directly dependent on the net performance of chlorophyll fluorescence parameters.

Maize (*Zea mays* L.) soybean (*Glycine max* L. Merr.) intercropping system is the main planting pattern for cereal and legume production (Rahman et al., 2017). Within the maize-soybean intercropping system – described here as the deliberate sowing of soybean crop within the intercropping strip area of maize planting – the presence of maize crop adds a level of complexity in terms of light environment dynamics for resource-use. Importantly, intercropping system of maize and soybean is used extensively in many parts of China and farmers are achieving a land equivalent ratio of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

1.3-1.4 that is much higher than other relay-intercropping systems in the world (Liu et al., 2018). However, soybean plants are extremely sensitive to shading conditions (Wolff and Coltman, 1990; Feng et al., 2018) and in this system soybean plants suffered from severe maize shading during their vegetative growth period from germination to maturity that increased the seedling height of soybean plants and it became more susceptible to lodging as the intensity of shade increases (Li et al., 2014a,b). Physio-morphological responses of soybean plant under shade conditions have also been considered. A number of studies have shown the significant reduction in stomatal conduction, photosynthetic rate, number of nodes, number of seeds and eventually seed yield (24~50%) under shade conditions (Yang et al., 2017). Therefore, great emphasis is placed on crop management with an aim to make plants more efficient in light use and enhance yield under shade conditions. However, the comprehensive understanding of physiology, morphology and gene expression related to carbon metabolism (SS and SPS) is still unclear because they are highly influenced by environmental factors under changing conditions especially intercropping systems.

To investigate the role of shading in regulation of soybean morphology and reproductive development, studies of shade treatments on soybean plants were carried out to (i) determine the impact of different shade treatments on morphological characteristics of soybean plants, (ii) study the effect of different shade treatments on photosynthetic and chlorophyll fluorescence parameters of soybean plants, and (iii) to suggest the threshold level of light intensity required by soybean plants for utilizing the available resources adequately to produce higher soybean seed yield under intercropping conditions. Overall, the main objective of this paper is to provide information for agronomists to develop appropriate planting patterns in which soybean plants receive optimum light intensity for their growth.

Materials and Methods

Plant Material and Growth Condition

Pot experiment was conducted at the greenhouse characterized by a 12 h dark/12 h light photoperiod, 28°C day and 25°C night temperature and approximately 60% relative humidity (in 2018). Experiment was complete randomized design with shade tolerant cultivar of soybean (*Glycine max* L.) Nandou-12 bred by NAAS (Nanchong Academy of Agricultural Sciences) was selected from 30 soybean varieties recognized on the basis of shade tolerance. The ten seeds were sown in pots with dimension of (30 cm diameter, 20 cm height). Soil was collected from the tillage layer of 0-10 cm depth at farm land located in Wenjiang, China. Soil textural class is silty clay loam or silty clay. Chemical properties were: 29.0 g kg^{-1} of SOC, 2.10 g kg^{-1} of total N, 0.78 g kg^{-1} of total P, 16.0 g kg^{-1} of total K, 154 mg kg^{-1} of available K and 7.11 of pH (1 : 5 v/v). The soil was air-dried and sieved through a 4-mm sieve. Basal nutrients were applied at the following rates (mg kg^{-1}): 210 urea, 215 KH_2PO_4, 160 $CaCl_2.2H_2O$, 45 $MgSO_4.7H_2O$, 8 Fe-EDTA, 7 $ZnSO_4$ and 4 $CuSO_4$, 0.8 H_3BO_3, 6.8 $MnSO_4.H_2O$, 11 $ZnSO_4.7H_2O$, 3 $CuSO_4$ .5H_2O, 0.5 $CoSO_4.7H_2O$, 0.4 $Na_2MoO_4.2H_2O$. All the plant-nutrients were mixed thoroughly with sand and soil. After the germination of soybean seeds, three plants per pot were maintained up to maturity and every treatment had 15 pots. In addition, when the first trifoliate leaves developed the soybean seedlings were transferred to four different shade treatments (Shao et al., 2014): T_{75} (75% shade); T_{50} (50% shade); T_{25} (25% shade); T_0 (0% shade, normal sunlight) and the shaded

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

conditions were provided by a covering the pots with black nylon net. The experimental period duration was 110 days. Plants were harvested at maturity and seeds were air-dried for seed yield and yield related parameters.

Measurements and Sampling

Plant Morphological Traits

Plants were collected from each replicate for morphological measurements, the plant height; stem diameter and plant biomass were measured at stages V_4 (four trifoliate of vegetative growth), R_1 (flower initiation at the start of reproductive growth) and R_5 (seed filling at the start of seed formation) of soybean because these are the critical stages which determines the growth and development of soybean. All the plant samples of soybean plants were placed to 105°C for 1 hour and dried to constant weight at 75°C plant biomass. Leaf area was measured by using (CI-203 CID Bio-Science Portable Instruments for Precision Plant Measurement Inc. 1554 NE 3rd Ave, Camas, WA USA). The basal internode (first internode) was used to determine the stem breaking strength of soybean plants by using the digital plant lodging tester (YYD-1, Zhejiang Top Instrument Co. Ltd., Hangzhou, China) according to the previously described method (Liu et al., 2016).

Photosynthetic Pigment Concentration

Latest fully expanded leaves from each treatment were sampled. Chlorophyll (Chl a, Chl b, and Chl a + b) was extracted by grinding samples with chilled mortar and pestle in 10 ml of 80% acetone and centrifuged 1000 rpm for 3 min at 4°C. The samples were placed in ice-cold 10 ml of 80% aqueous acetone solution in the dark for 24 h at room temperature. The supernatant was then separated and analyzed by spectrophotometer (UV-2250, Kyoto, Japan) at wavelengths of 663, 645, and 470 nm as described by Fan et al. (2018) to evaluate the Chl a, Chl b, and Chl a + b content, respectively (Fan et al., 2018).

Photosynthesis

Three fully expanded leaves of soybean plants from each treatment were selected at V_4, R_1 and R_5 stages of soybean. Photosynthetic parameters including net photosynthetic rate (P_n), transpiration rate (T_r) and intercellular CO_2 concentration (C_i) were measured with the help of portable photosynthesis system (*Model LI-6400, LI-COR Inc.,* Lincoln, NE) between 10:00 and 11:00 am. The following settings were used: PARi = 1,000, stomatal ratio = 0.5, flow = 500 µmol mol^{-1} and reference CO_2 concentration = 400 µmol mol^{-1}.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurement was taken by using Fluor Technologia operated using the Fluor Images software. Fully expanded leaf samples from each treatment were taken and immediately preserved in plastic bags and placed in ice box preventing from the direct light. Then by using above mention software, samples were passed to fluorescence analyzing device. We examined maximum quantum yield (Fv/Fm), effective quantum yield of photosystem (ϕPSII), photochemical quenching (qP) and electron transport rate (ETR) by placing 20 min under light and dark

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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conditions. Previously published method was followed by using FluorImager software, Technologia LTD version 2.2.2.2 (Pan et al., 2017).

Rubisco Analysis

For the measurements of Rubisco activated enzyme, frozen leaf samples (1 g) of soybean plants were ground with a mortar and pestle in an ice box and 2 ml of extraction buffer solution was used. The extracted solution was centrifuged 7000 rcf at 4°C for 15 min. Double antibody sandwich method was used to determine the level of plant Rubisco activase (RCA).The Rubisco activase (RCA) antibody was encapsulated by the micropore plate to form solid phase antibody, which was added to the micropore of the monoclonal antibody successively. 40 µl of sample diluent was added first and then 10 ml of sample solution in the micropore plate. The micropore plate was sealed with a plastic film for incubation at 37°C for 30 min and this incubation was repeated 5 times. Body - antigen - enzyme - labeled antibody complex, after thorough washing and substrate TMB color. TMB is transferred under the catalysis of HRP enzyme. Turn it into blue and turn it into the final yellow color under the action of an acid. After adding the stop solution, the absorbance (OD value) was measured within 15 min at 450 nm wavelength by enzyme marker and the sample was calculated by standard curve. The Rubisco activity was expressed as U/g (Seemann, 1989).

Biomass Accumulation and Distribution

For plant biomass accumulation and partitioning in different soybean plant organs, six soybean plants of two pots, were destructively sampled at V_4, R_1 and R_5 stages of soybean. Then all the collected soybean plants were separated into root, stem, leaves, and pod, and placed in oven for one hour at 105°C to kill the fresh-tissues and then dried the samples at 65°C to measure the constant weight before weighing of each plant organ of soybean for total biomass accumulation and partitioning analysis.

Carbon Status and Real-time Quantitative PCR Verification

To measure the starch and soluble sugar content soybean leaves were collected at V_4 stage at the end of the day and analyzed by following the previous method (Cross et al., 2006). Meanwhile, the expanding leaves of soybean plants at V_4 from all the treatments were collected for the determination of RNA abundance. All the leaves were labeled and frozen in liquid nitrogen immediately. Total RNA was extracted with RNAiso Plus (Takara, Japan). Reverse transcription and amplification of cDNA were performed using SuperScript II First-Strand Synthesis for qRT-PCR (Takara, Japan). Real-time quantitative PCR was conducted in CFX 96™ Real-Time System (Bio-Rad, USA) and $2^{-\triangle\triangle CT}$ method used for data analysis.

Seed Yield and Yield Parameters

In the current experiment, all the remaining pots (9 pots, 27 plants) were collected from every treatment at maturity. All these collected sampled were utilized to measure the seed yield and yield components. All the sampled plants were dried in sun-light for seven days, threshed by hand and weighed to measure the seed yield of every treatment and then converted into g plant^{-1}. The seed number plant^{-1} was counted for all the sampled plants and average seed number plant^{-1} was calculated. Three lots of 100 seeds were collected from bulk seed lot of every treatment and dried in oven at 65°C till

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constant weight achieved, and then seed weight (mg) was measured by using an electrical balance and mean weight was calculated.

Statistical Analysis of Data

All the data recorded for every parameter was analyzed using computer software Statistix (version, 8.1. Statistix, USA) (Raza et al., 2018a). Analysis of variance (ANOVA) technique and least significance difference (LSD) test were employed to assess the effect of shading treatments on measured parameters, and all the means were compared at 5% probability level. Moreover, Microsoft Excel program was used for the graphical presentation of data using standard error (± SE).

Results

Morphological Characteristics

Different light treatments considerably changed the morphological characteristics of soybean plants at all growth stages (V_4, R_1, and R_5). *Figure 1* shows the morphological characteristics of soybean plants under different light treatments.

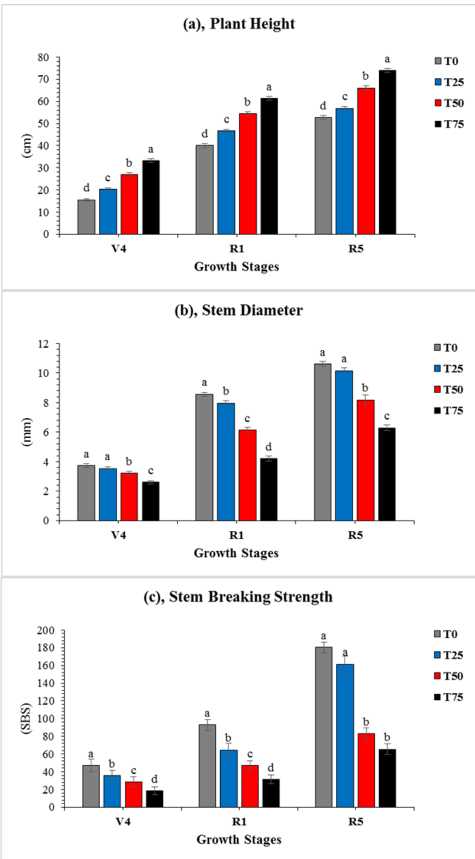

Figure 1. *Changes in plant height (a), stem diameter (b), and stem breaking strength (c) of soybean plants as affected by different photosynthetically active radiations treatments at V_4 (four trifoliate stage), R_1 (flower initiation stage), and R_5 (seed formation stage). T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively. Means are averaged over three replicates. Bars show ± standard errors, (n = 3). Within a bar, different lowercase letters show a significant difference (p ≤ 0.05) between treatments*

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In our study, different light treatments had a significant ($P < 0.05$) impact on plant morphological parameters, the plant height (PH) of soybean plants were substantially increased from T_0 to T_{75} at all growth stages. Specifically, the highest PH 33.2, 61.3, and 73.9 cm of soybean plants were recorded under T_{75}, and the lowest PH 15.6, 40.0, and 52.7 cm were measured in T_{75} at V_4, R_1, and R_5, respectively. Meanwhile, opposite findings were measured for stem diameter (SD) and stem breaking strength (SBS). Compared with T_{75}, SD and SBS significantly increased under T_{25} and T_0, and the SD and SBS of T_{25} and T_0, respectively improved by 41% and 60%, and 38% and 64% at R_5, than those in T_{75}. Importantly, our findings show that the shade from 25% (T_{25}) to 0% (T_0) significantly improves the morphology of soybean plants by increasing the SD and SBS.

Chlorophyll Content

In our experiment, the different light treatments significantly ($P < 0.05$) affected the chlorophyll (Chl a, Chl b, and Chl a + b) content of soybean leaves at all stages. The contents of Chl a, Chl b, and Chl a + b of soybean leaves under T_{75}, T_{50}, T_{25}, and T_0 were measured, as presented in *Figure 2*.

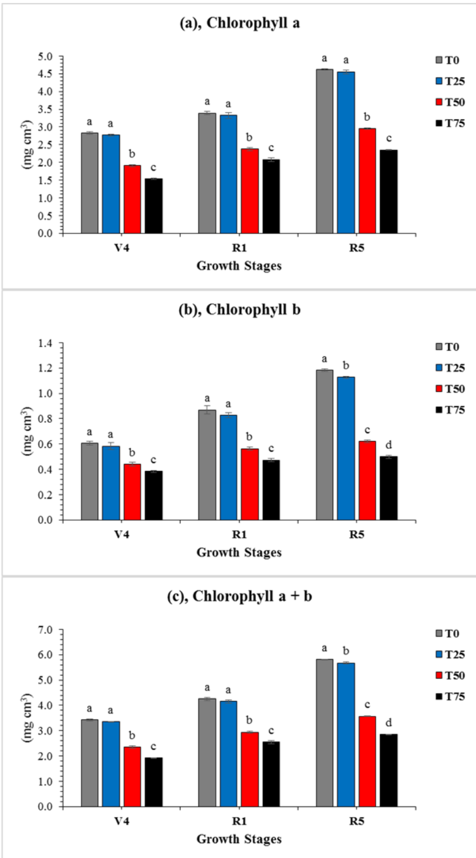

Figure 2. *Changes in chlorophyll a (a), chlorophyll b (b), and chlorophyll a +b (c) content of soybean plants as affected by different photosynthetically active radiations treatments at V_4 (four trifoliate stage), R_1 (flower initiation stage), and R_5 (seed formation stage). T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively. Bars show ± standard errors, (n = 3). Within a bar, different lowercase letters show a significant difference ($p \leq 0.05$) between treatments*

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We observed that decreasing shade from T_{75} to T_0 increased the Chl a, Chl b, and Chl a + b contents at all measured growth stages. Interestingly, the Chl a, Chl b, and Chl a+b contents of soybean leaves were found non-significant between T_{25} and T_0 treatments, but the contents of Chl a, Chl b, and Chl a+b were always found higher under T_{25} and T_0 than those of in T_{75} treatment. Overall, at R_5, the Chl a, Chl b, and Chl a+b contents increased by 48%, 56%, and 50%, under T_{25} in comparison with T_{75}, respectively, suggesting a direct link of chlorophyll contents with the changes in available light intensity.

Photosynthetic and Chlorophyll Fluorescence Characteristics

Table 1 presents the photosynthetic characteristics of soybean plants in response to different light treatments. Relative to T_{75} treatment, photosynthetic rate (Pn) and transpiration rate (Tr) enhanced considerably from T_{50} to T_0 treatments, while stomatal conductance (Gs) and intercellular CO_2 concentration (Ci) of soybean plants were reduced at all V_4, R_1, and R_5 (*Table 1*). The Pn of T_{50}, T_{25}, and T_0 respectively improved by 12%, 31%, and 32% at V_4, 11%, 27%, and 28% R_1, and 7%, 17%, and 22% at R_5 as compared to those under T_{75}. Additionally, all the photosynthetic parameters of soybean plants at R_5 followed the same pattern to that of V_4 and R_1. Importantly, at V_4 and R_1 soybean plants exhibited the non-significant differences for photosynthetic characteristics under T_{25} and T_0, suggesting that shade of 25% to 0% was enough to maintain the optimum photosynthetic rate for better growth and development.

Table 1. *Effect of different photosynthetically active radiations treatments on photosynthetically characteristics and enzymatic activity Rubisco of soybean plants at V_4 (four trifoliate stage), R_1 (flower initiation stage), and R_5 (seed formation stage)*

Stages	**Treatments**	**Photosynthetic Characteristics**				**Rubisco**
		Photosynthetic Rate (μmol CO_2 $m^{-2}s^{-1}$)	Transpiration Rate (mmol H2O $m^{-2}s^{-1}$)	Intercellular CO_2 Concentration (μmol CO_2 m^{-2} s^{-1})	Stomatal Conductance (mol H_2O m^{-2} s^{-1})	Activated (μmol CO_2 g^{-1} FW min^{-1})
V_4	T_{75}	10.17c	0.93c	275.87d	0.37a	0.17c
	T_{50}	11.53b	1.52b	316.50c	0.35a	0.22b
	T_{25}	14.68a	1.77a	319.64b	0.28b	0.27a
	T_0	15.04a	1.81a	367.10a	0.24c	0.28a
	LSD	0.74	0.07	1.10	0.03	0.04
R_1	T_{75}	13.84b	1.92d	324.51d	0.63a	0.20c
	T_{50}	15.53b	2.24c	365.15c	0.56b	0.28b
	T_{25}	19.01a	2.72b	368.29b	0.47c	0.33a
	T_0	19.13a	3.11a	415.75a	0.36d	0.35a
	LSD	2.51	0.12	1.10	0.02	0.04
R_5	T_{75}	18.54d	1.98d	305.70d	0.75a	0.21c
	T_{50}	20.03c	2.65c	372.33c	0.60b	0.31b
	T_{25}	22.41b	3.31b	445.7b	0.51c	0.36a
	T_0	23.85a	3.75a	456.70a	0.46d	0.38a
	LSD	1.00	0.11	2.35	0.04	0.04

T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively

The fate of absorbed solar energy in leaves of soybean at three different growth stages was investigated under changing light conditions, and shows in *Table 2*. In the present study, the chlorophyll fluorescence parameters including F_v/F_m, Φ_{PSII}, qP, and ETR were changed significantly under different light treatments. The F_v/F_m, Φ_{PSII}, qP,

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and ETR of soybean leaves in T_{25} and T_0 were found significantly higher than those under T_{75}. Moreover, treatment T_{25} and T_0, at R_5 significantly increased the chlorophyll fluorescence values of F_v/F_m, Φ_{PSII}, qP, and ETR by 4% and 5%, 17% and 21%, 11% and 13%, and 17% and 21%, respectively, as compared to T_{75} treatment, indicating that adequate light intensity (T_{25} and T_0) plays the fundamental role in improving the chlorophyll fluorescence parameters and photosynthetic capacity of soybean plants, which can help to maintain optimum growth and development under changing light conditions. In addition, the dynamics of chlorophyll fluorescence parameters at V_4 and R_1 stages under different light treatments showed the consistent pattern with those of at R_5 stage.

Table 2. *Effect of different photosynthetically active radiations treatments on chlorophyll fluorescence characteristics (Fv/Fm; quantum yield, ΦPSII; effective quantum yield of photosystem, qP; photochemical quenching, and ETR; electron transport rate) of soybean plants at V_4 (four trifoliate stage), R_1 (flower initiation stage), and R_5 (seed formation stage)*

Stages	Treatments	Chlorophyll Fluorescence Characteristics			
		ΦPSII	qP	ETR	FV/FM
V_4	T_{75}	0.21c	0.40c	92.93c	0.76b
	T_{50}	0.22b	0.42b	97.61b	0.77b
	T_{25}	0.24a	0.43b	102.00a	0.78a
	T_0	0.24a	0.45a	105.54a	0.79a
	LSD	0.01	0.01	4.35	0.00
R_1	T_{75}	0.28d	0.60c	122.26d	0.76d
	T_{50}	0.30c	0.62b	130.62c	0.78c
	T_{25}	0.32b	0.64a	136.99b	0.79b
	T_0	0.33a	0.65a	143.08a	0.80a
	LSD	0.01	0.01	5.40	0.00
R_5	T_{75}	0.31d	0.60c	132.46d	0.77d
	T_{50}	0.34c	0.63b	148.47c	0.78c
	T_{25}	0.37b	0.68a	160.37b	0.80b
	T_0	0.39a	0.69a	166.88a	0.81a
	LSD	0.00	0.01	3.47	0.00

T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively

Carbon status, Rubisco Activity and Gene Expression

To further study the impact of shade treatments on soybean growth, we measured the total soluble sugar and starch content of soybean root and shoot at the end of day. As expected, total soluble sugar and starch content were considerably increased with decreasing shade treatments (from T_{75} to T_0) in both root and shoot. The highest total soluble sugar content 2.45 mg g^{-1} and 6.47 mg g^{-1}, and starch content 0.37 mg g^{-1} and 0.69 mg g^{-1} were determined under treatment T_0 followed by T_{25} (total soluble sugar content 1.95 mg g^{-1} and 6.25 mg g^{-1}, and starch content 0.32 mg g^{-1} and 0.65 mg g^{-1}) in root and shoot, whereas minimum total soluble sugar and starch content were measured in T_{75} treatment (*Figure 3*). In this experiment, a significant difference in Rubisco activity was measured in all treatments. Acceleration in the activity of Rubisco occurred in all treatments from T_{75} to T_0, the amplitude of increase was higher under T_0 than T_{75}, T_{50}, and T_{25} treatments. However, non-significant differences were observed between T_{25} and T_0 treatment for Rubisco activity. Overall, the Rubisco activity of soybean plants under T_{25} was increased by 39%, 40% and 42% at V_4, R_1, and R_5, respectively

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with respect to those under T_{75} treatment. These results indicate that the activity of Rubisco under T_{25} and T_0 showed the similar trend and shade of 25% can be effective at the enzymatic activity of Rubisco. Homologues of soybean sucrose phosphate synthase (GmSPS1*T1* and GmSPS2*T2*) and sucrose synthase (*GmSS*) were selected after blast against Arabidopsis to determine gene expression under different treatments. All three genes for sucrose phosphate synthase and sucrose synthase were up-regulated with increasing light at V_4 stage. Relative to T_{75} treatment, the expression of GmSPS1T_1 and *GmSPS2T2* were increased by 0.34 and 0.42 folds in T_{25}, respectively. While the expression of *GmSS* was enhanced by 2.3 folds in treatment T_{25} than T_{75} *Figure 4*.

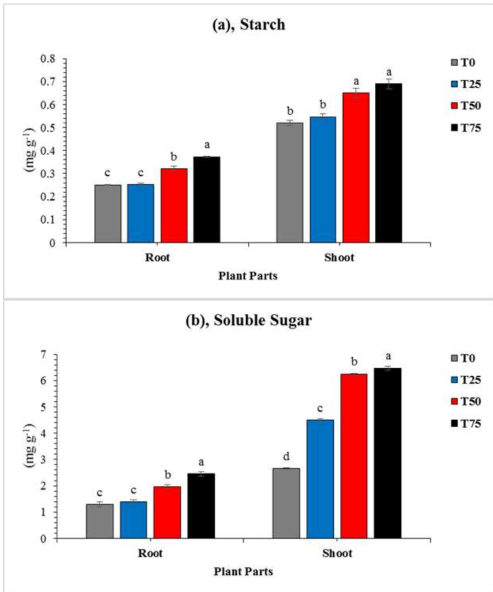

***Figure 3.** Changes in starch content (a) and soluble sugar content (b) of soybean leaves as affected by different photosynthetically active radiations treatments at V_4 (four trifoliate stage). T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively. Means are averaged over three replicates. Bars show ± standard errors, (n = 3). Within a bar, different lowercase letters show a significant difference ($p \leq 0.05$) between treatments*

Biomass Accumulation and Distribution

Changing light conditions significantly affected the total biomass accumulation (TBA) (g $plant^{-1}$) of soybean plants at various stages. In our study, the maximum TBA was 6.7, 22.4, and 35.5 under treatment T_0, while minimum TBA 4.1, 8.5, and 19.4 was observed in T_{75} treatment at V_4, R_1, and R_5, respectively (*Table 3*). However, soybean plants under T_{25} produced 86%, 76%, and 85% of the T_0 soybean biomass at V_4, R_1, and R_5, respectively. Furthermore, we also measured the biomass distribution among root, stem, leaves, and pods at different growth stages of soybean in response to changing light conditions (*Table 3*). At V_4 and R_1 stages of soybean, the maximum biomass partitioning (g $plant^{-1}$) was recorded in stem (3.8 and 13.4) followed by leaves (2.7 and 8.3) and root (0.25 and 0.65) under T_0, while at R_5 biomass distribution pattern was changes and the highest biomass was found in leaves (16.2) followed by stem (14.9), pods (2.6) and root (1.7) in T_0 treatment. Compared with T_{75} treatment, soybean plants under T_{25} and T_0 treatments, respectively obtained 40% and 35% higher pod biomass at R_5 stage.

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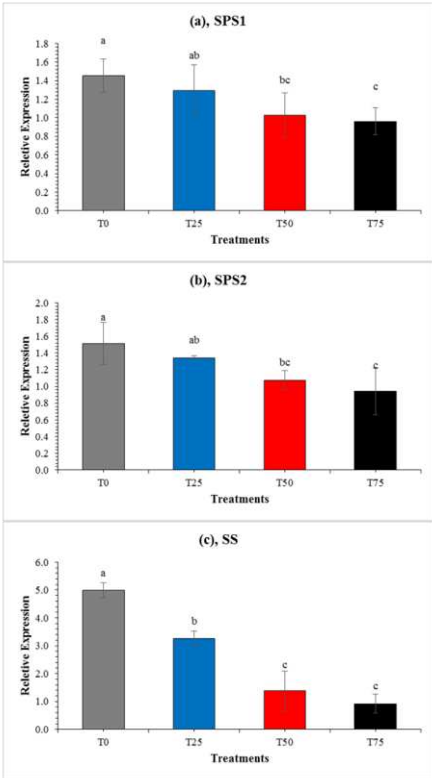

Figure 4. *Relative expression of sucrose phosphate synthase Gmsps1, (a), Gmsps2, (b) and sucrose synthase Gmss (c) genes of soybean leaves as affected by different photosynthetically active radiations treatments at V_4 (four trifoliate stage). T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively. Means are averaged over three replicates. Bars show ± standard errors, (n = 3). Within a bar, different lowercase letters show a significant difference ($p \leq 0.05$) between treatments*

Table 3. *Effect of different photosynthetically active radiations treatments on biomass partitioning (g plant^{-1}) among different plant parts and total biomass accumulation (g plant^{-1}) of soybean plants at V_4 (four trifoliate stage), R_1 (flower initiation stage), and R_5 (seed formation stage)*

Stages	Treatments	Biomass Partitioning				TBA
		Roots	Stem	Leaves	Pod	(g plant^{-1})
V_4	T_{75}	0.19d	2.43d	1.39d	-	4.01d
	T_{50}	0.22c	2.60c	1.72c	-	4.54c
	T_{25}	0.23b	3.38b	2.20b	-	5..81b
	T_0	0.25a	3.80a	2.70a	-	6.75a
	LSD	0.00	0.15	0.25	-	0.33
R_1	T_{75}	0.38c	5.16d	2.95d	-	8.50d
	T_{50}	0.41c	6.43c	4.28c	-	11.13c
	T_{25}	0.53b	10.46b	6.13b	-	17.13b
	T_0	0.65a	13.43a	8.32a	-	22.41a
	LSD	0.03	0.28	0.17	-	0.42
R_5	T_{75}	0.74d	7.73d	9.33c	1.59d	19.39d
	T_{50}	0.91c	9.49c	11.27b	2.05c	23.73c
	T_{25}	1.31b	11.48b	15.04a	2.45b	30.29b
	T_0	1.68a	14.92a	16.23a	2.65a	35.49a
	LSD	0.02	0.75	1.38	0.12	1.58

T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively

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Yield and Yield Components

In our study, there was a significant impact of different light treatments on seed yield of soybean plants *Figure 5*. The highest seed yield, 15.1 g plant^{-1}, was recorded in T_0 treatment. Relative to T_{75}, soybean plants under T_{25} and T_0 produced the 22% and 31% higher seed yield. Yield parameters also varied among different light treatments. The effects of light treatments on pod number (plant^{-1}), seed number (plant^{-1}) and seed weight (mg) were significant, and pod number and seed number under T_0 treatment were found significantly higher than that in T_{75}, T_{50}, and T_{25}. Meanwhile, seed weight was found considerably heavier in T_{75} as compared to treatment T_0. Overall, light treatment T_{25} and T_0 increased the pod number and seed number by 37% and 44%, and 38% and 45% as compared to treatment T_{75}, respectively, while T_{75} treatment enhanced the seed weight of soybean seeds by 21% than T_0 treatment *Figure 5*.

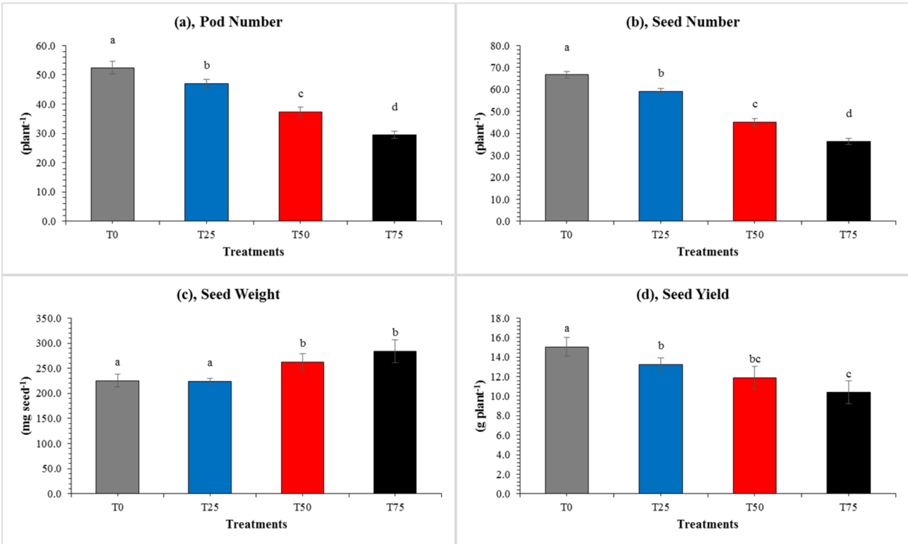

Figure 5. *Changes in pod number (a), seed number (b), seed weight (c), and seed yield (d) of soybean plants as affected by different photosynthetically active radiations treatments at V_4 (four trifoliate stage). T_{75}, T_{50}, T_{25}, and T_0 refer to 75%, 50%, 25%, and 0% shade, respectively. Means are averaged over three replicates. Bars show ± standard errors, (n = 3). Within a bar, different lowercase letters show a significant difference ($p \leq 0.05$) between treatments*

Discussion

The plant morphology has certain manipulability, and corresponding adaptation mechanisms present in changing environmental conditions (Gong et al., 2015). Use of higher planting density is the effective method for enhancing seed yields (Liu et al., 2015a). However, this practice is typically obstructed by impair light conditions (Li et al., 2014a). Numerous experiments have reported that shading conditions favor the upward growth of stem while reducing the stem breaking strength and stem diameter (Kurepin et al., 2007; Gommers et al., 2013; Yang et al., 2014). However, very few studies have Paidattention on the effect of changing shade treatments on soybean morphology to understand the threshold level of shade which will not affect the optimum soybean growth. In this study, a gradual decrease in shade (from T_{75} to T_0) significantly increased the stem diameter and stem breaking strength by maintaining the

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DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

optimum plant height of soybean plants (*Figure 1*). These findings showed that any variation in available light at soybean canopy directly affect the morphological characteristics of soybean plants and improved light availability positively improved the soybean growth by enhancing stem breaking strength which reduced the soybean lodging (Liu et al., 2016). Importantly, soybean plants under treatment T_{25} obtained superior morphological characters than T_{75} and T_{50}, suggesting that under 25% of shade soybean plants can maintain their optimum growth. Sun-light stimulates the plant growth and development; by photosynthesis process, plants use sun-light to convert H_2O and CO_2 into carbohydrate, photosynthetic pigments (Chl a, Chl b, and Chl a+b) playan important role in changing the solar energy to chemical energy (Liang, 2000; Yuncong et al., 2007). In changing light conditions, the study of Chl a, Chl b, and Chl a+b helps as an indices for sun-light absorption (Fan et al., 2018). In past reports, researchers have confirmed that Chl contents significantly affected by changes in light availability and decrease with the reduction in light (Li et al., 2014a,b). Similarly, significant variations were measured for Chl a, Chl b, and Chl a + b contents in all shade treatments, and chlorophyll contents were enhanced from 2.4 to 4.6 mg cm^3 with the decrease in shade from T_{75} to T_0, respectively, our results are consistent with previously reported results (Wittmann et al., 2001; Fan et al., 2018). On the other hand, several scientists have claimed that Chl a, Chl b, and Chl a + b contents increase with the increase in shade, especially Chl b contents (Li et al., 2014a).

In addition to the impacts of changing light conditions on morphology and chlorophyll contents our results showed that deleterious effects of increase shade eliminated by adequate light availability (treatment T_{25} and T_0). There are many causes why soybean plants under high shade conditions produced less carbohydrate. For instance, researchers have concluded that assimilate demands of soybean plants increased while photosynthetic rate decreased under increased shade conditions (Su et al., 2014; Yang et al., 2017). In this experiment, optimum light (T_{25} and T_0) at soybean canopy led to increase the photosynthetic rate and transpiration rate while stomatal conductance and intercellular carbon dioxide levels were decreased. Therefore, this showed that the enhanced photosynthetic characteristics increased the carbon gain and improved the soybean growth (Liao et al., 2005). Moreover, these findings indicating that the increased photosynthetic rate under T_{25} and T_0 treatments may be associated with the higher chlorophyll contents (i.e. higher Chl a, Chl b, and Chl a+b contents at V_4, R_1, and R_5, (*Figure 2*) of soybean leaves (Feng et al., 2018). Improved photosynthetic capacity is always accompanied with high quantity of electrons passing through PSII (Yao et al., 2017). Chl fluorescence parameters are one of the major factors in regulation of photosynthesis and crop responses to environmental conditions because of its sensitivity and convenience (Dai et al., 2009). In addition, total soluble sugar and starch content are the direct measure of high photosynthetic rate (Iqbal et al., 2018a). Crops translocate carbohydrate from source leaves to different plant parts that decides the plants fitness under changing environments (Amiard et al., 2005). Numerous previous experiments confirmed the role of light intensity for the production of total soluble sugar and starch content in plants (Preiss, 1982; Michalska et al., 2009). Similarly, we found that the total soluble sugar and starch content were considerably improved by 33% and 144% in T_0 than T_{75}. These results were in agreement with past reports (Pilkington et al., 2015). Previous experiments have confirmed that heavy shade results in reduce photosynthesis due to the decrease in qP, PSII and ETR (Huang et al., 2011; Yao et al., 2017; Feng et al., 2018). In this experiment, similar results were

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obtained, however, improved Chl fluorescence characteristics were measured under T_{25} and T_0 treatments. These results reveals that optimum light intensity (treatment T_{25} and T_0) improves the efficiency of PSII and ETR that could enhance the photosynthetic capacity of soybean plants by improving the energy transport from PSII to PSI, our results are consistent with previously reported results (Yang et al., 2018a). The loss of rubisco activity was recognized to be very early and fast response of crop plants to shade stress (Servaites et al., 1986). While, at R_5, in current research the activity of rubisco was significantly accelerated in T_0 (0.38 μmol CO_2 g^{-1} FW min^{-1}) and T_{25} (0.36 μmol CO_2 g^{-1} FW min^{-1}) as compared to T_{75} (0.19 μmol CO_2 g^{-1} FW min^{-1}) (*Table 1*), our findings are consistent with past reports (Carmo-Silva and Salvucci, 2013). This higher rubisco activity under decreased shade treatments (T_{25} and T_0) exhibited that the higher photosynthetic rate of soybean plants directly correlated with rubisco activity under changing environments (Zhang et al., 2002).

This study provides the important data on biomass accumulation in soybean plants in different shade treatments (*Table 3*). Increased photosynthetic rate is one of the main factors for plant biomass production (Raza et al., 2018b). Previously, researchers have found that biomass accumulation is directly associated with the availability of light intensity (Kiniry et al., 2004) and reductions in light decreased the biomass production (Maddonni and Otegui, 2004). Our findings proposed that an adequate light availability (T_{25} and T_0) at soybean canopy can increase biomass accumulation by capturing and utilizing sun-light for their biochemical and physiological processes which in turn increased availability of carbohydrates for seed formation and this finding was similar to previous result (Liu et al., 2015b). Moreover, the accelerated dry matter production in soybean may be related to the utilization of major nutrients (Raza et al., 2018a) as the nutrient uptake ability of crops increased with improve light conditions (Yang et al., 2017). Additionally, we also measured the biomass partitioning in leaves, stem, pods, and seed of soybean plants in response to different shade treatments (*Table 3*). The dry matter partitioning changed considerably at V_4, R_1, and R_5 in soybean in all treatments. At V_4 and R_1, when reproductive parts were the weak sink the highest allocation of plant-biomass was determined in stem followed by leaves (Jasdanwala and Khan, 1988; Srinivasan et al., 2017). After that, at R_1 and R_5, the plant-biomass distribution changed and the most of plant-biomass assimilated to the leaves and pods in all treatments (Couch et al., 2017). Similar to our findings researchers have reported that optimum light intensity increased the assimilation of plant-biomass to economic parts, while decreased the dry matter partitioning to vegetative organs (Jasdanwala and Khan, 1988). Nevertheless, severe shading conditions (T_{75} and T_{50}) increased the retention of plant-biomass in stems (Wu et al., 2017) which did not promote the seed formation process and decreased the seed yield (*Figure 5*). These findings of plant-biomass partitioning demonstrated that accelerated translocation of photo-assimilates from the stem for pod formation occurred in soybean plants of treatments T_{25} and T_0, while severe shading conditions was not favorable to plant-biomass partitioning to pod.

Previously, it has been found that severe shading conditions significantly decreased the soybean yield and yield components (Wu et al., 2016; Iqbal et al., 2018b). Similarly, in our study, treatment T_{75} (severe shading) had significant negative effect on seed yield and yield related parameters, and minimum pod number (29.9 $plant^{-1}$) and seed number (36.4 $plant^{-1}$), and seed yield (10.4 g $plant^{-1}$) of soybean were noticed in T_{75} treatments. This is might be due to the lower photosynthetic rate and biomass accumulation, similar to our results in another study of relay-intercropping scientist has reported that soybean

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

seed yield of soybean plants significantly decreased in severe shading conditions as compared to normal conditions (Yang et al., 2017). Moreover, light enrichment treatments significantly increased the pod number and seed yield of soybean. Hence, pod number (52.4 and 46.9 plant^{-1}) and seed number (66.7 and 59.1 plant^{-1}) of soybean plants might be improved in adequate light (T_{25} and T_0, respectively), these results implied that optimum light availability at soybean canopy can significantly improve the morphological parameters, photosynthetic and chlorophyll florescence characteristics which in turn considerably increased the seed yield of soybean plants by increasing the pod number and seed number.

Conclusion

The significant impacts of shade treatments on soybean have been studied previously, but rarely researchers have investigated the effects of different shade treatments on soybean plants to understand the threshold level of shade for the better growth and development. In this experiment, we showed that by selecting the shade tolerant variety soybean plants can cope shade up to 25% (T_{25}). Increased shade (T_{75}) considerably impaired the morphological characteristics (*Figure 1*), enzymatic activity of key enzyme (Rubisco) by down-regulating the important sucrose-synthase-genes (*Figure 4*). In addition, as compared to T_{75}, treatment T_0 and T_{25} significantly improved the photosynthetic and chlorophyll florescence characteristics of soybean plants especially quantum yield of PSII which in turn considerably increased the seed yield and yield-components (*Figure 5*). Overall, these results implied that agronomist should have to develop an appropriate intercropping planting pattern where the maximum shade density ranges from 20 to 30% to obtain higher seed yield of soybean crop under intercropping-system. This could be achieved by developing the narrow-wide row planting pattern in which maize plants can grow under narrow rows and soybean plants can grow in wide rows by maintaining the appropriate distance in which soybean plants can receive enough light for their optimum growth. In addition, developing the long term and environment friendly agronomic approaches to improve the seedling growth of soybean under intercropping systems by reducing the shade density in maize soybean intercropping system is an important direction for future research.

Conflict of interests. The authors have declared no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25512569

EFFECT OF SELECTED HEAVY METAL IONS ON THE GROWTH OF ENTOMOPATHOGENIC FUNGI FROM THE GENUS *ISARIA*

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(Received 12th Nov 2018; accepted 25th Jan 2019)

Abstract. This paper examines the effects of selected heavy metals: zinc, nickel, copper, cadmium and lead, on the growth of three species of entomopathogenic fungi of the genus *Isaria* Pers.: *I. farinosa* (Holmsk.), *I. fumosorosea* Wize, and *I. tenuipes* Peck. Ions of metals were added to the Sabouraud medium in three concentrations: 1× - concentration corresponding to the natural content of the given metal in Polish soils; 10× - concentration 10 times higher, and 100× - concentration 100 times higher than the natural content. Heavy metal ions in concentration 1× did not significantly restrict the growth of fungus colonies, and in some cases caused a slight growth stimulation. The greatest toxic effect of heavy metal ions on the entomopathogenic fungi was observed when their concentration was 100 times higher than the natural content. Nickel had the greatest inhibitory effect on the growth of fungal colonies, while lead showed the least effects. The fungal species most sensitive to the presence of heavy metals in the medium was *I. tenuipes*, while *I. fumosorosea* had the highest tolerance.
Keywords: *insect-pathogenic fungi, metal-resistance, mycelial growth, Hypocreales, soil*

Introduction

Heavy metals are considered one of the most dangerous environmental pollutants created as a result of human economic activities, both agricultural and non-agricultural (Kabata-Pendias and Pendias, 2001; Goyer, 2001; On et al., 2005; Micó et al., 2006). In the soil they react with other chemicals, accumulating in different forms with differentiated bioavailability to plants (Singh and Kalamdhad, 2011).

The solubility of heavy metals in soil, and hence their biological effects, decreases with increasing content of organic matter or clay minerals, and increases under higher acidity (Kabata-Pendias and Pendias, 1999, 2001). Metals in natural concentrations are often essential in the functioning of living organisms, but often have toxic effects when present in excessive amounts (Badura and Piotrowska-Seget, 2000; Singh and Kalamdhad, 2011). Additionally, their presence in soil has a major impact on cellular structures, growth and development, and biological activity of soil microorganisms (Badura and Piotrowska-Seget, 2000; Tscherko et al., 2007; Hassn et al., 2014).

Although heavy metals naturally occur in the soil environment, presently there are many factors significantly contributing to their increase such as industrial activities, and perhaps most importantly, the intensification of agricultural production using chemical-dependent farming methods (Lenart and Wolny-Koładka, 2013). The European Parliament and Council Regulation (EC) No 1107/2009, states the use of integrated plant protection natural alternatives should be considered a priority instead of the use of chemical ones (Tomalak, 2010). The use of products with biological origin based on

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25712582

micro-organisms such as entomopathogenic fungi are becoming increasingly important (Sheeba et al., 2001; Meyling and Eilenberg, 2007; Tkaczuk, 2008; Batta et al., 2011; Schemmer et al., 2016; Showket et al., 2017).

Entomopathogenic fungi are a very important component of the environment and infect many species of arthropods causing disruption of physiological processes in the host followed by mortality (Hajek and Leger, 1994; Chandler et al., 2000; Quesada-Moraga et al., 2007). They occur primarily in the soil and constitute an essential part of the organic biomass (Ferron, 1981; Keller and Zimmermann, 1989; Tkaczuk, 2008). Soil often provides the substrate for the maintenance of a natural reservoir of many entomopathogenic fungi. Soils can be inoculated with these fungi either by an infected insect entering the soil or by the deposition of spores on the soil surface by natural dispersion mechanisms (Hajek, 1997). Presence and pathogenicity of many entomopathogenic fungi depend on interactions with host organisms, prevailing climatic conditions, and other biotic and abiotic factors.

Laboratory studies have shown that heavy metals impact the growth, metabolism, and pathogenicity of entomopathogenic fungi (Ropek and Para, 2003; Baldrian, 2003; Pečiulytė and Dirginčiutė-Volodkienė, 2012; Hassn et al., 2014). Ecotypes of specific species of entomopathogenic fungi accumulate heavy metals in different amounts leading to diverse effects on growth and pathogenicity (Popowska-Nowak et al., 2004; Tkaczuk, 2008).

Some of the hypocralean fungi most commonly found in the soil environment that are highly parasitic to insects include species of the *Isaria* Pers. genus (Zimmermann, 2008; Ropek et al., 2014). *Isaria farinosa* (Holmsk.) is frequently responsible for natural epizootics among butterflies overwintering as pupa especially in soil and forest litter (Vänninen, 1996; Chandler et al., 1997). *Isaria fumosorosea* Wize is used to produce a bio-insecticide to control whiteflies infesting greenhouse production plants (Gonzales et al., 2016). *Isaria tenuipes* Peck. causes epizootics of larval and pupal stages of several lepidopterous pests. After the death of the host, the fungi produces fascicles of hyphae, referred to as synnemata, which emerge from the cadaver and produce great amounts of conidia (Fukatsu et al., 1997).

The purpose of this paper is to investigate the effect of selected heavy metal ions on the growth of entomopathogenic fungi of the *Isaria* genus under laboratory conditions.

Materials and methods

Fungal isolates

Laboratory tests examined the effects of five selected heavy metal ions: zinc, nickel, copper, lead, and cadmium, on the growth of *Isaria farinosa, I. fumosorosea*, and *I. tenuipes*. The fungal material was obtained from stock collections maintained at the Department of Plant Protection and Breeding, Siedlce University of Natural Sciences and Humanities, Siedlce, Poland.

Initial isolates of *I. farinosa* and *I. tenuipes* were obtained from infected pupae of an unidentified butterfly species found in leaf litter of mixed woods in the vicinity of Siedlce. The isolate of *I. fumosorosea* was obtained from the soil of cultivated fields and collected using the ‘insect bait’ method.

The fungi were identified on the basis of morphological characteristics, using identification keys (Inglis et al., 2012; Humber, 2012). Prior to treatments, isolates were

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applied to Petri-plates with Sabouraud medium and maintained at 20 ± 2 °C for 7 days in total darkness.

Preparation of media with metal ions

Heavy metal ions were added to sterile Sabouraud medium in three concentrations: 1× - concentration corresponding to the mean content of that metal in Polish soils, 10× - concentration 10 times higher and 100× - 100 times higher than the natural content. Treatment concentrations are listed in *Table 1*. Metal ions were added to the culture media in the form of various salts.

Table 1. *Characteristics of heavy metal salts used in the experiment*

Ion	Salt used	Concentration of ion corresponding to the natural content in soil (mg/l)
Cu	$CuSO_4 \times 5H_2O$	6.5
Zn	$ZnSO_4 \times 7H_2O$	33.0
Ni	$Cl_2Ni \times 6H_2O$	6.5
Pb	$Pb(NO_3)_2$	13.8
Cd	$CdCl_2 \times 2H_2O$	0.22

The Sabouraud medium was treated with $H_2PO_4^-$ and HPO_4^{2-} as a buffer to maintain pH of 6.4. Plates were centrally inoculated with a mycelial disc of 3-4 mm in diameter, and were stored in darkness at 20 °C. The colony diameter was measured 5, 10, 15 and 20 days after inoculation. Each experimental combination and the control with no metals were replicated four times. All laboratory experiments were achieved at the Siedlce University of Natural Sciences, Department of Plant Protection and Breeding, Siedlce, Poland in 2017.

Statistical analysis

Size of fungus colonies, expressed as a percentage in relation to the control, were treated as variables. The results were processed statistically using the Statistica 12 program. Tukey's test and single-factor analysis of variance at a significance level of $\alpha = 0.05$ were used to compare group means.

Results

Metal ions of zinc, nickel, copper, lead, and cadmium affected the growth of *Isaria* genus in various ways, with the reaction dependent on the type of metal as well as concentration. Levels of zinc, copper, and cadmium at the levels found in native Polish soils caused a slight stimulation of colonies of *Isaria farinosa* (*Table 2*).

After 20 days all colonies *I. farinosa* on the media with 1×, 10×, and 100× heavy metal concentrations grew, respectively, 110.8%, 115.8%, and 102.8% of the control colony diameter. Twenty days after the application of ionic cadmium and lead with the concentration 10 times higher than the natural content (10×) that the diameter of the *I. farinosa* colony was 32.9% smaller than the control for cadmium and 30% smaller for lead. Other heavy metals added to the media at 10× concentration did not significantly affect the growth of the colonies. Of all the heavy metals cadmium and nickel ions at

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DOI: http://dx.doi.org/10.15666/aeer/1702_25712582

the concentration 100 times higher than natural the most strongly inhibited the growth of the *I. farinosa* cultures.

Table 2. *The size of colonies of the fungus Isaria farinosa on medium in the presence of heavy metals ions (expressed in % of control)*

Metal	Con[1]	Date of observation in days			
		5	10	15	20
Zn	1×	103.7±0.2 b	103.7±0.8 a	119.8±0.1 b	110.8±0.0 c
	10×	106.9±0.4 b	91.7±2.1 a	111.6±0.8 b	96.7±0.0 a
	100×	93.1±0.3 a	88.6±0.8 a	103.0±0.1 a	100.5±0.0 b
Control		100 ab	100 a	100 a	100 b
Ni	1×	106.4±0.3 b	109.2±0.5 b	105.3±0.5 b	91.3±0.0 b
	10×	98.4±0.8 b	107.1±0.4 b	110.0±0.6 b	95.8±0.0 bc
	100×	49.5±0.8 a	68.6±0.5 a	78.6±0.1 a	75.8±0.0 a
Control		100 b	100 b	100 b	100 c
Cu	1×	102.6±0.3 b	97.8±0.8 ab	119.3±0.4 b	115.8±0.0 c
	10×	108.0±0.3 b	109.2±0.8 b	117.4±0.6 b	103.0±0.0 b
	100×	89.4±0.4 a	93.2±0.1 a	105.8±0.2 a	91.7±0.0 a
Control		100 b	100 ab	100 a	100 b
Cd	1×	102.1±1.8 b	96.8±1.4 c	101.5±1.7 c	102.8±0.8 c
	10×	81.4±0.8 a	71.3±0.9 b	69.3±1.2 b	67.1±1.1 b
	100×	78.9±3.0 a	66.7±1.4 a	58.9±0.8 a	54.6±0.6 a
Control		100 b	100 c	100 c	100 c
Pb	1×	101.5±0.9 b	98.4±1.4 a	98.2±2.0 b	97.8±0.7 c
	10×	96.9±2.0 ab	91.3±1.9 a	82.7±1.5 a	70.0±0.7 a
	100×	92.8±2.0 a	97.3±1.2 a	93.1±3.0 b	85.7±1.8 b
Control		100 b	100 a	100 b	100 c

Con[1] - concentration; 1× - the concentration corresponding to the natural content of the metal in the Polish soils; 10× - the concentration of 10-times higher than the natural content; 100× - the concentration of 100-times higher than the natural content; means in columns fallowed by different letters within the given metal differ significantly at $P < 0.05$

After 20 days of incubation fungal colonies on the media with ions of those two metals were, respectively, 54.6% and 75.8% of the control diameter. In the case of the other heavy metals: lead, copper, and zinc, those values were 85.7%, 91.7%, and 100.5%, respectively.

The ions of heavy metals at the concentration of 1× and 10× did not influence significantly the growth of *Isaria fumosorosea* colonies (*Table 3*). Only cadmium and lead ions reduced the growth by 6.6% when compared to the control. With respect to cadmium ions this difference was statistically significant. Other ions of heavy metals at both 1× and 10× concentrations slightly stimulated the development of *I. fumosorosea*.

Ions of nickel and zinc added to the medium at the concentration 100 times higher than the natural content in Polish soils limited fungus growth after 20 days the most, by 20.3% and 15.3%.

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Table 3. *The size of colonies of the fungus Isaria fumosorosea on medium in the presence of heavy metals ions (expressed in % of control)*

Metal	**Con[1]**	**Date of observation in days**			
		5	**10**	**15**	**20**
Zn	1×	110.2±0.9 c	112.8±0.4 c	109.7±1.0 c	105.2±1.8 b
	10×	112.9±0.0 c	114.2±0.7 c	111.4±1.5 c	104.2±1.1 b
	100×	80.6±0.0 a	88.8±0.3 a	82.9±1.8 a	84.7±0.5 a
Control		100 b	100 b	100 b	100 b
Ni	1×	98.4±1.2 b	105.7±1.4 b	107.3±1.3 b	100.4±2.5 ab
	10×	102.1±0.0 b	102.4±0.8 b	98.3±2.2 b	104.5±6.3 b
	100×	69.9±1.2 a	73.2±0.2 a	76.8±0.7 a	79.7±0.3 a
Control		100 b	100 b	100 b	100 ab
Cu	1×	103.8±1.1 a	110.9±1.5 a	109.5±3.0 a	103.7±2.0 b
	10×	106.4±1.1 a	110.6±1.2 a	107.7±2.3 a	101.6±1.8 b
	100×	101.1±1.1 a	99.7±1.4 a	98.6±1.8 a	90.3±0.8 a
Control		100 a	100 a	100 a	100 b
Cd	1×	92.4±0.4 a	92.5±0.8 a	93.9±0.3 a	93.4±0.6 a
	10×	105.0±1.2 ab	103.7±1.9 b	102.7±1.5 b	103.3±0.5 b
	100×	112.6±0.5 b	107.7±1.2 b	102.8±0.8 b	101.0±0.2 b
Control		100 ab	100 ab	100 ab	100 b
Pb	1×	89.9±0.2 a	88.3±0.4 b	90.4±0.8 ab	93.4±0.4 a
	10×	85.8±0.5 a	81.5±0.6 a	91.9±1.0 b	93.8±0.3 a
	100×	79.8±0.4 a	80.3±0.6 a	86.2±0.6 a	94.7±0.4 a
Control		100 b	100 c	100 c	100 b

Con[1] - concentration; 1× - the concentration corresponding to the natural content of the metal in the Polish soils; 10× - the concentration of 10-times higher than the natural content; 100× - the concentration of 100-times higher than the natural content; means in columns fallowed by different letters within the given metal differ significantly at $P < 0.05$

This difference was statistically significant. It was found that cadmium 100× concentration did not affect adversely the development of the *I. fumosorosea* colony, while in the case of lead and copper the colonies were only about 5.9% and 9.7% smaller than the control.

Discussing the experiment findings it should be noted that so far there have been no studies on the effects of heavy metal ions on the growth of *Isaria tenuipes*. This paper, therefore, is the first one in scientific literature devoted to the subject. The experiment showed that 20 days after heavy metal ions were added to the media at the concentration corresponding to their natural content in the Polish soils (1×) and 10-times higher (10×), they did not significantly inhibit *I. tenuipes* growth (*Table 4*). It was found that only in the presence of nickel were the diameters of fungus colonies around 10% smaller than the control, while in the case of them growing on the medium with cadmium and lead there was even a slight growth stimulation. With the concentration 100 times higher than their natural content in Polish soils, zinc, nickel, and copper ions were the most toxic to *I. tenuipes*. Within the first 5 days of the experiment the culture with nickel ions had not grown, and within 10 days the *I. tenuipes* colony had reached only 16.1% of the control diameter. 20 days after the experiment had started fungal colonies on media

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containing zinc, nickel, and copper at their highest concentration (100×) were, respectively, 29.5%, 33.8% and 69.1% of the size of the control culture.

Table 4. *The size of colonies of the fungus Isaria tenuipes on medium in the presence of heavy metals ions (expressed in % of control)*

Metal	Con[1]	Date of observation in days			
		5	10	15	20
Zn	1×	99.6±0.9 b	99.6±1.4 b	97.8±1.2 b	98.2±1.8 bc
	10×	100.0±0.9 b	95.9±2.7 b	91.5±2.0 b	93.8±5.6 b
	100×	33.2±5.6 a	22.5±0.8 a	24.9±0.7 a	29.5±1.1 a
Control		100 b	100 b	100 b	100 c
Ni	1×	79.9±5.5 a	80.9±4.4 b	87.8±2.2 b	90.5±5.9 b
	10×	69.7±1.5 a	74.5±4.8 b	89.8±0.5 b	93.0±3.1 b
	100×	No growth	16.1±1.6 a	24.9±4.4 a	33.8±3.2 a
Control		100 b	100 c	100 b	100 c
Cu	1×	102.4±0.0 b	100.0±1.2 b	97.8±1.1 b	99.4±2.0 b
	10×	104.5±1.8 b	103.9±2.3 b	101.0±2.5 b	95.3±8.0 b
	100×	66.8±3.8 a	64.7±2.0 a	64.7±0.8 a	69.1±1.8 a
Control		100 b	100 b	100 b	100 b
Cd	1×	105.2±2.0 b	108.1±1.2 c	110.2±1.0 b	105.9±0.7 b
	10×	96.0±1.6 a	97.4±2.4 ab	97.6±0.8 a	99.1±0.6 ab
	100×	96.0±1.3 a	92.9±1.8 a	90.8±0.9 a	91.3±1.2 a
Control		100 ab	100 b	100 ab	100 b
Pb	1×	108.0±0.0 c	111.6±0.0 b	113.5±0.6 b	106.8±0.4 b
	10×	108.0±0.0 c	113.2±1.2 b	113.5±0.5 b	107.4±0.4 b
	100×	86.0±0.9 a	98.0±0.6 a	104.5±0.3 a	106.2±0.2 b
Control		100 b	100 a	100 a	100 a

Con[1] - concentration; 1× - the concentration corresponding to the natural content of the metal in the Polish soils; 10× - the concentration of 10-times higher than the natural content; 100× - the concentration of 100-times higher than the natural content; means in columns fallowed by different letters within the given metal differ significantly at $P < 0.05$

With respect to the fungi *I. farinosa* and *I. fumosorosea* this difference was statistically significant. However, after 20 days the presence of cadmium in the medium only to a low degree decreased the size of the *I. tenuipes* colony, by 8.7%, while lead ions stimulated its growth. It should be noted that out of the entomopathogenic fungi of the *Isaria* genus, *I. tenuipes* proved the most sensitive to the presence of zinc, nickel and copper ions used in the concentration 100 times higher than their natural content in Polish soils (*Table 5*).

Discussion

Heavy metals are highly toxic to living organisms. They contribute to the deterioration of soil chemical properties and restrict the number of soil microorganisms (Kabata-Pendias and Pendias, 2001; Šmejkalová et al., 2003; Ahmad et al., 2005; Tscherko et al., 2007; Lenart and Wolny-Koładka, 2013).

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Table 5. *ANOVA of the data for effect of heavy metal ions on the growth of entomopathogenic fungi from the genus Isaria after 20 days incubation*

Source	df	Metals									
		Zn		Ni		Cu		Cd		Pb	
		F	P	F	P	F	P	F	P	F	P
Main effects *I. farinosa* Error	3 12	55.7	0.00	108.1	0.00	54.2	0.00	73.6	0.00	100.4	0.00
Total	15										
Main effects *I. fumosorosea* Error	3 12	38.8	0.00	5.25	0.00	7.27	0.00	12.9	0.00	11.2	0.00
Total	15										
Main effects *I. tenuipes* Error	3 12	553.1	0.00	361.7	0.00	51.8	0.00	8.39	0.00	28.4	0.00
Total	15										

Studies performed in vitro suggest certain species of entomopathogenic fungi, and as well as individual strains within species, exhibit different sensitivity to heavy metal ions (Jaworska et al., 1996; Ropek and Para, 2003; Keller et al., 2003; Tkaczuk, 2003, 2005; Gorczyca, 2005; Quesada-Moraga et al., 2007; Pečiulytė and Dirginčiutė-Volodkienė, 2012; Hassn et al., 2014). Some of the metals are inhibitory, while others, such as lead or zinc, can stimulate biomass growth of entomopathogenic fungi at certain levels (Bajan et al., 1998).

Our studies showed the presence of zinc, copper, and cadmium in a medium with concentrations similar to the natural content in Polish soils caused a slight stimulation of *I. farinosa* colony growth. Those findings were confirmed by the studies of Popowska-Nowak et al. (2000), who found that zinc ions added to a medium had stimulating effects in the initial phase of *I. farinosa* fungus growth. By examining various species of entomopathogenic fungi in the soil of Sweden Arnebrandt et al. (1987) and Nordgren et al. (1985) observed that two species: *I. farinosa* and *B. bassiana,* had the greatest tolerance to soil contaminated with copper and zinc. El-Sharouny et al. (1988) found in their study that *Isaria* and *Metarhizium* Sorokin were resistant to high content of zinc in the soils of Egypt. Hassn et al. (2014) studied the effect of metal ions on the growth of *Isaria javanica* (Bally) Samson and Hywel-Jones (= *Paecilomyces javanicus*) found that zinc, copper, and cadmium in amounts corresponding to their natural content in soils, as well as cadmium in concentration 10 times higher than the natural content, did not affect colony growth of this fungus significantly. Other species of entomopathogenic fungi have been shown to be tolerant and resistant to high contaminations of the soils, by heavy metals (Arnebrandt et al., 1987; Fomina et al., 2007). Trevors et al. (1986), Gaad (1993), and Zimmermann and Wolf (2002) found that entomopathogenic fungi display relatively high resistance to heavy metals, or produce mutations capable of tolerating high concentration. Additionally, Popowska-Nowak et al. (2004) observed that entomopathogenic fungi can employ adaptive mechanisms in areas heavily contaminated by heavy metals. Pečiulytė and Dirginčiutė-Volodkienė (2012) also

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observed higher concentration of copper ions added to a medium stimulates the number of infectious units produced by *Peacilomyces inflatus* (Burnside) J.W. Carmich. They also found by extending the breeding time, tolerance to copper and zinc increased, with some species starting growth on the first day of the experiment, and others as late as after 57 days. Fungus species belonging to *Beauveria, Isaria* or *Metarhizium* genus are among entomopathogenic fungi with the highest tolerance to high concentrations of heavy metals in soils, having an ability to accumulate them (Kameo et al., 2000; Fomina et al., 2007; Tkaczuk, 2008). Gorczyca (2005) reported selected metal ions, including cadmium, lead, copper, manganese, magnesium, and zinc stimulated fungal biomass growth of *B. bassiana* (Bals.) Vuill. strain Bb5.

Heavy metals in high concentrations, can significantly limit growth, spore germination, and pathogenicity of fungi for in vitro studies. Lead has a great capacity to accumulate in the environment can be particularly toxic to fungus cells (Ropek and Para, 2003; Jaworska and Gorczyca, 2004; Tkaczuk, 2005; Hassn et al., 2014). Ropek and Para (2003) observed that the heavy metals they used restricted the growth of *Paecilomyces farinosus* (= *Isaria farinosa*) significantly, and the ions of lead, cadmium, zinc, and copper had the most toxic effects on this fungus isolate.

In our studies concentrations of cadmium and lead at levels 10 times higher than the natural content in Polish soils showed an inhibitory influence on the growth of the *I. farinosa*. Levels of cadmium and nickel at 100 times higher than the natural soil content greatly reduced fungal growth. Tkaczuk (2005) showed nickel had the greatest inhibitory effects on the germination of spores of *Pandora neoaphidis* (Remaud and Hennebert). Hassn et al. (2014) reported that cadmium, copper, and zinc ions at the concentration 100 times higher than the natural content in the soils of Iraq, regardless of the period of observation, completely inhibited the growth of an *I. javanica*. Tkaczuk (2008) reported that cadmium was only slightly toxic to entomopathogenic fungi *B. bassiana* and *M. anisopliae* tested in vitro studies, but significantly reduced the number of colony forming units (CFU) of these fungi in soil. Jaworska and Gorczyca (2004) observed that only very high soil contamination with heavy metals (321 mg Cd, 1294 mg Cu, 1974 mg Pb and 3427 mg Zn × kg^{-1}) caused a decrease in *I. farinosa*, *B. bassiana* and *M. anisopliae* pathogenicity to *Galleria mellonella* larvae.

Heavy metal ions added to the medium at the concentration corresponding to the natural content in Polish soils and 10 times higher did not affect significantly the growth of *I. fumosorosea.* The relatively high resistance of *I. fumosorosea* to the effects of some heavy metals is confirmed by research of Gorczyca and Ropek (2000), Tkaczuk (2003, 2008), and Pečiulytė and Dirginčiutė-Volodkienė (2012).

We have shown that cadmium and lead in the highest concentration caused only a small reduction in the growth of the *I. fumosorosea.* Our results that indicated growth of the *I. fumosorosea* was inhibited by high levels of nickel and zinc were similar to studies of Jaworska et al. (1996) and Tkaczuk (2003). It is worth noting that in our studies cadmium ions showed no significant inhibitory effect on the growth of this fungus. Our results were confirmed by Tkaczuk (2008), who concluded that cadmium used for in vitro studies showed low toxicity to *Isaria* fungi. However, Gorczyca and Ropek (2000) reported that adding cadmium to a medium not only limited the growth of *P. fumosoroseus* (= *I. fumosorosea*), but also reduced the amount of its biomass. They further reported that zinc and copper has limited negative effects. Gorczyca and Ropek (2000) reported the accumulation of metal ions was up to 3 to 5 times higher in *P. fumosoroseus* biomass than in the culture medium.

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Heavy metals affect growth, morphology or metabolism of soil organisms (Niklińska and Chmiel, 1997). Pečiulytė and Dirginčiutė-Volodkienė (2012) reported fungi with the greatest resistance to heavy metals do not accumulate them in their biomass but bind them to the surface of their cells. It is known that fungi can accumulate significant amounts of metals. The cell walls of fungi are composed of polysaccharides, proteins and lipids which contain functional groups with potential metal complexing capacities (Trevors et al., 1986; Gaad, 1993).

Metals in soil are present as free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides (Leyval et al., 1997). The mobility of metals in soil is dependent of their speciation, which is controlled by hydrochemical variables (pH, redox potential, presence of complexing inorganic and organic an-ions, ionic strength) as well as by their interactions with solid surface. The toxicity of metals in soil depends on their bioavailability, which, according to Berthelin et al. (1995), is a function not only of their total concentration but also of physico-chemical and biological (e.g. biosorption, bio-accumulation and solubilization) factors. The negative effects of heavy metals on entomopathogenic fungi on metabolism, growth, germination and differentiation may vary, depending on the fungal species, metal concentration, and other physico-chemical factors such as soil organic matter and clay content (Gaad, 1993; Tobin et al., 1994).

Conclusions

Heavy metal ions added to fungal culture medium in concentrations corresponding to the natural content found in soils of Poland did not significantly affect the growth of cultures of *Isaria* ssp. When compared to control cultures, slight increases were noted. At a concentrations 10 times higher than the natural content in Polish soils, heavy metal ions had little influence on the growth of the *Isaria* fungal colonies. Cadmium and nickel has the highest inhibitory effects *Isaria.* Heavy metal concentrations of 100 times greater than levels found naturally had the greatest toxic effects. Nickle was the most toxic and lead was the least toxic. *Isaria tenuipes* showed the greatest sensitivity to heavy metals *I. fumosorosea* having the highest tolerance. Strong pollution of soil by some heavy metals could be a restrictive factor of development of entomopathogenic fungi in the environment. Further studies on heavy metal resistance of different isolates within the particular genus of these fungi can be performed to search of virulent strains to be applied as biopesticides.

Acknowledgements. The results of the research carried out under the research theme No. 360/13/S were financed from the science grant granted by the Ministry of Science and Higher Education.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25712582

SYNTHESIS, CHARACTERIZATION AND INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES FROM *CYDONIA OBLONGA* LEAF

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(Received 12th Nov 2018; accepted 16th Jan 2019)

Abstract. In this study, leaf extract of *Cydonia oblonga* were used to synthesize silver nanoparticles. Silver nanoparticles have been successfully synthesized by environmentally friendly and economical methods. Characterization of synthesized AgNPs used UV-visible spectroscopy (UV-Vis.), thermogravimetric and differential thermal analysis (TGA-DTA), X-ray diffraction spectroscopy (XRD), scanning electron microscopy (SEM), electron diffraction X-ray (EDX), Fourier transform infrared spectroscopy (FT- IR). XRD results demonstrated that the crystal structure of AgNPs was cubic and had a size of 27.30 nm. AgNPs were found to be spherical in SEM images. The element composition was also revealed by EDX. Antimicrobial activity of silver nanoparticles; Gram-negative *Escherichia coli* was tested on gram positive *Staphylococcus aureus* and *Candida albicans* yeast. The concentration values of 0.0552, 0.1535 and 0.0383 mg L^{-1} were determined as MIC (minimum inhibitory concentration), respectively. It was evaluated that the commercial antibiotics used were more effective at low concentrations compared to colistin, vancomycin and fluconazole.
Keywords: *XRD, SEM, TGA-DTA, silver nanoparticles, Cydonia oblonga*

Introduction

Particles with a size of 1-100 nm are called nanoparticles. These particles can be obtained by biological, physical and chemical means. They are used in different areas such as energy, pharmacology, biomedical, cosmetics, textiles, food and agriculture etc. (Chaudhry et al., 2018). Compared to physical and chemical methods, the advantages of biological methods such as ease of application and control, eco- friendliness and lack of toxic chemicals make this method important (Pantidos and Horsfall, 2014). Due to the fact that it is cheap and easy to obtain, the use of vegetable resources for synthesis is becoming increasingly common. In the synthesis of silver nanoparticles, phytochemicals in plant leaf extracts form AgNPs by reducing the Ag^+ ions in the media to the Ag^o form, and these phytochemicals also provide stability (Prakash et al., 2013). Silver nanoparticles (AgNPs) were synthesized using plant extracts and these particles showed good antimicrobial activity (Ahmed et al., 2018).

In this study, AgNPs were obtained in a very short time with stable, energy-free and room conditions with *Cydonia oblonga* leaf extract and without any additional chemicals/and or physical steps. The resulting nanoparticles showed a strong antimicrobial effect. In this study a simple and rapid green synthesis of AgNPs is presented.

AuNPs using leaves extract of R. rugosa have been reported. Silver and gold nanoparticles can be prepared with lower amounts of leaf extract and without any additional chemicals/and or physical steps. The effect of leaf extract quantity and concentration of metal solution were also evaluated to optimize the synthesis route producing the metal nanoparticles.

Materials and methods

Silver nitrate ($AgNO_3$) 99.98% was obtained from Sigma-Aldrich. Vancomycin, fluconazole and colistin were commercially purchased for antimicrobial applications.

Preparing Cydonia oblonga leaf extract

The leaf extract of *Cydonia oblonga* was washed with distilled water and dried at room temperature. 25 g was taken and the size reduced. It was mixed with 500 ml distilled water and boiled at 85 °C. The extract was filtered through Whatmann No.1 filter paper and cooled to room temperature. Stored at 4 °C for use in the study.

Synthesis of AgNPs

1 mM $AgNO_3$ aqueous solution was used for silver nanoparticles synthesis. It was left to react at room temperature with 125 ml of plant extract and 500 ml of silver nitrate solution was added to 1000 ml of flask and allowed to react under room conditions. AgNPs formation was monitored primarily with a macroscopic method depending on the change of colour and was determined according to time with spectrophotometric measurements (Pugazhendhi et al., 2018). Composed dark colored solution was obtained with centrifuge (7500 rpm, 10 min). The top, liquid portion was removed, while the remaining solid segment was washed with distilled water. The resulting AgNPs were left to dry at 65 °C and stored in the dark until use in the characterization process.

Characterisation techniques

Synthesised silver nanoparticles (AgNP) UV-Vis. Specrtrums were obtained on a spectrophotometer (UV-1601 220 V SHIMADZU). Scanning electron microscope (EVO 40 LEQ) was used to measure AgNPs dimension and morphology of AgNPs crystal construction was analysed with X-ray diffractometer (RadB-DMAX II) between $3° \leq 2\theta \geq 80°$ spaces. SEM- energy dispersive X-ray (EDX) spectroscopy was used to demonstrate AgNPs existence and morphology. FTIR (Perkin Elmer ATR-FTIR) analysis was used to determine those functional groups that has a role in the reduction in the plant extract in the range of 4000-400 cm^{-1}. Thermogravimetric analysis (TGA SHIMADZU DTG-60H) reported the degradation temperatures of AgNPs under the atmosphere of nitrogen gas at 25 °C min^{-1} and the atmosphere of nitrogen gas at constant heating rate.

Antimicrobial activity of AgNPs

The effects of the obtained particles on gram negative Escherichia coli ATCC 25922, gram positive Staphylococcus aureus ATCC 29213 bacteria and Candida albicans yeast were investigated. Minimum Inhibitory Concentration (MIC) was determined by microdilution method. In practice, the muller Hilton medium was added to the microplate wells and incubated overnight at 37 °C by addition of an appropriate amount of the microorganism mixture and the AgNP solution adjusted to 0.5 in turbidity according to the Mc Farland standard (El-Batal et al., 2018; Vishwasrao et al., 2018; Dhand et al., 2016). The next day the MIC was determined by the well in which the reproduction ended. In addition, vancomycin, colistin and fluconazole commercial

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antibiotics and 1 mM $AgNO_3$ solution were used for *S. aureus*, *E. coli* and *C. albicans* to compare the effects of AgNPs.

Result and discussion

XRD analysis with X-ray diffraction

The evaluation of the analysis of AgNPs XRD phase and crystal structure was shown in *Figure 1,* which was synthesised with green method. Crystal structures of silver nanoparticles' reflections (111), (200), (220) and (311) were calculated with the values 2θ (37.38°, 44.78°, 64.81° and 77.50°) on XRD analysis (*Fig. 1*). It was determined that AgNPs were of elemental (Ag^o) and cubic crystalline structure. The crystalline particle size of the AgNPs was calculated according to the Debye-Scherrer equation and found to be approximately 27.30 nm.

The Debye-Scherrer equation is (Narayanan and Sakthivel, 2011; Banala et al., 2015; Rolim et al., 2019):

$$D = K\lambda / (\beta \cos\theta)$$

Here, D is the crystal size of AgNPs (nm), k = Scherrer's constant, (0.90), λ = X-ray wavelength (1.5406° A), β = FWHM (full width at half maximum) of the peak located at 2θ = 37.38°, θ = Bragg's angle of diffraction.

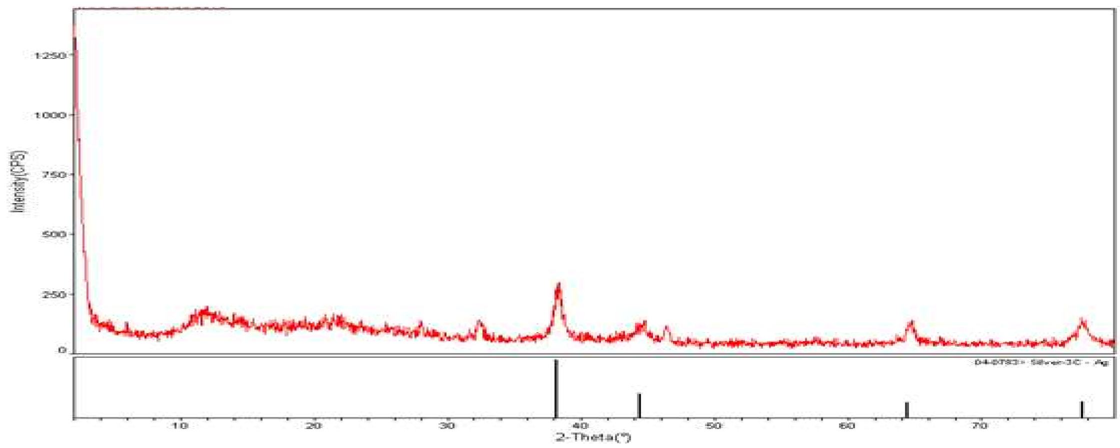

Figure 1. *XRD measurements of AgNPs confirming the cubic crystalline structure of AgNPs*

Analysis of UV-vis spectroscopy

In UV-vis spectroscopy analysis silver nanoparticles' formation was observed with samples taken at different times at 0, 5, 10, 15, 20, 25, 30 and 45 min. The color change to dark brown, which shows the formation of AgNPs, was observed very quickly. At about a maximum of 441.58 nm a sharp plazmon resonance indicated appropriately synthesised AgNPs (*Fig. 2*) Similarly, some other researchers which were near to our research found different spectrum values at 390-500 nm surface plazmon resonance (Al-Bahrani et al., 2017; Kalimuthu et al., 2008; Anbu et al., 2018; Khan et al., 2018; Rajakumar et al., 2017).

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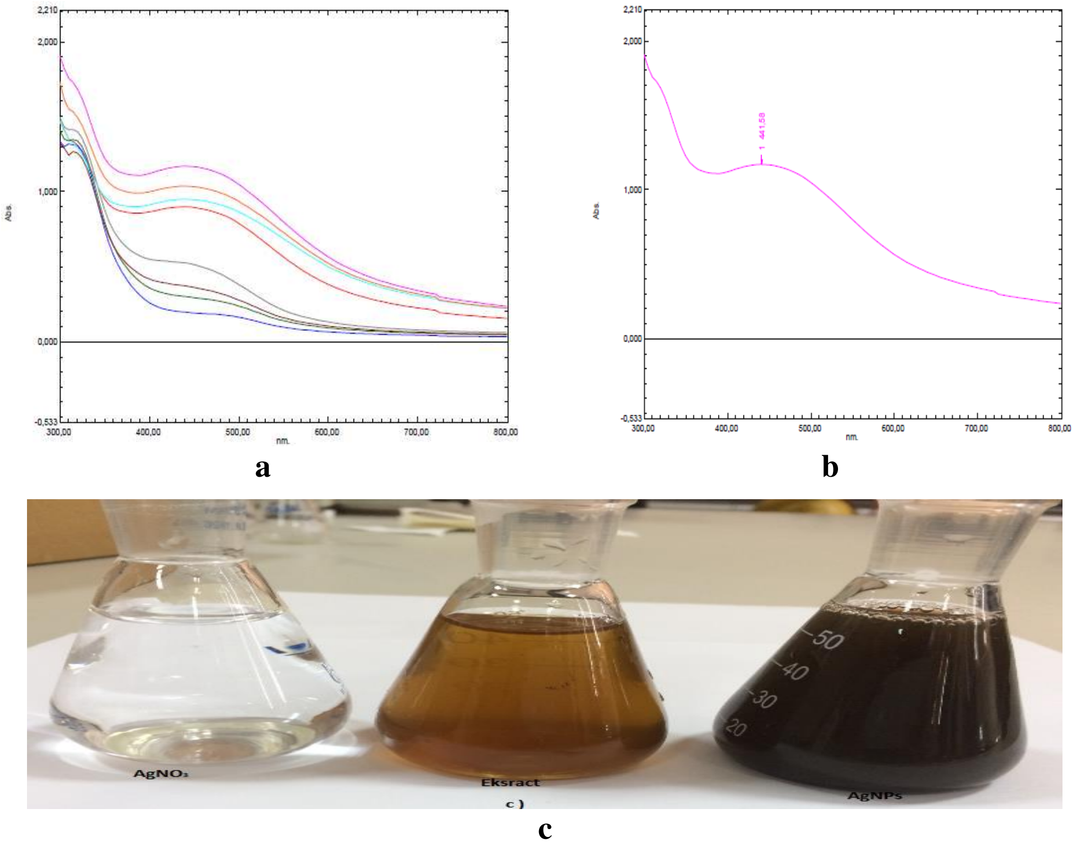

***Figure 2. a** The UV–Vis spectra of AgNPs synthesized by Cydonia oblonga leaf aqueous extract. **b** On UV-Vis spectroscopy maximum absorbance value synthesised AgNPs. **c** AgNPs stepwise thematic representation of the synthesis carried out*

Fourier transform infrared spectroscopy (FTIR) spectroscopy analysis

FT-IR spectrum provided information on which functional groups the reaction occurs in by comparing *Cydonia oblonga* leaf extract and synthesised AgNPs (*Fig. 3*). The characteristic peaks appeared at 3291 cm^{-1} and it represents the -OH stretching vibrations of free and hydrogen bound hydroxyl groups present in *Cydonia oblonga* extract. The peak obtained at 2081 cm^{-1} is due to -CH stretching vibrations of methylene groups, and furthermore, the sharp peak present at 16365 cm^{-1} can be assigned to non-hydrated C=O groups and it revealed that the asymmetric and symmetric stretching vibrations of carboxyl groups.

FTIR spectrum measurements which were synthesised on different studies showed the existence of intense band on 3314, 2119 and 1636 cm^{-1} (Pugazhendhi et al., 2018; Baran et al., 2018).

Analysis results of silver nanoparticles SEM and EDAX

Morphological specifications of AgNPs' which are obtained from leaf extract of *Cydonia oblonga* were examined by using electron microscope (SEM), shown in *Figure 4*. The results have shown the presence of silver nanoparticles (AgNPs). Morphological specifications which were obtained from leaf extract of *Cydonia oblonga* were examined by using electron microscope (SEM), shown in *Figure 4*. The results have

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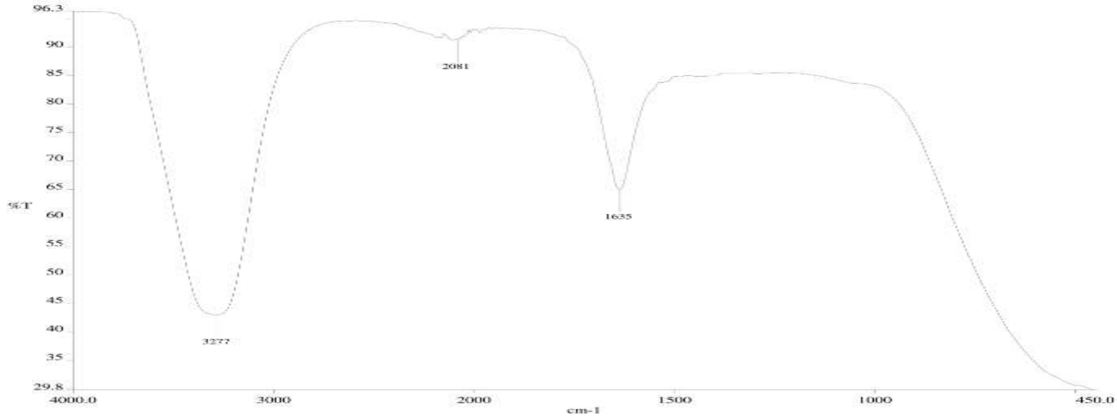
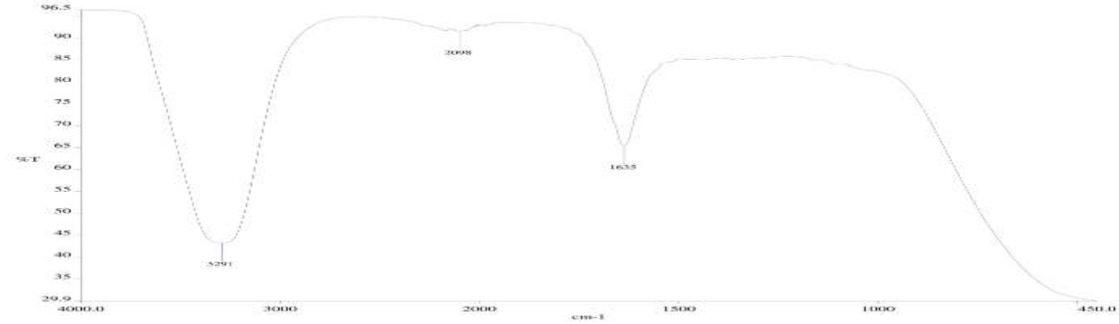
shown us clearly the existence of spherical shaped silver nanoparticles (AgNPs') under 100 nm. It was seen on XRD calculation that particles size was about 27.30 nm. Similarly, SEM analysis provided supplementary information about AgNPs size and morphological specifications (De Jesús Ruíz-Baltazar et al., 2017; Prakash et al., 2013; Jha and Shimpi, 2018).

Energy distributed spectrums of acquired AgNPs from EDX analysis have shown that there have been pure silver pieces (*Figure 5)*. The results obtained with AgNPs in other scientific studies support our findings (Veisi et al., 2018; Kumar et al., 2016; Ramkumar et al., 2017).

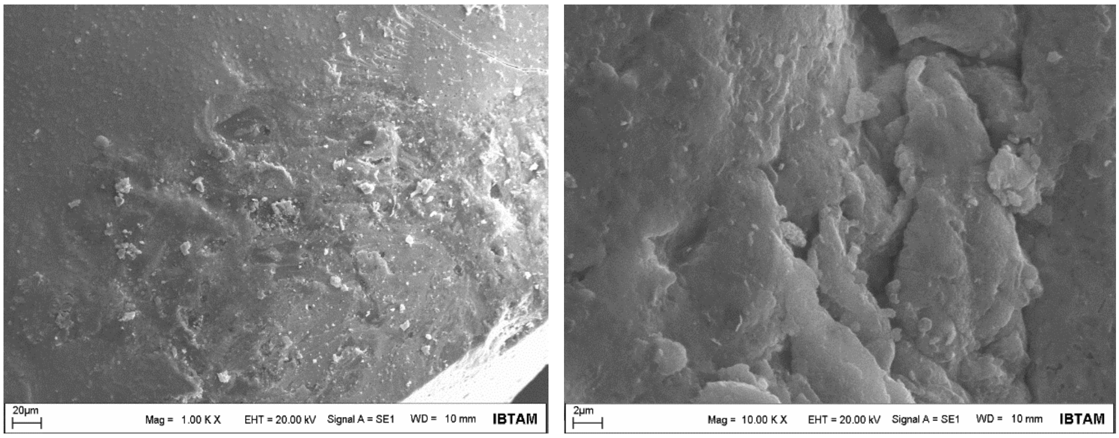


a



b

***Figure 3. a** FT-IR spectrum of plant extract spectrum.* ***b** FT-IR spectrum of synthesised AgNPs*



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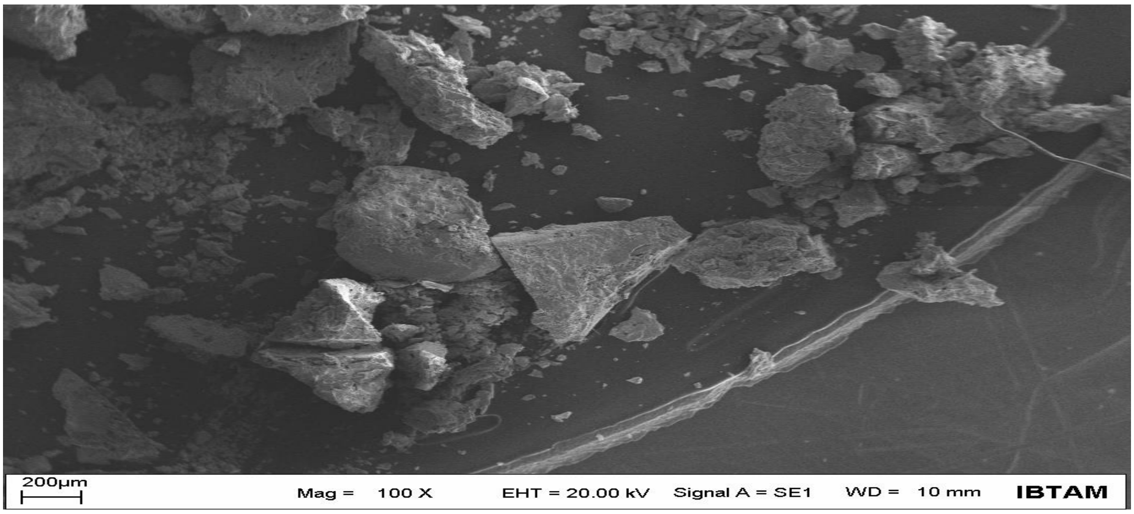

Figure 4. *Images of synthesised silver nanoparticles of SEM analyses*

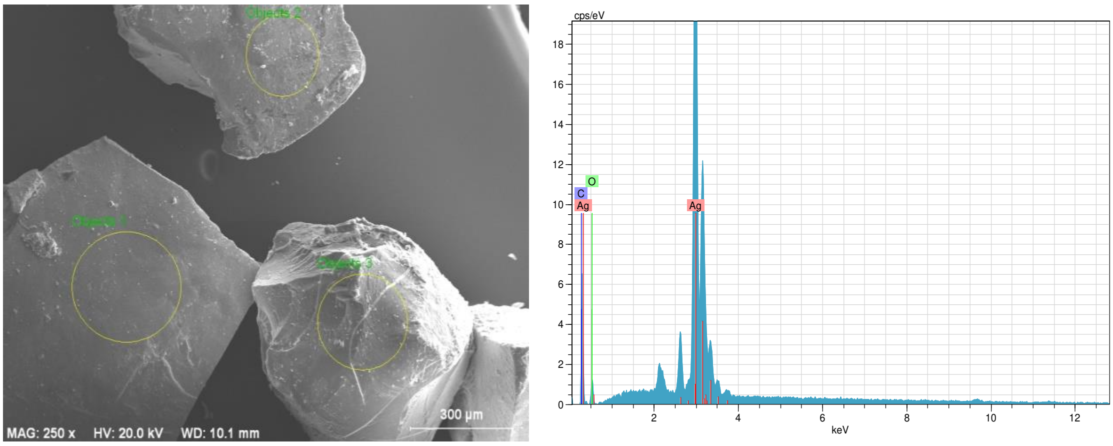

Figure 5. *EDX profile of AgNPs showing its elemental composition*

TGA-DTA findings

Nanoparticles which were prepared with green synthesis were analysed with the flow rate of 20 mL min^{-1} in N_2 (g) atmosphere with 10 °C min^{-1} heating rate between 31-900 °C TGA and DTA data. TGA bent shows specimen mass loss for thermal degradations. DTA bent determines maximum heat of dissociation at every stage of degradation (Baran et al., 2018).

It has been seen in *Figure 6* that at 31-257 °C mass loss is derived from moisture, at 257-333 °C there is a mass loss which is derived from cellulosic materials, at 333-900 °C the mass loss is derived from plant extract phytochemicals. TGA data were reported on the study about AgNPs synthesised mass loss. Similar studies support the results in this aspect (Baran et al., 2018).

Antimicrobial activity of synthesised AgNPs

Microorganisms are more and more resistant to the antibiotics used, which causes the struggle with them to fail. Therefore, the search for antimicrobial agent is more

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interested in combating infection. Antimicrobial activity studies with AgNPs may be a serious alternative to this condition. AgNPs increase the formation of reactive oxygen species (ROS) and disrupt the wall structure. These (ROS) to the affinity of the membrane structure and function changes are produced. They have an antimicrobial effect because they form a repressive process (Wang et al., 2017).

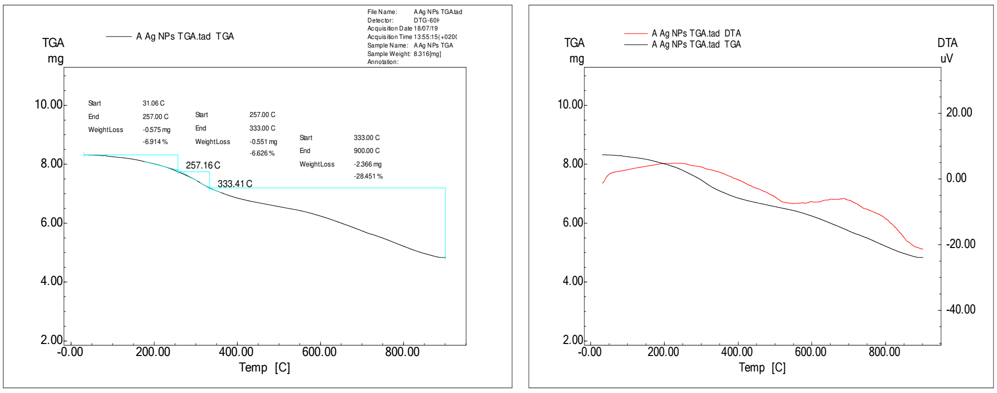

Figure 6. *TGA-DTA analysis result of synthesised silver nanoparticles*

In our study, the effect of AgNPs' antimicrobial effect is searched which are obtained from leaf extract of *Cydonia oblonga* by MIC method. Antifungal activities of AgNPs were studied on *C. Albicans* and probable antibacterial effect on gram positive *S. aureus ATCC 29213* and gram negative *E. coli ATCC 25922*. MIC worths were determined of AgNP and 1 mM silver nitrate. In our study, fluconazol, vancomisin and Colistin antibiotics were used. The synthesized silver nanoparticles have been shown to have a strong antimicrobial effect. The MIC of the AgNPs on *E. coli ATCC 25922*, *S. aureus ATCC 29213* and *C. albicans* was determined as 0.0552, 0.1535 and 0.0383 mg mL^{-1}, respectively. From the acquired results, which have been shown in *Table 1,* it is clear that AgNPs are more effective than antibiotics and 1 mM silver nitrate solution.

In order to determine the antimicrobial activity of AgNPs, in the other scientific studies, 53, 15 and 40 µg mL^{-1} MIC values were found for *E. Coli* (Sinsinwar et al., 2018; Rolim et al., 2019; Hemmati et al., 2019). Values of 100 and 11.60 µg mL^{-1} were calculated for E. coli (Ananda et al., 2019; Shao et al., 2018).

Table 1. *MIC values of synthesized silver nanoparticles (AgNPs) (mg mL^{-1}), silver nitrate and S. aureus, C. Albicans, vacomycin, fluconazole, colistin antibiotics for E. coli, respectively*

Organism	AgNPs	Silver nitrat	Antibiotic
S. aureus ATCC 29213	0.1535	0.500	0.50
C. albicans	0.0385	0.500	0.50
E. coli ATCC25922	0.0552	1.000	0.125

Conclusions

As a result, silver nanoparticles were characterized by UV-visible spectra, TGA-DTA, FTIR, SEM-EDX and XRD measurements. The nanoparticles were obtained by

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DOI: http://dx.doi.org/10.15666/aeer/1702_25832592

eco-friendly synthesis method with the use of *Cydonia oblonga* leaf aqueous extract, which does not involve simple, easy and toxic chemicals. In the case of waste, the use of the leaves as a vegetable source for synthesis provides a great advantage both for the recycling of the waste and for the cheap and easy production of the raw material for synthesis. AgNPs obtained by environmentally friendly methods can act as a powerful antimicrobial agent and their contribution to human health in medical industry can be a serious factor. The utilization of the leaf in waste form in this way and the synthesis will be quite interesting considering the transfer of the recycling to the industry. These nanoparticles can also be used for dye removal and bioremediation of waste water.

Acknowledgements. I would like to thank Mardin Artuklu University Scientific Research Project Unit for the support given to MAÜ.BAP.18.SHMYO.040.

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IDENTIFICATION AND CONTROL OF SALTWATER INTRUSION BY ADR APPROACH IN THE COASTAL AQUIFERS OF TUTICORIN, INDIA

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(Received 13th Nov 2018; accepted 16th Jan 2019)

Abstract. Due to industrial growth and urbanisation, excessive usage of groundwater resulted in a problem of saltwater intrusion in Tuticorin, India where control and management is very much essential. In the present study, groundwater samples are collected and analysed from 38 observation wells in years 2014 and 2018. Thirteen parameters namely pH, Electrical Conductivity (EC), Total Dissolved Solids (TDS), Total Hardness (TH), Calcium (Ca^{2+}), Magnesium (Mg^{2+}), Sodium (Na^{+}) Potassium (K^{+}), Bicarbonate (HCO_3^-), Chloride (Cl^-), Sulphate (SO_4^{2-}) Nitrate (NO_3^-) and Fluoride (F^-) are determined and considered in calculating the Water Quality Index (WQI) based upon weighted arithmetic index method. Geographical information system (GIS) is used to interpolate water quality data by inverse distance weighted method. Experimental investigation indicates saltwater intrusion in the coastal aquifer of Tuticorin is due to excessive withdrawal of groundwater. Potential intrusion of saltwater is studied with respect to distance of observation wells from seashore. Finite Element Modelling of Flow (FEFLOW) is used to select the optimum pumping and recharge rate to control saltwater intrusion. A model is calibrated with hydraulic head measured using piezometer in the observation well, as well as salt concentration. The model is simulated using three different groundwater scenarios such as Abstraction, Recharge and combined Abstraction, Desalination Recharge (ADR) method. The simulation results depicted that the planned ADR system accomplishes significantly better than using abstraction or recharge well.
Keywords: *GIS, groundwater management, FEFLOW, hydraulic head, water quality index*

Introduction

Groundwater extraction is required in several coastal areas where the freshwater supply from surface sources is not sufficient. However, extreme groundwater abstraction may lead to intrusion of saltwater into the aquifer, henceforth salinity increases making the groundwater unfit for human intake (Rao et al., 2004). Saltwater contamination arises due to vertical movement across interconnected aquifers through open well or bore well (Barlow and Reichard, 2010). Jebastina and Prince Arulraj (2016) analysed groundwater and determined the quality changes due to silicate weathering and dissolution of carbonate. Assessment of groundwater quality plays a vital role in our society, predominantly concerning health (Sener et al., 2009). One of the important and easy methods for evaluating intrusion of saltwater in coastal areas is periodic analysis of groundwater chemistry (Todd, 1980). The information of hydrochemistry is significant to assess the groundwater quality in Coimbatore in which the groundwater is used for drinking, agriculture, and industrial purposes (Jebastina and Prince Arulraj, 2017). In recent years water quality index (WQI) has become an

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

essential method to assess the quality of groundwater. Tavassoli and Mohammadi (2017) assessed the vulnerability of groundwater based on WQI values. WQI method handles complex data of water quality parameter and provides simplified output to understand water quality problem. Idowu et al. (2016) used geographical information system (GIS) as a prevailing tool to assess vulnerability of seawater intrusion and effective management of Groundwater.

Todd (1974) discussed various methods of preventing saltwater from polluting groundwater sources including; decrease of pumping rates, repositioning of extraction wells, use of sub-surface walls, recharge of water by natural and artificial methods, abstraction of saline groundwater and any combination of above techniques. El Mokhtar et al. (2018) developed model for planning and management of sustainable use of groundwater resources in the coastal aquifer of Fum Al Wad, Morrocco. Groundwater model evidenced that pumping of saline water from coastal aquifers would mitigate the rate at which saltwater flows into the aquifer so that quality of water improved. Hussain et al. (2015) suggested continuous Abstraction of brackish water, desalination and recharge techniques control saltwater intrusion effectively in coastal aquifers. Thomas et al. (2016) used simulation model to determine proper pumping strategy for the management of Coastal aquifers. Finite Element Modelling of Flow (FEFLOW) is a software used for groundwater modelling capable of simulating flow.

The main objective of this study is to evaluate the quality of groundwater using WQI in Tuticorin, India and thematically denote it using Geographical information system (GIS) for the better understanding of the current scenario at a glance. An effort has been made in the coastal aquifers of Tuticorin to protect the wells from seawater intrusion by FEFLOW code, using hydrogeological and hydro chemical data.

Location of the study area

The study area Tuticorin is situated in the southeast shoreline of Tamil Nadu, India and is positioned between 8° 19′ to 9° 22′ N latitude and 77° 40′ to 78° 23′ E longitude. The area is bound by the Gulf of Mannar in the east and Tirunelveli district, Tamil Nadu in the west. The main occupation of the people is agriculture. Many chemical industries, food processing industries, salt industries are situated in Tuticorin. Many industries are established after the construction of the port and Tuticorin became a district in the year 1986. After the formation, the economic development has been boosted and began to develop rapidly. Therefore, the urban expansion acquired in various areas of the district. The slope of the terrain is mild in the western part and flat in the eastern part. The location map of the study area is shown in *Figure. 1*.

Methodology

Sample collection

A total of 38 groundwater samples are collected from open and bore wells. The location of observation wells is shown in *Figure 2*. Each sample is collected in 1000 mL acid-washed polyethylene HDPE bottle. The bottle is fully filled with water such that no air bubble is stuck within the water sample. To avoid evaporation, the bottles are closed with double plastic caps. Precaution is also taken to prevent agitation of sample during transfer from field to the lab.

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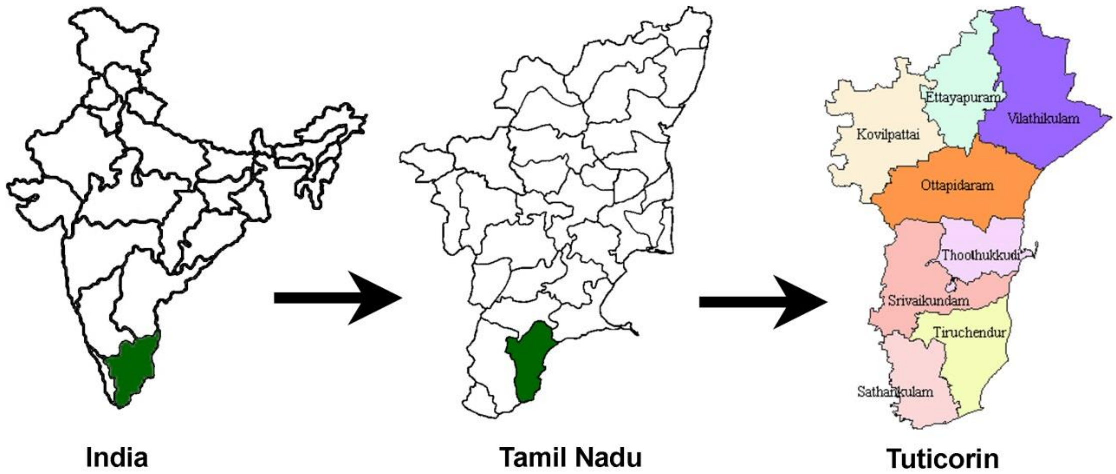

Figure 1. *Location map of study area*

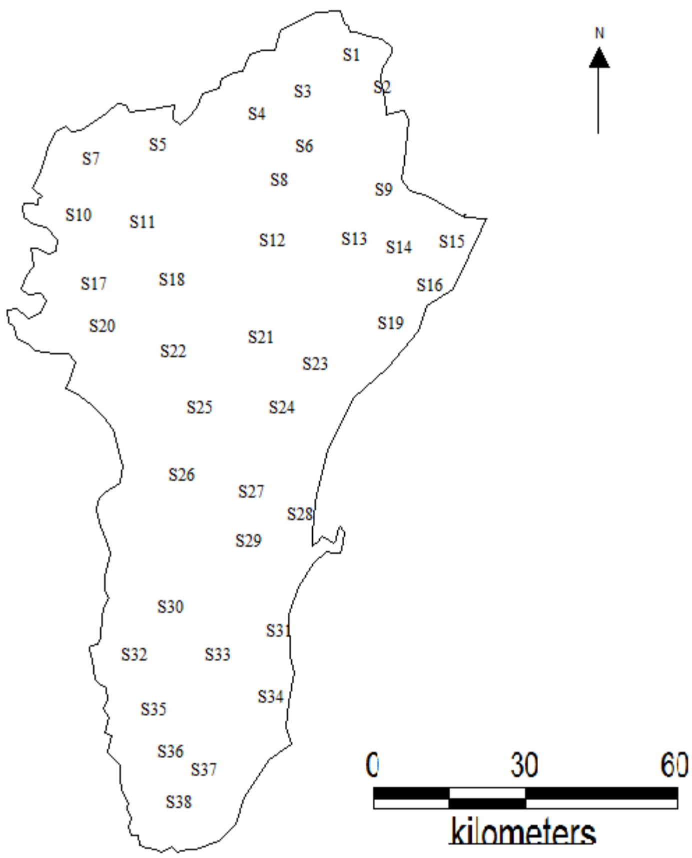

Figure 2. *Location of observation wells*

Laboratory measurements

The physico-chemical analysis was conducted using following methods and equipment as shown in *Table 1*.

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Table 1. *Method and instrument used in water quality analysis*

S.No	Parameter	Method	Instrument/equipment
1.	pH	Electrometric	pH meter
2.	EC (electrical conductivity)	Electrometric	Conductivity meter
3.	TDS (total dissolved solids)	Electrometric	Conductivity meter
4.	TH (total hardness)	Titration by EDTA	-
5.	Ca^{2+} (Calcium)	Titration by EDTA	-
6.	Mg^{2+} (Magnesium)	Titration by EDTA	-
7.	Na^{+} (Sodium)	Flame emission	Flame photometer
8.	K^{+} (Potassium)	Flame emission	Flame photometer
9.	HCO_3^- (Bicarbonate)	Titration by H_2SO_4	-
10.	Cl^- (Chlorine)	Titration by $AgNO_3$	-
11.	SO_4^{2-} (Sulphate)	Use wavelength of 420 nm	UV–VIS spectrophotometer
12.	NO_3^- (Nitrate)	Use wavelength of 220 nm	UV–VIS spectrophotometer
13.	F^- (Fluoride)	Ion-selective electrode (ISE)	pH meter

All concentrations are denoted in milligrams per litre (mg/L), excluding the pH and EC

Determination of water quality index (WQI)

To calculate WQI, thirteen parameters namely pH, EC, TDS, TH, Ca^{2+}, Mg^{2+}, Na^{+} K^{+}, HCO_3^-, Cl^-, SO_4^{2-} NO_3^- and F^- are considered into account. The following few steps are followed in calculating WQI.

Parameter selection

The selection of parameters depends on several aspects, such as the purpose of the index, the importance of the parameter, and the availability of data (Stigter et al., 2006). For the assessment of drinking water quality, priority should be given to those parameters which are known to be important to health and to be present in significant concentrations in the water source (Ramesh et al., 2010). *Table 2* gives the details of the weights and relative weights assigned to various parameters.

Table 2. *Weights for various parameters*

Water quality parameter	Units	WHO (2017)		Weight (W)	Relative weight (W_R)
		Most desirable limit	Maximum allowable limit		
pH	-	6.5	8.5	4	0.09
EC	µs/cm	780	3125	-	-
HCO_3	mg/L	-	300	1	0.03
TDS	mg/L	500	1500	5	0.11
F	mg/L	-	1.5	5	0.11
Cl	mg/L	200	600	5	0.11
NO_3	mg/L	45	-	5	0.11
SO_4	mg/L	200	400	5	0.11
Na	mg/L	-	200	4	0.09
Ca	mg/L	75	200	3	0.07
Mg	mg/L	30	150	3	0.07
K	mg/L	-	10	2	0.05
TH	mg/L	300	600	2	0.05
				$\sum W = 44$	$\sum W_R = 1$

Drinking water quality standards given by the World Health Organization (WHO, 2017)

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Weight assignment

The assignment of weight to water quality parameters is to represent the importance of each parameter in the overall water quality. Higher weightage implies greater importance of the variable with respect to public health (Song and Kim, 2009). Therefore, each of the selected parameters has been given a weight (W) 1 to 5, depending on their importance. The chosen weights are tabulated in *Table 2*. These weights are assumed based on the data available from previous studies (Serrekawo and Karoppannan, 2018; Ramakrishnaiah et al., 2009; Gebrehiwot et al., 2011; Kalpana et al., 2014).

Relative weight calculation

Relative weight (W_R) can be determined by dividing the individual weight of each parameter (W_I) by the sum of weight of all selected parameters (*W*) shown in *Equation 1*.

$$W_R = \frac{W_I}{W} \quad \text{(Eq.1)}$$

where W_R is the relative weight, W_I is the weight of the parameter under consideration.

Quality rating calculation

The fourth step is the calculation of quality rating (Q_I) for each parameter, is given in *Equation 2*.

$$Q_I = \frac{C_I}{S_I} \times 100 \quad \text{(Eq.2)}$$

where Q_I is quality rating, C_I (mg/L) is the concentration of each parameter in water sample, and S_I (mg/L) is the Bureau of Indian standard for chemical parameter.

Sub-index calculation

The sub-index for each chemical parameter is determined using *Equation 3*.

$$S_I = W_R \times Q_I \quad \text{(Eq.3)}$$

S_I is the sub-index of the *i*th parameter. It combines its quality rating as well as its assigned weight.

Calculation of water quality index

The overall WQI is calculated by summation of all sub index values of each groundwater sample as follows (*Eq. 4*):

$$WQI = \sum S_I \quad \text{(Eq.4)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Classification of water quality index

Higher WQI values indicate worse water quality, and lower values indicate excellent water quality. The detail description of WQI range and its classification is given in *Table 3*.

Table 3. *Classification of WQI values*

WQI range	Category of water
<50	Excellent
50–100	Good
100–200	Poor
200–300	Very poor
>300	Unfit for drinking purpose

GIS analysis

GIS is an effective method for spatial analysis and integration of the data to derive needed outputs and modelling. GIS can be beneficial for taking fast conclusions as graphical illustration would make visualization easy for policy makers. The study area of Tuticorin district is digitized from the survey of India toposheet using QGIS. The exact locations of observation wells are determined in the field using GPS and the precise longitudes and latitudes of observation wells are imported in GIS platform. Inverse Distance Weighted (IDW) interpolation method was used for spatial modelling of WQI and the parameter values are classified according to *Table 3*.

Model development (FEFLOW)

The first step in FEM analysis is the generation of mesh that fills the envisioned model domain with elements as shown in *Figure 3*. Based on the finite-element mesh, initial hydraulic head, boundary conditions and material properties are defined as given in *Table 4*.

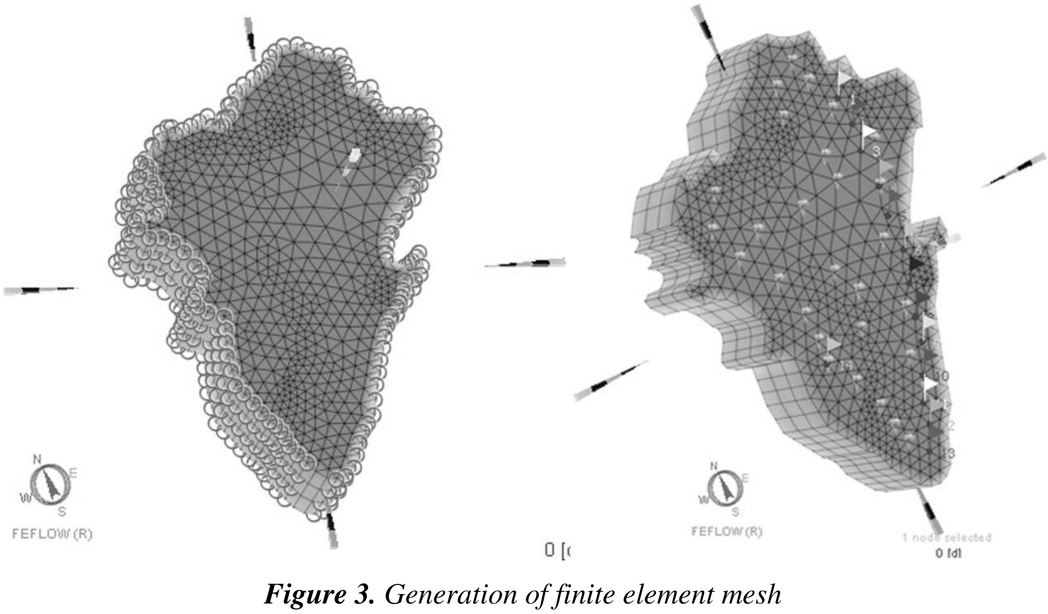

Figure 3. *Generation of finite element mesh*

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Table 4. *Data used for model development*

Parameter	Value
Physical parameters	
Mesh	
Element type	Triangle prism
Mesh element	3351
Mesh node	2496
Number of layer	1
Number of slice	2
Total model boundary areas	4621 km^2
Materials properties and boundary conditions (BC)	
Hydraulic conductivity K_X, K_Y, K_Z	4.5 × 10^{-4} m/s, 4.5 × 10^{-4} m/s, 1.5 × 10^{-4} m/s
Specific storage (fluid flux)	.002
Total porosity	.35
Longitude dispersivity	1
Transverse dispersivity	0.1 m
Density ratio	1025 kg/m^3
Constant head	0 m
Recharge concentration	0 mg/L
Constant head concentration	35000 mg/L
Initial concentration	20.22
Initial head	1.011 m

The model is calibrated by changing the aquifer properties and the boundary conditions in order to obtain a model appropriate to simulate the groundwater flow and the mass concentration distribution, within the calibration target. In the calibration process, a trial and error technique is carried out in order to reduce the variance between the simulated and the measured values. Once the model is calibrated; it is run to verify various scenarios as shown in *Table 5* where the extrapolative simulations are performed to minimize saltwater intrusion in the unconfined aquifer.

Table 5. *Three scenarios used for model simulation*

Simulation	Description
Scenario A	Abstraction of saline water 1000 m^3/day
Scenario B	Recharge of treated water at the rate of 500 m^3/day
Scenario C	Abstraction of saline water 1000 m^3/day, desalination and recharge of treated water at the rate of 500 m^3/day

Results

Laboratory measurements

The water quality characteristics of the groundwater samples during the year 2014 and 2018 are determined and the values are given in *Tables 6* and *7*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Table 6. *Physico-chemical values in 2014*

Well No	Latitude	Longitude	pH	EC	TDS	TH	Ca	K	Na	Mg	HCO_3	Cl	SO_4	F	NO_3	WQI 2014
S1	09°13'47"	78°08'12"	7.5	1360	763	370	54	9	145	57	342	220	33	0.38	17	130
S2	09°14'09"	78°01'50"	8	1440	823	420	60	4	145	66	354	149	154	0.38	15	121
S3	09°17'47"	78°03'56"	8.5	1190	749	305	84	3	124	23	73	21	442	0.65	1	86
S4	09°10'45"	77°52'25"	8.3	770	458	235	48	7	69	28	142	99	65	0.36	12	77
S5	09°08'30"	78°02'15"	8	3070	1985	550	80	12	460	85	366	355	624	1.04	42	258
S6	09°08'57"	77°42'10"	8.8	2930	1612	540	108	2	414	66	641	482	192	1.14	2	254
S7	09°09'00"	77°59'54"	7.8	1270	755	330	42	7	145	55	336	124	154	1.09	14	116
S8	09°07'45"	78°10'07"	8.2	3950	2290	770	148	5	552	97	647	709	384	1.57	16	362
S9	09°08'40"	77°47'15"	7.7	1410	1045	810	152	23	177	104	232	234	68	0.32	38	179
S10	09°04'30"	77°48'30"	8.8	690	399	185	8	14	69	40	155	53	96	0.26	4	63
S11	09°06'00"	78°00'40"	7.7	3610	2257	820	296	12	460	19	311	837	154	0.15	73	375
S12	08°33'00"	78°01'00"	8.2	740	394	280	40	4	41	44	74	170	29	0.46	5	89
S13	09°04'00"	78°10'35"	8.5	1730	1028	400	60	2	225	61	354	191	288	0.78	3	145
S14	09°13'47"	78°08'12"	8.6	2600	1606	370	96	16	442	32	689	326	216	0.63	27	215
S15	09°00'10"	78°11'50"	7.9	550	296	235	40	4	20	33	203	21	41	0.56	3	51
S16	09°03'35"	77°44'50"	8.6	1520	936	230	16	7	253	46	293	170	214	1.4	16	136
S17	08°59'40"	77°51'38"	7.9	730	425	285	50	9	35	39	109	117	27	0.29	18	81
S18	09°00'00"	77°58'00"	8	1610	966	240	20	1	276	46	421	206	144	1.02	14	139
S19	09°03'00"	77°54'00"	7.6	2330	1527	350	48	108	350	56	470	340	173	0.58	49	245
S20	09°01'10"	78°02'45"	8.4	2100	1376	260	40	88	331	39	439	234	264	1.48	35	212
S21	08°56'40"	77°52'00"	8.4	1740	1060	370	28	121	161	73	366	255	115	0.22	27	199
S22	08°54'45"	78°01'25"	7.6	3050	1752	1320	240	37	78	175	238	709	96	0.33	67	353
S23	08°52'30"	78°02'15"	7.5	2540	1598	540	100	66	304	70	329	404	178	0.1	70	252
S24	08°51'38"	77°54'00"	7.9	2530	1504	560	80	78	290	87	537	390	192	0.41	26	250
S25	08°47'25"	77°52'15"	8.6	590	315	200	42	20	32	23	212	32	25	0.17	2	55
S26	08°48'35"	78°01'25"	7.5	880	503	260	360	37	368	87	178	465	336	0.23	59	100
S27	08°48'00"	78°10'00"	8.2	250	145	100	24	3	10	10	59	28	22	0.35	2	34
S28	08°44'12"	77°59'45"	8.2	330	215	95	22	2	35	10	54	35	29	0.8	11	45
S29	08°38'00"	77°55'00"	8.4	660	391	170	34	13	69	21	151	82	48	0.2	6	66
S30	08°34'30"	78°05'45"	8	1550	914	320	24	100	161	63	445	191	125	1.45	6	178
S31	08°34'40"	77°49'15"	8.8	2530	1459	460	76	16	368	66	427	475	192	1.07	8	243
S32	08°26'40"	78°00'32"	8	2230	1302	240	48	18	391	29	702	255	192	1.03	4	178
S33	08°29'00"	78°01'30"	8	530	328	155	34	59	23	17	218	18	10	0.36	7	67
S34	09°05'00"	78°05'00"	8.3	480	249	125	18	2	55	19	211	7	13	0.86	1	43
S35	08°27'00"	78°01'00"	8.2	1470	799	420	48	11	143	73	214	347	58	0.6	3	164
S36	09°12'54"	78°12'30"	8	980	563	305	50	13	87	44	244	152	63	0.79	7	106
S37	08°25'00"	78°57'00"	7.8	830	473	340	64	4	32	44	114	131	43	0.16	19	86
S38	08°24'30"	77°52'00"	8.2	1090	616	300	50	7	115	42.5	293	181	35	0.74	9	142

Table 7. *Physico-chemical values in 2018*

Well No	Latitude	Longitude	pH	EC	TDS	TH	Ca	K	Na	Mg	HCO_3	Cl	SO_4	F	NO_3	WQI 2018
S1	09°13'47"	78°08'12"	8.2	1450	888	255	42	19	212	36	293	209	71	0.09	34	133
S2	09°14'09"	78°01'50"	8.2	1600	1022	430	92	20	166	49	329	163	125	0.06	55	250
S3	09°17'47"	78°03'56"	7.6	2640	1559	740	184	23	258	68	354	532	204	0.12	25	266
S4	09°10'45"	77°52'25"	8.2	610	352	165	28	4	67	23	207	28	27	0.23	11	50
S5	09°08'30"	78°02'15"	7.9	2680	1568	510	144	10	396	36	683	425	72	0.16	32	260
S6	09°08'57"	77°42'10"	8.1	190	99	80	18	1	7	9	74	7	7	0.32	1	24
S7	09°09'00"	77°59'54"	8.8	2040	1235	200	40	16	368	24	500	220	125	0.08	39	155
S8	09°07'45"	78°10'07"	8.2	3180	1886	670	72	47	414	119	268	737	250	0.06	25	335
S9	09°08'40"	77°47'15"	8.1	310	188	105	26	5	23	10	69	35	10	0.4	8	40
S10	09°04'30"	77°48'30"	8.2	920	555	210	46	7	113	23	299	67	42	0.17	24	76
S11	09°06'00"	78°00'40"	7.4	1890	1138	620	148	14	166	61	256	383	61	0.21	40	201
S12	08°33'00"	78°01'00"	7.9	1050	686	370	94	11	74	33	153	96	216	0.21	19	99
S13	09°04'00"	78°10'35"	8.1	9310	5581	1200	100	7	1610	231	732	1914	1344	0.85	2	837

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S14	09°13'47"	78°08'12"	8	5680	3268	1420	272	8	644	180	708	993	768	0.25	11	502
S15	09°00'10"	78°11'50"	8.1	3590	2125	660	40	11	552	136	403	695	456	0.92	8	338
S16	09°03'35"	77°44'50"	8.1	870	514	90	12	16	154	15	360	39	83	1.43	3	78
S17	08°59'40"	77°51'38"	8	120	73	40	8	7	7	49	35	14	6	0.43	1	32
S18	09°00'00"	77°58'00"	8.2	3150	1885	580	112	4	460	73	458	596	206	0.06	46	287
S19	09°03'00"	77°54'00"	8	250	145	100	24	7	10	10	99	7	10	0.07	4	28
S20	09°01'10"	78°02'45"	8.7	2970	1826	320	44	20	552	51	598	319	451	1.4	16	235
S21	08°56'40"	77°52'00"	8.2	1910	1136	340	32	3	294	63	500	248	165	0.51	18	160
S22	08°54'45"	78°01'25"	7.5	3380	1994	740	144	7	460	92	647	560	202	0.13	46	297
S23	08°52'30"	78°02'15"	8.1	1780	1068	490	68	19	173	78	281	305	160	0.12	28	175
S24	08°51'38"	77°54'00"	8.2	1380	786	250	54	5	207	28	488	152	71	0.11	6	108
S25	08°47'25"	77°52'15"	8.2	340	194	125	18	9	20	19	89	39	24	0.83	2	48
S26	08°48'35"	78°01'25"	8.2	990	599	230	24	63	92	41	256	121	84	0.46	10	114
S27	08°48'00"	78°10'00"	8.1	8410	5350	2120	36	7	1104	493	488	1773	1440	1.31	28	840
S28	08°44'12"	77°59'45"	8	970	560	210	18	6	124	40	244	121	83	0.4	10	88
S29	08°38'00"	77°55'00"	8.1	340	189	125	30	3	20	12	89	35	18	0.39	4	40
S30	08°34'30"	78°05'45"	8.2	1360	796	315	42	59	143	51	439	145	80	1.33	13	145
S31	08°34'40"	77°49'15"	8.2	840	499	100	12	4	147	17	217	60	62	0.66	14	68
S32	08°26'40"	78°00'32"	8.2	2140	1227	500	112	9	276	53	525	376	112	0.35	6	199
S33	08°29'00"	78°01'30"	7.8	1390	835	445	120	8	117	35	177	273	75	0.2	27	147
S34	09°05'00"	78°05'00"	8.2	490	286	210	54	4	20	18	108	60	50	0.32	3	55
S35	08°27'00"	78°01'00"	8	2150	1288	300	36	63	336	51	580	305	81	1.28	28	211
S36	09°12'54"	78°12'30"	8	470	274	110	16	6	58	17	129	53	30	0.09	3	46
S37	08°25'00"	78°57'00"	8.2	780	441	220	42	16	74	28	197	106	26	1.03	6	87
S38	08°24'30"	77°52'00"	8.2	1350	816	305	58	11	173	39	171	266	78	0.44	24	142

The water quality characteristics of the groundwater samples during the year 2014 and 2018 is statistically analysed and the results such as maximum, minimum, mean and standard deviation values are given in *Table 8*.

Table 8. *Statistical measures such as minimum, maximum, mean and standard deviation in 2014 and 2018*

Water quality parameters	**Jan 2014**				**Jan 2018**			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
pH	7.4	8.8	8.09	0.25	7.5	8.8	8.12	0.38
EC	120	9310	1972.89	2028.29	250	4180	1662.11	1045.56
HCO_3	35	732	328.93	199.79	54	702	311.63	175.17
TDS	73	5581	1182.39	1236.79	145	2503	996.74	637.78
F	0	1	0.46	0.43	0	2	0.64	0.42
Cl	7	1914	328.34	430.94	7	865	253.03	224.93
NO_3	1	55	17.89	14.64	1	73	19.42	20.54
SO_4	6	1440	194.08	322.85	10	624	145.63	133.62
Na	7	1610	265.55	317.25	10	552	196.18	154.98
Ca	8	272	64.79	56.65	8	360	74.53	75.39
Mg	5	493	62.32	86.10	10	175	52.34	31.98
K	1	63	14.71	16.17	1	121	24.84	33.06
TH	40	2120	418.42	412.23	95	1320	401.71	277.38

The values of water quality characteristics like pH, EC, HCO_3^-, TDS, F^-, Cl^-, NO_3^-, SO_4^{2-}, Na^+, Ca^{2+}, Mg^{2+}, K^+ and TH in 2014 and 2018 are compared with drinking water quality norms recommended by WHO (2017) and the samples beyond allowable limit during the years 2014 and 2018 are given in *Table 9*.

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Table 9. *Comparison of groundwater quality with WHO standards*

Water quality parameter	Units	WHO (2017)		Samples exceeding allowable limit in 2014	Samples exceeding allowable limit in 2018
		Most desirable limit	Maximum allowable limit		
pH	-	6.5	8.5	2	6
EC	µs/cm	780	3125	17	19
HCO_3	mg/L	-	300	16	20
TDS	mg/L	500	1500	16	17
F	mg/L	-	1.5	1	0
Cl	mg/L	200	600	11	13
NO_3	mg/L	45	-	3	5
SO_4	mg/L	200	400	2	5
Na	mg/L	-	200	15	16
Ca	mg/L	75	200	3	1
Mg	mg/L	30	150	1	3
K	mg/L	-	10	20	16
TH	mg/L	300	600	5	8

pH

A pH value 7 indicates neutral. If the value is lower than 7 it indicates acidity and higher than 7 indicates alkalinity. The values of pH ranges from 7.5 to 8.8 and 7.4 to 8.8 during 2014 and 2018 which specifies that the groundwater is alkaline in both 2014 and 2018 and some of the samples exceed the maximum permissible limits of WHO standards. The slight alkalinity is due to the existence of bicarbonate ions, which are formed by the free combination of CO_2 in water with the carbonates from the aquatic life, which affects the pH of the water (Azeez et al., 2000). The spatial distribution of pH is shown in *Figure 4* indicates all the samples are within the permissible limit of 6.5-8.5., except two samples in 2014 and six samples in 2018.

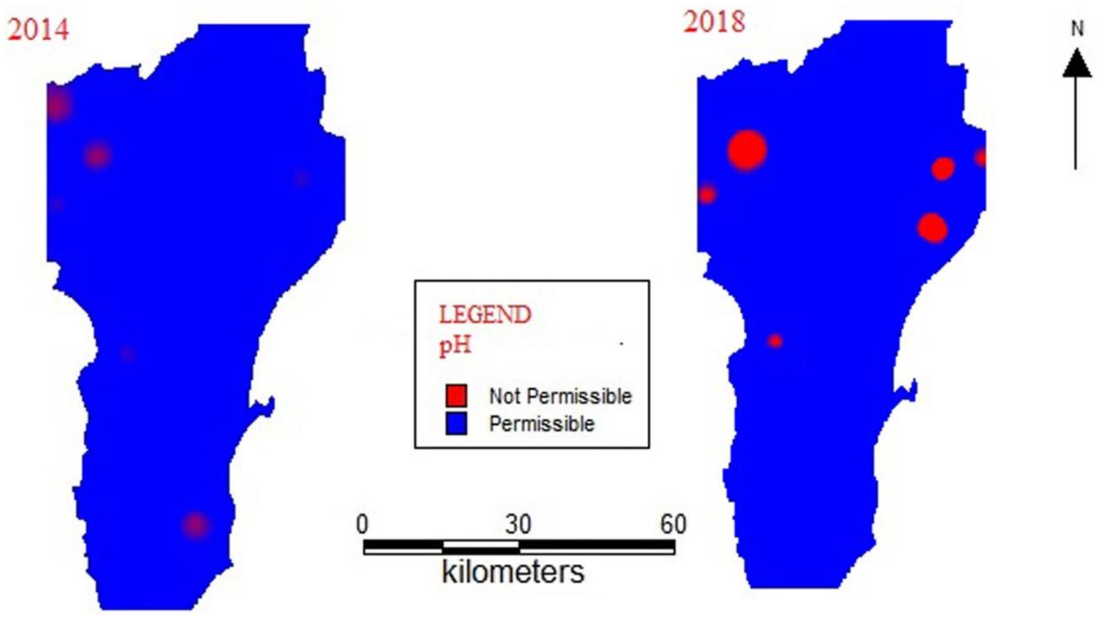

Figure 4. *Spatial distribution of pH*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Electrical conductivity

Electrical conductivity is the most significant parameter to delineate salinity hazard and suitability of water for irrigation purpose. The EC vary from 250 to 4180 µs/cm and from 120 to 9310 µs/cm during the years 2014 and 2018, respectively. The spatial distribution of EC is shown in *Figure 5*. Higher value of EC were noted during 2018 when compared to that of 2014. The classification of water salinity based upon Electrical conductivity shows that 45% in 2014 and 50% in 2018 falls within the permissible limit. To determine the salinity of groundwater, it is important to categorize the groundwater based on their EC values (Handa, 1969), which are represented in *Table 10*.

Table 10. *Classification of waters based on EC (Handa, 1969)*

EC (µs/cm)	Water salinity	2014		2018	
		Number of samples	Percentage of samples	Number of samples	Percentage of samples
0–250	Low	1	3	3	8
251–750	Medium	9	24	6	16
751–2250	High	17	45	19	50
2251–6000	Very high	11	20	8	21
6001–10000	Extremely high	-	-	2	5
10001–20000	Brines weakly concentrated	-	-	-	-
20001–50000	Brines moderately concentrated	-	-	-	-
50001–100000	Brines highly concentrated	-	-	-	-
>100000	Brines extremely highly concentrated	-	-	-	-
Total		38	100	38	100

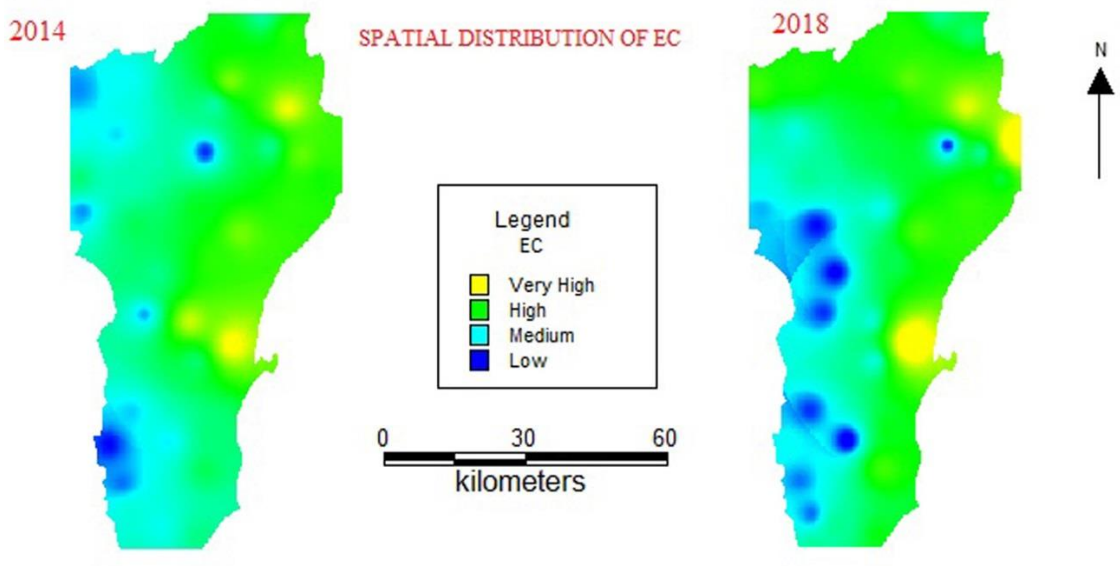

Figure 5. *Spatial distribution of EC*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Total dissolved solids

TDS of the groundwater are found to vary from 145 to 2503 mg/L during 2014 and 73 to 5581 mg/L during 2018. The Spatial Distribution of TDS is shown in *Figure 6*. Some groundwater samples having high value of TDS are may be due to presence of excess salts in the soil and also by manmade activities. Sewage waste from household may penetrate into the groundwater which also leads to raise in TDS values. To ascertain the groundwater, it is essential to categorize the groundwater based on their TDS values, which are presented in *Tables 11* and *12,* respectively.

Table 11. *Classification of groundwater based on TDS (Davis, 1966)*

Total dissolved solids (mg/L)	Classification	2014		2018	
		Number of samples	Percentage of samples	Number of samples	Percentage of samples
<500	Desirable for drinking	12	32	11	32
500–1000	Permissible for drinking	10	26	10	26
1000–3000	Useful for irrigation	16	42	14	37
>3000	Unfit for drinking and irrigation	0	-	3	8
Total		38	100	38	100

Table 12. *Classification of groundwater based on TDS (Freeze and Cherry, 1979)*

Total dissolved solids (mg/L)	Classification	2014		2018	
		Number of samples	Percentage of samples	Number of samples	Percentage of samples
<1000	Freshwater	16	42	15	39
1000–10000	Brackish water	22	58	23	61
10000–100000	Saline water	-	-	-	-
>100000	Brine water	-	-	-	-
Total		38	100	38	100

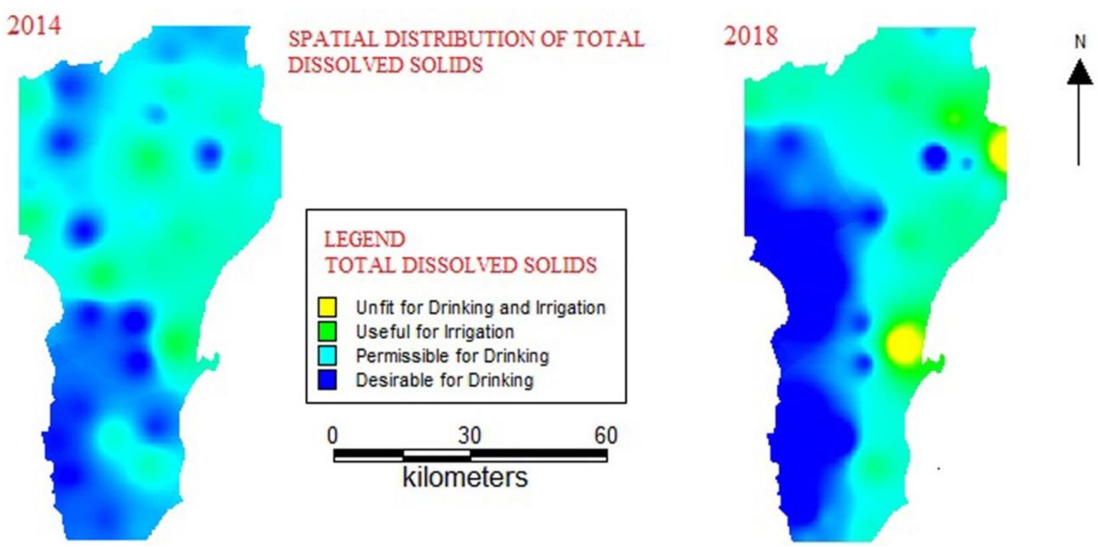

Figure 6. *Spatial distribution of TDS*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Total hardness

Total hardness is found to vary from 95 to 1320 mg/L with a mean value of 401.71 mg/L during 2014 and 40 to 2120 mg/L during 2018. According to WHO, the allowable limit of TH for drinking is 600 mg/L and the desirable limit is 300 mg/L. The Spatial Distribution of TH is as shown in *Figure 7*. The Total Hardness of the groundwater sample in the study area fell in the hard and very hard water category. Total Hardness means both temporary and permanent hardness. Hardness is a significant parameter in determining the need for industries and domestic purposes. Hard water does not produce much bad effect but it requires more detergent for cleaning and in some cases very hard water might be the reason for heart disease (Schoeller, 1965). The TH in mg/L is determined by *Equation 5* (Todd, 1959):

$$TH\,(mg\,/\,L) = 2:497Ca2^{+} + 4:115Mg2^{+} \quad \text{(Eq.5)}$$

Based on hardness groundwater is classified (Sawyer and McCarthy, 1967) and is presented in *Table 13*. In the study area, 22 samples were found in very hard class in 2014, whereas in 2018, 19 samples were found in very hard class. This reveals that Tuticorin experiences very hard water during both the years. High levels of hardness may affect water supply system, excessive detergent consumption and also health problem (Bhawan and Nagar, 2008).

Table 13. *Classification based on hardness (Sawyer and McCarthy, 1967)*

Total hardness as CaCo3 (mg/L)	Classification	2014		2018	
		Number of samples	Percentage of samples	Number of samples	Percentage of samples
<75	Soft	0	0	1	3
75–150	Moderately high	3	8	8	21
150–300	Hard	12	32	9	24
>300	Very Hard	22	58	19	50
Total		38	100	38	100

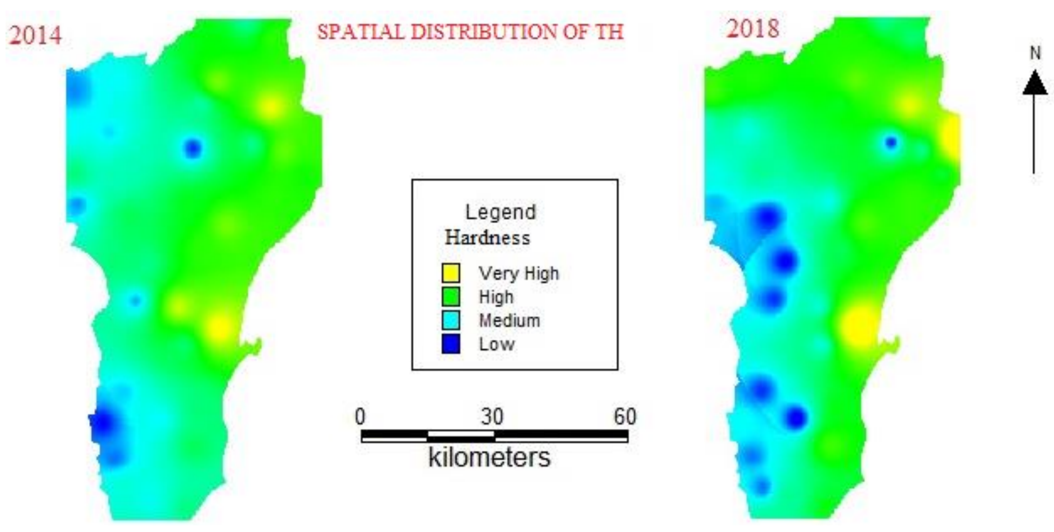

Figure 7. *Spatial distribution of TH*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Calcium

Calcium concentration is found to vary between 8-360 mg/L with a mean value of 74.53 mg/L for 2014 water samples and 8-272 mg/L with a mean value of 64.79 mg/L for 2018 samples. The allowable limit of calcium in groundwater is 200 mg/L as per WHO (2017). The Spatial Distribution of Calcium is shown in *Figure 8*. In 2014, 92% and in 2018, 97% of samples are up to the standard and only 8% samples exceed the permissible limit in 2014 and 3% samples exceed the permissible limit.

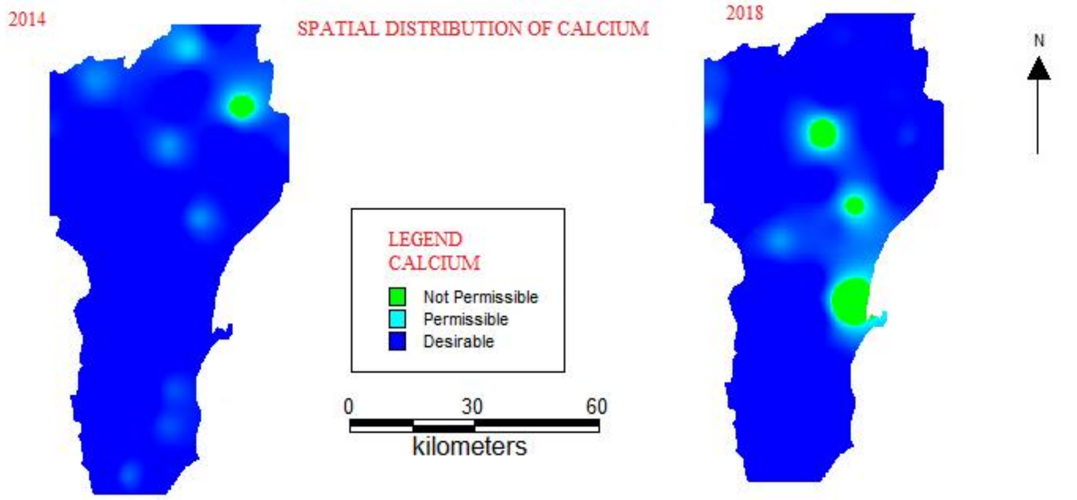

Figure 8. *Spatial distribution of calcium*

Magnesium

Magnesium vary from 10 to 175 mg/L with a mean value of 52.34 mg/L during the year 2014 and 5 to 493 mg/L with a mean value of 62.32 mg/L during the year 2018. The maximum allowable limit of magnesium in groundwater is 150 mg/L as per WHO (2017). The Spatial Distribution of Magnesium is shown in *Figure 9*. In 2014, only 3% of sample exceeds the permissible limit while in 2018 the 3% increased to 8% which revealed changes in water quality.

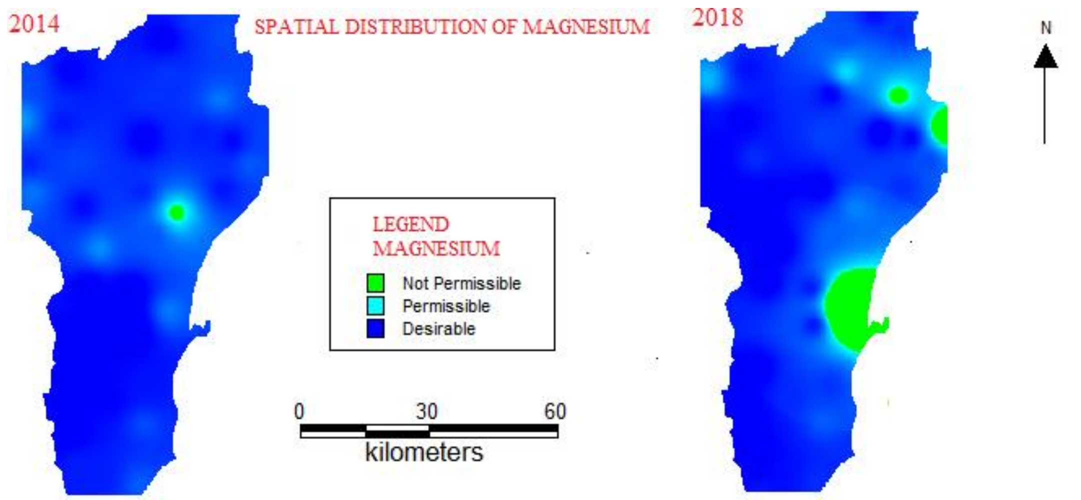

Figure 9. *Spatial distribution of magnesium*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Sodium

Sodium is found to vary from 10 to 552 mg/L with a mean value of 196.18 mg/L during the year 2014 and 7 to 1610 mg/L with a mean value of 265.55 mg/L during the year 2018. Sodium causes an increase in the hardness of soil as well as decrease its permeability (Tijani, 1994). The spatial distribution of sodium is shown in *Figure 10*. In 2014 and 2018 the high concentration of sodium is observed which may be due to saltwater intrusion.

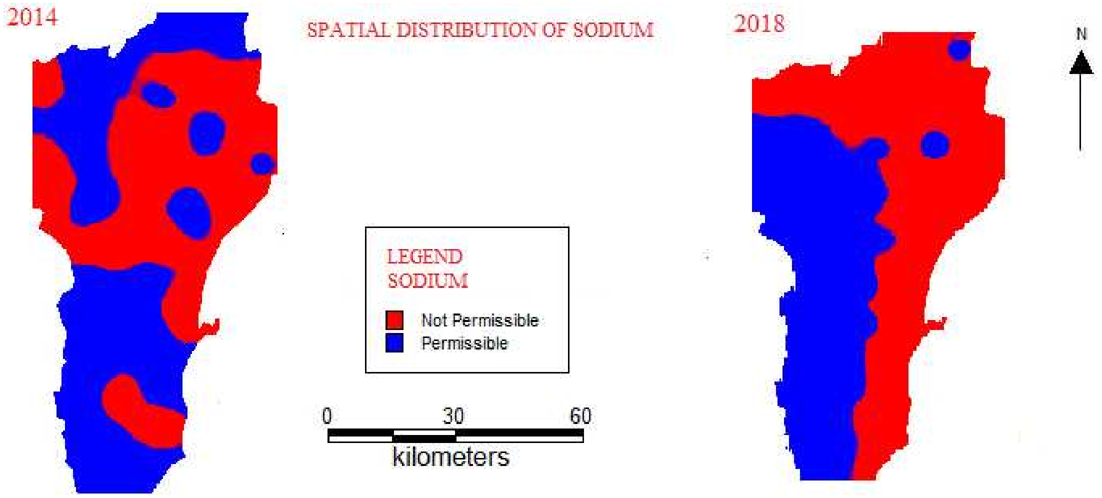

Figure 10. *Spatial distribution of sodium*

Potassium

Potassium ranges vary from 5 to 121 mg/L with a mean value of 24.84 mg/L during the year 2014 and 1 to 63 mg/L with a mean value of 14.71 mg/L during the year 2018. As per WHO (2017) standards, the allowable limit for potassium is 10 mg/L. The Spatial Distribution of Potassium is shown in *Figure 11*. Due to Salt pans, chemical fertilizer industries potassium concentration may be increased in the groundwater.

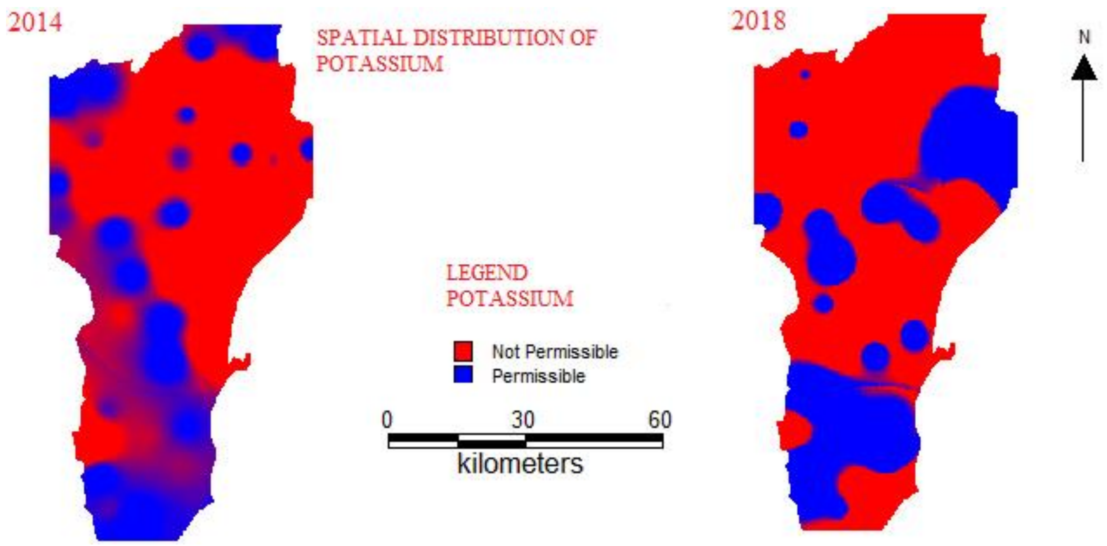

Figure 11. *Spatial distribution of potassium*

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Bicarbonate

Bicarbonate ranges from 54 to 702 mg/l with a mean value of 311.63 mg/L during the year 2014 and 35 to 732 mg/L with a mean value of 378.93 mg/L during the year 2018. Bicarbonate concentrations are somewhat higher in the year 2018 compared to the year 2014 indicating the influence of carbonate due to weathering processes. There is a major variation observed in the spatial distribution of bicarbonate as shown in *Figure 12* which may be due to saltwater intrusion (Selvam et al., 2013; Srinivasamoorthy et al., 2011).

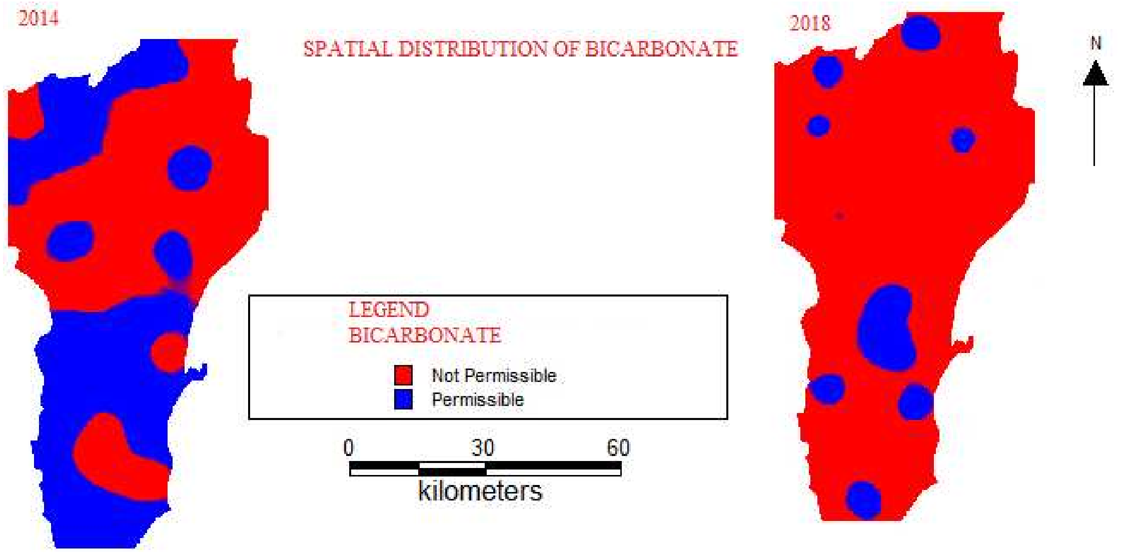

Figure 12. *Spatial distribution of bicarbonate*

Chloride

Chloride ranges from 7 to 865 mg/L with a mean value of 253.03 mg/L during the year 2014 and 7 to 1914 mg/L with a mean value of 328.34 mg/L during the year 2018. The allowable limit of Chloride is 600 mg/L. In Tuticorin 11% of sample exceed the permissible limit of chloride concentration in 2014. But in 2018 the percentage increases to 13%. Thus, high concentration of sodium and Chloride ions in groundwater may show a substantial effect of saltwater intrusion (Mondal et al., 2010). The Spatial Distribution of Chloride is shown in *Figure 13*. Higher concentration is noted during 2018 when compared with 2014 indicating domination of industrial activity and saltwater intrusion to the groundwater. High concentration of Chloride may be harmful to persons suffering from sicknesses of the heart and kidneys (Bhawan and Nagar, 2008).

Sulphate

Sulphate concentration vary from 10 to 624 mg/L with a mean value of 145.63 mg/L during the year 2014 and 6 to 1440 mg/L with a mean value of 194.08 mg/L during the year 2018. If the sulphate concentration exceeds allowable limit of 400 mg/L it will affect human organs. The sorting of groundwater based on sulphate is specified in *Table 2* and it is found that 5% of samples exceeded permissible limits during the year

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

2014 and 13% of samples are found to be exceeded the limits during the year 2018. Sulphate concentration in groundwater increases due to dissolution minerals and anthropogenic sources. The Spatial Distribution of Sulphate is shown in *Figure 14*.

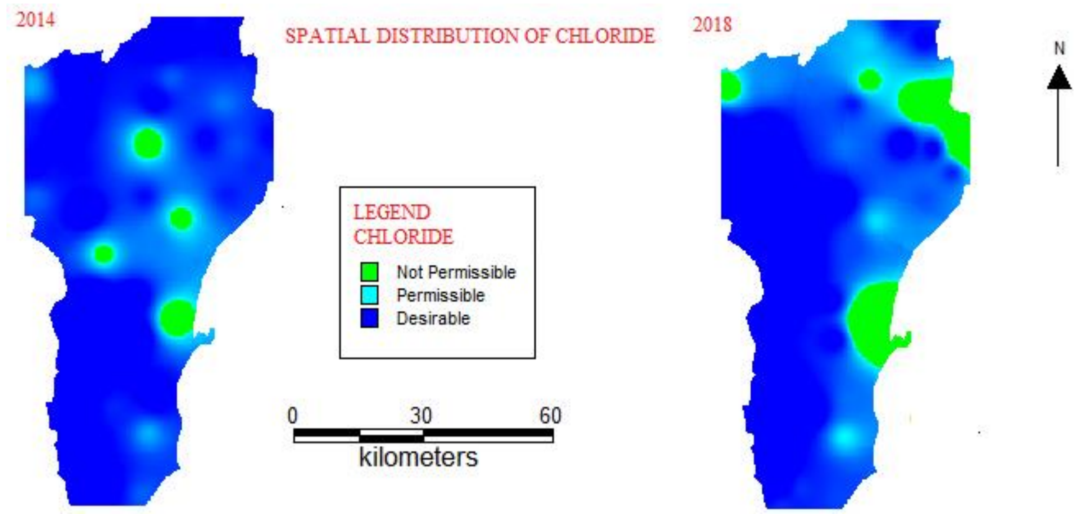

Figure 13. *Spatial distribution of chloride*

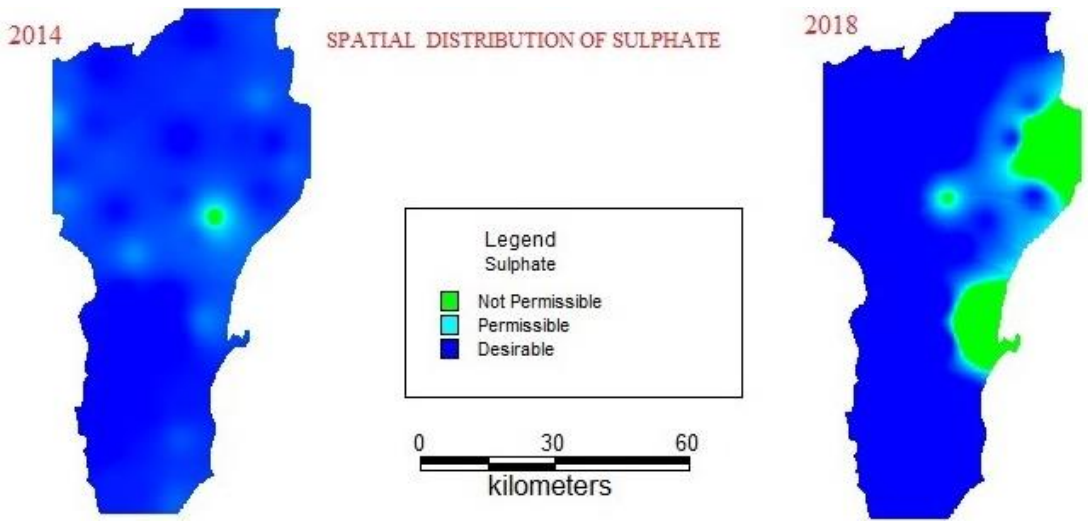

Figure 14. *Spatial distribution of sulphate*

Nitrate

Nitrate concentration vary from 1 to 73 mg/L with a mean value of 45.55 mg/L during the year 2014 and 1 to 55 mg/L with a mean value of 43.55 mg/L during the year 2018 allowable nitrate in drinking water as per WHO standards are 45 mg/L. The spatial distribution of nitrate is shown in *Figure 15*.

Fluoride

Fluoride concentration varies from 0 to 2 mg/L with a mean value of 0.64 mg/L during the year 2014 and 0 to 1 mg/L with a mean value of 0.46 mg/L during the year

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

2018. Allowable fluorides in drinking water as per WHO standards are 1.5 mg/L. Concentrations higher than allowable limit create many health problems (WHO, 2017). The spatial distribution of fluoride is shown in *Figure 16*.

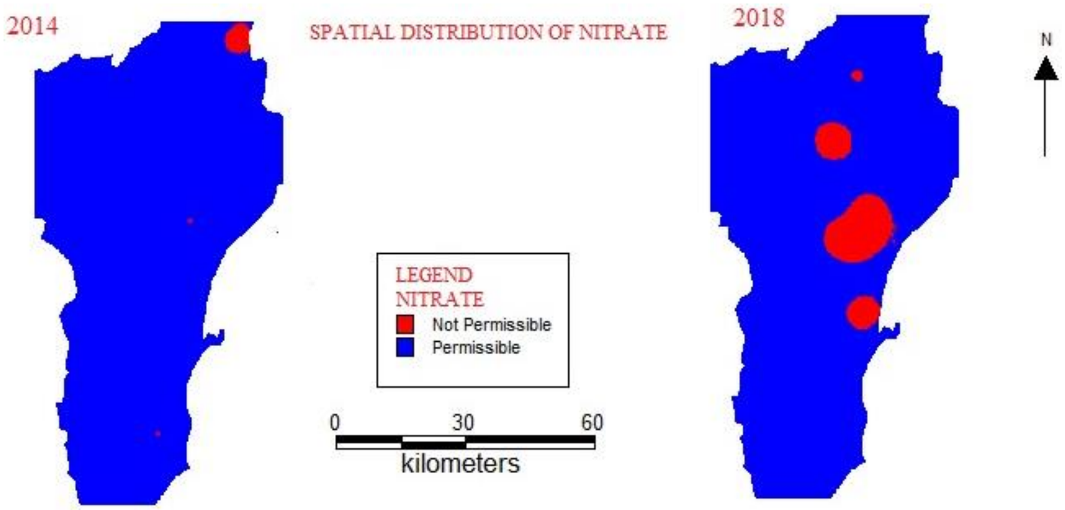

Figure 15. *Spatial distribution of nitrate*

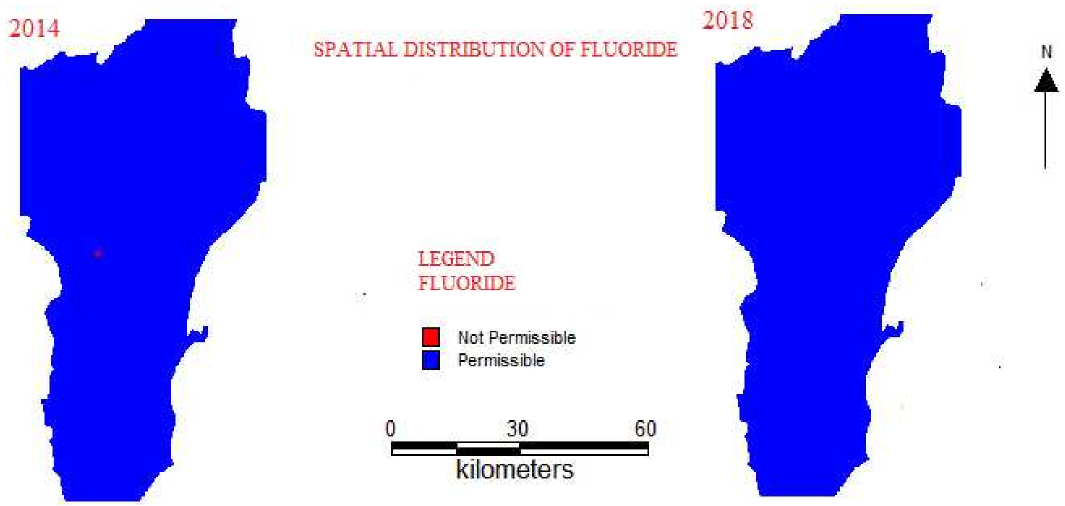

Figure 16. *Spatial distribution of fluoride*

According to salt concentration cations are characterized in ascending order as follows $Na^+ > K^+ > Mg^{2+} > Ca^{2+}$. From the above sorting it is clearly understood that Sodium and potassium concentration is prominent than the magnesium and calcium.

According to salt concentration anion are characterized in ascending order as follows $HCO^{3-} > Cl^- > SO_4^{2-} > F$. From the above sorting, Cl and HCO_3 showed a prominent role in Tuticorin than the concentrations of NO_3 and F.

Piper plot

Statistical diagram such as Piper plot is used to understand about hydro chemical processes functioning in the groundwater. The Piper diagram is used to recognize

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problems concerning the hydrogeochemical development of groundwater. The piper plot has three portions like two triangular portion and a diamond-shaped portion (Piper, 1944). The overall water quality is represented in the diamond-shaped field by extending the position of the plots in the triangular portion. Groundwater can be notable by their location, occupying certain spaces of the diamond-shaped portion. The experimental data obtained from the groundwater samples are plotted on a Piper trilinear diagram to understand the hydrogeochemical pattern in Tuticorin during the years 2014 and 2018.

The geochemical characteristics can be studied using Piper plot, which is separated into six sub groups (1) (Ca-HCO3 type); (2) (Na-Cl type); (3) (mixed Ca-Na-HCO3 type); (4) (mixed Ca-Mg-Cl type); (5) (Ca-Cl type) and (6) (Na-HCO3 type). The diagram *Figure 17* exposed that the samples during 2014 found in the group of Na-Cl and mixed Ca-Mg-Cl type. But during 2018, Ca-HCO3 type of water prevails in Tuticorin. Ca-Mg-Cl type of water falls under both years. It is detected that the alkalis (Na^+ and K^+) exceed the alkaline (Ca^{2+} and Mg^{2+}) and strong acids exceed weak acids. During 2014 and 2018, strong acid dominant over weak acid and HCO^{3-} and Cl^- have influence equal to Na^+, which indicates the severe saltwater intrusion occurred in Tuticorin.

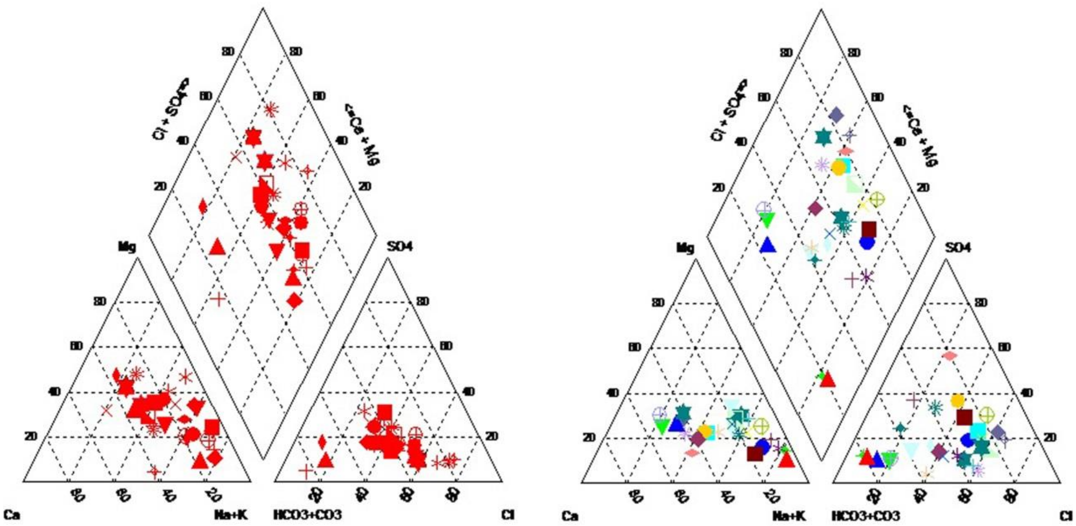

Figure 17. *Piper plot during the years 2014 and 2018*

Water quality index

The WQI for groundwater samples is exposed in *Tables 6* and *7*. Water collected from 38 observation wells are analysed for WQI. From the WQI value, it is found that villages like Keelairal, Kulathurterkku, Ottapidaram, Padanthapuli, Puthiyamputhur, Vilathikulam, Singathakuruchi, Vedanatham, Kachanavilai, Palangulam, Ariyanayagipuram, Srivaikundam, Thirukalur have showed improved water quality in 2018 compared with 2014. Water quality improvement may be due to the less withdrawal fresh groundwater and the rehabilitation of tanks. These villages located in the Taluk of Vilathikulam, Kovilpatti, Satankulam, Ettayapuram, Ottapidaram and Srivaikundam. The sample collected in villages present in Tuticorin Taluk like Melathattaparai, Marthadampatti have showed severe water quality deterioration

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occurred from 2014 to 2018. Since the fresh groundwater extraction is more in Tuticorin due to industries saltwater intrusion occurs in the coastal aquifer. It is clearly portrayed from WQI method that the saltwater intrusion mainly in the coastal belt of Tuticorin and moves inward.

The spatial and attribute data are integrated for the creation of the spatial WQI map. The WQI Maps for the year 2014 and 2018 are depicted in *Figure 18*.

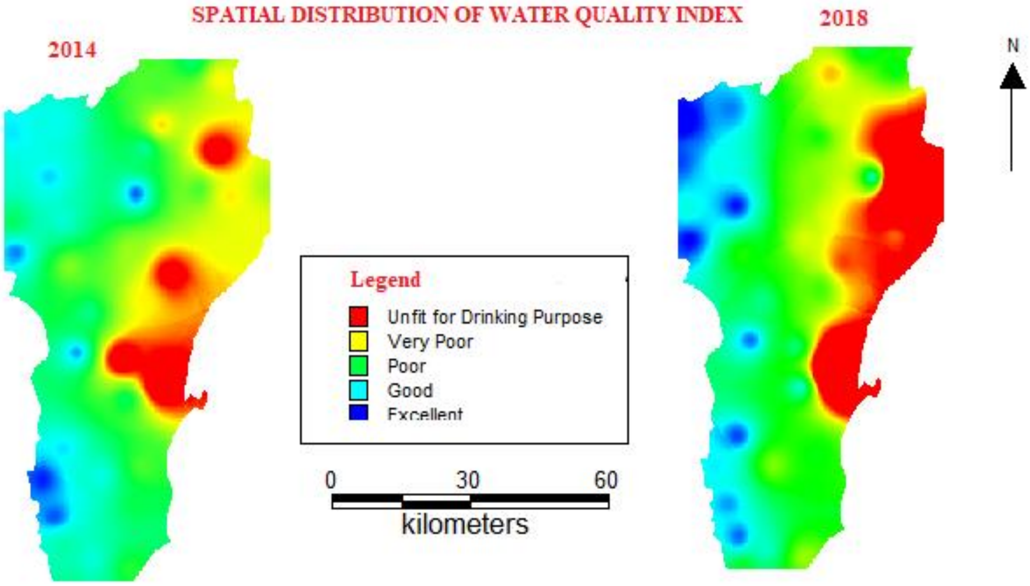

Figure 18. *Water quality index map for the year 2014 and 2018*

Comparison of WQI with the distance from sea

The computed WQI with respect to the distance of observation well from the sea is shown in *Figure 19*. From the figure it can be seen that water quality is poor in the observation wells found with in 15 km from sea shore during the year 2018 where as in 2014, observation wells found within 9 km are only affected. Similarly, in 2018 observation wells found with in 7 km is unfit for drinking. Hence the saltwater intrusion has moved inward from the seashore to the distance of 15 km during 2018.

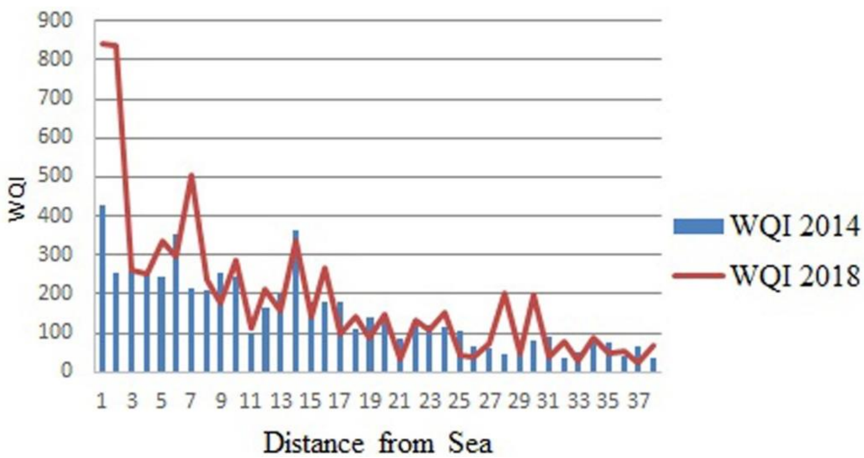

Figure 19. *WQI with respect to the distance from sea*

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Model calibration

The model is calibrated for steady state and then for transient state. In a steady state calibration hydraulic conductivity and the recharge concentration are adjusted until hydraulic head measured using piezometer in 15 observation wells in April 2018 become close to the computed hydraulic heads. The calibrated results are depicted in *Figure 20*. Good match is found between observed and computed hydraulic head. For transient state calibration longitudinal and transverse dispersivity are changed to match observed salt concentration with computed salt concentration. Final calibrated value of longitude dispersivity is 30 m and transverse dispersivity is 4 m.

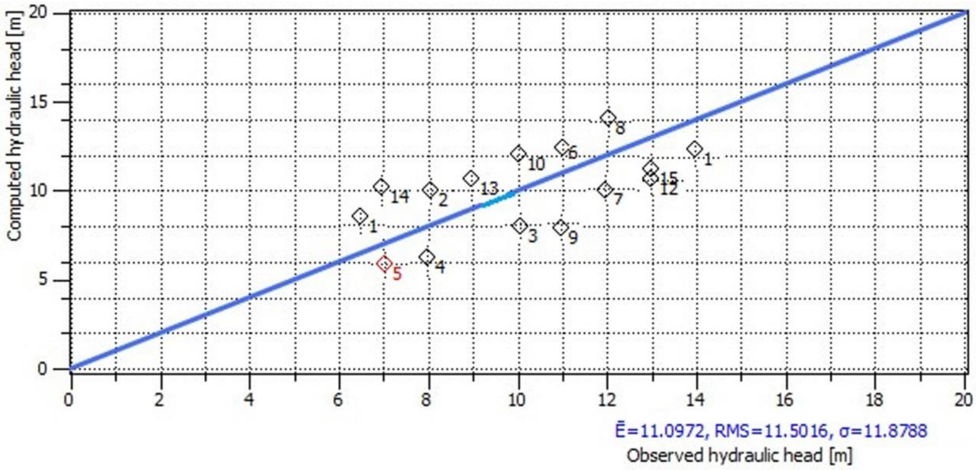

Figure 20. *Comparison of measured and computed hydraulic head*

Model verification

The Model developed is validated during the years 2000 to 2018 for the well located in Tuticorin by using data of salt concentration obtained from State Ground and Surface Water Resources Data Centre, Tamilnadu, India. A good match is found between the observed and simulated salt concentration in Tuticorin as shown in *Figure 21*.

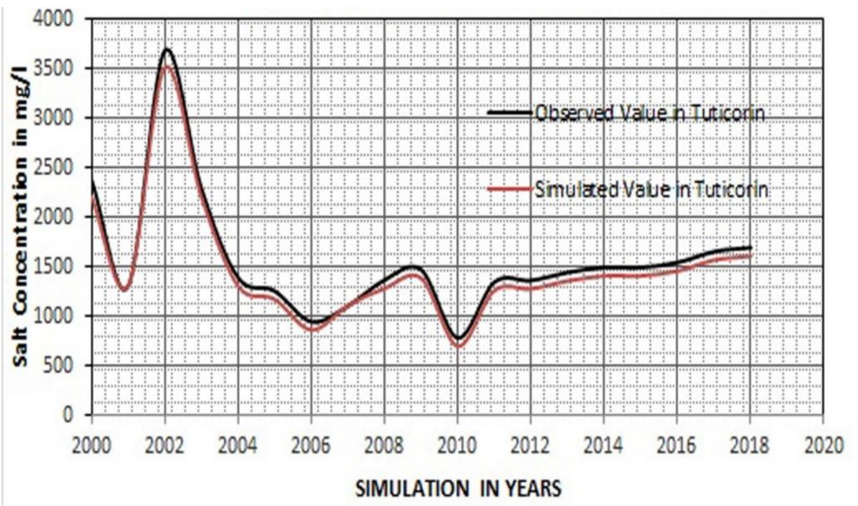

Figure 21. *Model verification for the well in Tuticorin*

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

Model simulation

The effect of Scenario A (Abstraction) Abstraction of saline groundwater using 15 pumping wells at a withdrawal rate of 1000 m^3/day per well at a short distance of 1500 m from the shoreline is showed a significant change in salt concentration in Tuticorin as shown in *Figure 22*.

The effect of Scenario A (Abstraction) Abstraction of saline groundwater using 15 pumping wells at a withdrawal rate of 1000 m^3/day per well at a short distance of 1500 m from the shoreline is showed a significant change in salt concentration in Tuticorin as shown in *Figure 22*. Simulated results of Scenario A (Abstraction) in the well in Tuticorin showed a decrease in groundwater salt concentration from 1600 mg/L in the year 2019 to 900 mg/L in the year 2030.

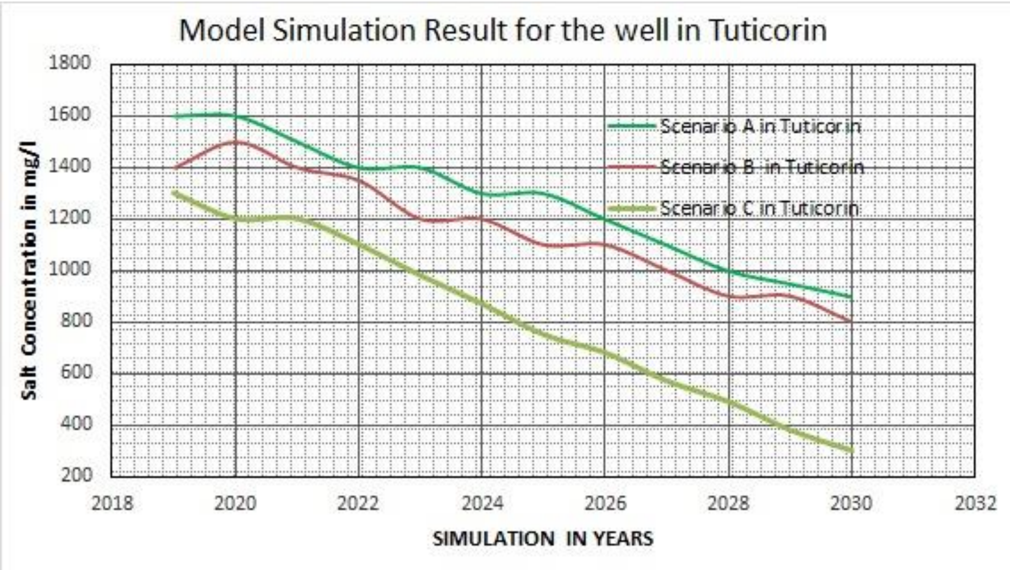

Figure 22. *Simulation result for the well in Tuticorin*

The Scenario B is the recharge of aquifer with treated saline water at the rate of 500 m^3/day, showed to have significant impact than the pumping Scenario A as depicted in *Figure 22*. Simulated results of Scenario B (Recharge) in the well in Tuticorin exhibited a decrease in groundwater salt concentration from 1400 mg/L in the year 2019 to 800 mg/L in the year 2030.

But in Scenario C abstracting saline groundwater 1000 m^3/day and recharge treated water at the rate of 500 m^3/day showed good control over the salt water intrusion in Tuticorin as revealed in *Figure 22*. Simulated results of Scenario C (ADR) in the well in Tuticorin exposed a steady decline in groundwater salt concentration from 1300 mg/L in the year 2019 to 300 mg/l in the year 2030.

From the simulation result it is understood that ADR method is good to control water quality in the well from saltwater intrusion.

Discussion

From the Spatial analysis of water quality parameters, WQI method, Piper plot diagram, it is understood that the severe saltwater intrusion is occurred in Tuticorin. The

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DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

current pumping rate would further decline the water quality and can lead to the destruction of groundwater resources.

An attempt is made in the study to determine the distance of seawater intrusion based on WQI. The analysis results give better knowledge of zone of dispersion. Water Quality became very poor in the observation wells found with in 15 km from sea shore in 2018. Hence the saltwater intrusion has moved inward from the seashore to a distance of 15 km. Pumping saline water from the zone of dispersion can reduce saltwater intrusion.

Several studies like Selvam et al. (2013) analysed the water quality parameter but the control measures not provided. In this research groundwater model is developed and three scenarios were applied to find the best method to minimise the seawater intrusion. The simulation results showed that ADR method is good to control from saltwater intrusion.

From the study, it is clearly depicted 15 pumping wells at a withdrawal rate of 1000 m^3/day per well at a short distance of 1500 m from the shoreline and Recharge treated water at the rate of 500 m^3/day required for the reduction, or displacement of the saline water towards the coast to eventually maintain or push the dispersion zone towards the coast is very important.

Conclusions

The effect of saltwater intrusion in coastal aquifer necessitates systematic investigation, especially when the extraction of groundwater in the coastal aquifer increases. The spatial analysis of groundwater using GIS, Water Quality Indices method, Piper plot diagram shows that the coastal aquifer is severely affected by saltwater intrusion. Potential intrusion of saltwater is studied with respect to distance of observation wells from seashore. In 2018 water quality in the observation wells located with in 15 km from the Seashore is very much affected. Three different simulation scenarios namely Abstraction, Recharge and a combination of system were applied to the observation well located in Tuticorin to reduce the seawater intrusion. FEFLOW model was used to evaluate these methods adopted to reduce seawater intrusion. The simulation results of FEFLOW show that the method ADR performs better than using abstraction or recharge wells. Integrating field study with developed model to determine the site selection of recharge well is a possibility for future work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_25932617

PHYTOREMEDIATION OF LANDFILL LEACHATE USING VETIVER (*CHRYSOPOGON ZIZANIOIDES*) AND CATTAIL (*TYPHA LATIFOLIA*)

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(Received 14th Nov 2018; accepted 25th Jan 2019)

Abstract. Several investigations have demonstrated the advantageous role that plants have in the phytoremediation of soils and waters contaminated by heavy metals. Therefore, under the present investigation, *Chrysopogon zizanioides* and *Typha latifolia* plants were tested for their effectiveness in absorbing 17 heavy metals (Al, B, Ba, Be, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, S, Se, Tl, V and Zn) coming directly from a landfill leachate. The translocation factor was quantified in both species, where *T. latifolia* recorded a FT >1 % of the metals V, Ni, Mn and B. While in *C. zizanioides*, the metals Tl, Se, Co and B obtained a FT >1 %. In general, extraction was achieved without noticeable toxicity despite the excessive level of metals. These results demonstrate the suitability of these hyperaccumulator plants and establish them as a comfortable and inexpensive alternative to reduce water and soil pollution problems.
Keywords: *leachate, plants, translocation factor, extraction, hyperaccumulator, pollution*

Introduction

The continuous industrial and commercial growth in many countries around the world, in the last decade have been accompanied by a rapid increase in the production of both municipal and industrial solid waste (Renou et al., 2008), encouraging the search for alternative methods for the final disposal or confinement of waste (Białowiec, 2011), with the sanitary landfill being one such large-scale method, especially for municipal solid waste (Del Moro et al., 2014; Koda et al., 2016; Wong et al., 2015; Remmas et al., 2017) and urban solid waste (Renou et al., 2008).

Landfilling involves particular methods and engineering works that control leachate leakage (SEMARNAT, 2012). Leachate is defined as the aqueous effluent generated as a result of the filtration of rainwater through the wastes and the inherent water content of the wastes themselves deposited in landfills (Renou et al., 2008; Zapata and Zapata, 2013). They are generally high in contaminants (Roongtanakiat et al., 2003), especially heavy metals and organic and inorganic matter (Bulc, 2006) that can contaminate groundwater and surface water in the area near the landfill (Khattabi and Aleya, 2007; Öman and Junestedt, 2008).

The effects on human health and ecosystems associated with heavy metal pollution (Vaverková et al., 2018) become even more worrying given their tendency to accumulate and magnify along trophic levels (Anning and Akoto, 2018). For example, due to their bioaccumulation they can have toxic effects on living organisms when they exceed a certain concentration (Nriagu et al., 1991; Gusiatin et al., 2013; Mahmoud et al., 2014; Sas et al., 2015), representing a risk to human health when transferred thought the food chain (Kelly et al., 1996; Mazur et al., 2013; Radziemska et al., 2013; Mahmoud et al., 2014; Roy and McDonald, 2015).

Faced with this problem, phytoremediation is an energy-efficient, cost-effective and aesthetically pleasing alternative to remediation sites with low to moderate levels of pollution (Mojiri et al., 2015). It is a set of technologies that reduce in situ or ex situ the concentration of various compounds from biochemical processes carried out by plants and associated microorganisms (Kelley et al., 2001; Cherian and Oliveira, 2005; Eapen et al., 2007; Cho et al., 2008). Some of the plants widely used in phytoremediation are *Chrysopogon zizanioides* and *Typha latifolia*, both of which are widely distributed around the world.

Chrysopogon zizanioides, commonly known as vetiver, is a perennial herb of the Poaceae family, originally from India (Truong et al., 2009). It has a high tolerance to elevated concentrations of heavy metals such as As, Cd, Cu, Cr, Pb, Hg, Ni, Se and Zn, showing its unique physiological characteristics (Truong, 2002; Chomchalow, 2003; Shu, 2003; Danh et al., 2009; Truong et al., 2010; Vargas et al., 2016). *C. zizanioides* is considered efficient in purifying leachate from a landfill (Xia et al., 2000). In terms of its morphological characteristics, vetiver has a complex root system that can grow between 3 to 4 m per year, so the root can penetrate much deeper into the soil and water. This massive root system can reduce and prohibit deep drainage, improve soil stability and nutrient uptake (Danh et al., 2009; Truong, 2000).

Regarding *Typha latifolia* (common cattail) of the Typhaceae family, it is an aquatic, rooted, perennial herbaceous plant that grows extensively in tropical and warm regions, measuring 1.6 to 2.2 m high (Ye et al., 1997; Bonilla and Arauz, 2012). This species is expansive and widespread both in shallow lowland waters and in lower mountainous regions (Klink et al., 2013). It is characterized by high biomass production and a high rate of decomposition (Podbielkowski and Tomaszewicz, 1982). *T. latifolia* is used in phytoremediation because of its ability to remove heavy metals from impacted sites and accumulate them mainly in its roots (Hernández et al., 2014) which has suggested that root tissue cells can tolerate high concentrations of metals (Carranza-Álvarez et al., 2008; Alonso-Castro et al., 2009; Leura-Vicencio et al., 2013).

In the present study, the phytoremediation capacity of *Chryzopogon zizanioides* and *Typha latifolia* in leachate from a municipal landfill was evaluated, determining the concentration of

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DOI: http://dx.doi.org/10.15666/aeer/1702_26192630

heavy metals, Aluminum (Al), Boron (B), Barium (Ba), Beryllium (Be), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn), Nickel (Ni), Lead (Pb), Selenium (Se), Thallium (Tl), Vanadium (V) and Zinc (Zn), by optical emission spectrometry with inductively coupled plasma (ICP-OES), and the translocation factor was calculated. The objective of the present study is to identify and measure the metals present in the heterogeneous mixture of the landfill leachate and to compare an introduced species that is *C. zizanioides* with *T. latifolia* native to Mexico. The originality of this work is centred on the type of contaminant in which the leachates are being used because most of the research focuses on a single metal or some metals in laboratory conditions without exploring how these metals would behave when they are in such a complex mixture as leachates without mentioning some other variants such as the type of soil being used. It is a pioneering work because the company opens the way for us to do research on these and other species found in the landfill.

Materials and methods

Samples

Collection of plants by simple random sampling of soil (contaminated and uncontaminated) as well as leachate was carried out at the sanitary landfill located in Leon, Guanajuato, Mexico (21°10’30.12” N, 101°46’29.25”). According to INEGI, 43% of the region’s surface is represented by dry and semi-dry climate, mainly in the northern region; 33% of the surface, towards the southwest and east, is sub-humid warm climate and the remaining 24% has sub-humid temperate climate. The average annual temperature is 18 °C. The rains are presented in summer, mainly in the months of June to September, the average precipitation of the state is approximately 650 mm annually. The collection of samples was during the month of October, in the dry period. The contaminated plants were taken from the landfill cells where they are irrigated with leachate that results from the decomposition of the deposited waste. The control plants were collected from the nursery located in the landfill facilities. At the collection points, 1 kg of rhizospheric soil and 300 mL of leachate from the leachate lagoon were also collected. They were stored at 4 °C until analysis.

Analysis of samples

The plants were washed and dried at room temperature. The shoots were separated from the roots, then cut and crushed in a mortar to obtain a particle size less than 2.0 mm (Farago et al., 1998). Subsequently, they were digested with 7 mL of HNO_3 and 3 mL of tridestilled H_2O. On the other hand, soil samples were placed under shade to remove excess water, ground in a mortar and sieved using stainless steel meshes of 9.51 mm and 2 mm in diameter. They were digested in tridestilled H_2O and 500 mL HNO_3 (1:1, v/v). Leachate was filtered using a 0.45 m filter (Farago et al., 1998) to remove particles that may interfere with the analyses. They were then digested with tridestilled H_2O and HNO_3, and a final volume of 25 mL with tridestilled H_2O was measured.

All previously digested samples were analyzed by means of an optical emission spectrometer with inductively coupled plasma (IRIS Advantage, Thermo Jarrell Ash Co.), with the software. A multi-element calibration curve was used including 16 chemical elements: Al, B, Ba, Be, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, Tl, V, Zn, prepared from a commercial standard Sigma Aldrich solution.

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Three repetitions per sample were made for the analysis of metals in plants, soils and leachate.

Translocation factor

The translocation factor (TF) is an indication whether or not a plant can be considered as a hyperaccumulator of chemical elements, it was determined based on the concentrations of metals in plants, soils and leachate, as well as their respective controls, according to the following formula (Mendieta and Taisigue, 2014) as shown in *Equation 1:*

$$\text{Translocation factor} = \frac{\text{Concentration of metals in shoots}}{\text{Concentration of metals in roots}} \qquad \text{(Eq.1)}$$

Statiscal analysis

An analysis of variance (ANOVA) was made using the statistical program SPSS 12 for Windows, used to discriminate between the means the procedure of Honestly Significant Difference (HSD) of Tukey, with a level of significance $\alpha = 0.05$.

Results

Concentration of heavy metals in the leachate

The concentrations of heavy metals in the leachate are presented in (*Table 1*) (average of the replicas performed) where the results are compared with those from a study carried out by Öman and Junestedt (2008) on leachate from Swedish municipal landfills.

Table 1. *Heavy metal concentrations (µg/L) of leachate of the site studied and the corresponding threshold limit values*

Metal	Concentration	Threshold Limit
Al	1.09 (± 0.0)	209
B	133.3 (± 86.0)	2580
Ba	810.8 (± 343.5)	280
Be	12.5 (± 22.9)	0.065
Co	12.5 (± 6.9)	7.7
Cr	27.34 (± 64.5)	15.3
Cu	4.52 (± 28.2)	23
Fe	249.9 (± 2.3)	6500
Mg	202.5 (± 13.4)	47,900
Mn	145.3 (± 13.6)	1152
Ni	36.01 (± 5.6)	31
Pb	8.5 (± 2.3)	4.4
S	825.9 (± 87.6)	90,800
Se	25.12 (± 0.1)	27
Tl	223.9 (± 35.7)	0.12
V	210.9 (± 38.4)	16
Zn	202.5 (± 13.4)	66

Values in parentheses are standard deviation

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DOI: http://dx.doi.org/10.15666/aeer/1702_26192630

Capture of heavy metals in T. latifolia and C. zizanioides

The concentration of the heavy metals found in *Typha latifolia* and *Chrysopogon zizanioides*, as well as their respective controls, are shown in *(Table 2)*.

Table 2. *Concentration of heavy metals in vetiver and cattail expressed in mg/kg*

Metal	*Cattail*		*Vetiver*	
	Contaminated	**Control**	**Contaminated**	**Control**
Al	4.92 (±0.5)	1.08 (±0.3)	3.70 (±0.4)	0.71 (±0.0)
B	7.23 (±1.1)	0.51 (±0.0)	4.23 (±0.0)	0.39 (±0.0)
Ba	205.24 (±15.4)	6.72 (±0.1)	76.00 (±2.6)	4.89 (±0.4)
Be	4.13 (±0.6)	0.29 (±0.1)	2.97 (±0.2)	0.26 (±0.0)
Co	0.25 (±0.0)	0.00 (±0.0)	0.37 (±0.1)	0.00 (±0.0)
Cr	1.07 (±0.0)	0.00 (±0.0)	0.55 (±0.0)	0.00 (±0.0)
Cu	1.57 (±0.2)	0.00 (±0.0)	1.15 (±0.1)	0.00 (±0.0)
Fe	95.18 (±31.7)	2.64 (±0.1)	105.67 (±14.8)	2.10 (±0.2)
Mg	75.98 (±8.3)	4.92(±0.5)	10.54 (±1.4)	3.10 (±0.1)
Mn	264.70 (±4.3)	2.47 (±0.3)	46.82 (±0.5)	1.92 (±0.1)
Ni	2.32 (±0.1)	0.20 (±0.1)	2.65 (±0.0)	0.06 (±0.0)
Pb	0.96 (±0.0)	0.30 (±0.2)	1.41 (±0.0)	0.07 (±0.0)
S	111.76 (±9.6)	0.00 (±0.0)	54.93 (±4.1)	0.04 (±0.0)
Se	0.79 (±0.2)	0.15 (±0.2)	0.80 (±0.0)	0.17 (±0.0)
Tl	16.14 (±0.2)	0.05 (±0.1)	17.23 (±0.2)	0.00 (±0.0)
V	18.54 (±1.5)	1.57 (±0.1)	18.79 (±0.0)	1.07 (±0.0)
Zn	15.40 (±3.3)	1.12 (±0.4)	6.54 (±1.0)	0.58 (±0.1)

Values in parentheses are standard deviation

Concentration of metals in biomass of species

A statistically significant difference ($p < 0.05$) was recorded in the absorbed concentration of each metal between the plants, as well as in their evaluated parts (shoots and roots). The concentration of metals was observed in the roots and in the shoots as shown in *Figures 1* and *2*.

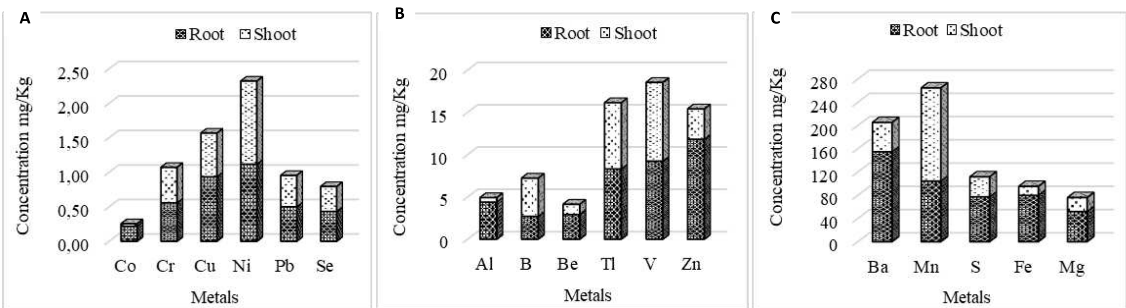

Figure 1. *Concentration of metals in cattail biomass*

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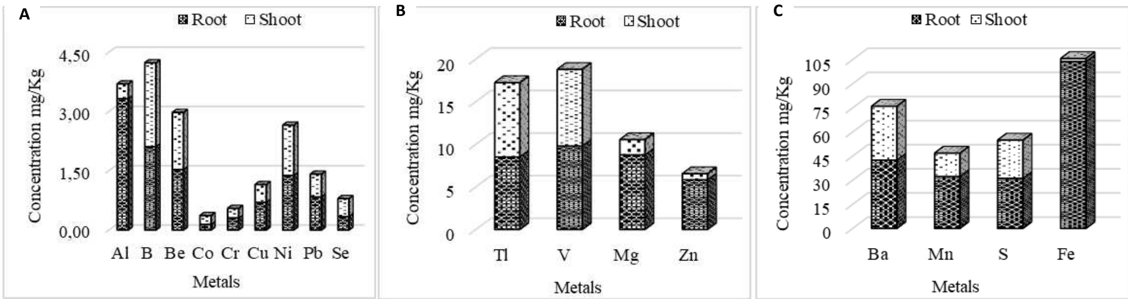

***Figure 2.** Concentration of metals in vetiver biomass*

Translocation factor

Successful phytoremediation evaluates results by emphasizing the ability to accumulate and transfer metals. While plant species may naturally accumulate heavy metals in their roots, a chemical amendment may be necessary to achieve effective translocation of such metals in plant shoots (Anning and Akoto, 2018). Baker (1981) points out that an FT above 1% indicates that a translocation from the root to the aerial part of the plant occurs. Plants with FT > 1 are considered accumulators or hyperaccumulators (if concentrations exceed 0.1%) (Mendieta and Taisigue, 2014), indicating that they are capable of accumulating excessive amounts of heavy metals.

Translocation factor (FT) values for all metals in cattail and vetiver are shown in *Figures 3* and *4.*

Discussion

Concentration of heavy metals in the leachate

Concentrations of heavy metals in the leachate exceeded the limits of the standard threshold *Table 1*. The threshold limits for metals come from a study conducted by Öman and Junestedt (2008) on leachate from Swedish municipal landfills. Heavy metal concentrations (µg/L) of leachate from the site studied and corresponding threshold limit values.

Seventeen heavy metals were identified: S, Ba, Fe, Tl, V, Mg, Zn, Mn, B, Ni, Cr, Se, Be, Co, Pb, Cu and Al, in descending order of concentration (µg/L). As can be seen, S is the metal with the highest absorption, with a concentration of 825.9 µg/L. But it failed to exceed the threshold concentration with a value of 90.800 µg/L. Aluminium was the least abundant metal with a concentration of 1.09 µg/L and differed with the reference site (209 µg/L). On the other hand, some metals (Ba, Be, Co, Cr, Ni, Pb, Tl, V and Zn) obtained a higher concentration than the threshold level. Ba obtained a concentration of 810.8 µg/L while the threshold level concentration was 280 µg/L. The concentration of Be (12.5 µg/L) and Co (12.5 µg/L) were higher than the threshold concentrations (0.065 and 7.7 µg/L respectively). In contrast, Tl, V and Zn showed relatively high concentrations (223.9, 210.9 and 202.5 µg/L respectively) compared to the corresponding threshold levels of 0.12, 16 and 66 µg/L. Ni reached a concentration of 36.01 µg/L, just above the threshold level (31 µg/L). The threshold concentrations of Cr and Pb were 15.3, 31 and 4.4 µg/L respectively, while the concentrations obtained in the study area were 27.34 and 8.5 µg/L respectively.

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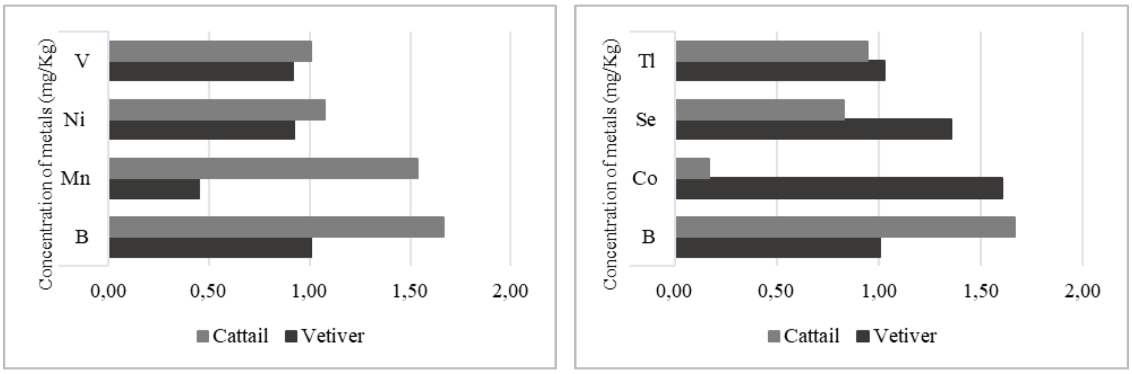

***Figure 3.** Translocation factor >1% of heavy metals in cattail and vetiver*

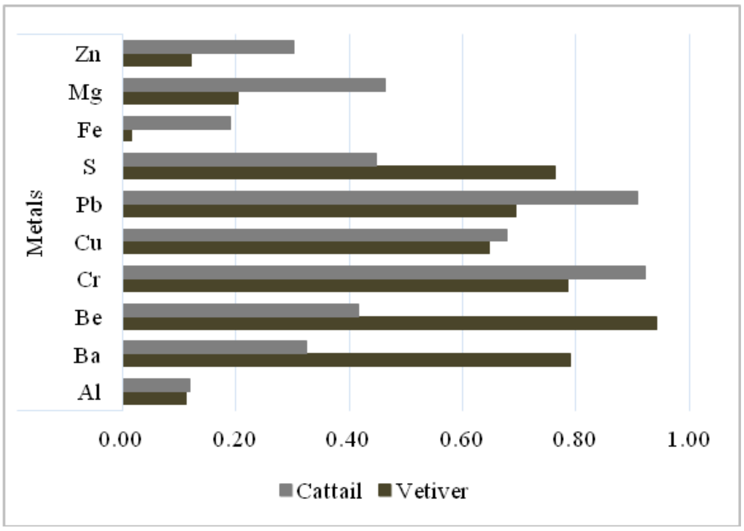

***Figure 4.** Translocation factor <1% in heavy metals in cattail and vetiver*

According to Méndez et al. (2004), the composition of a leachate from a sanitary landfill depends on factors such as: the initial degree of humidity of the residues, rainfall, atmospheric humidity, temperature and evaporation. It generally has a high content of pollutants, especially heavy metals and other harmful substances that become pollution in the area around landfills and in groundwater if they infiltrate (Roongtanakiat et al., 2003). As already mentioned, heavy metals are very harmful to the environment and human health due to their toxicity, persistence and non-degradability. These effects can be particularly problematic, requiring environmentally friendly and cost-effective remediation measures, such as phytoremediation (Ghosh and Singh, 2005; Ali et al., 2013).

Uptake of heavy metals in T. latifolia and C. zizanioides

In both plant species the 17 heavy metals also present in the previously analyzed leachate were identified. In the contaminated samples almost null concentrations (<1 mg/kg) up to high concentrations (>200 mg/kg) were detected. However, in both controls no values were recorded for Co, Cr and Cu, nor for S and Tl in cattail and vetiver, respectively. The highest uptake was in the cattail plant was for heavy metals:

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DOI: http://dx.doi.org/10.15666/aeer/1702_26192630

Mn, Ba, S, Mg, Zn, B, Al, Be, Cu and Cr, in descending order of concentration, and in vetiver were Fe, Tl, Ni, Pb and Co. For Se and V showed very similar concentration values in both contaminated plants analyzed.

Considering the relationship between the concentration present in the contaminated plant and its control, it was observed that vetiver had the highest uptake of Al, Fe, Ni, Pb and V; and for cattail the ones with the highest uptake were B, Ba, Be, Mg, Mn, Se, Tl and Zn. The analysis of variance showed a statistically significant difference ($p < 0.05$) between the concentration of each metal present in the contaminated plant and its respective control.

It is necessary to mention that some are essential elements, and are essential for the maintenance of the biochemical systems of all individuals (Yaman and Akdeniz, 2004). Several elements are essential for the growth, reproduction and/or survival of living organisms; others are of great economic and industrial importance and, from a toxicological point of view, their importance is enormous, presenting a multiplicity of toxic effects.

Based on the results, the cattail can be considered as an accumulator plant, reaching a higher uptake of heavy metals with respect to its control and vetiver. It has been reported the effectiveness of the cattail to capture Al, Cu and Zn (Margalef, 198 and Syukor et al., 2016,), as well as Mg, Cr and Mn (Sarkar et al., 2017), but not the uptake of Ba, S, Fe, V, Tl, B, Be, Ni, Pb, Se and Co.

With respect to vetiver, it seems to be a more effective accumulator of S, Fe, Ni, Pb, V, Al, Tl, Cu, Co and Cr. Truong (2000) reported for vetiver the uptake of Co, Ni and Se (50-100, 100, >74 mg/kg, respectively), in concentrations higher than those obtained in the present work (0.37, 2.65 and 0.8 mg/kg, respectively). The concentrations of Fe and Pb were higher (105.67 and 1.41 mg/kg, respectively) than those reported by Suelee et al. (2017) (0.08 and 0.31 mg/kg, respectively). It should be noted that the metals V and Tl have not been reported in investigations carried out in vetiver as a phytoremediation plant, being two of the elements with higher concentration (18.79 and 17.23 mg/kg, respectively) with respect to all those identified in this plant.

Concentration of metals in biomass of species

The highest concentration of metals was observed in the roots as shown in *Figures 1* and *2*. The metals with the highest concentration in the roots of both species evaluated were: Al, Ba, Be, Cr, Cu, Pb, S, V, Fe, Mg and Zn, with those of cattail showing the highest uptake of metals (Al, Ba, Be, Cu, Mg, S and Zn), compared to the roots of Vetiver (Fe, Ni, Pb and V). In the leaves the elements with the highest concentration were B, Co, Mn, Ni, Se and Tl. The belfry B, Mn and Ni, obtained higher concentrations in the leaves (4.51, 160.42 and 1.20 mg/kg). Vetiver leaves had higher concentrations of Co, Se and Tl (0.23, 0.46, 8.73 mg/kg, respectively).

Translocation factor (FT)

Successful phytoremediation evaluates results by emphasizing the ability to accumulate and transfer metals. While plant species may naturally accumulate heavy metals in their roots, a chemical amendment may be necessary to achieve effective translocation of such metals in plant shoots (Anning and Akoto, 2018). Baker (1981) points out that an TF above 1% indicates that a translocation from the root to the aerial part of the plant occurs. Plants with TF > 1 are considered accumulators or

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hyperaccumulators (if concentrations exceed 0.1%) (Mendieta and Taisigue, 2014), indicating that they are capable of accumulating excessive amounts of heavy metals. The translocation factor (TF) values for all metals in cattail and vetiver are shown in *Figures 3* and *4*.

In general, the translocation values of metals in plants presented a statistically significant difference ($p < 0.05$) between plants. The cattail recorded a TF >1% for V (1.01%), Ni (1.08%), Mn (1.54%) and B (1.66%), being higher for the latter. In the case of vetiver the metals that obtained a TF >1% were: B (1.01%), Tl (1.03%), Se (1.36%) and Co (1.60%). As can be seen in *Figure 3,* B presented 1.66 in the cattail and 1.01 in the vetiver.

The rest of the metals presented a FT <1%, for both evaluated plant species. According to Mendieta and Taisigue (2014), TF values between 0.1 and 1% indicate that plants are considered tolerant of these metals.

From the current results, the cattail seems to be a more effective accumulator and, to some extent, a translocator of V, Ni, Mn and B than the vetiver, which apparently has a greater affinity for Tl, Se, Co and B.

Research has established that both plants are able to absorb heavy metals from landfill leachate (in situ). Being the cattail, the species that achieved a higher concentration of heavy metals, specifically in their roots. As for vetiver, it successfully captured the metals, however, its concentrations are 10% lower than those of the cattail.

Conclusions

This study showed that the composition of the leachate analyzed from a sanitary landfill in Mexico, which due to its heavy metal content poses a risk to human health and the environment, therefore successful mitigation measures are required. The two plants, *T. latifolia* and *C. zizanioides*, studied in the phytoremediation of different heavy metals present in the previously characterized leachate, proved to be highly effective in capturing seventeen heavy metals present in the previously characterized sanitary landfill, which was supported by the TF values that were used as an indication of the suitability of these plants for phytoremediation. The use of these species indicates that it could be cosmopolitan due to its attributes of remediation of diverse pollutants being economic resources and of easy access. The results obtained will allow progress in the management of degraded spaces and their restoration through innovative phytoremediation techniques.

Recommendations based on the results, considerations should be taken, such as applying the process of phytoextraction assisted by chelating agents in situ, to evaluate and quantify the effect that these chelating agents have on the availability of metals in plant species. As well as to study the autochthonous microorganisms of the rhizospheric soil that participate during the removal of metals. On the other hand, to evaluate the relation plant-soil-microorganisms and to characterize the exudates that are produced in a natural way, for these conditions to define if these compete with organic acids of reactive grade.

Acknowledgements. This study was financed by Promotora Ambiental S.A.B. de C.V. Directed by Dr. Sugey López Martínez. I am also grateful for the scholarship granted by the National Council for Science and Technology (CONACyT) with scholarship No. 430983 during my postgraduate studies in Master's in Environmental Sciences (PNPC).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26192630

SPATIAL AND TEMPORAL DISTRIBUTION CHARACTERISTICS OF DROUGHT AND ITS INFLUENCING FACTORS IN HEILONGJIANG PROVINCE, CHINA FROM 1956 TO 2015

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(Received 14th Nov 2018; accepted 16th Jan 2019)

Abstract. In the context of global warming, extreme drought climate events show a trend of frequent occurrence. Studying the relationship between climate change and drought disasters by using the performance characteristics of climate change has become a new strategic focus of global change science. In this paper, we collected meteorological data from 14 meteorological stations from 1956 to 2015 in Heilongjiang Province and used a standard precipitation index (SPI) of meteorological drought to analyze temporal and spatial characteristics of droughts in the province. Multiple methods such as Linear regression analysis, Mann-Kendall trend test and Kriging interpolation were applied and analyzed to reveal temporal and spatial distribution patterns of drought frequency and drought intensity in different parts of the province. The results show that: (1) the precipitation in Heilongjiang Province has shown a downward trend in the past 60 years and the correlation between the monthly average precipitation and the monthly mean temperature is also apparent. (2) Since 1990s, the intensity of higher drought has escalated in the whole province and has gradually strengthened from south to north. (3) The frequency distribution of drought is the lowest in the central and southern regions, and the highest in the west. (4) The uneven precipitation, sandstorm and uneven distribution of evaporation caused by the monsoon in Heilongjiang Province are the main meteorological factors for the formation of drought in the province.
Keywords: *Heilongjiang Province, drought, spatial-temporal variation, standardized precipitation index (SPI)*

Introduction

Drought is one of the most complex and recurring natural disasters that is caused by intense and persistent shortage of precipitation. Precipitation is the main parameter that treats some environmental disasters as drought, and many other factors such as temperature, high winds, and low relative humidity also play a significant driving role in the occurrence of drought. Drought is considered to be one of the most serious natural disasters in the world because of its large spatial range and long duration, resulting in frequent occurrence of large-scale and intensive droughts around the world (Koutroulis et al., 2011; Okonkwo et al., 2013; Ngetich et al., 2014; Potop et al., 2014; Blauhut et al., 2015; Kazemzadeh and Malekian, 2016). China is deeply affected by the monsoon climate and topography, resulting in uneven distribution of water and heat leads to frequent droughts (Bi, 2007; Wang, 2007). Since 1950s, the drought disaster in northern China has been expanding and accelerating. According to the statistics, average area affected by drought in China is 1873.1 hm^2, with a disaster rate of 43%; the grain output reduced by 25-30 billion kg, accounting for more than 60% of the total

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26312650

losses caused by various natural disasters. Drought brings huge economic losses to China's national economy, especially agricultural production (Jiang and An, 2011).

The problem of aridification in northern China has always been the focus of academic circles. In particular, research on two typical arid regions in North China and Northwest China has received much attention (Sun and Gao, 2000; Li et al., 2007; Hao et al., 2010; Yi et al., 2012). The study on drought in northern China has mainly concentrated on the characteristics, causes and aridification of drought evolution. For example, Spinoni et al. (2014) conducted a statistical analysis of the frequency, duration and intensity of drought events worldwide based on the SPI index. Lu et al. (2010) based on the run-length theory, extracted the drought duration and drought intensity from the comprehensive meteorological drought index sequence, and established the joint distribution of the two copula functions. Corti et al. (2011) constructed a regional drought loss assessment model. At present, the research has mostly evaluated drought and set drought research indicators from the perspective of the causes and mechanisms of drought, and the main indexes involved are standardized precipitation index (SPI), Z index, standardized precipitation evapotranspiration index (SPEI), Palmer drought index (PDSI), surface water supply index (SWSI), Wald Wasser Haushalts Modell (WAWAHAMO), water deficit index (WDI) and meteorological drought index (CI) (Mishra and Singh, 2011). Due to the complexity of the drought itself, there is currently no drought index that can comprehensively describe the changing characteristics of different types of drought. The several drought indices mentioned above have relative rationality in different geographical areas and specific timescales. Among them, the standardized precipitation index (SPI) has been accepted by meteorological scientists all over the world because of its simplicity and multi-scale advantages based only on precipitation data. It has been widely used in drought analysis and assessment work in various places (Patel et al., 2010; Kumar et al., 2016; Sujitha and Shanmugasundaram, 2017; Yang et al., 2017; Guhathakurta et al., 2018). In recent years, due to the influence of multiple factors, the frequency of drought in Heilongjiang Province has increased, and the scope of drought has expanded, spreading from the Mudanjiang area to the whole province. Except for the Heilongjiang River coast and the Greater Xing'an Mountains, most of the counties and cities have experienced different degrees of drought (Zhang et al., 2014). However, most of the studies on drought in the north have been analyzed of a regional perspective, while there are few studies on the analysis of local drought from smaller scales. In the Heilongjiang region, especially on higher latitudes, analysis of annual and seasonal drought characteristic is very infrequent. Based on the standard precipitation index (SPI), this paper implemented data analysis or geographic interpolation from interannual, interdecadal, monthly, seasonal, and abrupt changes by calculating drought frequency and drought intensity, and analyzed the spatial and temporal distribution characteristics and formation mechanism of drought in Heilongjiang Province. Through these efforts, we hope to provide a theoretical reference for the scientific understanding of regional drought characteristics and effective defense against drought disasters.

Study area

Heilongjiang Province is located in the northeastern part of China, and the total land area is 473000 km^2. It is an important passage from the Asia-Pacific region to the Russian Far East and the European continent (*Fig. 1*). The Heilongjiang River Basin is

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mainly composed of four major river systems: Heilongjiang, Songhua River, Wusuli River and Suifen River. Regarding topography, the northwest, north and southeast of the province are relatively high, while the northeast and southwest are relatively low. Heilongjiang Province belongs to temperate continental monsoon climate with an annual average temperature between -5 and 5 °C, decreasing from south to north, roughly the 0 °C contour of Nenjiang and Yichun lines (Zhang et al., 2014). The annual precipitation is mostly between 400 and 650 mm, with more precipitation in the central mountainous area, less precipitation in the western and Northern regions, and precipitation resources are relatively stable. As an important province in Northeast China, Heilongjiang Province has a dense population and a large number of large and medium-sized cities, and it has a significant strategic position in China’s political economy. However, the frequency of drought in Heilongjiang Province is at the forefront of China, and the problems caused by drought have seriously restricted the social and economic development of Heilongjiang Province.

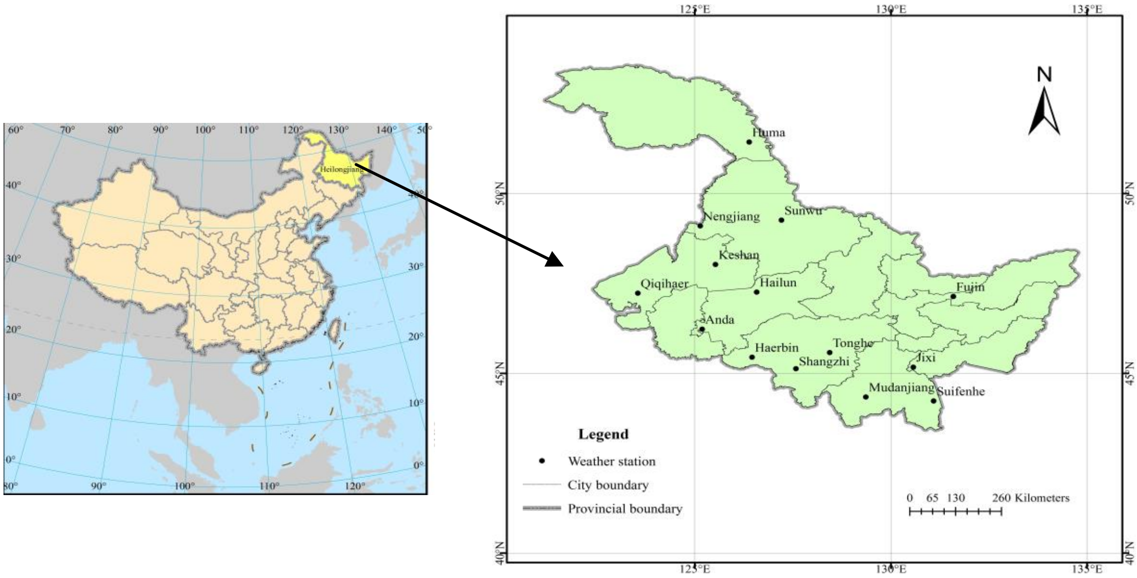

Figure 1. *Location of Heilongjiang Province and distribution of typical weather stations*

Materials and methods

Data

The meteorological data is provided by the China Ground International Exchange Station of China Meteorological Data Network about 14 Station which was used 1950-2015 monthly site data, including precipitation, maximum temperature, minimum temperature, average temperature, sunshine hours, wind speed and relative humidity, as well as latitude and longitude and elevation. Because bound by the site incomplete data due to site changes and lack of measurement caused by some reasons, 14 stations of Heilongjiang which has complete data were selected (*Fig. 1*). All data have undergone rigorous quality control and error correction. The GDEMV2 30M resolution digital elevation data used in this paper is from the Geospatial Data Cloud website. China’s basic administrative divisions and water system map data come from the Resource and Environmental Science Data Center of the Chinese Academy of Sciences.

Study methods

Linear regression analysis

Linear regression analysis describes the linear dependence between the dependent variable *y* and the independent variable *x*. If the independent variable has only one *x*, then this linear regression is called a one-way linear regression. The task of unary linear regression is to find a linear regression equation describing the two variables *x*, *y* based on several observation points (x_i, y_i) (*i = 1, 2, 3...n*). When judging the goodness of fit of a regression equation, the R^2 decision coefficient is a significant indicator. The calculation formula of the correlation coefficient *r* of the regression equation is (*Eq. 1*):

$$\mathrm{r} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \qquad \text{(Eq.1)}$$

The closer the absolute value of the correlation coefficient *r* is to 0, the worse the correlation between the two variables. Conversely, the closer the absolute value of the correlation coefficient *r* to 1, the better the correlation between the two variables *x* and *y*, and the closer the linear regression equation to a flat line parallel to the x-axis.

Standardized precipitation index (SPI)

The standardized precipitation index was proposed by Mckee in 1993 when studying the drought conditions in Colorado, USA. By first calculating the Γ distribution probability of precipitation in a certain period, and then normalization, the cumulative precipitation frequency distribution is used to divide the drought level. Due to the standardized treatment, the SPI index eliminates the spatial and temporal distribution of rainfall, has multiple time scale characteristics, is sensitive to drought response, and can effectively reflect drought and flood conditions in different regions and different time periods (Yuan and Zhou, 2004; Kim et al., 2015). The SPI calculations for this study used the computational procedures of the University of Nebraska approved by the World Meteorological Organization. After calculating the SPI index, we can classify the drought level according to *Table 1*. The SPI level is above the light drought (including light drought), indicating that the drought occurred in the area.

Drought evaluation indicators

1) Drought frequency

Drought frequency refers to the ratio of the number of times the drought occurs (the level corresponding to the SPI index is above light drought) to the total number of observations during that period (Song and Singh, 2010). The calculation formula is as follows (*Eq. 2*):

$$P_i = \frac{n}{N} \times 100\% \qquad \text{(Eq.2)}$$

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where: N is the total time point (year, season, month) of observation data at a station, and n is the number of time points (year, season, month) when drought occurs (SPI drought level is above light drought).

Table 1. *Classification of standardized precipitation index drought level*

Grade	Type	Value of SPI	Extent of the effects of drought
1	No drought	-0.5<SPI	Precipitation is normal or higher than normal years; moist surface, no signs of drought
2	Light drought	-1<SPI≤-0.5	Precipitation is less than normal years; the surface air dry, the soil appear moisture mild deficiencies
3	Moderate drought	-1.5<SPI≤-1.0	Precipitation continued below normal years; soil surface is dry, soil water shortage, surface of plant leaves daytime wilting phenomenon
4	Serious drought	-2.0<SPI≤-1.5	Soil appeared sustained severe lack of moisture, Soil appear thicker dry soil, wilting plants, dry leaves and fruit shedding, cause a serious impact on crops and ecological environment and industrial production, drinking water
5	Special serious drought	SPI≤-2.0	Soil appeared a serious shortage of water for a long time, surface plant withered or death, cause a serious impact on crops and ecological environment,, have a greater impact on drinking water and industrial production

SPI: standardized precipitation index

2) Drought intensity

Drought intensity is the average of the absolute values of the SPI index at a site or time period in which drought (above SPI drought levels are above light drought) occurs over a certain period of time (Chang et al., 2012). Calculated as follows (*Eq. 3*):

$$I = \frac{1}{m}\sum_{i=1}^{m}\left|SPI_i\right| \qquad \text{(Eq.3)}$$

The drought intensity was not obvious when $S_{ij} < 0.5$; $1 > S_{ij} \geq 0.5$ was a mild drought; when $1.5 > S_{ij} \geq 1$, it was a moderate drought; when $S_{ij} \geq 1.5$, it was severe drought.

Kriging interpolation

The Kriging interpolation method was first proposed by South African mining engineer D. G. Krige in 1951 and later developed by the famous French geographer G. Matheron. Based on the spatial autocorrelation, the Kriging method uses the original data and the semivariance function as the structure to perform unbiased optimal estimation of the unknown sample points of the regionalized variables (Li et al., 2013). The Kriging method not only considers the distance, but also considers the spatial distribution of known sample points and the spatial orientation relationship with

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unknown samples through variogram and structural analysis. The interpolation formula is (*Eq. 4*):

$$z(x_0) = \sum_{i=1}^{n} \lambda_i z(x_i) \quad \text{(Eq.4)}$$

where: $z(x_0)$ is the estimated value at x_0, $z(x_i)$ is the observed value at x_i, λi is the Kriging weight coefficient, and n is the number of observation points.

Mann-Kendall test

The Mann-Kendall test can be used to determine if there is an anomaly in the hydrometeorological data arranged in time series and to determine the time of the mutation. It is often used for the detection of precipitation and drought trends affected by climate change (Luo et al., 2008). Its calculation method is:

1) Calculate the order column S_k of the sequential time series and calculate UF_k according to the equation.

The order of the time series x with n sample sizes is listed as (*Eq. 5*):

$$s_k = \sum_{i=1}^{k} r_i \quad r_i = \begin{cases} 1, x_i > x_j \\ 0, x_i \le x_j \end{cases} \quad j = 1,2,\cdots,i \quad \text{(Eq.5)}$$

It can be seen that the order column S_k is the cumulative number of the number of values at the *i*-th time when the value is greater than *j*. Assuming the time series are randomly independent, the statistics are defined (*Eq. 6*):

$$UF_k = \frac{s_k - E(s_k)}{\sqrt{Var(s_k)}} \quad k = 1,2,\cdots,n \quad \text{(Eq.6)}$$

where: $UF_1 = 0$, $E(s_k)$, $Var(s_k)$ are the mean and variance of the cumulative number S_k, respectively. When $x_1, x_2, ..., x_n$ are independent of each other and have the same continuous distribution, they can be calculated by (*Eq. 7*):

$$E\ (s_k) = \frac{n(n+1)}{4} \quad Var(s_k) = \frac{n(n-1)(2n+5)}{72} \quad \text{(Eq.7)}$$

2) Calculate the order column S_k of the reverse sequence of time, and calculate UB_k according to the equation.

Repeat the process in 1) by the time series x reverse order x_n, x_{n-1}, ..., x_1, while making $UB_k = -UF_k (k=n, n-1,...,1)$, $UB_1 = 0$. Given a significance level of $\alpha = 0.05$, the critical value $U_{0.05} = \pm 1.96$. The two statistical sequence curves UF_k and UB_k and ±1.96 two critical lines are plotted on the same graph. If the value of UF_k or UB_k is greater than 0, it indicates that the sequence has an upward trend. Conversely, if the value of UF_k or UB_k is less than 0, it indicates that the sequence has a downward trend. If there is an intersection between UF_k and UB_k, and the intersection is between the two critical lines, the time corresponding to the intersection is the moment when the mutation starts. If the two curves exceed the critical value, it indicates that the trend of rising or falling

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is significant, and the range exceeding the critical line is the time zone in which the mutation occurs.

Results

Characteristics of drought time in Heilongjiang Province within the year

Analysis of monthly drought characteristics

The monthly temperature and precipitation data of 14 meteorological stations in Heilongjiang Province were statistically analyzed, and the correlation curves of monthly mean temperature and monthly mean precipitation in Heilongjiang Province were plotted (*Fig. 2a*).

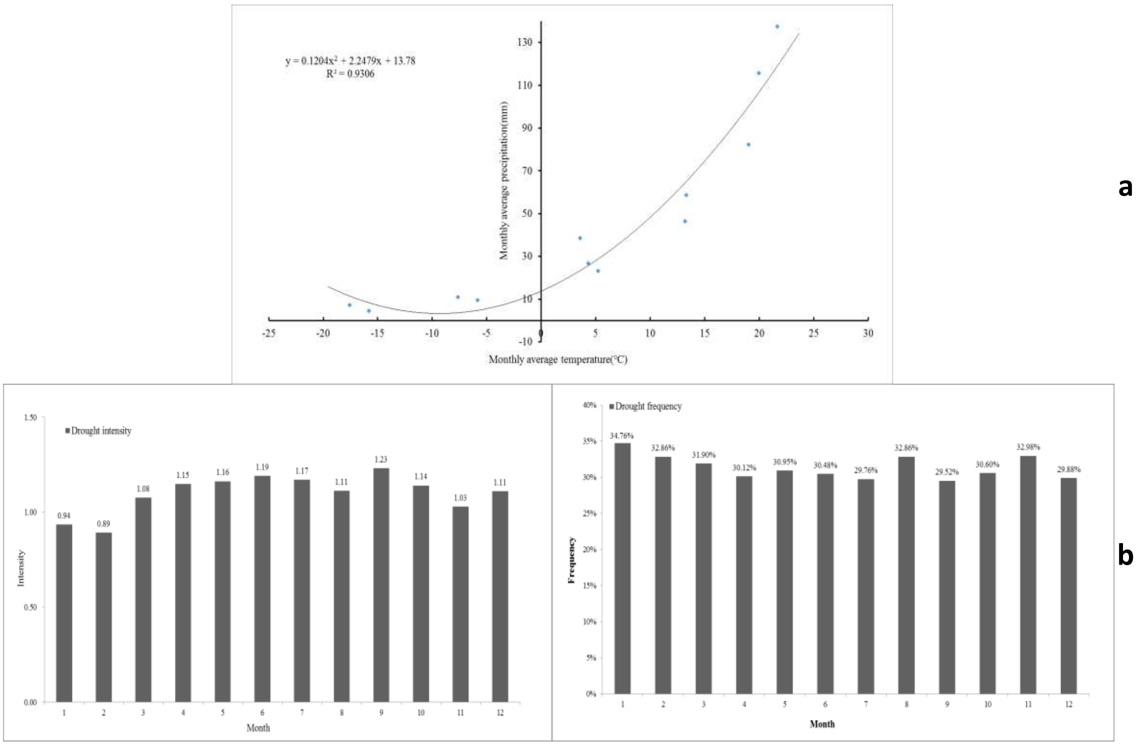

Figure 2. *(a) Analysis curve of monthly mean temperature and monthly mean precipitation in Heilongjiang Province. (b) Monthly drought frequency and intensity histogram of Heilongjiang Province*

It can be seen from the figure that there is a very obvious correlation between temperature and precipitation in Heilongjiang Province, and the R^2 value of the correlation analysis curve reaches 0.9417. This shows that in the global warming environment, the precipitation in Heilongjiang Province is greatly affected by the temperature and even changes with the temperature. Calculate the corresponding SPI index, and classify the data. It can be seen that the monthly drought frequency in Heilongjiang Province is not much different, and fluctuates around 31%. The month with the lowest frequency of drought is September, and the month with the highest frequency of drought is January. The annual intensity of the drought is relatively stable, and fluctuates between light and moderate levels. Among them, the intensity in January

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and February is 0.5~1, which is light drought. From March to December, it is between 1 and 1.5, all of which are in the middle drought. The month with the highest drought intensity is September, with a value of 1.23. The month with the lowest drought intensity was February, with a value of 0.89 (*Fig. 2b*). It can be seen that the drought in Heilongjiang Province is common during the year, but the intensity is relatively light and stable.

Analysis of seasonal drought characteristics

The precipitation in the four seasons of Heilongjiang Province varies greatly, showing typical monsoon precipitation. In winter, the icing period has adversely affected the water cycle of the four major river systems. Together with the combination of the northwest monsoon, the phenomenon of low precipitation in winter and high frequency of drought occurred in Heilongjiang Province. Comparing *Figure 3,* the seasonal drought frequency and drought intensity in Heilongjiang Province showed a reverse trend. The summer drought frequency is the lowest, but the drought intensity is the highest; the winter drought frequency is the highest, but the drought intensity is the lowest. This is due to the abundant precipitation in summer, which leads to a low frequency of drought. However, the terrain and water system in Heilongjiang Province are complex, and the summer is susceptible to the southeast monsoon, which is fragile and sensitive to drought. Moreover, global warming has caused frequent high temperatures in Heilongjiang in summer, greatly increasing the intensity of drought. Therefore, in summer, compared with winter, it is more susceptible to high temperature and forms a greater drought intensity.

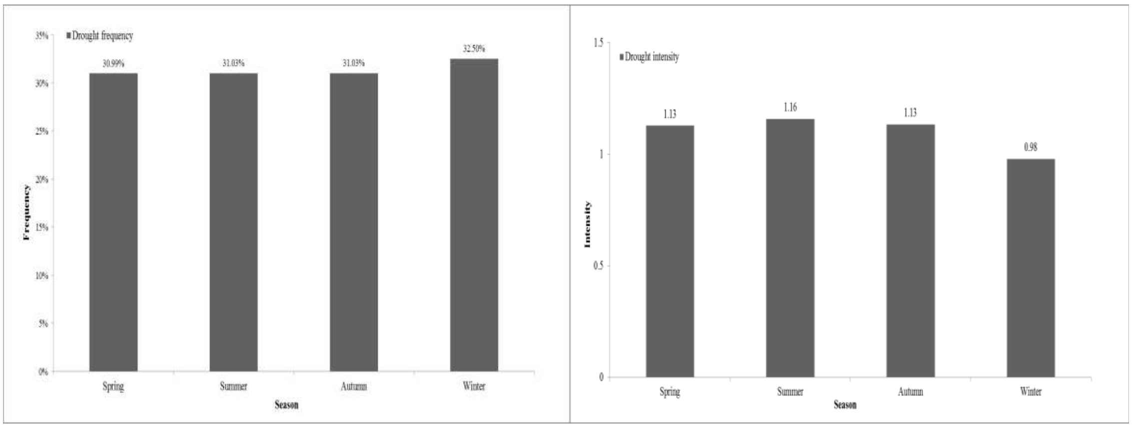

Figure 3. *Heterogeneous frequency and intensity histogram of four seasons in Heilongjiang Province*

Analysis of interannual and interdecadal drought characteristics in Heilongjiang Province

Time change trend analysis

Using linear regression method to analyze the trend of annual average precipitation in Heilongjiang Province, it can be seen that the precipitation in Heilongjiang Province showed a downward trend in the whole 60 years, and maintained a fluctuation of about 500 mm, but it did not pass the significance test ($P > 0.05$). The results show that the R^2

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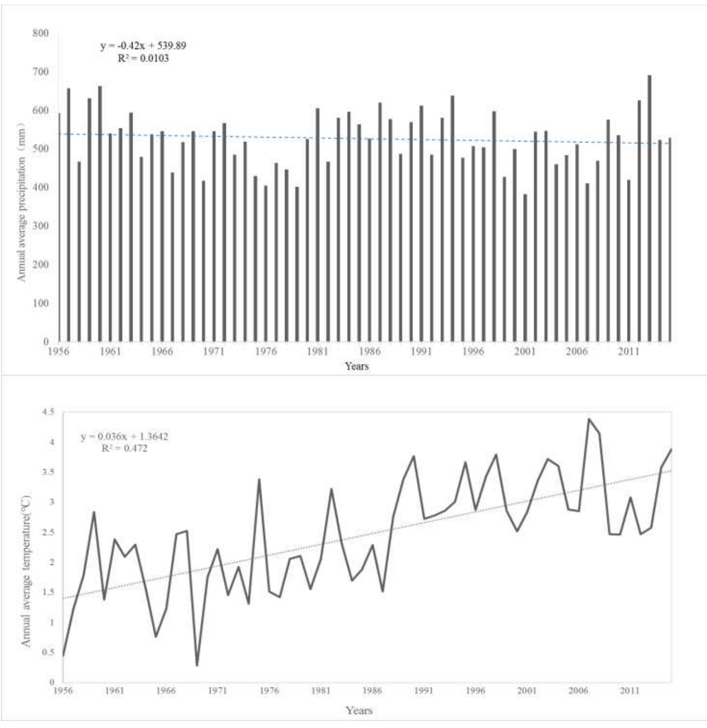
value is only 0.0103, which indicates that the downward trend is not significant, and the overall precipitation is relatively stable. The decadal trend map of precipitation (*Fig. 4a*) shows that during the ten years from 1956 to 1965, Heilongjiang Province had abundant precipitation, and the average precipitation was 572.07 mm. However, in the 20 years after 1966, the precipitation decreased sharply, and the average precipitation between 1966 and 1975 was only 501.6 mm. In these 20 years, the precipitation was low and lasted for a long time, which was very likely to lead to drought, and the most typical example was the national drought that occurred in 1978~1983. From 1986 to 1995, the precipitation increased and reached to 557.76 mm. During the ten years between 1996 and 2005, interdecadal precipitation ushered in a sharp drop and fell to the lowest point in 60 years (495.81 mm). This is closely related to the severe drought that lasted for 4 to 5 years in North China and Northeast China around 1997. In 2006~2015, the precipitation has rebounded (529.55 mm), but compared with the previous stage, it still shows a downward trend.

By analyzing the variation trend of annual mean temperature in Heilongjiang Province in the past 60 years, it can be seen that the temperature in Heilongjiang Province has been fluctuating and rising in general during the past 60 years, but it did not pass the significance test ($P > 0.05$). The R^2 value is 0.472, reflecting a relatively significant upward trend in temperature (*Fig. 4b*). Affected by global warming, the annual average temperature in Heilongjiang Province has gradually increased over the past 60 years, making the intensity of drought increasingly intensified. It can be seen from *Figure 8* that in the 30 years from 1956, the annual average temperature rose slowly at a lower rate; by the decade 1976-1985, the annual average temperature has increased to 1.98 °C. In the next ten years, the temperature increased significantly at a higher rate. The average temperature in the decade from 1986 to 1995 reached 2.87 °C. After that, the growth rate has slowed down noticeably. In the 20 years after 1996, the temperature in Heilongjiang Province has reached a state of constant stability, with an average temperature of 3.19 °C in two decades.



a

b

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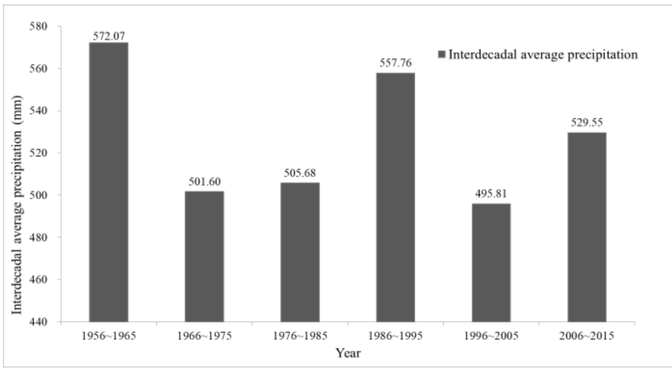

c

Figure 4. *(a) Trends in annual average precipitation in Heilongjiang Province; (b) trends in interdecadal precipitation in Heilongjiang Province; (c) trend pattern of decadal precipitation in Heilongjiang Province*

By calculating the SPI index, the drought frequency and drought index since 1956 in Heilongjiang Province were classified and calculated. It can be seen from *Figure 5a* that the drought frequency always had large fluctuations, but it was basically the same as the overall trend of 60 years. In 1957 and 2013, there were less than 12% of the frequency, and in 1971 and 2011 there were nearly 50% of the high frequency. It can be obviously seen from *Figure 5b* that the intensity of drought in Heilongjiang Province has fluctuating upward in 60 years, and passed the significance test ($P > 0.05$). The intensity was between light drought and moderate drought. This was related to the persistently high temperature in Heilongjiang Province in the past 20 years and was also a response to the deterioration of the global climate.

Mutation analysis

Using the Mann-Kendall test to test the precipitation sequence from 1956 to 2015 in Heilongjiang Province, we can find that the UF curve is greater than 0 in 1956~1957 and 1959~1961, but all passed the $\alpha = 0.05$ significance test, which indicates that during these two time periods, the precipitation in Heilongjiang Province has increased, but it is not significant (*Fig. 5c*). Between 1957 and 1959, the UF curve and UB curve intersected, and both of them were between the significant horizontal line of alpha = 0.05. This indicates that the precipitation in Heilongjiang Province showed two abrupt changes in the trend of rising-falling-rising between 1956 and 1961. In 1961, the UF curve intersected the UB curve again, after which the UF curve began to fall less than 0 and continued to fall. In 1974, it broke the $\alpha = 0.05$ significance test horizontal line, and then returned to the $\alpha = 0.05$ significance test level line in 1984. This showed that 1961 was an extremely important drop point in the precipitation sequence. From 1961, the annual average precipitation in Heilongjiang Province began to decline, and the decline between 1974 and 1984 was very significant. After 1984, the UF curve was still less than zero but rising. This showed that after 1984, the precipitation in Heilongjiang was decreasing, but its rate gradually slowed down. In 1993-1995, the UF curve exceeded zero again, and the precipitation increased. However, after 1995, the UF curve continued to decrease from zero again, which represents a decreasing trend of precipitation. This drop in precipitation for the Northeast region has brought about a drought lasting about five years, the Northeast people’s production and life has brought serious impact.

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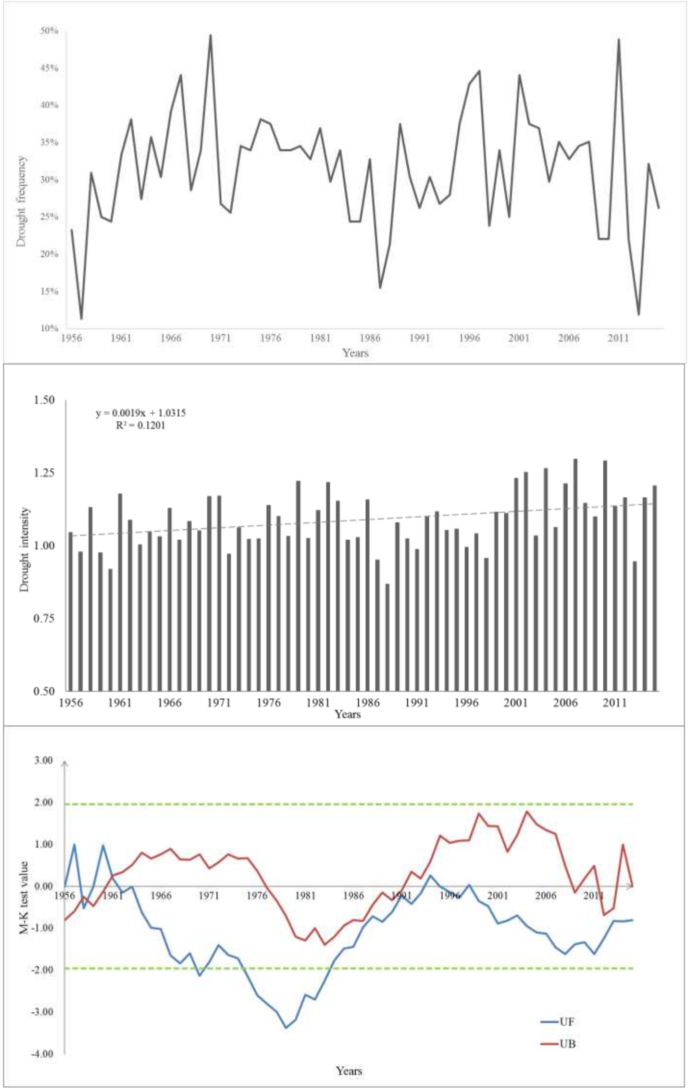

Figure 5. *(a) Trend pattern of drought frequency in Heilongjiang Province from 1956 to 2015; (b) trend pattern of drought intensity in Heilongjiang Province from 1956 to 2015; (c) annual average precipitation M-K test curve in Heilongjiang Province*

Analysis of spatial characteristics of drought in Heilongjiang Province

Interannual drought spatial distribution

According to the SPI index of 14 weather stations, the drought frequency and drought intensity in 60 years were calculated. According to the analysis of *Figure 6,* the highest drought frequency in the province was centered on Qiqihaer and has spread to Anda and Harbin, with values of 33.61%, 32.22% and 31.94%, respectively. Because of the existence of Zhangcailing and Mudanjiang in the south-central region, the drought frequency in the region centered on Tonghe River was less than 29.86%. The drought frequencies in the west of Jixi and Suifenhe were 32.36% and 32.08%, respectively. The variation of drought intensity in Heilongjiang Province was contrary to that of drought frequency. The drought intensity was small in areas with high drought frequency, but large in areas with low drought frequency. Drought events with high intensity were often rarer than those with small intensity.

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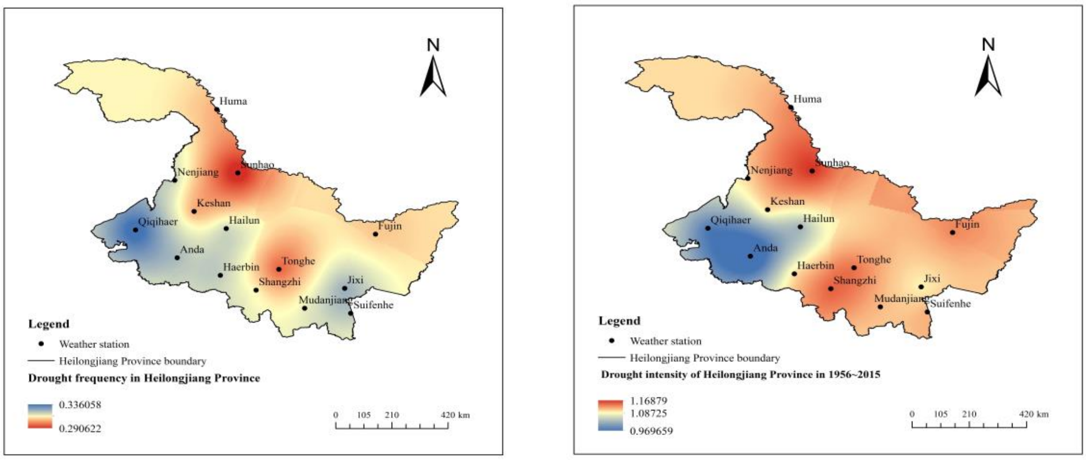

Figure 6. *Drought frequency and intensity distribution map of Heilongjiang Province*

Spatial evolution of interdecadal drought

According to the analysis of *Figure 7a,* in the 1956-1965 period, the Suifenhe River in the west and the Suifen River in the southeast formed the two most intense central points. In the 1966-1975s, one of the centers of gravity shifted, forming the central point of the Tonghe River in the south-central part and the Suifen River in the southeast. The overall drought intensity was characterized by weak southwest and strong southeast. From 1976 to 1986, the two central points of drought intensity were distributed on both sides of the East and West again, which was similar to the distribution from 1956 to 1965, but their scope was obviously expanded. The center of drought intensity in the West changed from one point of Nenjiang River to a larger enclosure formed by Nenjiang River, Sun Wu and Keshan, occupying the northwest of Xiaoxing'an Mountains. Moreover, the northern part of the Greater Khingan Range was also affected by the encirclement. The drought center in the south of China shifted from Suifen River to Mudanjiang area, which was not far from the southwest of Suifen River. In the 1986~1995 era, the southern drought intensity center continued to move westward to Harbin. The other drought intensity center moved from the northwest of the Xiaoxing'an Mountains in the last decade to Fujin in the eastern part of Heilongjiang Province, and almost covered the whole Sanjiang Plain area with Fujin as the center. Since 1996, global warming has caused long-term persistence in the Heilongjiang region to be affected by high temperatures, and the spatial distribution of drought intensity has changed significantly. In the decade from 1996 to 2005, the lowest drought intensity in Heilongjiang Province jumped from the previous drought to moderate drought, and formed the two major centers of Sun Wu and Tonghe. The high drought intensity ranged over the severe situation in central Heilongjiang Province. In the 2006-2015 period, the high drought intensity distribution area of Heilongjiang Province has spread throughout the province, gradually increasing from south to north. Different from the local point distribution in the previous high-intensity intensity areas, during this period, the drought intensity was generally above the moderate drought, and the higher drought intensity range has been extended to the province. The drought frequency distribution (*Fig. 7b*) of 14 meteorological stations in Heilongjiang Province from 1956 to 2015 was relatively stable, basically fluctuating around 30%, and the drought frequency of 14 stations showed similar distribution patterns in each age.

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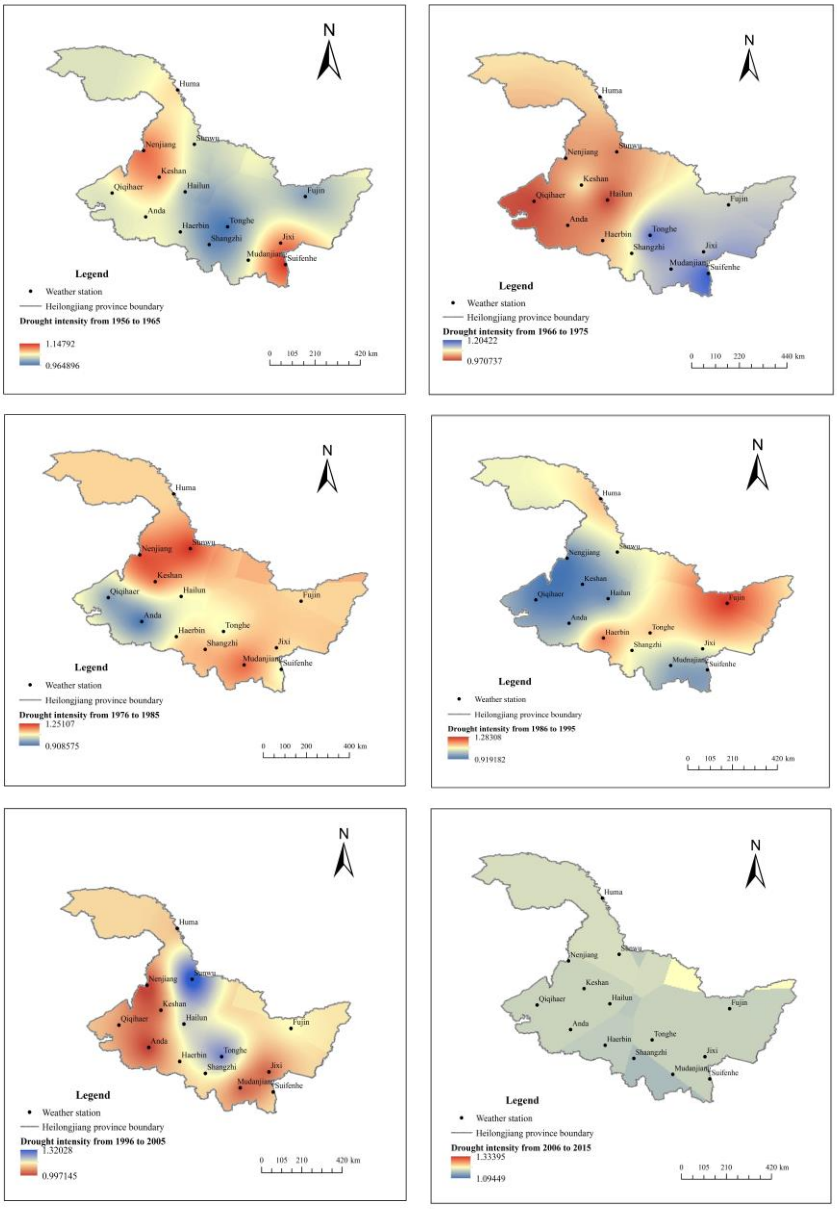

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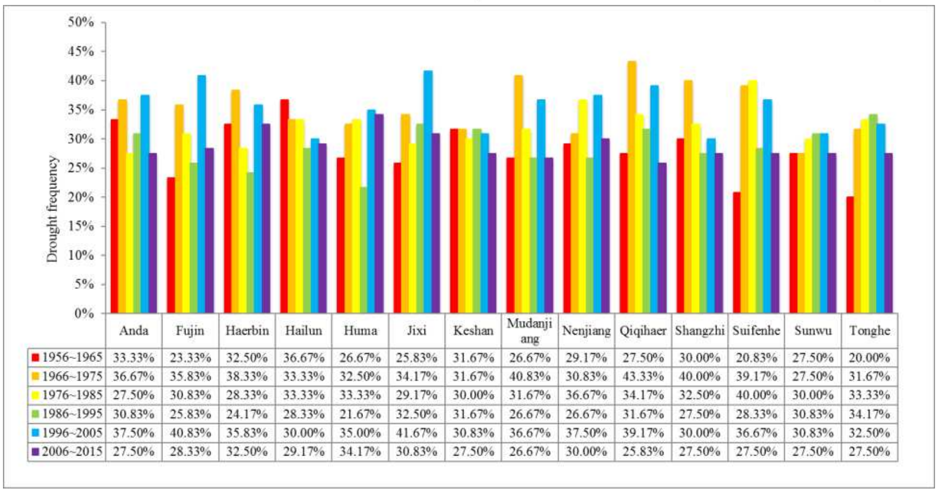

	Anda	Fujin	Haerbin	Hailun	Huma	Jixi	Keshan	Mudanjiang	Nenjiang	Qiqihaer	Shangzhi	Suifenhe	Sunwu	Tonghe
1956~1965	33.33%	23.33%	32.50%	36.67%	26.67%	25.83%	31.67%	26.67%	29.17%	27.50%	30.00%	20.83%	27.50%	20.00%
1966~1975	36.67%	35.83%	38.33%	33.33%	32.50%	34.17%	31.67%	40.83%	30.83%	43.33%	40.00%	39.17%	27.50%	31.67%
1976~1985	27.50%	30.83%	28.33%	33.33%	33.33%	29.17%	30.00%	31.67%	36.67%	34.17%	32.50%	40.00%	30.00%	33.33%
1986~1995	30.83%	25.83%	24.17%	28.33%	21.67%	32.50%	31.67%	26.67%	26.67%	31.67%	27.50%	28.33%	30.83%	34.17%
1996~2005	37.50%	40.83%	35.83%	30.00%	35.00%	41.67%	30.83%	36.67%	37.50%	39.17%	30.00%	36.67%	30.83%	32.50%
2006~2015	27.50%	28.33%	32.50%	29.17%	34.17%	30.83%	27.50%	26.67%	30.00%	25.83%	27.50%	27.50%	27.50%	27.50%

b

Figure 7. *(a) Spatial evolution map of interdecadal drought in Heilongjiang Province; (b) interdecadal drought frequency histogram of Heilongjiang Province*

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Spatial distribution of seasonal drought

It can be seen from *Figure 8a* that the intensity of drought in Heilongjiang Province was generally large and the distribution was the widest. The areas with high drought intensity were mainly concentrated in the central and southeastern mountainous areas, the Daxing'an Mountains in the north and the west of Nenjiang River. The plains have alleviated the drought to some extent due to the pooling of rivers and snow and ice. The two major centers with higher drought intensity in summer were Sun Wu in the north of Xiaoxing'an Mountains and Fu Jin in the north of Sanjiang Plain, and the drought intensity was strong in the north and weak in the southeast. This was due to the influence of the southeast monsoon in Heilongjiang Province in summer, which lead to more precipitation in Southeast China. The Wanda Mountain in the East and the mountains in the middle of Heilongjiang Province blocked the intrusion of warm and humid air currents, which formed a typical monsoon precipitation distribution in summer. The distribution pattern of drought intensity in autumn in Heilongjiang Province was similar to that in the past 60 years. Nenjiang-Sunwu-Keshan and Mudanjiang-Suifenhe were the two high drought intensity centers, which were moderate drought, and other areas were light drought. The distribution of drought intensity in Heilongjiang Province in winter was closely related to the 60-year average precipitation distribution. The drought intensity of the Shangzhi-Tonghe area with large precipitation was large, while the Qiqihar-Anda area with less precipitation had less drought intensity. This was due to the cold weather in Heilongjiang in winter, which caused the river to freeze, affecting the water cycle, and the precipitation was also reduced to the lowest in the four seasons. The Heilongjiang region was sensitive to drought due to its complex topography and water system. The area with more average precipitation had more water demand, so it was more sensitive to the lack of water and formed a higher drought intensity. It can be seen from *Figure 8b* that the drought frequency distribution of the four seasons in Heilongjiang Province was relatively uniform, and the drought frequency of the four stations in the four stations was stable at around 30%.

Discussion

Impacts of topography and basic meteorological elements on the formation of drought

The uneven precipitation, sandstorm and uneven distribution of evaporation caused by the monsoon in Heilongjiang Province are the main meteorological factors for the formation of drought in the province. The precipitation in Heilongjiang Province is greater in the east than that in the west, but the sand and evaporation are both greater in the west than those in the east. This is affected by the temperate continental monsoon climate of Heilongjiang Province. The humid monsoon in the southeast of summer is blown in from the southeast, but it cannot be penetrated inland by the middle and southern mountains (Zhou et al., 2011). In the winter and spring, the dry monsoon from south of Siberia carries the sands of Mongolia from Songnen Plain to the west of the central mountains. This has led to an increase in evaporation and increased drought in the western region. Based on the above factors, the overall drought frequency in Heilongjiang Province is higher than that in the east.

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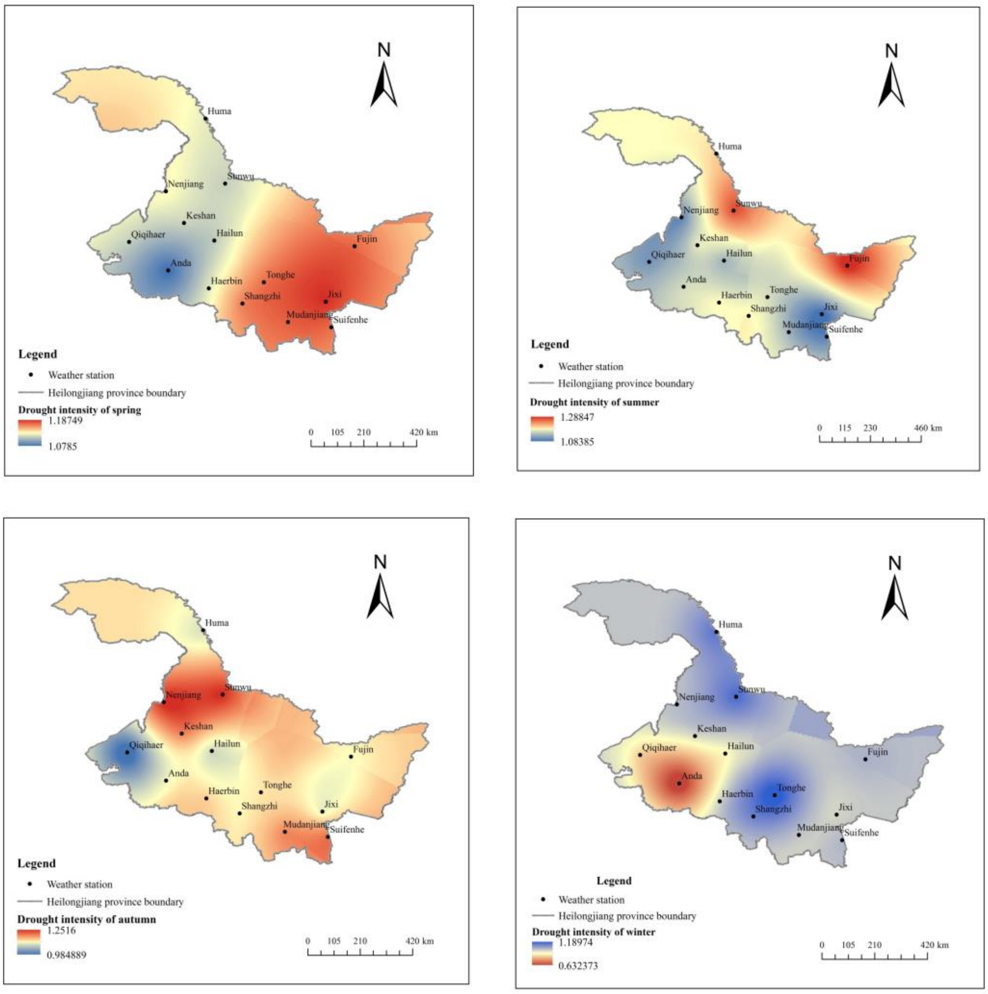

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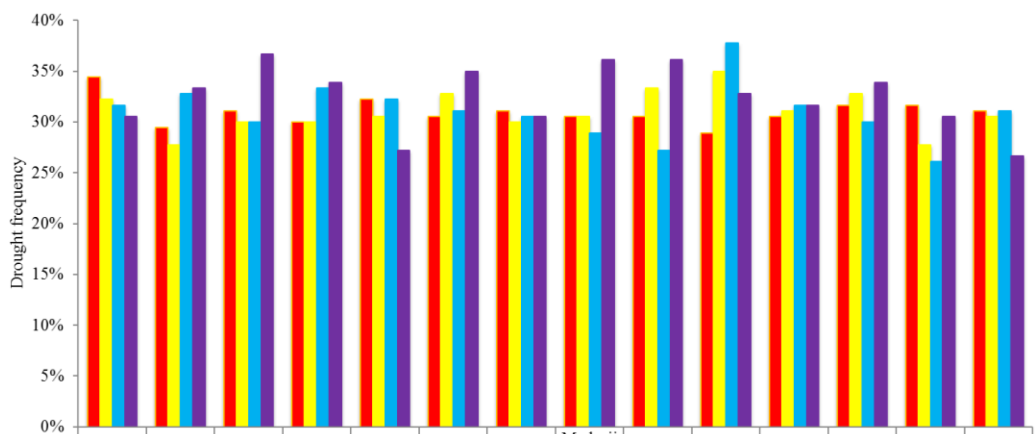

	Anda	Fujin	Haerbin	Hailun	Huma	Jixi	Keshan	Mudanjiang	Nenjiang	Qiqihaer	Shangzhi	Suifenhe	Sunwu	Tonghe
Spring	34.44%	29.44%	31.11%	30.00%	32.22%	30.56%	31.11%	30.56%	30.56%	28.89%	30.56%	31.67%	31.67%	31.11%
Summer	32.22%	27.78%	30.00%	30.00%	30.56%	32.78%	30.00%	30.56%	33.33%	35.00%	31.11%	32.78%	27.78%	30.56%
Autumn	31.67%	32.78%	30.00%	33.33%	32.22%	31.11%	30.56%	28.89%	27.22%	37.78%	31.67%	30.00%	26.11%	31.11%
Winter	30.56%	33.33%	36.67%	33.89%	27.22%	35.00%	30.56%	36.11%	36.11%	32.78%	31.67%	33.89%	30.56%	26.67%

b

Figure 8. *(a) Spatial distribution map of four seasons drought intensity in Heilongjiang Province. (b) Histogram of four seasons drought frequency in Heilongjiang Province*

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Interannual climate anomaly signals affecting drought in northern China

There are many complex factors which affect the climate change in northern China. Since summer precipitation in most parts of China accounts for the majority of total annual precipitation, the interannual anomaly signals affecting summer precipitation in China are discussed. Analysis of factors affecting summer precipitation include sea surface temperature (ENSO phenomenon), snow cover, ground temperature and other underlying surface thermal factors and Asian monsoon, tropical convection, equatorial convergence, cross-equatorial flow, Qinghai-Tibet high, western Pacific subtropical high, Mid-latitude block high pressure, polar vortex, teleconnection type, quasi-two-year oscillation (QBO), three major oscillations and other atmospheric activity centers or atmospheric circulation systems, as well as solar activities, astronomical factors, geophysical factors, etc. (Chen et al., 2001; Wang et al., 2005; Sha et al., 2013; Feng et al., 2014). These factors cover a wide range of aspects, including not only the Earth system ocean circle, the ice and snow circle, the atmosphere, the lithosphere, but also the solar activities outside the Earth system.

Impacts of climate warming and atmospheric circulation on drought

Studies have shown that precipitation in a particular place has a certain relationship with changes in atmospheric circulation (Yan and Zhang, 1994; Salinger and Mullan, 1999; Ramadan and Beighley, 2012). From the atmospheric circulation situation, it can be seen that there are significant differences between the rainy and rainy periods. During the rainy period, in the middle and high latitudes of East Asia, the westerly winds of the westerly winds are large, the high pressure of Yakutsk is strong and stable, and there is a clear north ridge in the eastern part of Lake Baikal, so that the eastern part of the northeast is at the front of the average trough of the south branch. Frequent low pressure and cold vortex move eastward, forming rainy weather and increasing precipitation. On the contrary, there is less precipitation (Sun and Zhou, 2010; Hanssen-Bauer, 2015).

The precipitation in Heilongjiang showed a significant downward trend in the 60 years of 1956~2015. After 1990s, there was a sharp decline in large-scale drought. The persistent drought in these years is closely related to the background of global warming. Global warming is an important reason for the frequent drought in Heilongjiang after the 80s of last century. Sustained high temperature makes the arid area enlarged; the surface evaporation increased; the soil organic matter decreased; the wetland area narrowed, destroyed the ecological diversity and stability of Heilongjiang Province, made the ecological climate environment of Heilongjiang Province fragile, land desertification, salinization, desertification greatly aggravated. Therefore, Heilongjiang is more vulnerable to drought. In addition, the precipitation in Heilongjiang Province has almost the same interdecadal oscillation trend with atmospheric circulation, vorticity field and divergence field. Since the 1980s, the East Asian westerly circulation affecting Heilongjiang Province has a strong latitude, the Yakutsk high is weak, there is no polar vortex center in the eastern Ural Mountains, and the eastern part of Heilongjiang Province is under the Northwest Airflow behind the ridge-trough, which often leads to high temperature and drought weather. At this time, the north of China is in the situation of weakening the convergent updraft caused by the weakening of positive vorticity and stronger divergent subsidence caused by the weakening of divergence, which is not conducive to the formation of precipitation.

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The mechanism for the formation of the drought are complex, there are a lot of factors that impact worthy of further study, such as El Nino phenomenon, sunspot activity and Heilongjiang drought pending further analysis. Warm and cold years the drought disasters variation and related mechanism need to be further clarified.

Conclusion

1) The correlation between monthly mean precipitation and monthly mean temperature in Heilongjiang Province is apparent. The drought in Heilongjiang Province occurred during the year, but its intensity was relatively light and it was relatively stable. The seasonal drought frequency in Heilongjiang Province was negatively correlated with drought intensity.

2) The precipitation in Heilongjiang Province in the whole 60 years showed a downward trend, but the downward trend was not significant, and the overall situation was relatively stable. Interdecadal precipitation fluctuated greatly, with two large declines in the 1960s and 1990s, and a very significant drop in 1961. Although the frequency of drought has been fluctuating greatly, the overall trend of 60 years has remained basically the same. The intensity of drought is fluctuating and the intensity is between mild drought and moderate drought.

3) The distribution of drought frequency in Heilongjiang Province is the lowest in Xiaoxing’anling in the central part and the Songhua River and Mudanjiang in the south, the highest in the Songnen Plain in the west. The spatial distribution of drought frequency in Heilongjiang Province is relatively stable in 60 years, and it basically fluctuates around 30%. The interdecadal drought intensity shows a periodical cycle of localized point-like distribution with strong intensity in the eastern and western regions in the previous decade and strong intensity in the eastern part of the decade. Since the 1990s, the intensity of drought in the entire central region has increased. After entering the 21st century, the distribution of higher drought intensity has spread throughout the province, and the lowest intensity has reached moderate drought, and gradually increased from south to north.

4) The intensity of drought in Heilongjiang Province is generally large and the distribution is the widest. In the other three seasons, only local areas have greater drought intensity. The drought frequency distribution of the four seasons in Heilongjiang Province is relatively uniform, and the drought frequency of the four weather stations in the four seasons is stable at around 30%.

5) The uneven precipitation, sand and evaporation distribution caused by the monsoon in Heilongjiang Province are the main meteorological factors for the formation of drought in the province. Global warming is an important reason for the frequent drought in Heilongjiang after the 1980s. Heilongjiang Province is prone to high temperature and dry weather due to the influence of the East Asian Westerly circulation, the Yakutsk high pressure and the northwest airflow. Moreover, the weaker positive vorticity caused by the weaker positive vorticity in Heilongjiang Province and the weaker divergence caused by the divergent downdraft flow are not conducive to the formation of precipitation.

The climate of Heilongjiang Province has the typical characteristics of the northeast region. Its temperature change and extreme weather events in recent years have been some change, and its causes are still unable to be defined. The aim of research in the

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future is seeking the regional disaster response to the global climate change and mechanism between them.

Acknowledgements. We express our sincere appreciation to the anonymous reviewers for their constructive suggestions. The work was supported by the special program for the National Natural Science Foundation of China (41471160, 41602356). The authors would also to thank the reviewers for their very valuable comments, which greatly improved the quality of the paper.

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MODELING OF GROUNDWATER LEVEL USING ARTIFICIAL INTELLIGENCE TECHNIQUES: A CASE STUDY OF REYHANLI REGION IN TURKEY

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(Received 22nd Nov 2018; accepted 28th Jan 2019)

Abstract. Determination of the change in groundwater level in terms of planning and managing resources is important**.** In this study, the groundwater level of Reyhanlı region in Turkey was predicted using multi-linear regression (MLR), adaptive neural fuzzy inference system (ANFIS), Radial basis neural network (RBNN), support vector machines with radial basis functions (SVM-RBF) and support vector machines with poly kernels (SVM- PK) methods. Models were carried out using 192 data of monthly ground water level, monthly total precipitation and monthly average temperature values measured for 16 years between 2000 and 2015. Comparisons revealed that the SVM–RBF and SVM-PK models had the most accuracy in the groundwater level prediction.
Keywords: *groundwater level prediction, multi-linear regression, support vector machines, adaptive neural fuzzy inference system, radial basis neural network*

Introduction

Groundwater level (GWL) drops due to excessive water withdrawal are serious threats to the sustainability of drinking water basins. Due to global warming and the growing population, the increase in agricultural activities negatively affects the groundwater especially in rural areas. In Turkey, many basins are experiencing problems in terms of the groundwater potential. Increasing water demand, adverse conditions created by climate change and lack of planning related to underground water management in the basin have increased these problems. There are a number of studies that have examined groundwater level reductions due to various reasons. In the field of hydrology, models of groundwater level require knowledge or estimation of the hydrologic parameters of the basin. Prediction of groundwater level is important for effective planning and sustainable groundwater management.

In recent years, artificial intelligence approaches have been widely used in water resource management and hydrological projects (e.g., Kaya et al., 2011, 2016; Mohanty et al., 2013; Li et al., 2012; Shiri et al., 2013; Demirci and Baltaci, 2013; Üneş et al., 2015a, b, 2017; Demirci et al., 2015a, b; Tasar et al., 2017, 2018; Feng and Tian, 2018; Yılmaz et al., 2018; Kankal et al., 2018; Nacar et al., 2018; Wunsch et al., 2018).

Many investigations have been also conducted to predict groundwater level fluctuations with using artificial intelligence techniques. Emamgholizadeh et al. (2014) studied the potential of artificial neural network (ANN) and adaptive neural fuzzy inference (ANFIS) for the groundwater levels (GWL) predictions. Heesung et al. (2011) studied two nonlinear time-series models for predicting the groundwater level (GWL) fluctuations using ANNs and SVM. Nourani et al. (2015) used feed-forward neural network (FFNN), Auto Regressive Moving Average (ARIMAX) models for GWL forecasting of the plain of

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Ardabil, Northwestern Iran. Guzman et al. (2015) used Artificial Neural Networks (ANN) and Support Vector Regression (SVR) in daily groundwater levels predictions. According to their results ANN and SVR have good accuracy to predict daily groundwater levels. Yoon et al. (2016) used a weighted error function approach to improve the performance of artificial neural network (ANN)- and support vector machine (SVM)-based recursive prediction models for the long-term prediction of groundwater levels in response to rainfall. Zhou et al. (2017) proposed a data-driven prediction model combining discrete wavelet transform (DWT) preprocess and support vector machine (SVM) for groundwater depth forecasting. Ebrahimi and Rajaee (2017) investigated the effect of wavelet analysis of ANN, MLR and SVR approaches using GWL data which recorded over in the Qom plain, Iran. Zare and Koch (2018) used new hybrid Wavelet-ANFIS model with several combinations of inputs and mother wavelets to simulate and predict GWL-fluctuations in the Miandarband plain, Iran. The results showed that all model approaches could be used with acceptable accuracy. Other authors also found that artificial neural network in general are exceptionally well suited to predict groundwater levels for different geological settings (Coulibaly et al., 2001; Nayak et al., 2006; Mohammadi, 2008; Sreekanth et al., 2009; Adamowski and Chan, 2011; Trichakis et al., 2011; Chitsazan et al., 2015; Khalil et al., 2015; Chang et al., 2016; Bargezar et al., 2017; Yu et al., 2018).

The main purpose of the present study is to analyze the performance of the MLR, ANFIS, RBNN, SVM-RBF and SVM-PK techniques in monthly groundwater level forecasting using the Monthly Mean Precipitation (MP), Monthly Average Temperature (MT), Monthly Ground Water Level (GWL+1) data. The predictions of the models were compared with those of observed values using te statistical analysis. The monthly groundwater level data measured from DSI (Turkish General of State Hydraulic Works) between 2000 and 2015 and the monthly total precipitation and monthly average temperature data recorded by Antakya Meteorological Station were used to determine groundwater level.

Material and methods

Study area

In this study, groundwater level of Reyhanlı region on Amik plain is studied (*Fig. 1*). Amik plain is the most efficient plain of Turkey in terms of productivity. It is covered with thick alluvial soil layer and is known as the most efficient basin in terms of agricultural potential. It forms the largest flat land of Hatay Province. Amik Plain nourishes its soil primarily with Asi River, Afrin and Karasu Stream. The plain which locates in the basin of Asi has an area of approximately 65 000 ha. The groundwater level in Amik Plain reduces by approximately 5-6 m each year. Due to drying of Amik Lake, global climate changes and unconscious irrigation, there is a very serious water shortage in the plain which lead to several problems especially for farmers. For these reasons, using existing water resources more effectively has become inevitable.

The DSI observation-well with number 15768 used in the present study is in Reyhanlı region and located at 36.2685 latitude and 36.5676 longitude. It has a depth of 50 m. The monthly groundwater level data taken from DSI (Turkish General of State Hydraulic Works) data and the monthly total precipitation and monthly average temperature data recorded by Antakya Meteorological Station were used to determine groundwater level. Models were carried out using 192 data of monthly ground water level, monthly total precipitation and monthly average temperature values measured for 16 years.

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Figure 1. Location of Reyhanlı region

The monthly groundwater level fluctuation between the years 2000 and 2015 is shown in *Figure 2*. In the Reyhanlı region, generally, high precipitations occur in early and end of spring. However, drought is observed in the summer months. It is observed that consequent groundwater level fluctuations are entirely dependent on meteorological conditions, which affect the drainage basin. It can be seen from this figure that the vertical axis of *Figure 2* shows well gauge readings located in Amik region and the level fluctuations range in the order of 27 m.

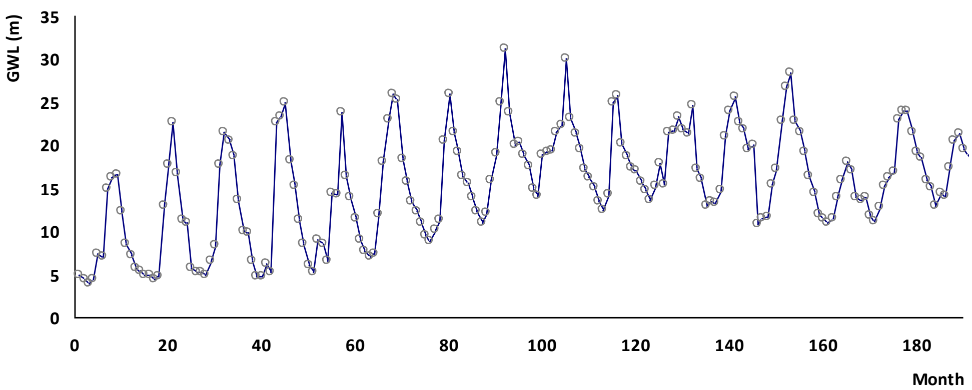

Figure 2. *Groundwater level fluctuations of Reyhanlı region between the years 2000 and 2015*

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In all of the models, Monthly Mean Precipitation (MP), Monthly Average Temperature (MT), Monthly Groundwater Level (GWL+1) were used for estimating of the Ground Water Level fluctuations. The models are first trained by using the first set of data, namely training data comprising the first 144 data of 192 months' observations. Once the training stage was completed, the models were applied to the testing data which comprise the last remaining 48 months' observations. For applied each model, the mean square error (MSE), mean absolute error (MAE), and the determination coefficient between the model estimations and the observed values are computed. The results are used to compare the performance between model prediction and observed well data. The MSE and MAE are given as in *Equations 1* and *2:*

$$MSE = \frac{1}{N}\left(\sum_{i=1}^{N} Yi_{observed} - Yi_{forecast}\right)^2 \tag{Eq.1}$$

$$MAE = \frac{1}{N}\sum_{i=1}^{N}\left|Yi_{observed} - Yi_{forecast}\right| \tag{Eq.2}$$

where N and Yi denote the number of data sets, groundwater level data respectively. Different number of input combinations were tried for the best performance of using models were compared with each other.

Multiple-linear regression (MLR)

This method is used to find out how much a dependent variable is affected and the value of the independent variables in which it is affected and associated.

In multiple regression analysis, dependent variable y indicates the relationship between independent variables $x_1, x_2, ..., x_p$ and it can be written as in *Equation 3:*

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + ... + \beta_j x_j + ... + \beta_p x_p + \varepsilon \tag{Eq.3}$$

Here, β_0, β_1, β_2, β_j, .., βp are called the regression coefficients. Any βj regression coefficient gives the expected change in the y variable versus a unit change in the xj variable when other variables are kept constant (when the effect of the other variables is eliminated).

Adaptive neural fuzzy inference system (ANFIS)

Fuzzy rules are expressed in form of if-then statements between system inputs and outputs depending on the linguistic variables. ANFIS was first represented by Jang (1993). ANFIS works by a learning algorithm derived from neural network functional rules. The parameters of the fuzzy inference system are determined by the neural network learning algorithms in a fuzzy rule-based system. Fuzzy Inferences System can be classified into two types; Mamdani's system and Sugeno's system. ANFIS techniques are based on the Sugeno's system. ANFIS with Sugeno type works according to "If-Then" rule and the ANFIS structure uses the Sugeno-Fuzzy rules.

The Sugeno NF system, generated by two rules using three inputs, is shown in *Figure 3a*. Where, w_1 or w_2 is obtained by weighted mean of individual rule outputs. NF

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structure is shown in *Figure 3b*. NF is connected via directional links and contains several nodes. Every node has a node function that can be constant or adjustable parameters. In the figures, A_1, B_1, C_1 and A_2, B_2, C_2 are linguistic labels (such as “low”, “medium” or “high”), f_1 and f_2 denote, respectively, output functions of 1. and 2. rule, $\{p_i, q_i, r_i, s\}$ specified as result parameters. Sugeno’s system is more compact and computationally more efficient (Jang, 1993). For more information, it can be looked at Jang (1993).

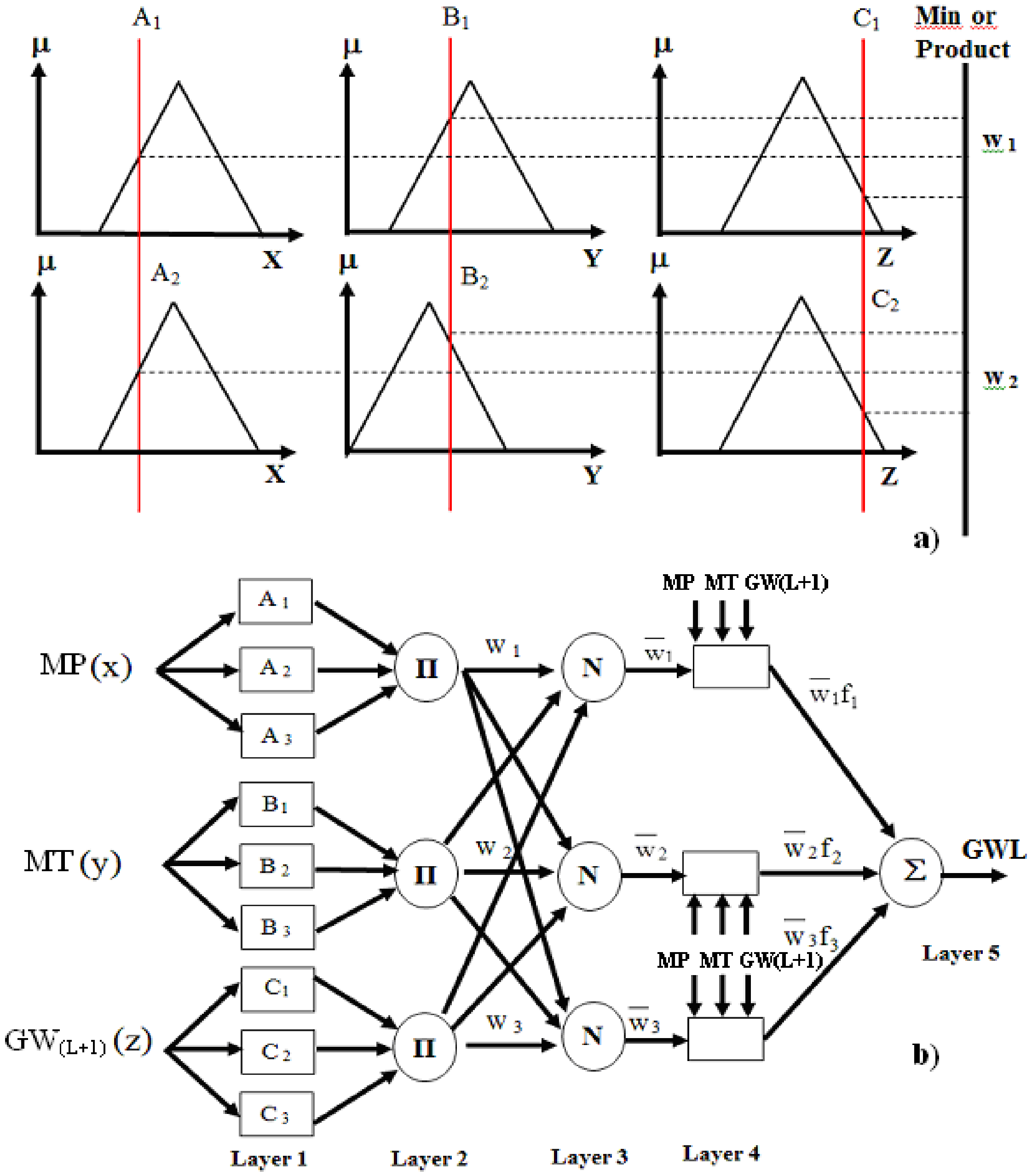

Figure 3. a *Sugeno type fuzzy model.* ***b*** *Neuro-fuzzy (NF) system structure for three inputs. In this presented fuzzy model, input data were taken as monthly mean precipitation (MP), monthly average temperature (MT), monthly ground water level (GWL+1), and ground water level (GWL) were estimated as output data*

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Radial basis neural network (RBNN)

Radial basis neural network was first presented into the ANN literature by Broomhead and Lowe (1988). The RBNN models consist of two layers whose output nodes form a linear combination of the basis functions. The learning scheme of RBNN is fundamentally different from that of the feed-forward ANN. The RBNN assumes a radially symmetric function, typically the Gaussian function, for its hidden units. The relation between inputs and outputs is demonstrated in *Figure 4*. Detailed information about RBNN theory can be found in the works of Haykin (1998).

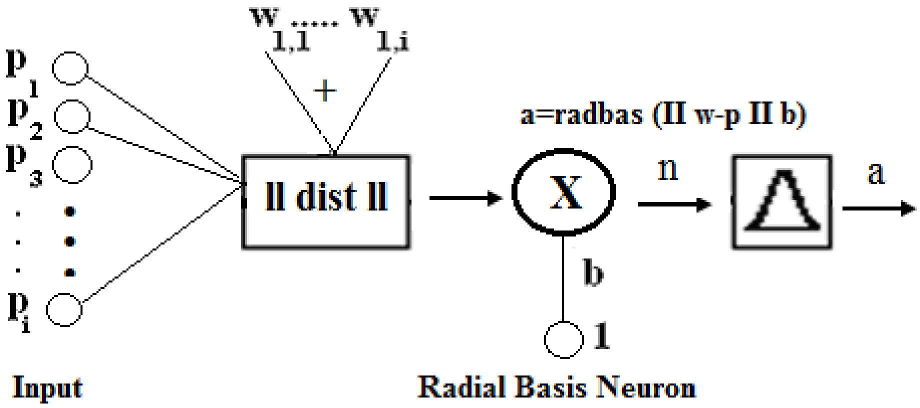

Figure 4. *Schematic representations of RBNN*

In *Figure 4,* the net input to the radbas transfer function is the vector distance between its weight vector w_i and the input vector p_i, multiplied by the bias b. The || dist || box in this figure accepts the input vector p and the single row input weight matrix, and produces the dot product of the two. The radial basis function has a maximum of 1 when its input is 0. As the distance between w and p decreases, the output increases. Thus, a radial basis neuron acts as a detector that produces 1 whenever the input p is identical to its weight vector w. The transfer function for a radial basis neuron is given as *Equation 4:*

$$radbas(n)= e^{-n^2} \quad \text{(Eq.4)}$$

Support vector machines (SVM)

The SVM has become a relatively novel and promising estimator in data-driven research fields, of which basic concept and theory have been introduced by Vapnik (1998). The generalization ability of the SVM is considered to be better than ANN, in the sense that it is based on the structural risk minimization rather than the empirical risk minimization of ANN. The main process of SVM model building consists of selecting support vectors which support the model structure and determining their weights. *Figure 5* shows the SVM models schematic representations. The idea behind SVM is to find a hyperplane that separates two classes in the transformed feature (input) space with a maximum distance. SVM aims to find the optimal regression hyperplane, that all training samples lay within an ε-margin around it and is also as flat as possible (Schölkopf and Smola, 2002). SVM models are closely related to artificial neural

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networks and use a sigmoid kernel function while having a two-layer, forward-feed artificial neural network (Haykin, 1998).

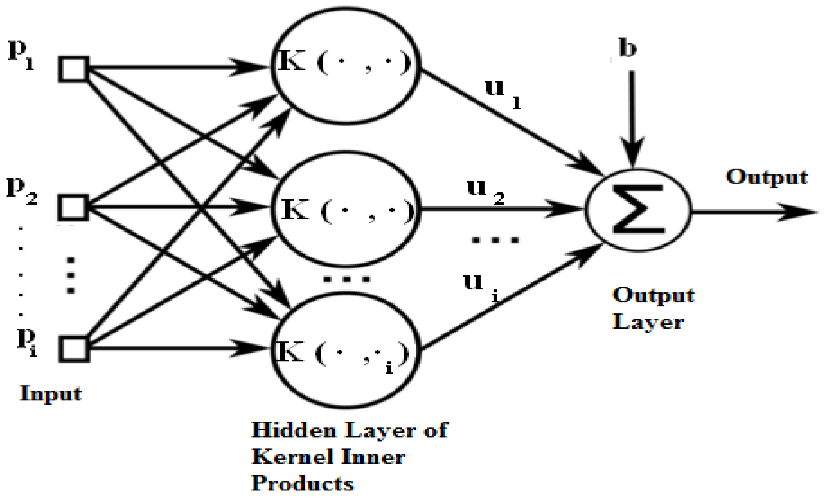

Figure 5. *Schematic representations of SVM*

An interesting feature of SVM is that by minimizing the average error rate on the data set from the empirical risk minimization principle derived, statistical learning can be achieved.

SVM defines how to draw this boundary between variable groups. SVM studies according to statistical learning theory. A set of training data [(p_1, y_1), (p_2, y_2),..... (p_n, y_n)], where "p_i" value indicates the input space of the sample and has a corresponding target "y_i" value.

The SVM function is expressed as in *Equation 5:*

$$y = (K_{pi}.W_{jk}) + b \qquad \text{(Eq.5)}$$

where the Kernel function is K_{pi}, b is bias term of SVM network and W_{jk} is called as the Lagrange multipliers that obtain the significance of the training data sets for the output data. The kernel function of non-linear radial basis (Hsu et al., 2003) is in *Equation 6:*

$$K_{xi} = e^{-\gamma \|p_i - y_i\|^2} \qquad \gamma > 0 \quad \text{and} \quad i = 1,2,3,...n \qquad \text{(Eq.6)}$$

where γ is a user-defined parameter. The kernel function of polynomial (Hsu et al., 2003) is in *Equation 7:*

$$K_{xi} = (p.y + c)^d \qquad i = 1,2,3,...n \qquad \text{(Eq.7)}$$

SVM differs from the other classification methods significantly. Its intent is to create an optimal separating hyperplane between two classes to minimize the generalization error and thereby maximize the margin. SVM is an approximate implementation of structural risk minimization approach. Structural risk minimization method described that the error rate of learning machine on test data is bounded by the sum of training error rate and a term that based on Vapnik–Chervonenkis dimension (Haykin, 1998).

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Results and discussion

In order to demonstrate the models capability for predicting groundwater level variations, this section will discuss the validation process of the optimal MLR, RBNN, RBNN, SVM-PK and SVM-RBF models. The correlation coefficient (R), mean square error (MSE) and absolute mean error (MAE) for the performance evaluation of all models are calculated. Comparison parameters of MSE, MAE and R obtained from testing data are shown in *Table 1*. Results are used to compare the performance of model prediction and the observation data.

In *Figure 6a,* results of MLR model shows that the correlation coefficient is high and the groundwater level estimate is closer to the actual values. MLR models performed less efficiently than the other models based on MSE, MAE and R (5.44-1.73-0.84) criteria shown in *Table 1*.

For RBNN model, the distribution and scatter graphs are shown in *Figure 6b*. When scatter graphs for testing data are analyzed, RBNN model results also showed a good performance. RBNN estimated values are close to the actual values. Correlation coefficient was obtained as R = 0.88. According to the MSE, MAE and R (4.64, 1.62, 0.88) criteria, compared to MLR model, the RBNN model results showed better performance.

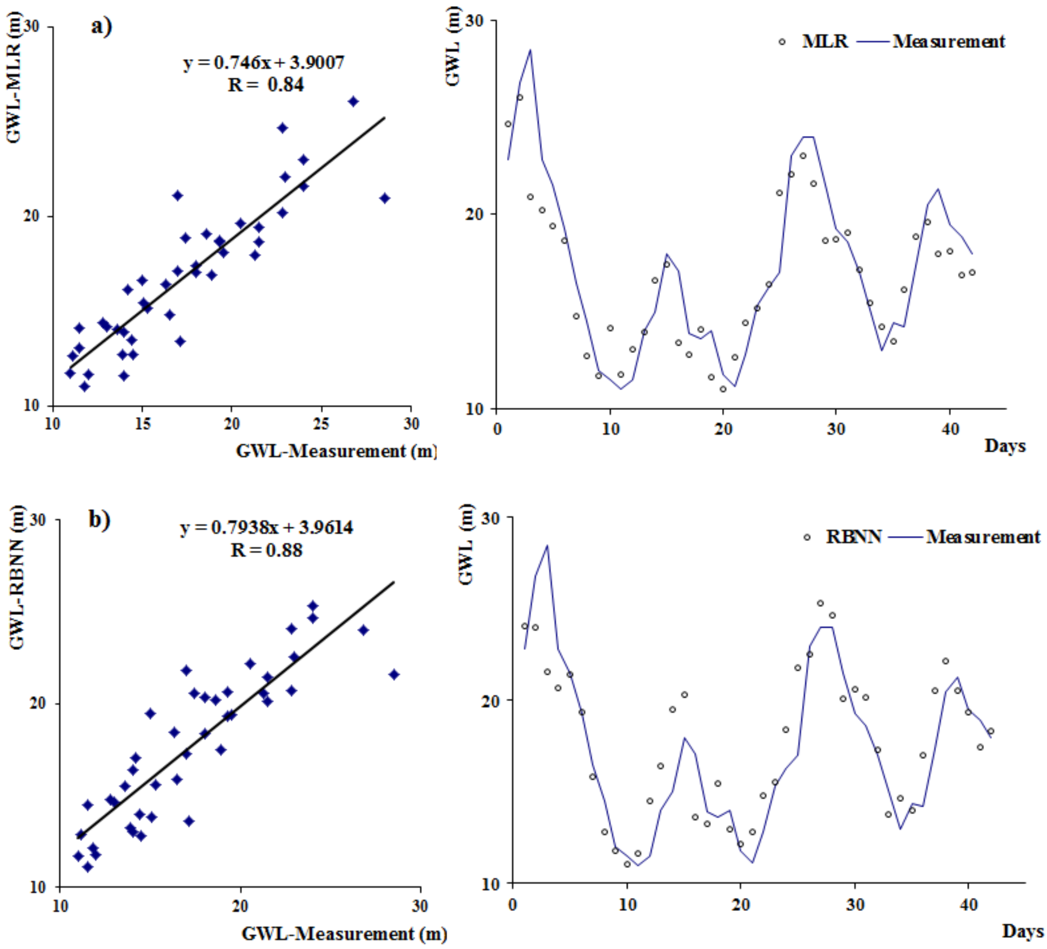

Figure 6. ***a*** *Measurement and MLR model scatter and distribution.* ***b*** *Measurement and RBNN model scatter and distribution*

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16 years data were evaluated for ANFIS model, and the results are defined as follows. In ANFIS models, distribution and scatter graphs for testing data are shown in *Figure 7a*. Correlation coefficient were obtained as R = 0.87. When distribution and scatter charts are analyzed, estimated values are close to the actual values and ANFIS results showed similar results according to RBNN results. When we looked MSE, MAE and R (4.82-1.63-0.87) criteria values shown in *Table 1,* The ANFIS estimated values gave better results than the MLR values

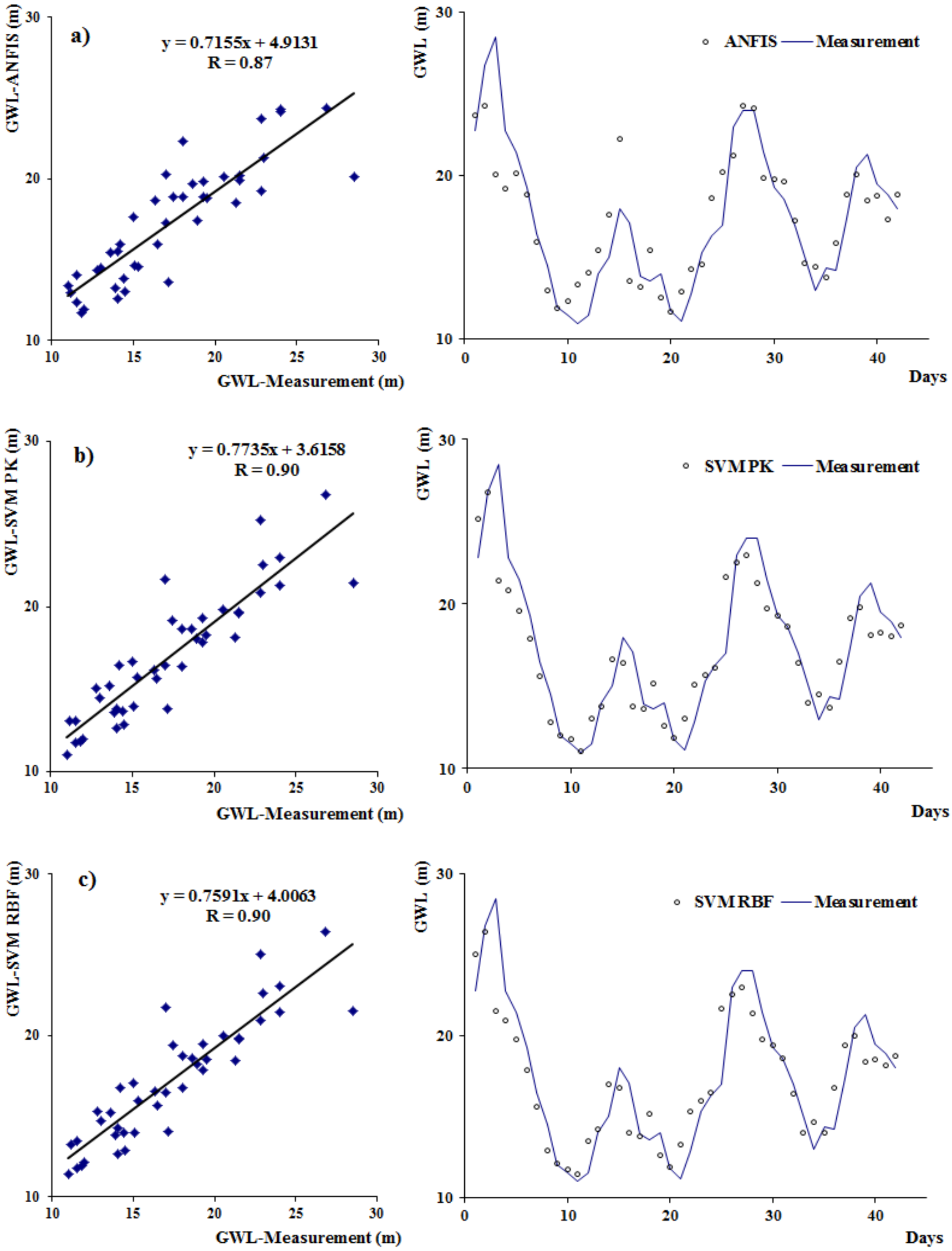

Figure 7. ***a*** *Measurement and ANFIS model scatter and distribution.* ***b*** *Measurement and SVM-PK model scatter and distribution.* ***c*** *Measurement and SVM-RBF model scatter and distribution*

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Table 1. *Model results according to statistical criteria*

MODELS	INPUTS	MSE	MAE	R
MLR	MP,MT, GWL(L+1)	5.44	1.73	0.84
RBNN	MP,MT, GWL(L+1)	4.64	1.62	0.88
ANFIS	MP,MT, GWL(L+1)	4.82	1.63	0.87
SVM-PK	MP,MT, GWL(L+1)	3.81	1.41	0.90
SVM-RBF	MP,MT, GWL(L+1)	3.78	1.42	0.90

MSE: mean square error, MAE: mean absolute error, R: correlation coefficient, MP: monthly mean precipitation, MT: monthly average temperature, GWL+1: monthly groundwater level

The SVM Poly Kernels (SVM-PK) and SVM Radial Bases Function (SVM-RBF) models were also applied to estimate the ground water level. Estimated testing results for SVM models are shown in *Figure 7b* and *c*. The SVM Poly Kernels (SVM-PK) and SVM Radial Bases Function (SVM-RBF) estimated values gave closer results according to the RBNN values. The correlation coefficient was obtained for both model to be R = 0.90 as seen in *Figure 7b* and *c*. SVM model results data are close to the actual values shown in figures. As seen from the scatter plots, the SVM model estimates are less scattered in relation to the other models.

According to MSE, MAE and R, the MLR (5.44-1.73-0.84) model has the lowest success rate. ANFIS approach (4.82-1.63-0.87) and RBNN approach (4.64-1.62-0.88) have the higher success rate than the MLR model. SVM-PK (3.81-1.41-0.90) and SVM-RBF (3.78-1.42-0.890) models were found to perform better than the other models at all performance evaluations.

Conclusions

In this study, groundwater level fluctuations of Reyhanlı region in Turkey were predicted with using MLR, ANFIS, RBNN, SVM-RBF and SVM-PK approaches.

The accuracy of all models in groundwater level estimation was also investigated, and the results were compared with each other model. Comparisons revealed that the SVM –RBF and SVM-PK had the closer accuracy in the groundwater level prediction. In general, MLR model has the smaller lowest success rate. ANFIS and RBNN models gave better results than the MLR model. According to the MSE, MAE and R criteria, the best results were obtained in SVM-RBF and SVM-PK models. The results of the case study are satisfactory and demonstrate that SVM-RBF and SVM-PK models can be a useful prediction tool in the area of groundwater hydrology.

It is recommended for future studies that authors should study the use of new methods of artificial intelligence techniques in daily, weekly, or yearly groundwater level forecasting for different geographical regions.

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THE EFFECT OF PHOSPHORUS AND ZINC ON YIELD AND ON SOME AGRONOMIC CHARACTERISTICS OF COTTON (*Gossypium hirsutum* L.)

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(Received 22nd Nov 2018; accepted 25th Jan 2019)

Abstract. This study was conducted to determine the effect of Phosphorus (P) i.e. (P_0= Control, P_{40}= 40, P_{80}= 80, and P_{120}= 120 kg ha^{-1}) and Zinc (Zn) i.e. (Zn_0= Control, Zn_{05}= 5, Zn_{10}= 10, and Zn_{15}= 15 kg ha^{-1}) on yield and on some agronomic characteristics of cotton (*Gossypium hirsutum* L.) Adiyaman, Turkey. The field experiments were arranged in a randomized complete block with a split plot design with three replications in years of 2011 and 2012. P and Zn treatments were applied in main and sub-plots, respectively. Cotton cultivar of Stoneville-453 was used in the experiment. Based on the results, phosphorus and zinc applications increased the seed cotton yield. The highest plant height was obtained from the interaction of P_{80} x Zn_5 and the highest number of bolls was taken from the interaction of P_{80} x Zn_{10}. In terms of the number of monopodial branches, statistically significant differences were found in both years, but this significance was not stable over the years. It was concluded that the combined treatments of phosphorus and zinc did not have a significant effect on the boll weight, boll seed cotton weight and ginning outturn. The effect of fertilizers on the number of sympodial branches and 100 seed weight were significant in the first year but not significant in the second year.
Keywords: *sympodial branch, agronomic traits, ginning, interaction, replications*

Introduction and literature review

In addition to being the main source of natural fiber, cotton is a plant that has an important place in global agriculture and trade with its oil, meal, and other by-products obtained from its seed. Cottonseed pulp is also used in animal nutrition. Along with the developing textile industry, the importance of cotton production in the world economy is increasing gradually. Fertilizer is an important input in cotton production and it has become a necessity for farmers to use for a profitable production. An important component of profitable cotton production is an adequate and balanced fertilization. In the case of cotton and most plants various yields are obtained due to the various growing conditions in different regions. It is necessary to apply cultural measures such as fertilization, tillage, and diseases and pest control as well as sowing, seedbed preparation, and other managements for higher yielding.

Today, the most applied fertilizer of cotton plant is nitrogen, the second one, phosphorus (P) is also one of the essential elements for plant development. Zinc and Phosphorus are important plant nutrients affecting plant growth directly. Phosphorus increases boll development by affecting the flower formation and fertilization biology in cotton. Despite the continuous addition of phosphorus from the organic and inorganic sources to the heavily cultivated soils, the phosphorus level of the soil is insufficient for profitable crop production. P is an essential element for metabolic functions and vital structures in plants. It is especially vital in early root development, photosynthesis, cell division, energy transfer, early boll development and early maturity (Stewart, 1998).

Phosphorus deficiency leads to deterioration of plant membranes and decreases energy transfer in the plant (Oosterhuis et al., 2007). P deficiency is one of the most

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

important factors limiting the yield in plant production, especially in calcareous alkaline soils. Particularly above pH 7, phosphorus in the soil is fixed by cations such as calcium in the soil to form insoluble salts (Zhou et al., 2001). The phosphorus, which turns into salt, becomes useless for the plants (Castro and Torrent, 1995).

Phosphorous fertilizers, unlike nitrogen fertilizers, are fixed very tightly in the soil. Plants rarely intake more than 20% of the applied phosphorus. However, this rate is as high as 60% in nitrogen. Phosphorous fertilizers turn out to be beneficial for plants faster in soils with a pH of 5.5-7. The plant cannot sufficiently utilize it, as it is fixed too tightly under these pH degrees. These fertilizers are more beneficial when supplied with lime to acidic soils. As the lime prevents phosphorus to be fixed by minerals like iron and aluminum (Aydemir, 1982).

Phosphorous fertilizers can affect significantly the increase of the number of flowers and bolls, the size of bolls, and early maturity rather than the vegetative growth of cotton. It is claimed that phosphorus helps blooming, bearing fruits, and increases the yield in cotton, and that phosphorus deficiency in the soil results in the reduction of plant height as well as seed cotton yield by 30% (Şenel, 1980).

Crop yield is limited by the low levels of micronutrient minerals such as zinc (Zn) especially in arid and semi-arid regions of calcareous soils (Cakmak et al., 1999). Zinc is the most preferred micronutrient element to cotton. That is important in regulating the chlorophyll content and assimilation rate of the cotton leaf (Weir et al., 1996). Zinc plays an important role in some enzyme systems and catalyzes the process of disintegration in the plant cell. Moreover, it is also vital in the structural change of carbohydrates, chlorophyll formation and growth-promoting substances. Zinc also prevents withering by promoting water intake (Price, 1970). It was reported that the critical limit of zinc in the field soil is 0.5 ppm (Linsday and Norwel, 1978), and application of zinc as $ZnSO_4$ increases seed cotton yield significantly comparing non-applied control plot and a 25 kg ha^{-1} $ZnSO_4$ application gives the highest yield (Sharma and Gupta, 1988), furthermore, NPK+Zn application significantly increases the seed cotton yield and the number of bolls (Prasad and Prasad, 1994). 44 and 75 kg ha^{-1} phosphorous fertilization with 40 ppm foliar zinc application 75 and 90 days after sowing resulted in an increase in the number of bolls, boll weight, 100 seed weight, and seed cotton yield in cotton plants (Sawan et al., 1997). In addition, Soomro et al. (2000) also reported that due to a 5 kg ha^{-1} zinc sulfate application the cotton increased the number of bolls, boll weight, seed index, and seed cotton yield compared to the control. Zinc treatment was reported to increase the cotton yield compared to the control (Sawan et al., 2001) and phosphorus and zinc applications increase100 seed weight, seed cotton yield, and fiber yield (Elwan et al., 2002). Nevertheless, it was also stated that zinc application increased plant height the same treatment in soil with high pH and phosphorus content did not affect the yield (Ören and Başal, 2006). Panayotova et al. (2017) observed that 0, 40, 80, 120, and 160 kg ha^{-1} P applications, increased the seed cotton yield from 5.02 to 11.71%, the number of bolls from 3.0 to 25.9%, and plant height significantly.

Foliar zinc application twice i.e. (0 and 58 g ha^{-1}) 70 and 85 days after sowing, and the foliar phosphorus application twice i.e. (control, 576, 1152, and 1728 g ha^{-1}) 80 and 95 days after sowing increased seed cotton yield, number of bolls, 100 seed weight, and fiber yield (Sawan et al., 2008). Zinc treatments of the 0, 5, 7.5, 10, and 12.5 kg ha^{-1} doses a 7.5 kg ha^{-1} was stated to be the most effective zinc dose, and this zinc ratio increased 100 seed weight and seed cotton yield (Ahmed et al., 2010).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

This study aimed to assess the effect of phosphorus and zinc applications on the yield and agronomic characteristic of cotton in South East Anatolia.

Materials and methods

Field trials were carried out in zinc deficient soils of Old Hosni Mansur (Eskihüsnümansur) village of Adiyaman in Turkey in 2011 and 2012 growing seasons. Stoneville 453 cotton cultivar was employed as plant material.

Field trials were set up at the same location in both years, but on the different parts of experimental field showing Zn deficiency. Some soil properties of the experimental area were given in *Table 1*.

Table 1. *Soil properties of the field*

Soil properties	2011	2012
Saturation (%)	55	57
$Ec_{25}10^3$ (mmhos/cm)	0.083	0.079
pH (1:2:5)	7.60	7.45
P_2O_5 (kg ha^{-1})	8	21
K_2O_5 (kg ha^{-1})	799	691
Organic matter (%)	1.4	2.03
Lime Sodium N (me/L)	23.9	24.1
Cu (mg/L)	2.48	2.43
Mn (mg/L)	3.18	3.08
Fe (mg/L)	7.05	6.94
Zn (mg/L)	0.27	0.25

Anonymous, 2012a

Adiyaman is located at the transitional zone between coastal and continental climate conditions. Adiyaman province is located at 37°45’ north latitude and 38°16’ east longitude with an altitude of 672 m. Field trials were located at 37°41’47’ north latitude 38°20’55’ east longitude with a 547 m altitude from sea level (*Figure 1*).

Figure 1. *The map of the experimental area*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

In the 2011 growing season, the average temperatures were between 10.2°C to 32.3°C and the maximum temperatures were between 20.2°C and 42.5°C, while in the 2012 cultivation season the average temperatures were between 10.4°C to 32.5°C and the maximum temperature was between 20.5°C and 43.7°C (*Table 2*).

Table 2. *The official record of Meteorology Directory, Adiyaman, Turkey.*

	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Annual
2011										
Av. Temp.	10.2	16.1	21.3	26.4	32.3	31.4	25.4	17.6	10.4	17.6
Max Temp.	21.4	27.9	33.0	37.2	42.5	41.3	36.0	31.2	20.2	42.5
Min Temp.	0.4	2.7	5.7	13.3	18.0	20.7	15.3	5.8	0.0	-3.8
Total Precip.	80.6	25.4	36.5	13.5	0.0	3.5	0.4	44.1	70.8	457.7
2012										
Av. Temp.	10.8	16.7	21.8	29.1	31.1	32.5	25.6	19.4	10.4	17.9
Max Temp.	21.7	27.2	37.1	40.2	41.0	43.7	38.3	32.4	20.5	43.7
Min Temp.	2.0	4.0	9.0	15.3	20.4	20.8	12.9	10.0	1.7	-6.3
Total Precip.	62.3	64.9	33.9	0.1	0.0	0.1	0.3	82.8	90.7	678.0
Average temperatures for 10 years period (2002-2012)										
Av. Temp.	9.8	14.7	20.2	26.3	30.6	30.1	25.4	15.8	11.8	16.7
Max Temp.	24.7	30	36	40	44	43.5	40	35	28.2	44
Min Temp.	-6	-2	6	10.6	16.7	16.3	10.2	2.2	-3.2	-14.6
Total Precip.	111.3	82.9	51.4	5.9	1.5	0.8	3.8	30.6	76.7	798.1

Anonymous, 2012b

In the field trials, phosphorus (Triple Super Phosphate), and zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (22%) were used as treatments. The experiments were carried out by split plot in randomized complete blocks with 3 replications. P doses i.e. (P_0= Control, P_{40}= 40, P_{80}= 80, and P_{120}= 120 kg ha^{-1}) formed main plots and Zn treatments i.e. (Zn_0= Control, Zn_{05}= 5, Zn_{10}= 10, and Zn_{15}= 15 kg ha^{-1}) formed sub-plots. Each plot had a length of 12 m and consisted of 6 rows. There were 2 m alleys between plots, and 3 m between blocks 70 cm inter rows and 15 cm intra row spacing were allowed. Sowing was practiced on 22nd April in 2011, and 27th April in 2012, by a pneumatic drill. In total, a 160 kg ha^{-1} pure nitrogen was applied. Half of the nitrogen was given during the sowing and the remaining half was given by the lister tool just before the first irrigation. All of the phosphorus was given during the sowing by drill and the Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) was manually given into neighboring rows located 5-6 cm apart and depth from cotton seeded rows.

Irrigation was practiced seven times in 2011 and 8 times in 2012 applying 840 mm and 960 mm for consecutive years, respectively. Furrow irrigation method was performed. Agronomic managements were carried out based on common practices employed in the region. No spraying was carried out against pests in either year.

Seed cotton harvests were done manually (handpicking) on 23th September 2011 and 17th September 2012, respectively. Seed cotton yield (kg plot^{-1}) was scored taking into account two rows in the middle of 10 meters in each plot (10 m x 1.4 m = 14 m^2) excluding the one-meter area from both ends of the plot. The ginning outturn (%) was determined by the ginning of 0.5 kg of seed cotton collected from each plot. Plant height (cm), number of monopodial branches (per plant^{-1}), number of sympodial branches (per plant^{-1}), and number of bolls (per plant^{-1}) were calculated by scoring in 10 randomly selected plants in each plot. Boll weight (g), boll seed cotton weight (g) and

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DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

100 seed weight (g) were scored by employing 35 boll samples randomly selected from plots just before harvest (Worley et al., 1976).

The data obtained from the experiment was analyzed by using the MINITAB 18 (Minitab Inc., USA) statistical software and analysis of variance was performed according to the split plot test in randomized complete block design and the means were grouped by the Tukey HSD ($p \leq 0.05$) test.

Results and Discussion

In the analysis of variance (ANOVA) according to the combined years of the properties examined in the trial, statistical differences were found between years. Due to that the results of each year were analyzed separately.

F values obtained from the analysis of variance of the traits under study in the experiment were given in *Table 3*.

Table 3. *Statistical significance (F) of some traits in the ANOVA*

	Seed Cotton Yield		**Plant Height**		**Monopodial Branches**		**Sympodial Branches**		**Number of Bolls**	
	2011	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**
P	**	**	**	ns	**	ns	**	ns	**	**
Zn	**	**	**	*	**	**	ns	ns	ns	*
PxZn	**	**	**	**	**	**	**	ns	*	**

	Boll Weight		**Boll Seed Cotton Weight**		**Ginning Outturn**		**100 Seed Weight**	
	2011	**2012**	**2011**	**2012**	**2011**	**2012**	**2011**	**2012**
P	ns	ns	ns	ns	ns	ns	*	ns
Zn	ns	ns	ns	ns	ns	ns	ns	ns
PxZn	ns	ns	ns	ns	ns	ns	*	ns

* ($p \leq 0.05$), ** ($p \leq 0.01$), ns: non-significant

Seed cotton yield (kg ha^{-1}), plant height (cm), number of monopodial branches (no. $plant^{-1}$), number of sympodial branches (no. $plant^{-1}$), number of bolls (no. $plant^{-1}$), boll weight (g), boll seed cotton weight (g), ginning outturn (%) and 100 seed weight (g) were given in *Tables 4, 5 and 6*.

Seed Cotton Yield (kg ha^{-1})

Table 4 showed that the lowest seed cotton yield in 2011 was obtained from the control plot of P_0 x Zn_0 with as 3401 kg ha^{-1} and the highest yield was provided by the P_{120} x Zn_{15} interaction with 4595 kg ha^{-1}, while the lowest seed cotton yield in 2012 was again obtained from the control plot, of P_0 x Zn_0, with 2993 kg ha^{-1} and the highest yield was provided by the P_8xZn_1 interaction with 4189 kg ha^{-1}.

The lowest yield provided by the control plots in both years showed that phosphorus and zinc had some positive effects on yield. However, the highest yield varied in both years, and this fluctuation was based on various climatic and soil factors.

Sawan et al. (2006) found that phosphorus application at three concentrations (i.e. 600, 1200, and 1800 ppm P) significantly increased the seed cotton yield compared to control (9.49% -17.12%). Meanwhile, Singh et al. (2013) indicated that phosphate deficiency decreased plant growth and photosynthesis, thereby reduced biomass

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DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

accumulation and yield. Russell (2001) claimed that phosphorus, as part of the cell nucleus, was important for cell division and the development of meristematic tissue, and therefore had a stimulating effect to increase the number of bolls and flowers per plant.

Table 4. *Means and the groups of seed cotton yield (kg ha^{-1}), plant height (cm) and monopodial branches (per plant)*

P X Zn Interactions		Seed Cotton Yield (kg ha^{-1})		Plant Height (cm)		Monopodial Branches (per plant)	
		2011	2012	2011	2012	2011	2012
P_o	Zn_0	3401 e*	2993 h*	74.4 f*	73.8 a*	0.8 de*	0.5 a*
	Zn_5	3787 cde	3188 fgh	76.2 cdef	68.2 bcd	1.0 bcd	0.1 e
	Zn_{10}	3461 e	3340 ef	75.6 def	67.8 bcd	1.3 ab	0.3 bcd
	Zn_{15}	3434 e	3127 fgh	74.6 ef	68.2 bcd	1.3 ab	0.2 cde
P_{40}	Zn_0	4136 abcd	3213 fgh	77.4 b-f	72.7 ab	0.8 de	0.2 cde
	Zn_5	4158 abcd	3068 gh	76.9 b-f	70.2 abc	0.9 cde	0.3 bcd
	Zn_{10}	3706 de	3006 h	75.4 def	65.6 cd	1.2 ab	0.2 cde
	Zn_{15}	3888 bcde	3575 cd	78.6 abcd	65.5 cd	0.8 de	0.3 bc
P_{80}	Zn_0	4046 bcd	3259 efg	79.9 abc	64.2 d	1.1 abc	0.2 cde
	Zn_5	3978 bcd	3748bc	82.4 a	73.3 a	1.0 bcd	0.3 bc
	Zn_{10}	4182 abcd	4189 a	78.9 abcd	69.8 abc	1.4 a	0.3 bcd
	Zn_{15}	4340 ab	3904 b	78.0 b-f	70.1 abc	0.7 e	0.3 bc
P_{120}	Zn_0	4246 abc	3452 de	79.1 abcd	71.2 ab	1.1 abc	0.4 ab
	Zn_5	3736 cde	3671 cd	78.5 a-e	67.8 bcd	1.4 a	0.3 bcd
	Zn_{10}	3761 cde	3659 cd	75.5 def	70.3 abc	1.0 bcd	0.3 bc
	Zn_{15}	4595 a	3329 ef	80.3 ab	70.9 ab	1.3 ab	0.2 de
C.V. %		4.09	4.60	2.14	5.91	10.22	18.07

*Means followed by different letters within columns are significantly different ($p \leq 0.05$)

Zinc might have a positive effect on the photosynthetic activity of leaves (Welch, 1995), which enhances the mobilization of photosynthetic assimilates and directly affected the boll weight. In addition, zinc was required for the synthesis of tryptophan, a precursor of indole-3-acetic acid (Oosterhuis et al., 1991). This is the main hormone that prevents the separation of bolls and bract leaves of the plant. Thus, this increases the number of bolls in the plant, consequently, the seed cotton yield (Rathinavel et al., 2000).

Similar findings suggested that zinc and phosphorus applications increased the seed cotton yield (Sharma and Gupta, 1988; Sawan et al., 1997, 2001; Soomro et al., 2000; Elwan et al., 2002; Mamatha, 2007; Ahmad et al., 2009; Ahmed et al., 2010; Saleem et al., 2010; Emara, 2016; Sawan, 2018).

Plant Height (cm)

Table 4 showed that the lowest plant height in 2011 was obtained from the control plot of P_0 x Zn_0, with 74.4 cm and the highest plant height was provided by the P_{80} x Zn_5 interaction with 82.4 cm, while the lowest plant height in 2012 was obtained from the plot P_{80} x Zn_0 with 64.2 cm and the highest plant height was provided by the control plot of P_0 x Zn_0 and the plot of P_{80} x Zn_5 interaction with 73.8 cm and 73.3 cm, respectively.

The results revealed that phosphorus and zinc had a statistically significant effect on plant height. The P_{80} x Zn_5 interaction taking the first rank in both years indicated that this treatment increased plant height. This result is supported by the Neilsen and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Hogue's (1986) finding claiming that in phosphorous-rich soils phosphate had low solubility forms, such as the compound $Zn_3(PO_4)_3$ which prevents the transport of zinc to the above-ground organs of the plant, hence, the growth decreases due to the lesser amounts of Zn in the plant tops. Ören and Başal (2012) and Mamatha (2007) observed that zinc applications and Saleem et al. (2010) observed that P applications increased plant height compared to control.

Monopodial Branches (Per Plant)

Table 4 showed that some statistically significant differences were found in both years of experiment in terms of the number of monopodial branches. In 2011, the lowest number of monopodial branches was in P_{80} x Zn_{15} (0.7 per plant) interaction and the highest number was in P_{80} x Zn_{10} (1.4 per plant) and P_{120} x Zn_5 (1.4 per plant). In 2012, the lowest number of monopodial branches was obtained from P_0 x Zn_5 (0.1 per plant) interaction, while the highest number of monopodial branches was obtained from P_0 x Zn_0 (0.5 per plant) interaction.

In terms of the number of monopodial branches, statistically significant differences were found in both years of experiment. Nonetheless, this difference was not stable but varied depending on years. The difference between the experimental years might be due to soil properties and climatic conditions in the experimental years. Saleem et al. (2010) reported that 90 kg ha^{-1} P application decreased the number of monopodial branches compared to the control.

Sympodial Branches (Per Plant)

Statistically significant differences were in 2011, but no difference was found in 2012. The lowest number of sympodial branches in 2011 was from the P_{40} x Zn_0 (11.5 per plant), while the highest number of that was from the P_{120} x Zn_{15} (14.77 per plant) and P_0 x Zn_5 (14.4 per plant) interactions, whereas in 2012, the lowest number of sympodial branches was obtained from the interaction of P_0 x Zn_5 (7.1 per plant), while the highest number of sympodial branches was obtained from P_8 x $Zn_{0.5}$ and P_8 x Zn_1 (11.2 per plant) interactions (*Table 5*).

The results indicated that phosphorus and zinc applications were important in 2011 but they were non-significant in 2012 for sympodial branches, and this difference derived from climate and soil conditions. This showed that there was no stability derived from the effects of phosphorus and zinc on the number of sympodial branches. Ahmad et al. (2009) and Mamatha (2007) reported that zinc applications, both 34 kg ha^{-1} and 50 kg ha^{-1} increased the number of sympodial branches per plant compared to control.

Number of Bolls (Per Plant)

From *Table 5*, it is evident that the lowest number of bolls occured from P_{40} x Zn_0 (12.7 per plant) interaction and the highest number from P_{80} x Zn_{10} (17.7 per plant) interaction in 2011, while in 2012, the lowest number of bolls was obtained from P_{40} x Zn_5 (8.8 per plant) interactions and the highest number was obtained from P_{80} x Zn_5 (11.40 per plant) and P_{80} x Zn_{10} (11.0 per plant) interactions.

These results revealed that phosphorus and zinc interaction had a statistically significant effect on the number of bolls and the highest effect was obtained from the interactions of P_{80} x Zn_5 and P_{80} x Zn_{10}. Phosphorus and zinc applications can lead to an

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

increase in the number of bolls by having a positive effect on flower formation and fertilization biology. The results in the current study number of bolls was similar to that from Prasad and Prasad (1994), Sawan et al. (1997), Soomro et al. (2000), Sawan et al. (2008), and Emara (2016). Furthermore, Ahmad et al. (2009) reported that phosphorus application of 34 kg ha^{-1} increases the number of bolls and Mamatha (2007) showed that 50 kg ha^{-1} of zinc application increased the number of bolls by 18.88% compared to control.

Table 5. *Means and the groups of sympodial branches (no. plant^{-1}), number of bolls (per plant) and boll weight (g)*

P X Zn Interactions		Sympodial Branches (per plant)		Number of Bolls (per plant)		Boll Weight (g)	
		2011	2012	2011	2012	2011	2012
P_o	Zn_0	13.8 ab*	11.0 ns	15.9 abc*	9.4 de*	6.2 ns	5.9 ns
	Zn_5	14.4 a	7.1	15.2 a-d	9.1 ef	6.3	6.0
	Zn_{10}	13.7 ab	10.3	15.4 abc	9.8 cd	6.4	6.1
	Zn_{15}	13.5 ab	10.9	15.3 abc	9.8 cd	5.9	6.2
P_{40}	Zn_0	11.5 c	10.7	12.7 d	9.1 ef	6.6	6.0
	Zn_5	13.2 abc	10.4	15.1 a-d	9.7 cd	6.5	6.4
	Zn_{10}	12.3 bc	10.2	14.0 cd	8.8 f	6.1	6.1
	Zn_{15}	13.2 abc	10.5	15.2 abc	10.2 bc	6.1	6.0
P_{80}	Zn_0	13.3 abc	9.7	16.9 ab	9.7 cd	6.1	6.3
	Zn_5	13.4 ab	11.2	16.8 ab	11.4 a	6.4	6.3
	Zn_{10}	13.1 abc	11.2	17.7 a	11.0 a	6.1	6.1
	Zn_{15}	13.0 abc	9.4	16.5 abc	10.4 b	6.3	6.1
P_{120}	Zn_0	13.8 ab	10.3	15.1 a-d	9.9 bcd	6.3	6.4
	Zn_5	14.0 ab	9.3	15.6 abc	9.1 ef	6.4	5.9
	Zn_{10}	13.1 abc	9.7	14.8 bcd	9.9 cd	6.2	6.4
	Zn_{15}	14.7 a	9.8	15.1 a-d	8.7 f	6.4	6.4
C.V. %		4.41	6.63	5.38	8.88	4.29	6.86

*Means followed by different letters within columns are significantly different ($p \leq 0.05$) ns non-significant

Boll Weight (g)

In 2011 and 2012, no significant effect of phosphorus and zinc on the weight of bolls was found (*Table 5*). Boll weights ranged from 5.9 to 6.4 g in both years of experiment. Therefore, it can be concluded that phosphorus and zinc had no significant effect on the weight of bolls in the present study.

Nonetheless, these results in terms of boll weight contradict partially with that of Soomro et al. (2000), Mamatha (2007), and Emara (2016) regarding zinc applications increase the weight of bolls. This may be due to the various cultivars employed in the study and zinc and phosphorus doses used in the trials.

Boll Seed Cotton Weight (g)

In both years, no statistically significant effect of phosphorus and zinc applications on the weight of boll seed cotton was found (*Table 6*). Boll seed cotton weights varied from 4.8 g to 5.4 g. These results showed that phosphorus and zinc had no significant effect on the weight of boll seed cotton.

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Ginning Outturn (%)

From *Table 6*, no statistically significant effect of phosphorus and zinc on ginning outturn was found in 2011 and 2012. The ginning outturn in 2011 and 2012 varied from 40.8 to 42.3%. These results revealed that phosphorus and zinc had no a significant effect on ginnıng outturn.

Table 6. *Means and the groups of boll seed cotton weight (g), ginning outturn (%) and 100 seed weight (g)*

P X Zn Interactions		Boll Seed Cotton Weight (g)		Ginning Outturn (%)		100 seed weight (g)	
		2011	2012	2011	2012	2011	2012
P_o	Zn_0	4.9 ^{ns}	4.7 ^{ns}	41.7 ^{ns}	41.9 ^{ns}	9.9 b*	9.9 ^{ns}
	Zn_5	4.9	4.6	41.7	41.8	9.9 b	9.9
	Zn_{10}	5.0	4.8	41.4	41.6	9.9 b	10.3
	Zn_{15}	4.9	4.9	41.5	42.3	9.6 b	9.6
P_{40}	Zn_0	5.2	4.7	41.2	41.5	9.6 b	9.6
	Zn_5	5.2	5.4	41.2	40.8	9.9 b	9.6
	Zn_{10}	4.8	4.8	41.4	42.1	9.8 b	10.3
	Zn_{15}	4.8	4.7	41.8	42.1	9.5 b	9.8
P_{80}	Zn_0	5.0	4.6	42.0	42.3	9.4 b	9.8
	Zn_5	5.0	5.0	42.2	41.9	9.9 b	9.8
	Zn_{10}	4.9	4.8	41.1	41.0	10.0 b	9.7
	Zn_{15}	5.0	4.8	41.4	41.0	9.8 b	10.0
P_{120}	Zn_0	5.0	5.1	41.9	41.7	10.1 ab	10.0
	Zn_5	5.1	4.6	41.3	42.2	9.7 b	9.5
	Zn_{10}	5.0	5.1	41.1	42.4	9.9 b	9.5
	Zn_{15}	5.0	5.1	42.0	41.7	10.7 a	10.6
CV %		3.37	8.41	1.62	2.16	2.11	6.57

*Means followed by different letters within columns are significantly different ($p \leq 0.05$) ns non-significant

Similar results were reported by Emara (2016) that zinc had no significant effect on ginning outturn. However, Mamatha (2007) observed that zinc application increased the ginning outturn while Ahmad et al. (2009) reported that phosphorus had little effect on ginning outturn. This might be due to the differences in the plant material and the doses of zinc and phosphorus used in the experiments. In addition, climatic conditions might affect the formation and development of fibers and might influence the formation of various Zinc and P ginning outturns.

100 Seed Weight (g)

It was observed in *Table 6* that there were differences among factors affecting 100 seed weight only in 2011 but not in 2012. In 2011, the lowest 100 seed weight was obtained from the interaction of P_{80} x Zn_0 (9.4 g) and the highest 100 seed weight from the interaction of P_{120} x Zn_{15} (10.7 g). In 2012, the 100 seed weight varied from 9.5 g to 10.6 g. The presence of the difference between the years might be due to climate and soil factors. Sawan et al. (2001) and Emara (2016) reported that Zn applications increased 100 seed weight. In this case, the increase in 100 seed weight might be due to increasing photosynthesis activity by Zn application (Welch, 1995).

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DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

Conclusion

Mineral nutrients are one of the most important factor affecting plant growth by contributing the formation of dry matter by affecting the biochemical processes occurring in plants. It was found that phosphorus and zinc management had a significant effect on seed cotton yield, plant height, number of bolls, and number of monopodial branches in both years of the experiment. Both plant nutrients had significant effect on the number of sympodial branches and 100 seed weight only in the first year but not in the second year. These inputs had no significant effect on boll weight, boll seed cotton weight, and ginning outturn in both years.

It was concluded that, depending on the distribution of plant nutrients in the soil, 80-100 kg of phosphorus and 10-15 kg of Zinc applications per hectare can increase in seed cotton yield. Validity of these results must be verified via multi location tests in future studies.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26652676

POSSIBILITIES OF USING FODDER GALEGA IN THE ENERGY SECTOR AND AGRICULTURE

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(Received 22nd Nov 2018; accepted 25th Jan 2019)

Abstract. This paper presents changes in basic energy parameters of fodder galega (*Galega orientalis* Lam.) cultivated for five years as a potential energy crop under the effect of mineral fertilization. Additionally, the chemical composition of ash obtained after burning the fodder galega biomass was examined for its possible use as a liming agent. The field experiment was conducted between 2005 and 2009 in the experimental plots of the Soil Science and Plant Nutrition Department at the Siedlce University of Natural Sciences and Humanities, Poland (52°17′N and 22°28′E). Five levels of fertilization were included in the study: without fertilization, NP, NK, PK, NPKCa (0 – control, N–20, P–50, K–150, Ca–150 $kg \cdot ha^{-1}$). Significantly highest values of heat of combustion (18.95 $MJ \cdot kg^{-1}$) and calorific value were found for galega fertilized with nitrogen (20 $kg \cdot ha^{-1}$) and potassium (150 $kg \cdot ha^{-1}$). The highest dry matter yields and the highest yield energy value, as well as the most amount of ash obtained from biomass combusting were found for galega harvested from objects fertilized with NPKCa (20; 50; 150; 150 $kg \cdot ha^{-1}$). The content of macroelements in galega ash followed a decreasing sequence of: Ca > K > P >Mg > S > Na.

Keywords: *Galega orientalis Lam., heat of combustion, calorific value, ash, macroelements*

Introduction

In recent years, a great interest in new species of energy crops has been observed all over Europe (An et al., 2018; Drazic et al., 2017; Haines et al., 2015; Jureková et al., 2015; Kalembasa and Symanowicz, 2003; Maw et al., 2017; Tumminello et al., 2018; Wang et al., 2018).

According to the EU Climate and Energy Package of 17.12.2008, by 2020, Member States should reach the level where 20-30% of all energy obtained is from renewable energy sources (Zegada-Lizarazu et al., 2010). The research conducted in central-eastern Poland, concerning adaptation of fodder galega (*Galega orientalis* Lam.), a plant originating from the Caucasus, demonstrated the multi-directionality of its cultivation. It can be cultivated for animal fodder in the form of green forage, silage, dried feed, hay or protein concentrate. This is a perennial, leguminous plant (cultivation for even 16-18 years), rich in macro- and microelements. Biomass of the species can also be co-fired with coal (Brodowska et al., 2018).

The research conducted in the Podlasie region (Kalembasa and Symanowicz, 2003), with the application of ^{15}N isotope, proved the high possibilities demonstrated by fodder galega to biologically reduce atmospheric nitrogen (380 $kg \cdot ha^{-1}$). Preliminary research

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

also showed the possibilities of cultivating fodder galega as an energy crop. Therefore, the introduction of an additional plant, apart from *Miscanthus sacchariflorus, Miscanthus x giganteus* (Jeżowski et al., 2011; Maksimović et al., 2016); *Sweet Sorghum, Maize, HBS* (Maw et al., 2017); *Sorghum hybrids* (Pannacii and Bartolini, 2016); *Salix spp*., (Stolarski, 2008) and *Sida hermaphrodita* (Krzyżaniak et al., 2015) is recommended and related to the possibility of increasing biomass production, with a view to binding atmospheric N_2. An additional benefit is the possibility of using ash from biomass combustion as a liming agent in agriculture. To evaluate the usefulness of biomass for energy purposes, the following values must be examined: moisture, ash content, sulphur content, heat of combustion and calorific value coal (Brodowska et al., 2018). The calorific value of biofuels is 5-8 $MJ \cdot kg^{-1}$ for biomass of 50-60% moisture content, 15-17 $MJ \cdot kg^{-1}$ for pre-dried biomass (15-20% moisture content) and about 20 $MJ \cdot kg^{-1}$ for completely dried biomass.

The potential of biomass production is enormous and is of importance for bioenergy in the future. Eastern galega provide a relatively anknown input feedstock for conversion of biomass into biofuel especially when grown on marginal lands. Field studies carried out 10 years ago are still valid. The search for new potential energy crops, especially perennial with low crop costs, continues (Foster et al., 2017).

The aim of this paper was to determine the optimum conditions for cultivating fodder galega in terms of usefulness for energy purposes and the fertilizing value of ash obtained after biomass combustion.

Material and methods

In the years between 2005 and 2009 the field experiment was set up with a completely randomized method in three replications, on the experimental plots of the Soil Science and Plant Nutrition Department at the Siedlce University of Natural Sciences and Humanities, Poland - 52°17′N, 22°28′E (*Figure 1*).

Figure 1. *Location of the study area [https://contour maps.com.pl] in the own modification*

Five levels of fertilization were included in the study: without fertilization, NP, NK, PK, NPKCa. The field experiment was conducted on loamy sand soil containing 31.5 $g \cdot kg^{-1}$ of carbon compounds, 1.66 $g \cdot kg^{-1}$ of total nitrogen, and pH in KCl $mol \cdot dm^{-3}$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

– 6.6. The content of available forms of phosphorus and potassium in soil determined with the Egner-Riehm’s method was high (80 mg·kg^{-1} P and 140 mg·kg^{-1} K) and the content of magnesium determined with the Schachtschabel’s method was average (50 mg·kg^{-1} Mg). According to the IUSS World Reference of Soil resources (2014), the soil on which the field experiment was carried out was classified as *Hortic Anthrosol*. The fodder galega seeds was sown in the amount of 24 kg·ha^{-1} (300-500 plants per 1 m^2). The germination was 45-60% and the 2.4 grams was TGW. Mechanically scarified seeds were sown into soil inoculated with *Rhizobium galegae* bacteria. In the subsequent years, the following fertilization scheme was implemented: N – 20 kg·ha^{-1} as 34% ammomium nitrate applied in early spring; P – 50 kg·ha^{-1} as triple superphosphate applied in autumn; K – 150 kg·ha^{-1} as 60% potassium salt at two doses: dose I (100 kg·ha^{-1} K) applied in early spring, dose II (50 kg·ha^{-1} K) applied after the first swath; Ca – 150 kg·ha^{-1} as dolomite calcium applied in autumn. During vegetation, agricultural procedures such as weeding were performed. The three swaths of fodder galega were harvested at budding in each,subsequent crop year (*Figure 2*).

***Figure 2.** Fodder galega (Galega orientalis Lam.) – budding phase*

In the samples marked plant analytical moisture, ash and combustion heat in accordance with Polish Norm PN-81/G-04513. The calorific value (according to Szyszlak-Bargłowicz and Piekarski, 2009) and energy value were calculated.

$$H_c = \frac{h_{cc}(t_h - t_l) - h_{rw} \cdot w_{rw}}{m_p} \quad \text{(Eq.1)}$$

H_c – heat of combustion value (MJ·kg^{-1});
h_{cc} – heat capacity of the calorimeter;
t_h – highest temperature of the main measurement period;
t_l – lowest temperature of the main measurement period;
h_{rw} – heat of burning the resistance wire;
w_{rw} – weight of the resistance wire;
m_p – mass of the plant sample.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

$$C_v = H_c - h_{vw} \cdot (m_a + f_{h/w} \cdot h_c) \quad \text{(Eq.2)}$$

C_v – caloric value ($MJ \cdot kg^{-1}$);
H_{vw} – heat of vaporization of water at 25°C;
m_a – analytical moisture;
$f_{h/w}$ – conversion factor of hydrogen to water (8.94);
h_c – hydrogen content in the analytical sample.

$$E_v = Y_b \cdot C_v \quad \text{(Eq.3)}$$

E_v – energy value ($MJ \cdot ha^{-1}$);
Y_b – yield of biomass;
C_v – caloric value.

The content of total macronutrients was determined with the ICP-AES method (Spectrometer Optima 3200RL, Perkin Elmer, Waltham, USA) in plant material after drying, comminution and dry mineralization (550°C).

The results obtained were subjected to statistical analysis using the analysis of variance ANOVA (Statistica 12 PL, Statsoft, Inc., 2018). The least significant difference (LSD) determined by the Tukey's test. The criterion for significance was set at $P \leq 0.05$. The linear regression equations and correlation coefficients between selected features were determined (Statistica 12 PL, Statsoft Inc., 2018).

The atmospheric conditions in the year 2005-2009 exerted a large impact on the content of total NPKCa in the sub-surface horizon (Ap) of the soil profile and on its uptake by the test plant (*Table 1*). Excessively high temperatures limited the uptake of macroelements whereas a large volume of precipitation caused extensive washing-out of these elements deep into the soil profile.

Table 1. *Meteorological conditions during the studies in 2005-2009 years. Reported by the measurement centre in Siedlce*

Years	Months												Mean/ Sum
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	
Means monthly air temperature (°C)													
2005	0.3	-4.0	-0.7	8.6	13.0	15.9	20.2	17.5	15.0	8.5	2.7	-0.9	8.0
2006	-7.7	-2.4	1.1	8.4	13.6	17.2	22.3	18.0	15.4	9.9	5.0	3.2	8.7
2007	-4.5	-0.7	5.8	8.3	14.5	18.2	18.5	18.6	13.1	9.3	5.1	1.2	8.6
2008	0.6	-0.7	4.6	9.1	12.7	17.4	18.4	18.5	12.2	8.5	3.4	0.1	8.7
2009	-4.5	-2.5	3.5	10.0	12.8	15.8	19.3	17.3	14.3	6.5	4.7	3.7	8.4
Multiyear average 1987-2000	-9.8	-9.9	3.8	7.8	12.5	17.2	19.2	18.5	13.1	7.2	3.6	-2.8	6.7
Total monthly rainfalls (mm)													
2005	13.2	13.2	11.7	12.3	64.7	44.4	86.5	45.4	15.8	0.0	13.8	32.9	353.9
2006	39.0	30.0	34.0	29.8	39.6	24.0	16.2	227.6	22.0	15.1	10.5	20.8	862.5
2007	28.6	34.1	29.6	21.2	59.1	59.9	70.2	31.1	67.6	12.8	7.5	15.4	437.1
2008	51.4	0.7	53.1	28.2	85.6	49.0	69.8	75.4	63.4	18.6	5.3	30.4	530.9
2009	10.9	29.0	33.5	1.8	19.9	54.5	18.8	31.9	4.5	17.5	12.2	16.5	251.0
Multiyear average 1987-2000	45.7	32.4	29.5	38.6	44.1	52.4	49.8	43.0	47.3	36.1	27.9	41.3	488.1

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DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

Results and discussion

Changes in the basic energy parameters of fodder galega (a potential energy crop) are presented in *Table 2*. The determined analytical moisture of fodder galega harvested from fertilized objects ranged from 6.33 to 6.49%. Fertilization applied and years of research did not significantly differentiate this parameter.

Mineral fertilization and years of research significantly differentiated ash content in the fodder galega biomass. The significantly highest amount of ash in biomass was found for the galega harvested from the control object (without fertilization) as compared to the fertilized objects under analysis. The significantly highest ash content was found for the biomass of fodder galega harvested in the fourth year of the research. The energy value of 1 kg of dry matter of entire fodder galega plants, obtained according to PN-81/G-04513, designed to determine heat of combustion in the analytical state, ranged from 16.88 to 18.95 $MJ·kg^{-1}$. The significantly highest values of heat of combustion were determined for fodder galega fertilized with nitrogen (20 $kg·ha^{-1}$) and potassium (150 $kg·ha^{-1}$). Similar values of heat of combustion (17.8-18.2 $MJ·kg^{-1}$) were determined in studies on *Miscanthus* fertilized with sludge in the doses of 0, 10, 20, 40, 60 $Mg·ha^{-1}$ dry matter (Kołodziej et al., 2016). In studies on sorghum hybrids fertilized 75 kg N and 75-80 kg K_2O the average values of HHV elevate 17.7-18.4 $MJ·kg^{-1}$d.m. (Pannacci and Bartolini, 2016). According to Pilon and Lavoie (2013) switchgrass calorific value was on the level 18.0-19.5 $MJ·kg^{-1}$. The amount of energy (higher and lower heating value) as well as the thermophysical and chemical composition of biomass (carbon, sulphur, hydrogen, nitrogen as well as ash and moisture content) are very important characteristics of biomass used for energy purpose and production of biomaterials. For example, ash formed in the process of biomass combustion (responsible for particulate emission and corrosion), affect the operation of boilers, installation safety and its later use or utilization. The composition of feedstock also influences the quality of bio-products obtained by thermochemical conversions (Wilson et al., 2013).

Table 2. *Energy parameters of the fodder galega (Galega orientalis Lam.)*

Treatment	Analytical moisture (%)	Ash (%)	Heat of combustion value ($MJ·kg^{-1}$)	Calorific value ($MJ·kg^{-1}$)	Yield of dry weight of biomass ($Mg·ha^{-1}$)	Energy yields value ($MJ·ha^{-1}$)
0[1]	6.33	5.82^b	18.03^b	17.88^b	7.81^a	139.64^a
NP	6.47	4.57^a	18.15^b	17.99^b	12.78^b	229.91^b
NK	6.36	4.40^a	18.95^c	18.79^c	14.10^b	264.94^c
PK	6.49	4.94^a	16.88^a	16.72^a	13.56^b	226.72^b
NPKCa	6.40	4.92^a	18.80^c	18.63^c	16.25^c	302.74^d
P	ns	*	*	*	*	*
2005[2]	6.03	5.06^a	18.13^b	17.98^b	7.19^a	129.28^a
2006	6.24	4.89^a	18.26^b	18.11^b	12.22^c	221.30^c
2007	6.28	4.63^a	17.56^a	17.40^a	11.40^b	198.36^b
2008	6.43	5.36^b	18.62^b	18.46^b	16.92^d	312.34^d
2009	6.39	4.91^a	18.93^b	18.77^b	16.78^d	314.96^d
P	ns		*	*	*	*
Fertilization/Years of research	ns	*	*	*	*	*

[1]0 – control, N–20, P–50, K–150, Ca–150 $kg·ha^{-1}$; [2]1, 2, 3, 4, 5 – years of research; *significant at the $P \leq 0.05$; ns – non significant; values with the same letters are not different at the significance level of $P \leq 0.05$

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DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

It should be assumed that for cultivation of leguminous plants intended for energy purposes, harvested in the budding phase, intensive fertilization with nitrogen and phosphorus is not necessary. Leguminous plants are able to biologically reduce atmospheric N_2 as a result of their symbiosis with bacteria of the *Rhizobium* genus. The highest values of combustion heat were obtained for fodder galega cultivated in the fifth year of research. The calorific value presented in *Table 2*, calculated on the basis of formulas (Szyszlak-Bargłowicz and Piekarski, 2009), was about 0.89% smaller than the heat of combustion determined. To obtain maximum total dry matter yield (3 crops during the vegetation period) of fodder galega (16.25 $Mg \cdot ha^{-1}$), it is necessary to supply soil with NPKCa. In the studies with *Miscanthus*, lower dry matter yields were obtained in the first year of research (Kohle et al., 2001; Jeżowski, 2008). The average dry weight of biomass sorghum was on the level 13.5-21.7 $t \cdot ha^{-1}$ after 141 days after emergence and 22.0-27.3 $t \cdot ha^{-1}$ after 127 days after emergence (Pannacci and Bartolini, 2016). In our own research conducted, the optimum balanced dose of mineral fertilizers was as follows: N-20, P-50, K-150, Ca-150 $kg \cdot ha^{-1}$. In subsequent years of cultivation, the dry matter yield of fodder galega significantly increased. It was related to a strongly developed root system, an increased process of biological reduction of molecular nitrogen due to soil infection with *Rhizobium galegae* bacteria and supplying basic nutrients to soil. Comparing the yield of fodder galega with its energy value in subsequent years demonstrates that the dry matter yield contained significant amounts of energy. The amount of energy obtained from 1 ha was directly proportional to the volume of the yield obtained. Total amount of energy obtained after five years of the experiment amounted to 1176.24 ($MJ \cdot ha^{-1}$). The energy value of the yield obtained under the effect of fertilization with NPKCa was over twice as high as the values obtained for the yield of fodder galega obtained in objects without fertilization. The energy value of the fodder galega yield under the effect of fertilization with NPKCa was 33% higher than the value obtained for *Miscanthus* fertilized with 20 $Mg \cdot ha^{-1}$ of dry matter sludge (Kołodziej et al., 2016). Based on the research conducted, significant relations were found between the calorific value (C_v) and heat of combustion (H_c) and between energy value (E_v) and dry matter yield of fodder galega (Y_b) for the fertilization applied (*Figure 3a and 3b*).

A statistical analysis also demonstrated a significant relation between dry matter yield (Y_b) of fodder galega and analytical moisture (M_a) for the years of research. The mean energy value (E_v) calculated for individual years of research was also correlated with analytical moisture (M_a). A positive correlation between heat of combustion (H_c) and calorific value (C_v) was demonstrated for the years of research and energy value (E_v) and dry matter yield of fodder galega (Y_b)obtained in subsequent years of research. The obtained results of our own research were at a similar level in comparison to the previous research carried out in the soil abundant in available nutrients (Kalembasa and Symanowicz, 2003).

The average content of macroelements in ash obtained after combusting fodder galega biomass followed a sequence of decreasing values: Ca > K > P > Mg > S >Na (*Table 3*). The fertilization applied and years of research significantly differentiated the content of phosphorus, potassium, calcium and sulphur in ash. Higher total amounts of macroelements were determined in ash obtained from fodder galega fertilized with NK and NPKCa and in the fourth year of research.

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DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

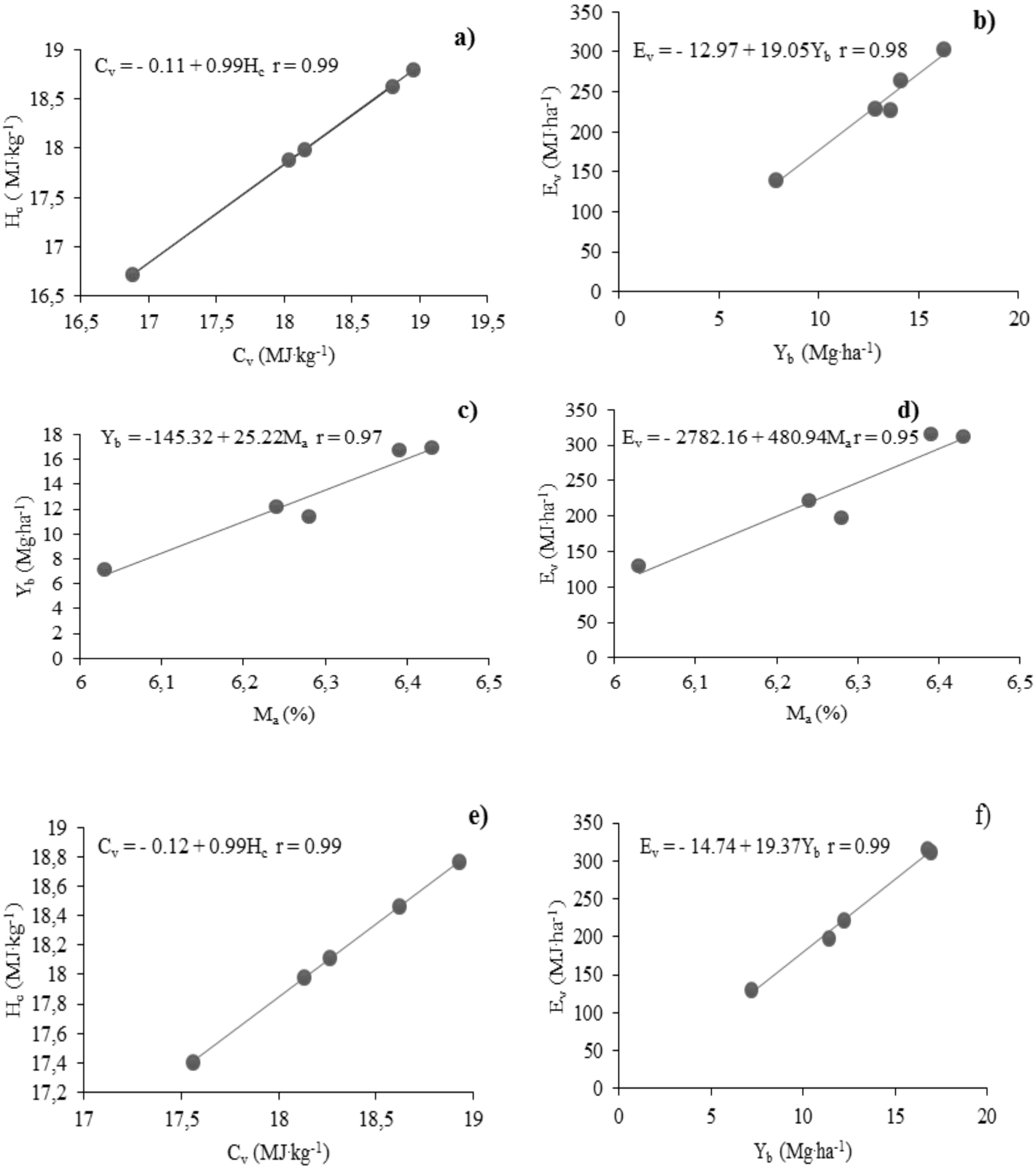

Figure 3. *The relations between heat of combustion (H_c), calorific value C_v), energy value (E_v), dry matter yield of fodder galega (Y_b), analytical moisture (M_a) for the fertilization applied – Fig.1a,b and for the subsequent years of research – Fig. 1c,d,e,f*

The amount of ash obtained after the combustion of the total yield of fodder galega biomass, and the amount of phosphorus, potassium, calcium, magnesium and sulphur was significantly differentiated for fertilizing objects and years of research (*Table 4*). The significantly highest amounts of the analysed parameters (ash, phosphorus, potassium, calcium, magnesium, sulphur and sodium) were obtained for fodder galega fertilized with NPKCa. Similar values of ash were obtained in the research with sugar miscanthus, while the content of macroelements determined in ash obtained after combusting sugar miscanthus was at a low level (Borkowska and Lipiński, 2007). In examining subsequent years of research, the highest percentage share of calcium, potassium, phosphorus and magnesium was found in the fourth year of research, and the highest share of sulphur and sodium was found in the fifth year.

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DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

Table 3. *The chemical composition of ash obtained from fodder galega (g·kg^{-1})*

Treatment	P	K	Ca	Mg	S	Na
0[1]	2.98[b]	13.47[b]	14.04[a]	2.56	2.36[b]	0.47
NP	2.60[a]	11.38[a]	17.89[c]	2.74	1.76[a]	0.41
NK	2.49[a]	13.06[b]	18.25[c]	2.56	1.53[a]	0.48
PK	2.41[a]	15.63[c]	16.60[b]	2.35	1.48[a]	0.48
NPKCa	2.79[a]	15.99[c]	17.78[c]	2.30	1.55[a]	0.43
P	*	*	*	ns	*	ns
2005[2]	2.64	17.09[d]	16.33[a]	2.42	2.08[b]	0.39
2006	2.53	12.22[b]	16.34[a]	2.15	1.49[a]	0.48
2007	2.73	13.97[b]	15.91[a]	2.57	1.55[a]	0.61
2008	2.96	15.40[c]	18.13[b]	2.96	1.73[a]	0.48
2009	2.41	10.85[a]	17.86[b]	2.41	1.83[a]	0.51
P	ns	*	*	ns	*	ns
Fertilization/Years of research	ns	*	*	ns	*	ns

[1]0 – control, N–20, P–50, K–150, Ca–150 kg·ha^{-1}; [2]1, 2, 3, 4, 5 – years of research; *significant at the $P \leq 0.05$; ns – non significant; values with the same letters are not different at the significance level of $P \leq 0.05$

Table 4. *The amount of ash and macronutrients obtained after burning the total biomass yield of the fodder galega per hectare (in kg)*

Treatment	Ash from biomass	P	K	Ca	Mg	S	Na
0[1]	454.5[a]	23.27[a]	105.2[a]	109.6[a]	20.00[a]	18.43[a]	3.67
NP	584.0[b]	33.23[b]	145.4[b]	228.6[b]	35.02[b]	22.49[b]	5.24
NK	620.4[c]	35.11[b]	184.1[c]	257.3[c]	36.10[b]	21.57[b]	6.77
PK	669.9[d]	32.68[b]	211.9[d]	225.1[b]	31.87[b]	20.07[b]	6.51
NPKCa	799.5[e]	45.34[c]	259.8[e]	288.9[d]	37.37[b]	25.19[b]	6.99
P	*	*	*	*	*	*	ns
2005[2]	363.8[a]	18.98[a]	122.9[a]	117.4[a]	17.40[a]	14.95[a]	1.37[a]
2006	597.5[c]	30.92[b]	149.3[b]	199.7[c]	26.27[b]	18.21[b]	5.86[b]
2007	527.8[b]	31.12[b]	159.2[c]	177.9[b]	29.30[b]	17.67[b]	6.95[b]
2008	906.9[e]	50.08[d]	260.6[e]	306.7[e]	50.08[d]	29.27[c]	8.12[b]
2009	823.9[d]	40.44[c]	182.1[d]	299.7[d]	40.44[c]	30.71[c]	8.56[b]
P	*	*	*	*	*	*	*
Fertilization/Years of research	*	*	*	*	*	*	ns

[1]0 – control, N–20, P–50, K–150, Ca–150 kg·ha^{-1}; [2]1, 2, 3, 4, 5 – years of research; *significant at the $P \leq 0.05$; ns – non significant; values with the same letters are not different at the significance level of $P \leq 0.05$

The results obtained were lower than those obtained in the research with other energy crops - miscanthus sinensis, virginia mallow, basket willow (Stolarski, 2008; Stypczyńska et al., 2017). The harvesting date of energy crops significantly affects the content of macroelements (in particular, of potassium) in biomass and ash. The diversified content of macroelements in energy crops is shown in studies, in which 5 times less sulphur was determined in the biomass of basket willows in comparison to our own research. The ash obtained from the combustion of fodder galega biomass, containing large amounts of alkaline elements, can be used for deacidification of

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DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

medium and heavy soils (Regulation Commission UE, 2013). The possibility of using ashes to increase soil alkalinity, to improve the properties of heavy soils and to increase the content of macroelement in soils was indicated in the research by other authors (Kowalczyk, 2017; Stypczyńska et al., 2017; Symanowicz et al., 2018).

Conclusions

A strong interest in alternative energy sources indicates the possibility of using fodder galega biomass as a source of renewable energy which does not produce such high SO_2 and NO_x emissions, as is the case while combusting hard coal.

The calculated calorific value of 1 kg of fodder galega dry matter, ranging from 16.72 to 18.79 MJ, is comparable to the data concerning of sweet sorghum, switch grass, *Miscanthus,* straw, bark and lignite.

Additionally, the waste by-product created in the process (ash) can be used for agricultural purposes as a liming agent of balanced chemical composition.

The highest dry matter yields and the highest yield energy value, as well as the most amount of ash obtained from biomass combusting were found for galega harvested from objects fertilized with NPKCa (20; 50; 150; 150 $kg \cdot ha^{-1}$).

Acknowledgements. The results of the research carried out under the research theme No 315/12/S were financed from the science grant granted by the Ministry of Science and Higher Education.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_26772687

GENOME SEQUENCE OF *BACILLUS VELEZENSIS* W1, A STRAIN WITH STRONG ACARICIDAL ACTIVITY AGAINST TWO-SPOTTED SPIDER MITE (*TETRANYCHUS URTICAE)*

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(Received 26th Nov 2018; accepted 28th Jan 2019)

Abstract. *Bacillus velezensis* W1, isolated from two-spotted spider mites that had died naturally, is a patented strain with strong capability to cause mortality of the phytophagous mite *Tetranychus urticae*. The whole genome of W1 was completely sequenced with a combination of an Illumina Miseq platform (400-bp paired-end) with 2 × 250 bases and a Pacific Biosciences (PaBio) RS II Single Molecule Real Time (SMRT) sequencing platform using a 20 kb SMRTbell™ template library. Here, we report the complete genome sequence of *B. velezensis* W1, including one circular chromosome of 4,237,431 bp encoding 4,352 genes with GC content of 45.84%, providing insights into the genomic basis of its acaricidal activity and facilitating its application in red spider mite biocontrol.
Keywords: *Bacillus, two-spotted spider mite, biocontrol, whole genome, Acaricides*

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, is one of the most polyphagous arthropod herbivores and feeds on more than 1,100 plant species belonging to more than 140 different plant families including species known to produce toxic compounds. It is a major pest in field crops, destroying both annual and perennial crops (Grbic et al., 2011). The use of chemical insecticides and acaricides are currently the primary control method of *T. urticae* (Jafari et al., 2016). The frequent application of these chemicals, paired with several biological aspects of this species, such as its short life cycle, high fecundity and arrhenotokous reproduction, has led to the mites developing resistance to the most pesticide groups (Minazzi et al., 2016). Additionally, chemical pesticides can have unintended consequences, impacting environmental quality, food safety, human health, and biodiversity (Yang et al., 2016).

Recently, biological control agents in the form of parasites, predators and pathogens have gained a lot of attention. Biological control of spider mites using predators or parasites is widely practiced, particularly the use of phytoseiid mites, which are currently sold worldwide (Funayama et al., 2015). Entomopathogenic fungi or entomogenous fungi such as *Hirsutella thompsonii* Fisher (El-Sharabasy, 2015), *Neozygites floridana* Weiser and Muma (Klingen et al., 2008), *Beauveria bassiana*

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

(Balsamo) Vuillemin (Ullah and Lim, 2015), *Verticillium lecanii* (Zimm.) (Seiedy, 2015), and *Isaria cateniannulata* (Zhang et al., 2016) are currently the most studied biological control agents of spider mites. Few bacteria, however, have been reported as biological control agents of spider mites owing to the piercing-sucking mouthparts of the mites, which make it difficult for bacteria to infect them. In recent years, researchers have concentrated on intracellular organisms such as *Wolbachia* that may cause distorted sex ratio in the mite offspring, thereby impacting population (Chen et al., 2016), and toxin-producing bacteria such as *Bacillus thuringiensis* that can produce crystal proteins called δ-endotoxins that are commonly used as a biological acaricides (Neethu et al., 2016). Additional research has been carried out on potential acaricide producing strains such as *Pseudomonas putida* (Aksoy et al., 2008).

Bacillus velezensis W1 (W1 hereafter), isolated from two-spotted spider mite that had died naturally, is a patented strain (Patent no; ZL201610096541.8) that has a strong capability to cause mortality of the phytophagous mite *T. urticae* (Li et al., 2018). This strain has the potential to be a safe and eco-friendly acaricide. To further explore its biocontrol ability and to reveal its acaricidal activity mechanism, we carried out the complete genome sequencing and analysis of W1.

Materials and methods

Bacterial strain and culture condition

B. velezensis W1 is a Gram-positive rod shaped bacterium averaging 2.5 µm in length and 1 µm in width (*Fig. 1a*). W1 spores are centrally located and average 1.3 µm in length (*Fig. 1b*). The bacteria could grow rapidly in Luria Bertani (LB) liquid medium reaching the stationary phase after 12 h at 35 °C. By comparison, the growth rate in LB solid medium was much slower with the stationary phase attained at 24 h. Optimum growth occur at a temperature 35 °C and pH 8.0 (*Table 1*). The colony morphology of strain W1 grown in solid LB medium is circular convex with undulate beige-opaque margins (*Fig. 1c*).

Phylogenetic analysis

Phylogenetic tree of the *B. velezensis* W1 and other related taxa was constructed with MEGA 7.0 (Kumar et al., 2016) using Neighbor-Joining method (Saitou and Nei, 1987) based on *gyrB* gene, and the distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), with 1000 replications in the bootstrap test. Bootstrap confidence levels > 30% are indicated at the internodes. The scale bar indicates nucleotide substitutions per nucleotide position.

Genome sequencing information

Genome project history

Strain W1 was selected for sequencing due to its ability to cause mortality of the phytophagous mite, *Tetranychus urticae*. The whole genome was deposited in GenBank under the accession number CP028375. Genome sequencing and assembly was performed at the Wuhan Genoseq Technology Co., Ltd, Wuhan, China. The summary of the project information is shown in *Table 2*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

***Table 1.** Classification and general features of Bacillus velezensis W1 (Field et al., 2008)*

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS (Woese et al., 1990)
		Phylum *Firmicutes*	TAS (Gibbonst and Murray, 1978)
		Class *Baclli*	TAS (Oren and Garrity, 2016)
		Order *Bacillales*	TAS (Skerman et al., 1980)
		Family *Bacillaceae*	TAS (Fischer, 1895)
		Genus *Bacillus*	TAS (Cohn, 1872)
		Species *Bacillus velezensis*	TAS (Dunlap et al., 2016)
		(Type) strain: *W1 (CP028375)*	
	Gram stain	Positive	IDA
	Cell shape	Rod	IDA
	Motility	Motile	IDA
	Sporulation	not reported	
	Temperature range	20-40 °C	IDA
	Optimum temperature	35 °C	IDA
	pH range; Optimum	5–9; 8	IDA
	Carbon source	Heterotrophic	IDA
MIGS-6	Habitat	Plant, spider mite	IDA
MIGS-6.3	Salinity	14% (w/v) NaCl	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
MIGS-15	Biotic relationship	free-living	IDA
MIGS-14	Pathogenicity	Not reported	
MIGS-4	Geographic location	China	NAS
MIGS-5	Sample collection	2015	NAS
MIGS-4.1	Latitude	25° 01' N	NAS
MIGS-4.2	Longitude	102° 19' E	NAS
MIGS-4.4	Altitude	1835	NAS

[a]Evidence codes – MIGS: The minimum information about genome sequence; IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project (Ashburner et al., 2000)

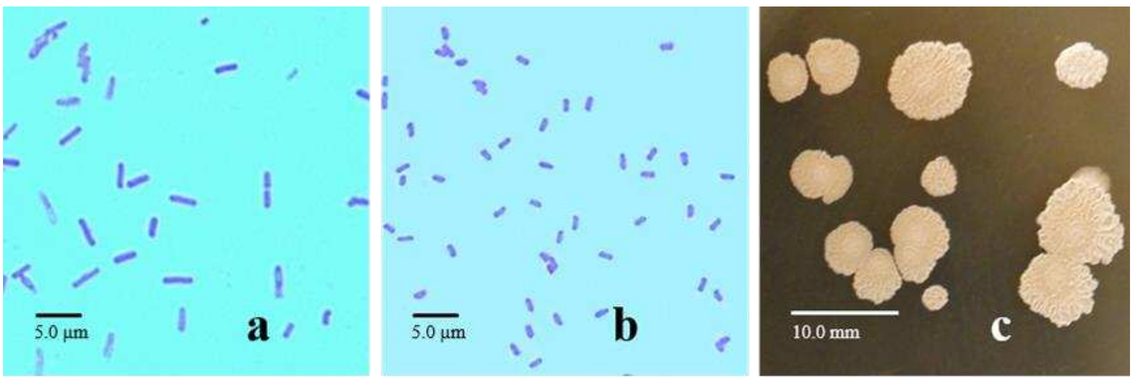

***Figure 1.** Cellular and colony morphology of Bacillus velezensis W1. **a** Vegetative W1 cells grown 72 h, Gram stained, and then at 100x magnification captured with light microscope. **b** W1 spores at 100x magnification captured light microscope. **c** Pure culture of W1 grown on nutrient agar plate*

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

Table 2. *Genome sequencing project information for Bacillus velezensis W1*

MIGS ID	Property	Term
MIGS 31	Finishing quality	Finished
MIGS 28	Libraries used	a 20 kb SMRTbell^{TM} template library
MIGS 29	Sequencing platforms	Illumina MiSeq + PacBio
MIGS 31.2	Fold coverage	305x
MIGS 30	Assemblers	A5-miseq version 20150522
MIGS 32	Gene calling method	NCBI Prokaryotic Genome Annotation Pipeline
	Locus Tag	/
	Genbank ID	CP028375
	Genbank Date of Release	June 28th, 2018
	GOLD ID	/
	BIOPROJECT	PRJNA445958
MIGS 13	Source Material Identifier	*Bacillus velezensis* W1
	Project relevance	Biocontrol, Agriculture

Growth conditions and genomic DNA preparation

The genomic DNA of W1 was extracted using a QIAamp DNA mini kit (Qiagen, USA), according to the manufacturer's protocols.

Genome sequencing and assembly

The whole genome of W1 was completely sequenced with a combination of an Illumina Miseq platform (400-bp paired-end) with 2 × 250 bases and a Pacific Biosciences (PaBio) RS II Single Molecule Real Time (SMRT) sequencing platform using a 20 kb SMRTbell^{TM} template library. Approximately 1,385.26 Mb with 4,661,344 reads were generated from the Illumina Miseq and PacBio sequencings, respectively. The quality of these reads was assessed by the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and low quality reads were filtered by Quake (Kelley et al., 2010) and AdapterRemoval (version 2.1.7) (Lindgreen, 2012). The clean reads were *de novo* assembled into a single contig with a genome coverage of 305 folds using A5-miseq version 20150522 (Coil et al., 2015), Canu v1.4 (Koren et al., 2017) and pilon v1.18 (Walker et al., 2014). Glimmer 3.02 (Delcher et al., 1999) was used to predict the open reading frames (ORFs).

Genome annotation

Software packages tRNAscan-SE v. 1.3.1 (Lowe and Eddy, 1997) and RNAmmer v. 1.2 (Lagesen et al., 2007) were used to predict tRNA and rRNA, respectively. The gene function annotations were based on BlastP similarity searches (E-Value < 10^{-6}) against 5 databases: evolutionary genealogy of genes: Non-supervised Orthologous Groups (http://eggnogdb.embl.de/), Kncyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), Non-Redundant GenBank Protein Database databases (www.ncbi.nlm.nih.gov/protein), Swiss-Prot (http://www.uniprot.org/), Gene Ontology Database (http://www.geneontology.org/). Circular genome map was created by cgview (Stothard and Wishart, 2005) with COG function annotation.

Results and discussion

Sequence analysis using gyrB

The phylogenetic analysis based on *gyrB* gene sequences using MEGA 7.0 [14] showed that *B. velezensis* W1 is evolutionarily positioned between *B. velezensis* and *B. amyloliquefaciens* (*Fig. 2*). In recent studies of genome sequencing and comparative genomics of *B. velezensis* NRRL B-41580, *B. methylotrophicus* KACC 13015 and *B. amyloliquefaciens* subsp. *plantarum* FZB42, it was established that these last two strains are heterotypic synonyms of *B. velezensis* (Dunlap et al., 2016), and based on our results, the classification of strain W1 was confirmed as a member of the species *B. velezensis*. The complete genome sequence of strain *B. velezensis* W1 is deposited at GenBank under accession number CP028375. The strain is available from China General Microbiological Culture Collection Center (CGMCC No. 11949).

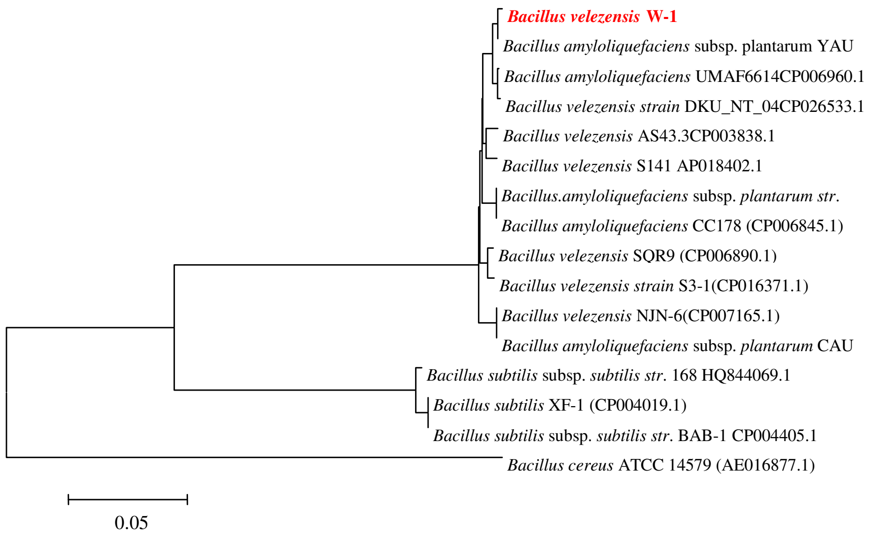

Figure 2. *Phylogenetic trees of the B. velezensis W1 and other related taxa*

Genome properties

The complete genome of W1 consisted of one 4,237,431 bp circular chromosome with an average GC content of 45.84% without a plasmid. Totally, 4,404 protein-coding genes were predicted (*Table 3; Fig. 3*), along with 85 tRNA genes and 27 rRNA genes (*Table 4; Fig. 3*). Among these 4,404 protein-coding genes 4,352 ones (98.82%) were annotated with predicted function (*Table 5*). There were 2879 (65.37%) genes assigned to COG database (*Table 6*).

Table 3. *Genome features of B. velezensis W1*

Features	Value
Genome size (bp)	4,237,431
Average G + C content (%)	45.84
Protein-coding genes	4,404
Longest Protein-coding genes	17,103
Total size of Protein-coding genes	3,761,991
Mean length of Protein-coding genes (bp)	854
Total size of Protein-coding genes % of Genome (%)	88.78

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

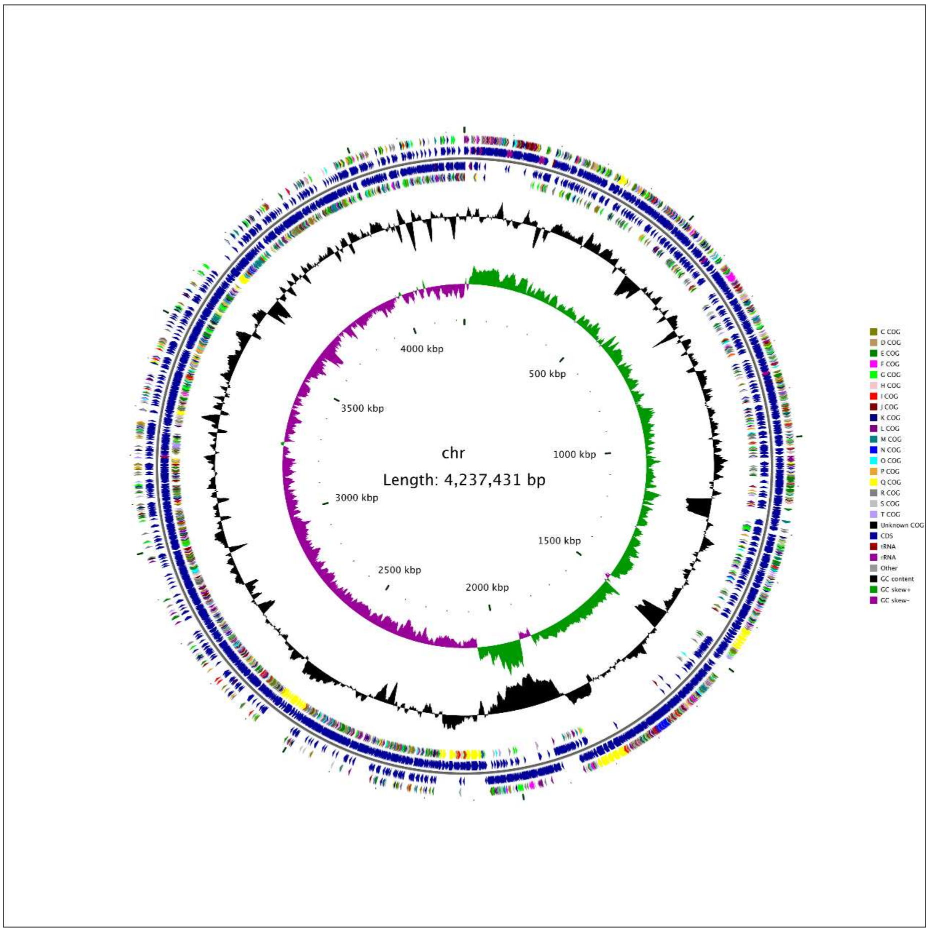

Figure 3. *Circular genome graph of Bacillus velezensis W1. From inside to outside, the first circle represents the scale; the second circle represents the GC Skew; the third circle represents the GC content; the fourth and seventh circles represent each CDS's COG; the fifth and sixth circles represent the position of CDS, tRNA and rRNA on the genome*

Table 4. *Statistics of ncRNA prediction of protein-coding genes of B. velezensis W1*

ncRNA type	Copy	Average length (bp)	Total length (bp)	% of genome
5s rRNA	9	115	1,033	0.024
16s rRNA	9	1,570	14,128	0.333
23s rRNA	9	2,934	26,402	0.623
tRNA	85	77	6,568	0.155
other ncRNA	9	115	1,033	0.024

Table 5. *The statistics of gene function annotation of B. velezensis W1*

Database	Annotated number	% of Genome
NR	4,352	98.82
eggNOG	2,879	65.37
KEGG	2,203	50.02
Swiss-Prot	3,785	85.94
GO	2,485	56.36

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

Table 6. *COG categories of B. velezensis W1*

Category	Description	Value	Percent (%)
B	Chromatin structure and dynamics	1	0.02
C	Energy production and conversion	177	4.02
D	Cell cycle control, cell division, chromosome partitioning	33	0.75
E	Amino acid transport and metabolism	340	7.72
F	Nucleotide transport and metabolism	83	1.89
G	Carbohydrate transport and metabolism	250	5.68
H	Coenzyme transport and metabolism	125	2.84
I	Lipid transport and metabolism	119	2.70
J	Translation, ribosomal structure and biogenesis	144	3.27
K	Transcription	279	6.34
L	Replication, recombination and repair	133	3.02
M	Cell wall/membrane/envelope biogenesis	179	4.06
N	Cell motility	57	1.29
O	Posttranslational modification, protein turnover, chaperones	98	2.23
P	Inorganic ion transport and metabolism	205	4.66
Q	Secondary metabolites biosynthesis, transport and catabolism	116	2.63
R	General function prediction only	451	10.24
S	Function unknown	297	6.74
T	Signal transduction mechanisms	159	3.61
U	Intracellular trafficking, secretion, and vesicular transport	49	1.11
V	Defense mechanisms	63	1.43
W	Extracellular structures	3	0.07
	Not in eggNOG	1,525	34.63

Insights from the genome sequence

The virulence factors of pathogenic bacteria (VFDB) of W1 were predicted using BLAST similarity searches (E-Value < 10−6) in Virulence Factors Database (http://www.mgc.ac.cn/VFs/) (Chen et al., 2012). W1 comprises several virulence factors (*Table 7*) which might all contribute to acaricidal activity, such as clpC, clpE, clpP, gale, lplA1, acpXL, bslA/yuaB, cps4I, and ureB.

There is a cluster of three collagen-related structural motif genes found in the genome, i.e., clpC, clpE and clpP. The genes of collagen-like proteins (clPs) have been identified in a broad range of bacteria, including some human pathogens and non-human pathogenic strains such as *B. cereus* and *B. amyloliquefaciens* which are reported to use as biocontrol agents (Zhao et al., 2015). The clPs are important for biofilm formation and bacterial adhesion to host cells and swimming motility (Zhao et al., 2016). In low-GC Gram-positive bacteria, the clpP protease is the main system involved in protein degradation (Vaz Cassenego et al., 2016), and play a role in both virulence and environmental adaptation, and acyldepsipeptides activated by ClpP core were effective in killing persister cells (Springer et al., 2016).

Biofilms are surface-associated bacterial aggregates, in which bacteria are enveloped by polymeric substances known as the biofilm matrix. Biofilms of biocontrol strains were considered as some significant biocontrol properties, which provide the necessary proximity to the host so that the secretion of secondary metabolites featuring biocontrol activity from biofilm forming cells may act directly on the target (Kröber et al., 2016; Vlamakis et al., 2008). *B. amyloliquefaciens* is a Gram-positive bacterium that forms biofilms, which are created by a heterogeneous population of motile, matrix-producing and sporulating cells. One characteristic feature of biofilms is the extracellular matrix built from EPS combined with macromolecules like proteins and nucleic acids (Kröber et al., 2016). The bslA/yuaB was considered to stand for biofilm surface layer protein and be responsible for the hydrophobic layer on the surface of biofilms (Kobayashi and Iwano, 2012).

The chromosome encodes six chitin deacetylases (chr_orf00199, chr_orf01107, chr_orf03856, chr_orf05798, chr_orf01368, chr_orf02151), which are insect chitin degradation enzymes that catalyze the deacetylation of chitin to form chitosan, and enable the degradation of chitin in the midgut peritrophic membrane of many insects and have been identified as insect virulence factors (Yang et al., 2018; Yu et al., 2016).

Table 7. *Predicted virulence factors of B. velezensis W1*

VFDB ID	Proteins	Annotation	Count
VFG000079 (gi:16802278)	chr_orf00119	clpC	1
VFG000080 (gi:16803037)	chr_orf01991	clpE	1
VFG013286 (gi:16272302)	chr_orf05807	galE	1
VFG002158 (gi:16802971)	chr_orf01464	lplA1	1
VFG011430 (gi:17987758)	chr_orf02383	acpXL	1
VFG045350 (gi:16080160)	chr_orf04548	bslA/yuaB	1
VFG000077 (gi:16804506)	chr_orf05117	clpP	1
VFG001373 (gi:15900286)	chr_orf05282	cps4I	1
VFG000270 (gi:15644702)	chr_orf05452	ureB	1

Conclusions

This report described the complete genome sequence of *B. velezensis* W1. The species has biotechnological potential due to its capability to cause mortality of the phytophagous mite, *Tetranychus urticae*. Its acaricidal activity might be related to function of the virulence factors of pathogenic bacteria, like clpC, clpE, clpP, gale, lplA1, acpXL, bslA/yuaB, cps4I, and ureB, as well as collagen-related structural motif genes clpC, clpE and clpP. Biofilm and chitin deacetylases have been identified as biocontrol properties and insect virulence factors. Moreover, this bacterial strain can be used as microbial acaricides to control more and more acari in the field and greenhouse.

Acknowledgement. This work was supported by the National Natural Science Foundation of China (31660536).

Conflict of interests. The authors declare that they have no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_26892699

DETERMINING THE EFFECTS OF SEWAGE SLUDGE AND *RHIZOBIUM* INOCULATION ON NUTRIENT AND HEAVY METAL CONTENT OF LENTIL (*LENS CULINARIS* MEDIC.)

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(Received 28th Nov 2018; accepted 28th Jan 2019)

Abstract. This study investigates the effects of increasing doses of sewage sludge with *Rhizobium* inoculation on lentil (*Lens culinaris* Medic.) straw and grain macro, micro and heavy metal contents in 2003-2004 and 2004-2005 growing seasons. Experiment was conducted as a randomized complete block design with 3 replications. At the end of the study, it was seen that *Rhizobium* inoculation affects the N, Mg, and Pb contents of the lentil straw on by P<0.05, P and Ca contents by P<0.01 in the first year; the N, Fe, and Zn contents on P<0.05 level, on the Pb content P<0.01 level in the second year. Lentil grains were affected only on the K, Fe, Pb, and Cd contents by P<0.05. Sewage sludge applications were used to detect the effects on the N, Mg, Fe, Mn and Cu contents of the lentil straw by P<0.01, Co content by P<0.05, and Pb content by P<0.01 in first year; on the N, K, Zn and Pb contents by P<0.01, P and Mn contents by P<0.01, on the Mg, Fe, Cu, and Co contents by P<0.05 in the second year. Effects received by the lentil grains are as follows: The N, K, and Cu contents by P<0.01, on the Pb, Fe, P, and Mg contents by P<0.01, on the Ca and Zn contents by P<0.05 in the first year; on the P, K, Ca, Mg, Fe, Mn, Zn, Cu and Co contents by P<0.01, on the Pb content by P<0.05 in the second year. The effects of inoculation on the lentil straw and grain, regarding the nutrient and heavy metal contents were determined to be insignificant. This result may be a result of weak inoculation application and the heavy metal content of the sewage sludge, as well as the negative effects of *Rhizobium* inoculation.
Keywords: *Cadmium, macro nutrients, legume, Rhizobium, municipal waste sludge*

Introduction

One of the preferred methods to remove sewage sludge in industrialized and populous countries is to use sewage sludge in agricultural production under controlled conditions. Particularly, sewage sludge derived industrial waste cause health problems because of the potential toxic substance like heavy metals, phenolics (Mueller et al., 1989; Van Assche and Clijsters, 1990). Moreover, the organic substance of soils is low. In order to achieve steady agricultural production, and taking into account the limited organic fertilizer resources – used for preserving or increasing the organic substance content of soils – scientists have been using sewage sludge.

High or low of organic substance extent of soils have effects on physical, chemical and biological specification directly and indirectly. The organic fertilizer source used includes nutrient elements (stable manure, poultry manure, refuse compost, sewage sludge) and the content of potential toxic elements affect the frequency of application in agricultural production. In addition, the pH of the soil affects the amount and place of application of the sewage sludge (Logan and Chaney, 1983).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Generally, it is preferred that sewage sludge is applied to the soil which has 6 pH or more. Because sewage sludge contains elements especially like Cd, Zn and Ni. These have high activity on acidic soils. Using sewage sludge in agricultural production improves physical characteristic of the soil and it functions as a source of nutrition elements for plants (Smith, 1996). The important thing here is that we should be careful in the harvest period after sewage sludge applied. Because after sewage sludge is applied, there should be a delay period before harvesting and therefore, sewage sludge applications must be done considering the harvest periods (Lovell et al., 1995).

As sewage sludge includes potential toxic substances, it has serious effects on macro and micro flora of soils (Sterritt and Lester, 1980; Brynhildsen and Rosswall, 1997; Athar and Ahmad, 2002). So, some researchers had investigated and found out what the sewage sludge effects on the soil development and soil microorganism are (Sönmez, 2003; Tüfenkçi et al., 2006a). According to the research, microorganisms had developed some mechanism against the toxic substances, but *Rhizobium* bacteria (Haferburg and Kothe, 2007), was discovered to be especially vulnerable to them (Chaudri et al., 1993, 2000). Therefore, soil organisms especially *Rhizobiums* where sewage sludge are applied face some important problems. In the area where sewage sludge is applied, heavy metal loading in different organs of plants has been monitored (Xian, 1989; Nellesen and Fletcher, 1993; Yürük and Bozkurt, 2006). The legumes used in the rehabilitation of contaminated areas with heavy metals (Zribi et al., 2012), and especially the presence of microbial density produced by plants in the rhizosphere and root exudates are very important (Glick, 2004). It has been reported that it has a negative effect on the inoculation of cadmium, copper and zinc *Rhizobium* in areas where treatment sludge has been applied for many years (Charlton et al., 2016).

In this study, we aimed to determine the effects of *Rhizobium* budding on the nutrition element of the straw and grain and monitor the changes on the heavy metal content after the application of sewage sludge on the lentil plant.

Materials and Methods

This study was carried out on the experiment area of Van Yüzüncü Yıl University in Eastern Anatolia in Turkey (between latitudes 35° 57’ 84 N and longitudes 42° 74’ 61 E at an elevation of 1725 m) in 2003-04 and 2004-05 growing seasons using one red-seeded nationally registered lentil cultivar (cv. Sazak-91) with 1000-seed weight of 30 g. Annual amounts of rainfall were 349.7 mm and 421.2 mm in 2003-04 and 2004-05, respectively. Average temperatures were 9.9^{0}C in 2003-04 and 9.6^{0}C in 2004-05 (*Table 1*). The results of the elemental analysis of the treatment area soil and the sewage sludge used in the experiment were given in *Table 2*.

Seeds were inoculated using peat inoculants which included a mixture of nodule-forming strains of *Rhizobium leguminosarum biovar viciae* specific to lentil. Peat inoculant was prepared with commercial peat cultures provided by the Soil and Fertilizer Research Institute, Ankara, Turkey, according to the method of Somesagaran and Hoben (1994). Peat inoculants were kept in a refrigerator at +4 ^{0}C until they were used. It was separately provided from the Soil and Fertilizer Research Institute in 2003-04 and 2004-05 winter seasons. Content and activity of peat inoculants were checked before the trials in 2003-04 and 2004-05. Peat inoculants was used after cell count was adjusted to 1 x 10^8 *Rhizobium* cells g^{-1} so the content of peat culture was standardized by diluting peat inoculants. The most probable number (MPN) method was used for estimating viable cells of *Rhizobium* (Somesagaran and Hoben, 1994).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Table 1. *Meteorological data for the growing seasons of 2003-2004, 2004-2005 and long-term averages in Van, Turkey (TSMS, 2006)*

Months	Precipitation (mm)			Average temperature (ºC)			Relative humidity (%)		
	2003-04	2004-05	LTA*	2003-04	2004-05	LTA*	2003-04	2004-05	LTA*
September	16.4	-	13.0	17.0	18.0	17.2	64.5	48.7	44.0
October	23.6	48.1	45.2	13.0	12.0	10.6	71.0	64.1	58.0
November	59.6	102.4	47.9	4.5	4.6	4.4	74.3	75.1	66.0
December	14.9	41.0	37.3	0.2	-3.7	-0.8	76.7	73.8	69.0
January	25.0	34.4	35.4	-0.9	-3.3	-3.6	78.8	77.1	68.0
February	39.6	27.2	32.5	-0.6	-3.3	-3.2	76.1	73.7	69.0
March	69.9	59.1	45.7	3.7	2.5	0.9	72.3	70.9	68.0
April	26.9	55.9	56.6	6.9	8.9	7.4	66.4	64.1	62.0
May	68.7	35.8	45.0	12.4	13.3	13.0	67.8	62.5	56.0
June	3.1	13.0	18.5	18.5	18.7	18.0	57.8	55.9	50.0
July	2.0	0.3	5.2	21.4	24.1	22.2	52.7	51.3	44.0
August	-	4.0	3.4	22.2	23.4	21.8	46.5	62.1	41.0
Total	349.7	421.2	385.7						
Average				9.9	9.6	9.0	67	64.9	57

*LTA = Long-term average (1979-2006)

Four doses of trial sewage sludge (SS0; 0 (control), SS10; 10, SS20; 20 and SS40; 40-ton ha^{-1}) were applied as inoculated and non-inoculated in parcels with 3 repetitions. Sowing was done by hand with 30 cm row spacing during late October in both years (20 October 2003 and 25 October 2004). The seeding rate was 140 kg ha^{-1} with the large-grained Sazak 91. A basal dose of 140 kg ha^{-1} di-ammonium phosphate (DAP) was given to each parcel at the time of sowing. Seeds were inoculated with *Rhizobium leguminosarum biovar viciae* bacteria at the predetermined rates before sowing in all parcels, except for the uninoculated control (Vincent, 1970). Application of the peat inoculant on the seeds was conducted using water which contains 2% sugar. The parcel size was 1.5 x 5 m. The experiment was carried out as rain fed. Parcels were hand-weeded twice each season. Plants were harvested during late June in both years (22 June 2004 and 27 June 2005).

The levels of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cobalt (Co), cadmium (Cd) and lead (Pb) were analyzed in dried and grinded samples that straw and grain extracts were obtained from each repetition to be in two parallel according to the methods reported by Kacar and İnal (2008) (*Table 3*).

At the end of the experiment, as in Jackson (1958), soil samples were taken from 0-20 cm depth on each repeating and brought to the laboratory and analyzed. The rest was done as follows: Texture, (Bouyoucous, 1951) soil reaction, (Jackson, 1958); total salt, (Richards, 1954): lime, (Hızalan and Ünal, 1966); organic substance, according to modified Walkley Black Method (Walkley, 1947); nitrogen, according to Kjehldahl Method (Kacar, 1994); available phosphorus, according to sodium bicarbonate method (Olsen et al., 1954); exchangeable potassium, calcium and magnesium, according to Thomas (1982) with 1 N ammonium acetate; micro elements and heavy metals found in soil samples were retrieved after shaking with 0.05 M DTPA (Diethylene Triamine Penta Acetic acid) adjusted to pH 7.3 (Lindsay and Norvel, 1978). These residuals were specified in Atomic Absorption Spectrometer. The values obtained at the end of the experiment were analyzed with Costat statistics program (Düzgüneş et al., 1987).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Table 2. *The results of the chemical and physical analysis of the testing area soil and the sewage sludge*

Source	Years	Salt	OM	Texture	Lime	N	pH	P	K	Ca	Mg	Fe	Mn	Zn	Cu	Cd	Co	Pb
		%			%			mg kg^{-1}										
Soil	1.Year	0.03	0.82	SL	4.99	0.08	7.93	3.92	360	2130	201	10.37	19.0	1.57	1.61	-	0.132	-
	2.Year	0.03	0.80	SL	5.01	0.07	7.95	3.87	351	2201	211	9.7	17.6	1.03	1.52	0.11	0.098	0.06
S.S.	1.Year	0.04	29.4	-	-	1.32	7.98	1544	2430	2100	567	956	440	560	149	0.96	4.56	2.89
	2.Year	0.05	30.2	-	-	1.40	8.01	1645	2301	2081	497	889	413	529	152	1.02	4.12	3.01
	Permissible limit (pH>6)*													300	140	3	20	300

SS: Sewage sludge, OM: Organic matter, *: Anonymous (2003)

Table 3. *Results of some chemical and physical analysis of application parcels after testing*

R	SS, ton ha^{-1}	Salt	OM	Lime	N	pH	P	K	Ca	Mg	Fe	Mn	Zn	Cu
		%					mg kg^{-1}							
R+	0	0.026*	0.85	6.2	0.071	7.49	3.7	499	2125	208	17.2	25.5	2.9	3.1
	10	0.027	0.94	3.7	0.115	7.49	3.8	470	2109	195	18.7	22.7	2.6	1.6
	20	0.023	0.97	5.2	0.119	7.47	4.3	327	2160	197	19.5	20.0	1.2	1.8
	40	0.025	1.03	4.2	0.136	7.58	6.0	411	2144	184	16.4	19.7	0.4	1.4
R-	0	0.021	0.82	5.0	0.080	7.73	3.9	360	2130	201	10.4	19.0	4.6	1.6
	10	0.020	0.92	3.6	0.098	7.83	4.2	305	2067	161	17.6	18.0	1.8	1.4
	20	0.023	0.96	2.7	0.106	7.63	4.6	311	2051	159	16.8	20.1	1.0	1.4
	40	0.018	1.04	2.7	0.132	7.73	5.3	352	2040	175	15.5	17.1	0.4	1.3

R: *Rhizobium*, R+: *Rhizobium* inoculate, R-: *Rhizobium* non-inoculate, SS: Sewage Sludge, OM: Organic Matter, *: values are the average of 3 replications

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Results

Effects of the application in the content of straw and grain macro elements

As it can be seen in *Table 4*, inoculation had a significant effect on both the first and the second year lentil straw N ($p<0.01$) and Ca ($p<0.01$) values, while it had a significant effect on P and Mg ($p<0.01$) values only on the first year. Its effect on potassium was found to be insignificant in both years. It was seen that while the sewage sludge affected the nitrogen ($p<0.01$) on both years, it was discovered that it affected P, K and Ca ($p<0.01$) only on the second year and Mg ($p<0.01$) only on the first year. It was observed that the interaction only affects nitrogen with a value of $p<0.05$ in both years (*Table 4*). While the sewage sludge application on the grain nitrogen content had a significant effect on the first year ($p<0.01$), it had a significant effect on the P, K, Ca, and Mg elements in both years, respectively as $p<0.01$, $p<0.01$, $p<0.05$ / $p<0.01$ and $p<0.01$. Inoculation had a significant in grain nitrogen and phosphorus in both years ($p<0.01$ and $p<0.05$), in potassium and calcium ($p<0.01$ and $p<0.05$), it had a significant effect only in the first year. It was detected that the interaction affected the K and the Mg content of the grain macro nutrients on the second year with a level of $p<0.05$ (*Table 5*).

Table 4. *Effects of Rhizobium and sewage sludge on macro element contents of lentil straw*

Treatments	Nitrogen (%)		Phosphorus (mg kg^{-1})		Potassium (%)		Calcium (%)		Magnessium (%)	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)										
SS0	1.189 c**	1.533 c**	1315 b ^{ns}	1300 b**	2.224 b ^{ns}	2.127 d**	3.95 b ^{ns}	4.60 b**	2.446 c**	2.522 b*
SS10	1.290 b	1.568 c	1420 ab	1417 b	2.419 ab	2.427 c	4.18 ab	4.92 b	3.507 bc	2.567 ab
SS20	1.270 bc	1.651 b	1452 ab	1762 a	2.530 ab	2.799 b	4.30 ab	5.32 a	3.538 ab	2.687 ab
SS40	1.402 a	1.728 a	1578 a	1907 a	2.597 a	2.931 a	4.48 a	5.48 a	3.578 a	2.598 a
***Rhizobium* (R)**										
R(+)	1.412 a*	1.693 a*	1701 a**	1610 ^{ns}	2.537 ^{ns}	2.594 ^{ns}	4.443 a**	5.32 ^{ns}	0.551 a*	2.568 ^{ns}
R(-)	1.164 b	1.547 b	1183 b	1580	2.347	2.548	4.018 b	4.79	0.484 b	2.559
Interactions (RxSS)										
R(-)xSS0	1.072 d *	0.433 d **	1110 ns	1291 ns	2.182 ns	2.089 ns	3.606 ns	4.503 ns	2.410 ns	2.515
R(-)xSS10	1.199 cd	0.455 d	1168	1402	2.343	2.362	3.938	4.750	2.475	2.551
R(-)xSS20	1.167cd	0.599 c	1205	1833	2.392	2.835	4.134	4.935	2.512	2.610
R(-)xSS40	1.185 cd	0.703 ab	1246	1794	2.473	2.906	4.393	5.008	2.540	2.562
R(+)xSS0	1.307 bc	0.633 bc	1520	1311	2.266	2.164	4.297	4.697	2.481	2.530
R(+)xSS10	1.349 b	0.682 ab	1673	1417	2.495	2.492	4.426	5.094	2.540	2.582
R(+)xSS20	1.372 b	0.703 ab	1700	1694	2.668	2.763	4.474	5.703	2.565	2.587
R(+)xSS40	1.619 a	0.752 a	1910	2023	2.721	2.957	4.575	5.955	2.616	2.575

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, #: Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

When the change in the content of straw macro element of lentil at sewage sludge application was investigated, it was seen that the contents of N, P, K, Ca and Mg increased considerably in both years according to control. The highest N, P, K, Ca and Mg values were reached respectively as 1.402%, 1578 mg kg^{-1}, 2.597%, 4.48% and 3.578% at the application of 40-ton ha^{-1} of sewage sludge in the first year (*Table 4, Figure 1*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Table 5. *Effects of Rhizobium and sewage sludge on macro element contents of lentil grain*

Treatments	Nitrogen (%)		Phosphorus (mg kg^{-1})		Potassium (%)		Calcium (%)		Magnessium (%)	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)										
SS0	4.339 c**	3.681 b ns	5139 b**	5328 c**	1.072 c**	1.277 d**	0.934 b**	1.135 c**	0.145 c**	0.126 b**
SS10	4.453 bc	3.840 ab	5689 a	5553 b	1.133 c	1.419 c	1.173 ab	1.450 b	0.188 b	0.135 b
SS20	4.481 b	3.960 ab	5866 a	5612 b	1.291 b	1.611 b	1.287 a	1.510 ab	0.201 b	0.136 b
SS40	4.629 a	4.170 a	5996 a	5833 a	1.463 a	1.846 a	1.416 a	1.589 a	0.254 a	0.157 a
***Rhizobium* (R)**										
R(+)	4.541 ns	4.057 ns	5844 ns	5638 ns	1.300 a*	1.519 ns	1.311 ns	1.459 ns	0.205 ns	0.140 ns
R(-)	4.411	3.768	5501	5532	1.179 b	1.558	1.093	1.503	0.189	0.138
Interactions (RxSS)										
R(-)xSS0	4.246 ns	3.607 ns	4880 ns	4208 ns	1.020 ns	1.228 b*	0.0797 ns	s0.1363 ns	0.132 ns	0.126 ns
R(-)xSS10	4.346	3.724	5699	4506	1.078	1.457 b	0.0988	0.1469	0.185	0.141
R(-)xSS20	4.411	3.862	5704	4561	1.298	2.708 a	0.1239	0.1544	0.187	0.143
R(-)xSS40	4.638	3.879	5722	4855	1.320	1.840 ab	0.1347	0.1634	0.251	0.149
R(+)xSS0	4.431	3.753	5398	4448	1.124	1.327 b	0.1070	0.1386	0.158	0.127
R(+)xSS10	4.560	3.956	5680	4601	1.188	1.382 b	0.1358	0.1431	0.191	0.129
R(+)xSS20	4.551	4.059	6027	4663	1.283	1.515 b	0.1334	0.1476	0.216	0.129
R(+)xSS40	4.620	4.461	6269	4840	1.605	1.852 ab	0.1484	0.1545	0.257	0.165

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, #: Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

Same results were reached also in the second year, but only the content of Mg value was reached as 2.687% at application of 20-ton ha^{-1} of the sewage sludge. At the application of 40-ton ha^{-1} of sewage sludge, the highest values of N, P, K, Ca and Mg were determined as 1.728%, 1907 mg kg^{-1}, 2.931%, 5.48% and 2.569%, respectively. The lowest value of both years was determined in control parcels (*Table 4, Figure 1).*

After statistical analyses, straw macro element of lentil in inoculated parcels increased in both years as compared to non-inoculated parcels. It was seen that N content in this increase was significant in both years; P, Ca and Mg are significant only in the first year. Increase in potassium content was not significant (*Table 4*). Inoculation resulted in significant increases in the nitrogen, phosphorus, calcium and magnesium content of lentils. A significant increase was detected for N in both years; for P, Ca and Mg it was only detected in the first year. It was seen that the decrease and increase in potassium content was statistically insignificant (*Table 4, Figure 1*).

At the application of 40-ton ha^{-1} of sewage sludge, the highest value was reached in both years. The lowest value in both years was determined in control parcels. The highest value on grain of N, P, K, Ca and Mg elements were respectively detected as 4.629%, 0.599%, 1.463%, 0.142% and 0.254% in first year, as 4.170%, 0.583%, 1.846%, 0.159% and 0.157%, respectively in second year (*Table 5, Figure 1*).

The effect of application to the straw and grain micro element content of lentil

The results of variance analysis and the results of the averages of the effect of straw and grain micro element content of lentil and *Rhizobium* applications are given in *Table 5, 6* and *Figure 2*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

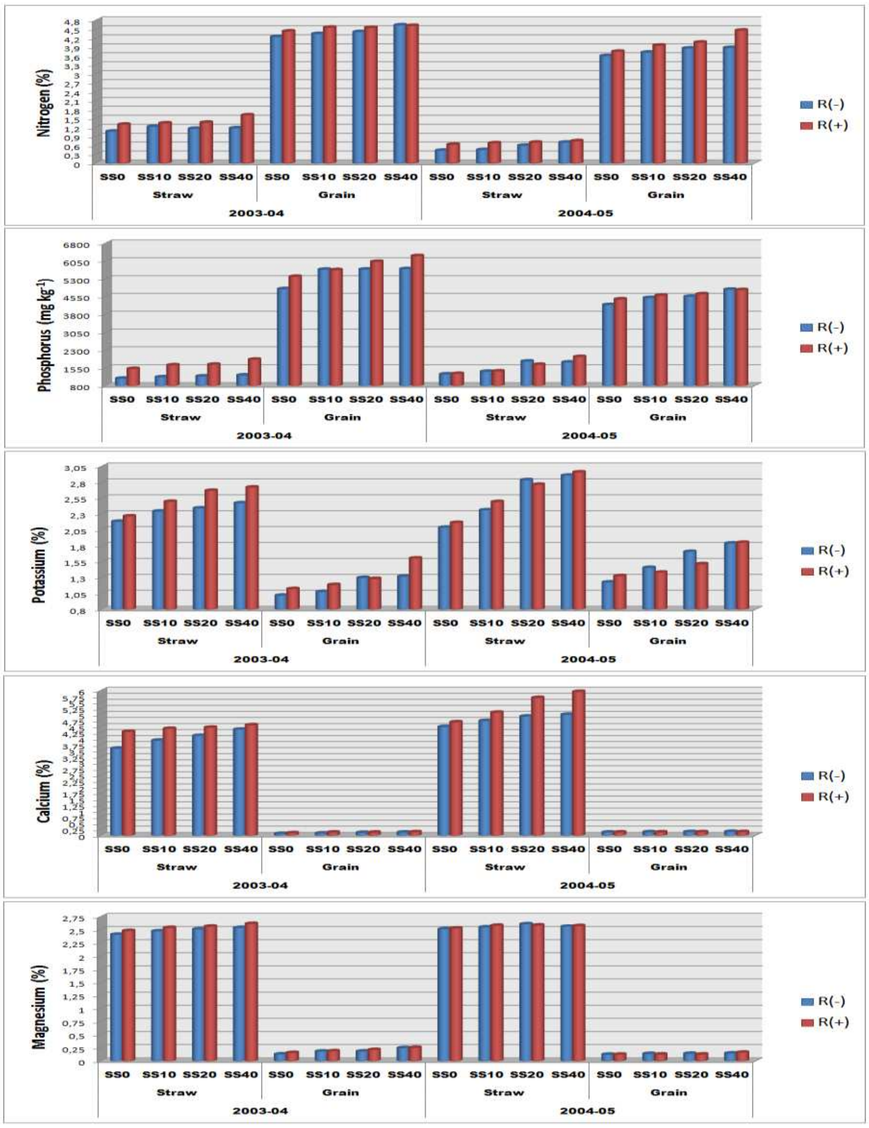

Figure 1. *Effects of applications on lentil straw and grain macro element contents SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R-: Rhizobium non-inoculation, R+: Rhizobium non-inoculation*

The application of sewage sludge affected the content of straw iron value of lentil by P<0.01 in first year, by P<0.05 in the second year. It affected the manganese content by P<0.01 in both years; zinc content by P<0.01 in the second year while it was insignificant in the first year. Copper content was affected by P<0.01 in the first year, by P<0.05 in the second year. Inoculation affected the content of manganese and copper in both years, while it affected the content of iron and zinc by P<0.05 in the second year. It was seen that its effect on iron and zinc application in the first year was insignificant (*Table 6*).

Inoculation effect on the grain micro element content was significant for Fe and Zn (p<0.01) in both years and for Mn in the second year (p<0.01). The sewage sludge application was significant for Fe, Zn and Cu (p<0.01 / p<0.05) in both years and for Mn (p<0.01) in the second year. The effect of the interaction was detected as significant in the second year only in Mn (p<0.01) (*Table 7*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Table 6. *Effects of Rhizobium and sewage sludge on micro element contents of lentil straw*

Treatments	Iron (mg kg^{-1})		Manganese (mg kg^{-1})		Zinc (mg kg^{-1})		Copper (mg kg^{-1})	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)								
SS0	50.6 c**	28.9 b*	54.8 b **	44.8 b**	6.07 a ns	6.29 c**	7.12 b**	7.84 b*
SS10	57.6 bc	34.4 ab	61.5 a	46.0 b	6.42 a	7.42 b	9.55 a	9.23 ab
SS20	68.3 ab	36.9 ab	64.2 a	46.7 b	6.53 a	8.03 ab	10.48 a	9.39 a
SS40	72.9 a	42.9 a	67.7 a	56.3 a	6.57 a	8.66 a	11.07 a	10.12 a
***Rhizobium* (R)**								
R(+)	63.5 ns	39.7 a*	65.2 ns	47.4 ns	5.79 ns	7.81 ns	9.54 ns	9.15 ns
R(-)	61.2	31.9 b	58.9	49.5	6.86	7.39	9.57	9.15
Interactions (RxSS)								
R(-)xSS0	49.5 ns	24.5 ns	52.5 ns	46.2 **	6.33 ns	6.26 ns	7.28 ns	8.01 ns
R(-)xSS10	54.2	31.9	56.4	47.7	6.56	6.97	10.13	9.09
R(-)xSS20	68.8	31.8	50.4	47.9	7.37	7.77	9.90	9.32
R(-)xSS40	72.3	39.5	66.3	56.2	7.18	8.55	10.97	10.15
R(+)xSS0	51.8	33.4	57.2	43.2	5.79	6.31	6.96	7.67
R(+)xSS10	60.8	36.9	66.6	44.4	6.3	7.87	8.97	9.37
R(+)xSS20	67.8	42.0	68.0	45.5	5.8	8.29	11.06	9.46
R(+)xSS40	73.2	46.4	68.9	56.4	5.38	8.76	11.17	10.09

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, #: Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

Table 7. *Effects of Rhizobium and sewage sludge on micro element contents of lentil grain*

Treatments	Iron (mg kg^{-1})		Manganese (mg kg^{-1})		Zinc (mg kg^{-1})		Copper (mg kg^{-1})	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)								
SS0	48.1 c**	67.9 d**	10.7 ns	11.0 c**	16.37 b*	11.85 c**	10.18 c**	7.85 c**
SS10	54.2 bc	95.2 c	11.3	14.3 b	17.39 ab	13.65 b	10.39 bc	8.42 b
SS20	58.8 b	123.3 b	11.2	14.8 b	17.81 ab	14.49 b	11.08 ab	9.09 a
SS40	81.8 a	166.2 a	10.0	16.7 a	18.98 a	16.57 a	11.70 a	9.16 a
***Rhizobium* (R)**								
R(+)	66.1a**	121.7 ns	10.8 ns	13.5 ns	18.41 ns	13.18 ns	10.87 ns	8.56 ns
R(-)	55.3 b	104.6	10.8	14.9	16.87	15.10	10.81	8.69
Interactions (RxSS)								
R(-)xSS0	42.8 ns	63.8 ns	10.7 ns	10.7 c**	15.17 ns	12.29 ns	9.83 ns	7.61 ns
R(-)xSS10	46.7	86.1	12.2	14.2 b	16.74	14.58	11.08	8.37
R(-)xSS20	53.6	107.9	9.8	15.5 b	17.35	15.17	10.35	9.33
R(-)xSS40	78.3	160.5	6.5	19.2 a	18.19	18.37	11.98	9.46
R(+)xSS0	53.5	72.0	10.6	11.4 c	17.57	11.40	10.54	8.08
R(+)xSS10	61.7	104.2	10.4	14.1 b	18.03	12.73	11.08	8.47
R(+)xSS20	64.0	138.6	11.7	14.3 b	18.28	13.81	10.42	8.85
R(+)xSS40	85.3	171.8	10.6	14.3 b	19.76	14.76	11042	8.85

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, # : Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

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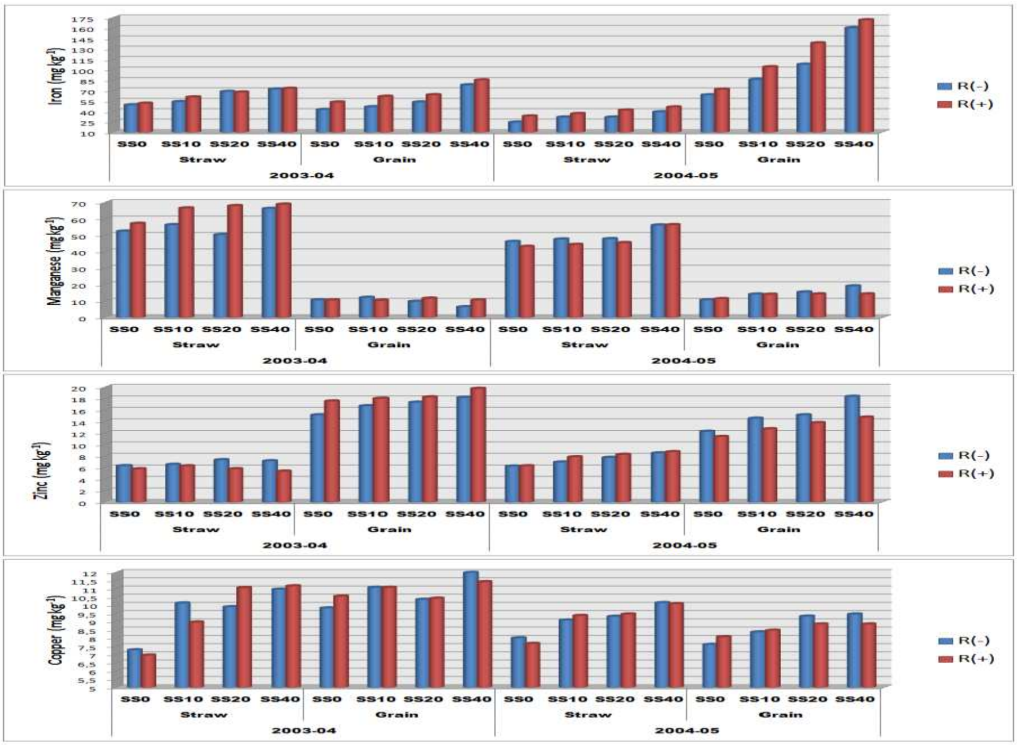

***Figure 2.** Effects of the applications on straw and grain micro element contents of lentil SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R-: Rhizobium non-inoculation, R+: Rhizobium non-inoculation*

The highest value of straw content of Fe, Mn, Zn and Cu were reached at the application of 40-ton ha^{-1} of sewage sludge in both years and the lowest value level was detected at control parcels. The highest value was reached as 72.9 mg kg^{-1}, 67.7 mg kg^{-1}, 6.57 mg kg^{-1} and 11.07 mg kg^{-1} respectively in first year; as 42.9 mg kg^{-1}, 56.3 mg kg^{-1}, 8.66 mg kg^{-1} and 10.12 mg kg^{-1} respectively in second year. The alteration in content of straw micro element depending on inoculation was variable. Others were found to be insignificant while only iron was insignificant in the second year (*Table 6, Figure 2*).

Even though inoculation had insignificant effects on the grain micro element content of lentil in both years, except for Fe, it was seen that sewage sludge had very important effects on Fe, Zn and Cu in both years, on Mn in the second year only (*Table 7, Figure 2*).

According to control, depending on applications of sewage sludge, Mn increased only in the second year, while Fe, Zn and Cu increased highly in both years. The highest value was reached at the application of 40-ton ha^{-1} of sewage sludge as 81.8 mg kg^{-1}, 18.98 mg kg^{-1} and 11.70 mg kg^{-1} respectively in the first year and 166.2 mg kg^{-1}, 16.7 mg kg^{-1}, 16.57 mg kg^{-1} and 9.16 mg kg^{-1} respectively in the second year (*Table 7, Figure 2*).

The effect of the applications to the straw and grain heavy metals content of lentil

The results of variance analysis and the results of the averages of the effect of sewage sludge and *Rhizobium* applications on the straw and grain heavy metal contents of lentil are given in *Table 8, 9,* and *Figure 3*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

Rhizobium application had a significant effect on the straw Co content of the lentil in the second year ($p<0.05$) and on the Pb content in both years ($p<0.01$). The effect of the sewage sludge application and interaction was significant only on Pb content in both years ($p<0.01$). While the inoculation on grain heavy metal content of lentil had a significant effect on the content of Pb ($p<0.01$) and Cd ($p<0.05$) in the first year, the sewage sludge application had a significant effect on Co in the second year ($p<0.05$) and on Pb ($p<0.01$/$p<0.05$) in both years. The effect of interference on grain heavy metal content was found to be insignificant (*Table 8, 9*).

As the doses of sewage sludge are increased, the content of straw heavy metal of lentil also increased. The highest value in Co, Pb, and Cd contents were reached at the application of 40-ton ha^{-1} of sewage sludge in both years as 2.714 mg kg^{-1}/0.999mg kg^{-1}, 0.618 mg kg^{-1}/0.602 mg kg^{-1}, 0.201 mg kg^{-1}/1.027 mg kg^{-1} respectively, and the lowest value was reached at control parcels. Inoculation only affected Pb content. Lead content decreased in the second year while it increased with inoculation in the first year (*Table 8, Figure 3*).

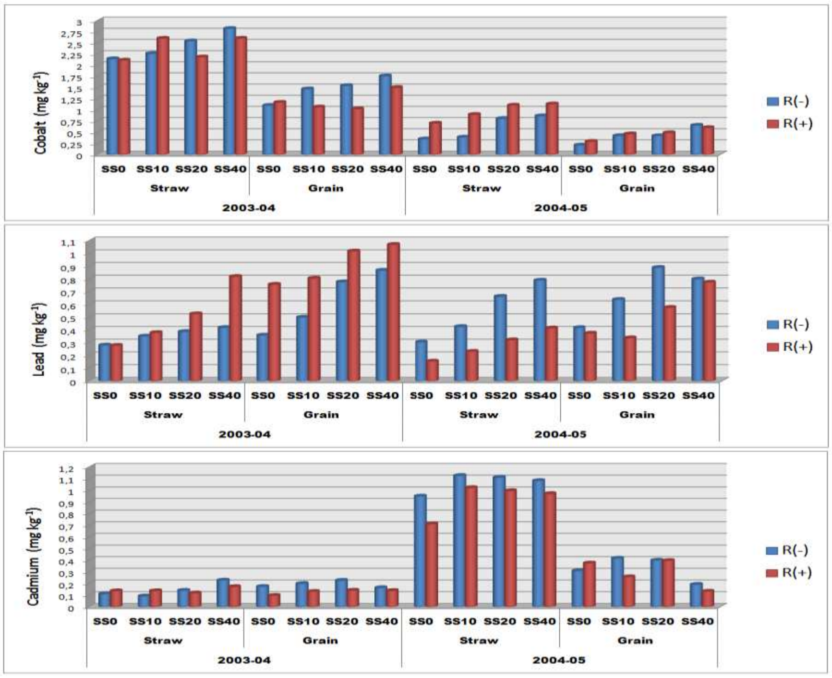

Figure 3. *Effects of the applications on straw and grain micro element contents of lentil SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R-: Rhizobium non-inoculation, R+: Rhizobium non-inoculation*

It was seen that sewage sludge had a statistically important effect on the grain heavy metal content of lentil with regards to Co in the second year and Pb in both years but its effect on Cd was insignificant (*Table 9, Figure 3*).

Co and Pb content increased significantly with the application of residual sewage sludge according to control in both years. The highest value was reached as 1.631 mg kg^{-1} and 0.968 mg kg^{-1} respectively in the first year; as 0.628 mg kg^{-1} and 0.686 mg kg^{-1} respectively in the second year. *Rhizobium inoculation* decreased the taking of Cd, while it increased the taking of Pb seed in the first year (*Table 9, Figure 3*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

***Table 8.** Effects of Rhizobium and sewage sludge on heavy metal contents of lentil straw*

Treatments	Cobalt (mg kg^{-1})		Lead (mg kg^{-1})		Cadmium (mg kg^{-1})	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)						
SS0	2.129 b*	0.524 b*	0.280 d**	0.230 d**	0.124 ab*	0.831 ns
SS10	2.432 ab	0.644 ab	0.365 c	0.329 c	0.115 b	1.075
SS20	2.364 ab	0.956 a	0.456 b	0.492 b	0.130 ab	1.053
SS40	2.714 a	0.999 a	0.618 a	0.602 a	0.201 a	1.027
***Rhizobium* (R)**						
R(+)	2.376 ns	0.959 ns	0.500 a*	0.281 b**	0.142 ns	0.925 ns
R(-)	2.443	0.603	0.359 b	0.546 a	0.143	1.068
Interactions (RxSS)						
R(-)xSS0	2.1437 ns	0.3483 ns	0.2808 d**	0.3047 d**	01117 ns	0.9500 ns
R(-)xSS10	2.2620	0.3897	0.3508 cd	0.4267 c	0.0925	1.1267
R(-)xSS20	2.5443	0.8067	0.3867 cd	0.6623 b	0.1408	1.1100
R(-)xSS40	2.8227	0.8657	0.4183 c	0.7889 a	0.2283	1.0833
R(+)xSS0	2.1133	0.7007	0.2783 d	0.1551 e	0.1367	0.7117
R(+)xSS10	2.6027	0.8973	0.3787 cd	0.2313 de	0.1375	1.0225
R(+)xSS20	2.1843	1.1043	0.5258 b	0.3218 d	0.1185	0.9967
R(+)xSS40	2.6043	1.1323	0.8183 a	0.4146 c	0.1733	0.9717

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, #: Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

***Table 9.** Effects of Rhizobium and sewage sludge on heavy metal contents of lentil grain*

Treatments	Cobalt (mg kg^{-1})		Lead (mg kg^{-1})		Cadmium (mg kg^{-1})	
	2003-04	2004-05	2003-04	2004-05	2003-04	2004-05
Sewage Sludge (ton ha^{-1}) (SS)						
SS0	1.133 b ns	0.253 c**	0.558 b**	0.393 c*	0.136 ns	0.342 ns
SS10	1.267 ab	0.443 b	0.652 b	0.488 bc	0.166	0.275
SS20	1.282 ab	0.454 b	0.897 a	0.732 ab	0.184	0.398
SS40	1.631 a	0.628 a	0.968 a	0.787 a	0.151	0.225
***Rhizobium* (R)**						
R(+)	1.188 ns	0.462 ns	0.912 a*	0.515 ns	0.128 b*	0.291 ns
R(-)	1.468	0.427	0.625 b	0.686	0.191 a	0.330
Interactions (RxSS)						
R(-)xSS0	1.0990 ns	0.2113 ns	0.3590 ns	0.4183 ns	0.174 ns	0.309 ns
R(-)xSS10	1.4693	0.4223	0.4992	0.6383	0.200	0.417
R(-)xSS20	1.5430	0.4190	0.7761	0.8888	0.227	0.400
R(-)xSS40	1.7603	0.6547	0.8667	0.7992	0.164	0.195
R(+)xSS0	1.1670	0.2937	0.7567	0.3743	0.098	0.375
R(+)xSS10	1.0637	0.4627	0.8050	0.3373	0.132	0.258
R(+)xSS20	1.0217	0.4880	1.0174	0.5758	0.142	0.397
R(+)xSS40	1.5010	0.6017	1.0690	0.7738	0.139	0.133

ns: not significant; *: Significant at P<0.05 level, **: Significant at P<0.01 level, #: Values followed by the different letters are significantly different, SS0: 0 ton ha^{-1}, SS10: 10 ton ha^{-1}, SS20: 20 ton ha^{-1}, SS40: 40 ton ha^{-1}, R(-): *Rhizobium* non-inoculation, R(+): *Rhizobium* inoculation

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Discussion

In our study, it was determined that the increasing applications of sewage sludge increase the amount of lentils' straw and grain macro, micro and heavy metal contents in both years. The highest values were reached in 40-ton sewage sludge ha^{-1} applied parcels. Increases in N, P, K, Ca, Mg, Fe, Mn, Cu, Co, and Pb contents of the lentil straw in the first year were respectively 17.9%, 19.7%, 16.8%, 13.4%, 5.4%, 44.1%, 23.5%, 55.5%, 27.5%, 120.7 and in the second year they 12.7%, 47.7%, 37.8%, 19.1%, 3.0%, 48.4%, 25.9%, 29.1%, 90.7%, 161.7%, respectively. The straw zinc content increased by 37.7% in the second year and the cadmium content increased by 62.1% in the first year. The first and second year increases in the grain nutrient and heavy metal contents were respectively N; 6.7%-13.3%, P; 16.5%-9.8%, K; 36.5%-44.6%, Ca; 52.7%-16.1%, Mg; 75.2%-24.6%, Fe; 70.1%-144.8%, Mn; 51.8%, Zn; 15.9%-39.8%, Cu; 14.9%-16.7%, Co; 43.9%-148.2% and Pb; 73.5%-100.3%. Even though there was a decrease in the grain Cd content, this was found to be statistically insignificant (*Table 4, Table* 5, *Figure 1*).

In similar studies, it has been reported that plants' nutrient and heavy metal contents go up as a result of increasing sewage sludge applications (Sönmez and Bozkurt, 2005; Tüfenkçi et al., 2006b; Yürük and Bozkurt, 2006; Singh and Agrawal, 2010). This is probably because of the changes in the nutrient element composition of soils with sewage sludge. Indeed, Antolin et al. (2005) reported in their study that with the addition of long-term sewage sludge, there is a decrease in the pH of the soil and increase in the total organic carbon and DTPA extractable heavy metal content. In other relevant studies, it was reported that there was an improvement in the physical and chemical properties of the soil with the addition of sewage sludge. The amount of product and the nutrient element contents increased too (Barzegar et al., 2002; Speir et al., 2003; Sönmez and Bozkurt, 2005; Tüfenkçi et al., 2006b). With a high rate of sewage sludge addition, the cation exchange capacity (CEC) of soils increases (Soon, 1981) and the bioavailability of heavy metals become limited (Kladivko and Nelson, 1979; Kim and Kim, 1999).

With *Rhizobium* inoculation; the phosphorus, potassium, calcium and magnesium contents of straw and grain nitrogen have increased within the years. These increases were 21.3%-9.4%, 44.1%-1.9%, 8.9%-1.8%-10.5%-11.9% and 2.6%-0.3% respectively in lentil straw and 2.9%-7.6%, 6.2%-2.0%, 10.3%-2.6%, 20.2%-3.4% and 8.5%-1.5% respectively in lentil grain (*Table 4, Table 5, Figure 1*). It is reported by some researchers that *Rhizobium* inoculation provides significant increases, especially in the nitrogen content, as well as in plant growth (Marschner, 1995; Al-Karaki, 1999; Kacar and Katkat, 1999; Soumaya et al., 2016; Tüfenkçi et al., 2006a). The physical and chemical properties of soils have a significant effect on microorganism activities (Ham, 1980; Lowendorf, 1980; Haktanır and Arcak, 1997). In addition to being a good source of organic fertilizers with high organic matter and nutrients, sewage sludge should be used with caution due to the high amount of heavy metals they contain (Fernandes, 2005; Bozkurt et al., 2006; Carbonell et al., 2009; Fernandez et al., 2009). Applied sewage sludge affects the physical, chemical and biological properties of soil and therefore affects the microorganism activities. This is also seen in the results reported by some researchers (Chaudri et al., 2008; El-Azhari et al., 2012; Charlton et al., 2016). As a matter of fact, Mattanaa et al. (2014) reported that the effects of sewage sludge on soil microorganisms are different according to the methods applied to the sludge before.

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Antolin et al. (2010) stated that the addition of sewage sludge has no significant effect on nodulation.

The effect of inoculation on grain and straw micro element content compared to non-inoculated plants is evident in iron values. Manganese and zinc were detected only in the grain in the second year. Iron increases in the first and second year straw and grain are respectively 3.8% -24.5% and 19.5% -16.4%. In the second year, 10.4% increase in grain manganese and 14.6% decrease in zinc were determined. It was seen that inoculation in copper did not make a significant difference (*Table 6, Table 7, Figure 2*). Zribi et al. (2012) reported that *Medicago sativa-sinorhizobium* symbiosis could be used for phytoremediation in medium polluted areas with Cd, Cu, Pb and Zn.

The significant effect of *Rhizobium* inoculation on the heavy metal content was determined as an increase in the cobalt content of the lentil straw in the second year and lead in both years by 59.0%, 39.3% and 94.3%, respectively. The effect of inoculation on the grain heavy metal content of lentil is designated in lead and cadmium contents. Despite the increase in lead content in both years, it was seen that only the first year was important. It was seen that there was an increase of 45.9% compared to non-inoculated plants. The cadmium content was decreased with inoculation by 49.2% in the first year and 13.4% in the second year. The decrease with inoculation in the cobalt content during the first year was 23.6%, but this decrease was deemed insignificant (*Table 8, Table 9, Figure 3*). Akhtar et al. (2018) reported that *Rhizobium* inoculation has a positive effect on metal contaminated areas. Ghnaya et al. (2018) reported that *Rhizobium* inoculation in cadmium-contaminated environments increased the root cd content of *Medicago sativa* L. and increased the transport to the surface of the plant.

Conclusion

Rhizobium inoculation is particularly recommended in legumes. Due to rapid population increase and technological developments, the amount of treatment sludge is increased. It is currently being investigated for the disposal of sewage sludge, which is an important problem for use in agricultural areas. In this study, the efficiency of treatment sludge use and *Rhizobium* inoculation in legumes was investigated. As a result, *Rhizobium* inoculation has been found to have a positive effect on both the lentil and the nutrient element content.

In increasing applications of sewage sludge, *Rhizobium* inoculation has been shown to have a more pronounced effect on the straw element content of lentils, especially in the first year trials than in *Rhizobium* non-inoculation.

The most important disadvantage of the use of sewage sludge is the risk of heavy metal contents of soil and plant. In this study, *Rhizobium* inoculation has been shown to have a negative effect on the absorption of heavy metals against increasing sludge applications. The chemical composition of the treatment sludge to be used in such studies should be considered and accordingly the application dose should be adjusted.

As a result, we have determined that the use of sewage sludge is not a risk for heavy metal in terms of both soil and plant and it can be used with *Rhizobium* inoculation.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27012717

RAINFALL EROSIVITY IN NORTH-CENTRAL ANATOLIA IN TURKEY

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(Received 25th Jul 2018; accepted 4th Feb 2019)

Abstract. This study was performed by the reevaluation of historical Universal Soil Loss Equation using field experiment data that was obtained between 1978 and 1995 in Tokat, Turkey. In the study area, the annual average rainfall was 452 mm and about 50% of erosive rainfall events were less than 10 mm. The duration of average erosive rainfall event was 6 h and the average event intensity was 3.83 mm h^{-1}. The average erosive rainfall time in daylight (06:00-18:00), night (18:00-06:00) and mix rainfall conditions were 223.1 and 191.6, 450.2 min, respectively. The maximum rainfall amount, event intensity, storm erosivity, the maximum intensity of 10 min (IM10), the maximum intensity of 15 min (IM15), the maximum intensity of 30 min (IM30) were mostly occurred in daylight erosive rainfalls. But the maximum soil loss and runoff produced by night erosive rainfall. This situation was attributed to the short-term effect on soil erodibility of day and night temperature differences in spring which is rainfall erosivity is high for the region. Higher correlation is observed between soil loss and IM10 compared to IM30. USLE R-factor showed a good correlation with Modified Fournier Index (MFI) for the region. The Mann–Kendal and Theil–Sen Slope estimator statistics to detect the direction and magnitude of an available monotonic trend in some erosivity parameters as well as soil loss and runoff. Trend analysis showed monotonic decreasing of all rainfall physical properties, soil loss and runoff.
Keywords: *rainfall erosivity, modified Fournier index, erosion index, trend analysis, soil loss, runoff, Turkey*

Introduction

Erosion is the most severe and widespread environmental problem in Turkey, as well as in worldwide. In the water erosion, the rainfall erosivity is the major factor for soil loses. The universal soil loss equation (USLE) was the most common prediction model and conservation planning tool in the United States and worldwide (Wischmeier and Smith, 1978; Beasley et al., 1986; Young et al., 1989; Sharpley and Williams, 1990). The rainfall erosivity factor (R) is the one of USLE factors and represents the multiplication of rainfall energy and maximum 30 min intensity (EI30) by erosive rainfall events in a year. This rainfall erosivity index is widely used for empirical soil loss prediction. Its calculation, however, requires high temporal resolution rainfall data that are not readily available in many parts of the world (Xie et al., 2014).

Rainfall erosivity is influenced by the rainfall type, amount, occurred time and intensity of storm. In many parts of world, pluviograph records are not sufficiently available in rural areas to calculate erosivity (Renard and Freimund, 1994; Yu and Rosewell, 1996a). This limitation has, however, been met by the estimation of R values from rainfall amount (Ferro et al., 1991; Salako et al., 1995). Several empirical equations such as the KE > 25 (Hudson, 1971), the AI_m (Lal, 1976), the Fournier index (Fournier, 1960), the Modified Fournier Index (Arnoldus, 1977) and the physically-based A index (Sukhanovski et al., 2002) have been setting to handle R factor values from rainfall amount (Richardson et al., 1983; Renard and Freimund, 1994; Yu and Rosewell, 1996b; Salako, 2008).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

In a study conducted in Santa Catarina, Brazil, daily precipitation graphs between 1989 and 2012 were used to calculate the various indices of precipitation erosion. The indices were correlated with the respective soil losses from the standard plot of USLE and the 30 min erosivity index (EI30) of rainfall recommended for the region (Jefferson et al., 2014).

Renard and Freimund (1994) suggest the use of the MFI for areas where long term data is not available. The Fournier index has been used to describe erosivity, in several countries, e.g. Germany (Sauerborn et al., 1999), Argentina (Busnelli et al., 2006), Spain (Angulo-Martínez and Beguería, 2009), Jordan (Eltaif et al., 2010), Cape Verde (Sanchez-Moreno et al., 2014), Greece (Efthimiou, 2018).

Despite the importance of the effect of rainfall characteristics (duration, occurrence time, etc.) on erosion occurrence and sediment delivery process, studies conducted on this subject have been limited in the past decades. In a study, the role of rainfall variability and extreme events were studied in long-term landscape development. The result showed that low-intensity rainfall events with long durations, large magnitudes and less return periods, contribute significantly to total soil erosion (Baartman et al., 2013). Rainfall duration is among the most effective characteristics of erosivity factor on runoff, splash and soil loss (Katebikord et al., 2017). In Masse (Central Italy) rainfall data were used to determine of erosive characteristics of the region between 2008-2012 years. Within the data period, the rainfall total durations varied from 0.3 to 97.3 h. A total of 228 single rainfall events was identified $p \geq 1$ mm 60 of them were named erosive events and the other 168 have not produced runoff and soil loss and were named non-erosive events. In the study, rainfall depth P, and duration D were determined in detail. In the 58 erosive storms have a range of P, and D, respectively of $7.6 \leq P \leq 148.6$ mm, and $0.4 \leq D \leq 97.3$ h, and the 51 non-erosive storms have a range of $1 \leq P \leq 19$ mm, and $0.3 \leq D \leq 19.01$ h. The rainfall of the region were defined certainly erosive if $D > 24$ h and $P > 20$ mm (Todisco, 2014).

The 30 min maximum intensity (IM30) value of individual rainfall event is widely used in various calculations. However, the effects of different rainfall intensity values on runoff and soil losses are investigated. In a study in Iran, the 18 erosion plots were placed on rangeland hill slopes and measured of runoff and soil loss from 2010 to 2011. Thirteen maximum rainfall intensities were calculated and some of them are presented as maximum intensity in 10, 20, 30, 40, 50, 60 and 90 min. The kinetic energy of storms was computed by USLE R factor computation procedure (Wischmeier and Smith, 1978). The relationship between rainfall intensity and soil loss were varied across intensities. In low rainfall intensities a linear function is fitted to soil loss-rainfall intensity, and in high rainfall intensities nonlinear functions are fitted to soil loss-rainfall intensity (Mohamadi and Kavian, 2015).

Runoff plot observations under natural rainfall conditions represent the primary method for conducting water and soil conservation studies (Araya et al., 2011; Phan et al., 2012). However, performing plot observations experiments is a time-consuming and resource-intensive process (Cerdan et al., 2010). In Turkey, a project was carried out to determine some factor values of USLE in 15 locations has different soil and climatic conditions. The research results of all study locations were reported in a guide (Oğuz et al., 2006). Since erosivity varies significantly from year to year, at least 15 years of data are required to obtain representative estimates of annual erosivity (Foster et al., 2003). In the project, the study duration were chosen about 20 years to better represent the climate and soil conditions of study locations. Therefore, the effect of humid, dry and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

normal climatic conditions on soil and water losses is considered to be sufficiently represented. Tokat region was one of the study locations of this national project. Soil losses and runoff records by erosive rainfall of the natural erosion plots were kept regularly between 1978-1995 years. The purpose of that study was only determine some USLE factor values of study locations so only a limited part of the data were presented. It was thought that the reevaluation of this long period natural erosion plot data could contribute to the solution of the current erosion problems of Tokat region. For this purpose this article is structured in three sections. The first part of this research we present rainfall characteristics of the study area such as several times maximum intensities, erosive rainfall duration and amounts, occurrence times of soil and runoff loss, and the correlation between soil loss, runoff and some several time maximum intensities.

Secondly, comparison of the Modified Fournier Index (MFI) and USLE R factor values for individual rainfall events.

Finally, we analyzed the trend of some rainfall characteristics, soil loss and runoff amounts and estimated the steepness of the slope used the Mann-Kendall for testing trend and Sen’s slope estimator to determine trends of variables in Tokat region for the study period.

Materials and methods

The study was based on reevaluated historical natural Universal Soil Loss Equation plot data between in 1978 and 1995 in Tokat, Turkey (40°18’N, 36°34’E). Tokat is located in North Central Anatolia and lies at an average altitude of 600 m. It is situated in the area of transition from Central Anatolia to the Middle Black Sea region. The study area contains plain to moderately steep area with high vegetation entities and/or under cultivation. The major vegetation types in plain area are vegetable, sugarbeat, potato and wheat. Where, orchard and wheat are common in mild steep areas, and grassland with Graminea and Fabaceae as dominant species in the moderate steep areas, other types being shrub and meadow. The mean annual temperature is 12.6 °C and the annual precipitation is 452 mm.

Study is conducted in Akis Soil Series, Typic Ustorthent which are moderate to well drained soils, and with standard USLE conditions at a site with the standard slope steepness of 9% and slope length of 22.13 m. Runoff plot was framed with a 20 cm high border, to keep precipitation and runoff within the plot area. The plot had a collecting tank and a container tank (0.56 m diameter and 1.00 m deep) at the lower end of the slope to catch runoff and sediment.

Hourly rainfall data were collected using a tipping bucket rain gauge with totals recorded every 24 h from 1978 to 1995. The rainfall charts from 1978 to 1995 were used to handle a data set of some rainfall characteristics such as amount (mm), duration (min) and time of occurrence (the 24 h day was separated as 06:00-18:00 h (daylight), 18:00-06:00 h (night) and if the rains occurred during both period we called mix). The maximum intensities of 10 (IM10), 15 (IM15) and 30 (IM30) min for each erosive rainfall were calculated from rain charts. Rainfall erosivity is computed using USLE-R Factor calculation procedure (Wischmeier and Smith, 1978) with the help of erosion index values of 30 min only (EI30). About 373 rainfall charts were analyzed for this purpose.

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):2719-2731.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

Rainfall erosivity was evaluated with USLE-R factor and Modified Fournier Indices. The R factor is calculated with the help of *Equations 1–4*.

$$E_u = 0{,}119 + 0.0873\, log\, log\, I \quad \text{(Eq.1)}$$

where I: Intensity, mm h^{-1}; E_u: Rainfall energy per unit of rainfall, MJ ha^{-1} mm^{-1}.

$$E_g = E_u * h \quad \text{(Eq.2)}$$

where E_g: Storm energy, MJ ha^{-1}, h: Rainfall amount, mm.

$$EI = E_g * IM30 \quad \text{(Eq.3)}$$

where EI: Single storm erosivity, MJ mm ha^{-1} h^{-1}; IM30: the maximum 30 min intensity, mm h^{-1}.

$$R = \sum (E_g * IM30) \quad \text{(Eq.4)}$$

where R: Annual erosivity which occurred within in a year (R factor), MJ mm ha^{-1} h^{-1} y^{-1}.

Fournier is developed an erosivity index (Fournier, 1960) and this index was modified by FAO (FAO, 1977) as follows (*Eq. 5*):

$$MFI = \sum_{i=1}^{12} \frac{pi^2}{P} \quad \text{(Eq.5)}$$

where pi is the rainfall in a month, mm and P is the annual rainfall, mm.

In the meteorological variables, parametric and non-parametric approaches have been used over years. The most frequently used tests for identifying the variations in meteorological variables have been nonparametric. The most popularized approach among them is Mann–Kendal test (Kendall, 1975; Mann, 1945). The Mann-Kendall nonparametric test was used to test for trend. This test is calculated as *Equation 6* (Mohtar et al., 2015; Yurekli, 2015):

$$S = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \mathrm{sgn}(x_j - x_i) \quad \text{(Eq.6)}$$

where n is the number of event, x_j and x_i are the observed events, and sgn is the sign function.

The variance is given by *Equation 7:*

$$\sigma_S^2 = 18^{-1}\left[n(n-1)(2n+5) - \sum_{i=1}^{m} t_i(t_i - 1)(2t_i + 5)\right] \quad \text{(Eq.7)}$$

where n is number of events, m is the number of tied events, ti is the number of events that are tied. For sample size greater than 10 the test can be given in *Equation 8:*

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DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

$$Z_{MK} = \begin{cases} \frac{S-1}{\sqrt{\sigma_S^2}} & \text{if } S > 0 \\ 0 & \text{if } S = 0 \\ \frac{S+1}{\sqrt{\sigma_S^2}} & \text{if } S < 0 \end{cases} \quad \text{(Eq.8)}$$

For increasing rainfall trends, the values of Z_{MK} is positive and for decreasing trend the value of Z_{MK} is negative.

Sen's estimator for slope is a nonparametric test for the steepness of the trend. For N pairs of data (*Eq. 9*):

$$Q_k = \frac{x_j - x_i}{j - i} \text{ for } k = 1, ..., N \quad \text{(Eq.9)}$$

where Xj and Xk are the values at times j and k (j > k), respectively. The N values of Qk are ranked from smallest to largest. According to condition that N is odd or even, the median concerning with total N values of is calculated by *Equation 10:*

$$Q_{med} = \begin{cases} Q_{\left[(N+1)/2\right]} & \text{if N is odd} \\ 2^{-1}\left\{Q_{\left[(N)/2\right]} + Q_{\left[(N+2)/2\right]}\right\} & \text{if N is even} \end{cases} \quad \text{(Eq.10)}$$

The confidence interval for Q_{med} was obtained to determine the significance of the slope.

Results and discussion

Rainfall distribution: The mean monthly rainfall and the coefficient of variation of the rainfall were recorded from 1978 to 1995 (*Table 1*). The annual average rainfall was 452 mm, the minimum rainfall occurred in August and the maximum in May. The maximum and minimum annual average rainfall occurred in 1993, with 604.5 mm and in 1994 with 311.7 mm, respectively.

The monthly rainfall was divided into 13 groups considering the frequencies. Monthly storm amount distribution between 1978 and 1995 in Tokat is given in *Figure 1,* which shows that about 78% of monthly rainfall amounts were less than 60 mm and only about 22% of monthly rainfall exceeded 60 mm. In the region, the monthly rainfall exceeding 70 mm was relatively low. 90-100 mm and 120-130 mm monthly rainfall amount group was observed quite low frequency. No frequency was observed in the 110-120 mm monthly storm amount group.

In the study period, totally 373 erosive single storm were occurred. Erosive single rainfall events distribution between 1978 and 1995 in Tokat is given in *Figure 2*. As shown in *Figure 2,* about 50% of erosive rainfall event were less than 10 mm and about 30% of erosive rainfall events were 20 mm. Only about 20% of erosive rainfall events

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

were between 30 mm to 130 mm. There is a negative correlation between erosive rainfall amount and frequency. This result showed that the erosive rainfall events of Tokat region is generally occurs in low amounts.

Table 1. *Monthly and annual averages of rainfall between 1978 and 1995 at Tokat city*

Month	Rainfall amount, mm	Coefficient of variation, %
Jan	44	54
Feb	35	36
Mar	35	66
Apr	59	50
May	66	52
Jun	37	68
Jul	13	125
Aug	6	114
Sep	17	87
Oct	42	79
Nov	54	63
Dec	42	43
Annual average	452	

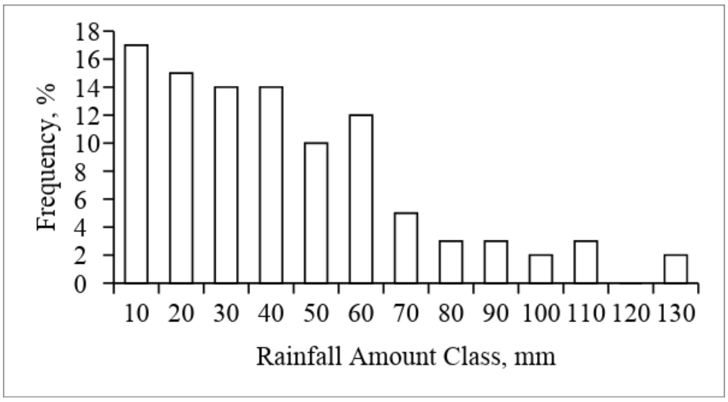

Figure 1. *Monthly storm amount distribution between 1978 and 1995 at Tokat city*

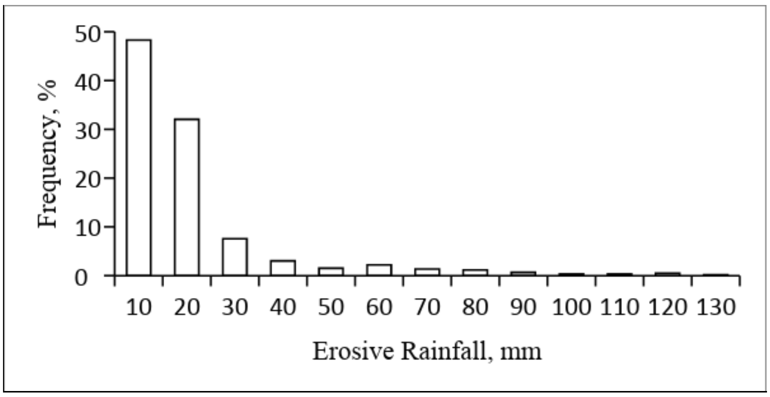

Figure 2. *Erosive rainfall amount distribution between 1978 and 1995 in Tokat*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

Rainfall duration, event intensity and time of occurrence: The average erosive rainfall event duration was 6 h and the average erosive event intensity (amount for an event/total duration) was 3.83 mm h^{-1}. Erosive rainfall was separated according to daylight, night and mix as presented in *Table 2*, where differences between rainfall characteristics and soil loss and runoff in different erosive rainfall time of occurrence, are shown. In the study period, the number of the erosive rainfall occurred in daylight, night and mix conditions were 74, 73 and 226, respectively.

Average erosive rainfall duration in daylight, night and mix rainfall conditions were 223.11, 191.58, and 450.18 min, respectively (*Table 2*). The average night erosive rainfall duration was observed shorter than the other types. Mix rainfall has been found having the longest rainfall duration. Jingi et al. (2011) studied the effect of rainfall duration (5, 10, 15, 20, 25, and 30 min) on separation of soil grains and splash erosion using splash collecting container, and their results indicated significant positive effect of rainfall duration on soil splash. But under natural rainfall conditions, an erosive rainfall has usually variable intensities. When a rain takes place in a prolonged period of time, it generally causes the variables intensities of this rain to achieve lower energy levels. Therefore, the long erosive rainfall duration mostly causes less soil loss and runoff than shorter erosive rainfall duration. The erosive rainfall duration of daylight took place between erosive rainfall duration of mix and night conditions. The average night erosive rainfall amount was also at the lowest levels. The daylight was moderate, and the mix had maximum rainfall amount.

Average erosive event intensity in daylight, night and mix rainfall conditions were 6.92, 4.51, 2.60 mm h^{-1} respectively. Maximum event intensity took place in daylight erosive rainfall and minimum was in mix rainfall. Likewise storm erosivity (EI) was 35.72, 23.80 and 18.65 respectively in daylight, night and mix rainfall (*Table 2*).

In the study, maximum rainfall intensities (IM10, IM15 and IM30) of each erosive rainfall are grouped for different time occurrences (daylight, night and mix rainfall conditions) and we observed the maximum average values in daylight rainfall (*Table 2*). Average erosive rainfall intensities (IM10, IM15 and IM30) were occurred the second in night and the third in mix rainfall.

Soil losses and runoff values are grouped for three different time occurrences (*Table 2*). The maximum soil loss and runoff are occurred in night rainfall. Even though in night erosive rainfall had less storm erosivity, event intensity and several time maximum intensities (IM10, IM15 and IM30) than daylight erosive rainfall, the most soil loss and runoff was observed. This result might be caused by day and night differences in soil conditions like infiltration, evaporation, etc. On the other hand, because of the longer mix rainfall duration more rain infiltrated in the soil by decreasing the runoff eventually the soil loss. Various rainfall properties such as the duration, intensity and its energy have more impact on the erosion compared to the rainfall amount. Although, the average erosive night rainfall amount is less than the mix erosive rainfall amount, the maximum runoff occurred in night rainfall because of soil hydraulic conditions.

The study showed that mix erosive rainfall characteristics were common in the Tokat region. However, because these rainfalls have lower event intensity, storm erosivity and several time intensities, they presented lower erosion risk. Interestingly, daylight rainfalls which have higher erosivity parameters (event intensity, storm erosivity and several time intensities) compared to night erosive rainfalls caused less soil loss. It is believed that this situation was caused by the day and night time temperature

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

differences causing short time changes in the soil erodibility. As a matter of fact, erosive rainfalls are widely observed during April, May and June, when the day and night time temperatures are quite high.

Table 2. *The relation between some properties of the erosive rainfalls and soil losses and runoff in Tokat*

Rainfall time	Descriptive statistics	Event duration min	Erosive rainfall amount mm	Event intensity mm h^{-1}	Storm erosivity MJ mm ha^{-1} h^{-1}	IM10 mm h^{-1}	IM15 mm h^{-1}	IM30 mm h^{-1}	Soil loss ton ha^{-1}	Runoff mm
Daylight rainfall	Average	223.11	10.15	6.92	35.72	18.31	14.25	9.68	3.87	2.43
	Minimum	18.00	0.55	0.15	1.03	1.80	1.60	1.60	0.001	0.12
	Maximum	732.00	35.30	60.51	768.36	199.80	134.00	78.80	19.32	13.72
	St. dev.	164.78	6.63	13.67	132.51	34.68	23.56	14.78	6.15	4.15
	Variance	27524.78	44.19	187.44	17658.08	1205.00	555.80	218.70	38.03	17.29
	CV	73.86	65.38	197.55	371.01	189.43	165.35	152.74	158.99	171.03
Night rainfall	Average	191.58	8.71	4.51	23.80	15.20	12.34	8.57	6.78	2.91
	Minimum	18.00	2.50	0.90	0.51	2.40	1.90	1.40	0.002	0.04
	Maximum	740.00	33.10	57.00	481.05	109.17	80.35	54.80	27.51	15.35
	St. dev.	142.94	4.98	6.81	63.53	17.66	12.95	8.40	10.02	4.74
	Variance	20715.69	25.11	47.04	4091.66	316.18	169.92	71.50	120.40	25.01
	CV	74.61	57.15	150.89	266.89	116.21	104.92	97.98	147.81	163.15
Mix rainfall	Average	450.18	12.44	2.60	18.65	10.73	8.72	6.32	0.79	1.66
	Minimum	25.00	0.20	0.07	0.49	0.42	0.30	1.00	0.0003	0.01
	Maximum	2172.00	44.30	20.67	279.35	85.23	62.97	43.00	8.22	15.92
	St. dev.	349.41	7.89	2.86	39.10	12.92	9.40	6.20	1.68	3.07
	Variance	122630.05	62.46	8.21	1535.35	167.54	88.80	38.67	2.90	9.59
	CV	77.62	63.38	110.00	209.64	120.41	107.80	98.19	213.82	184.45

Correlations between soil loss, runoff and several time maximum intensities (IM10, IM15 and IM30): Some descriptive statistics for soil loss, runoff and several maximum time intensities are presented in *Table 3*, which reveals that, the higher CV of variables occurred in IM10, IM15, IM30, soil loss and runoff respectively. When the maximum intensities are compared, the CV values of IM10 and IM15 are more changeable than IM30.

Table 3. *Descriptive statistics of soil loss, runoff and several time maximum intensities*

Properties	Min.	Max.	Mean	St. dev.	Variance	CV
Soil loss, ton ha^{-1}	0.0003	27.51	2.20	4.88	24.28	221.66
Runoff, mm	0.01	15.92	2.00	3.50	12.38	174.64
IM 10, mm h^{-1}	0.42	199.80	13.10	18.22	332.76	139.01
IM 15, mm h^{-1}	0.42	134.00	10.53	12.95	168.27	123.07
IM 30, mm h^{-1}	1.00	78.80	7.43	8.45	71.57	113.77

The correlations between soil loss, runoff, IM10, IM15 and IM 30 are presented in *Table 4*. According to correlation analyses, there is strong positive correlation between properties (soil loss and runoff) and IM10, IM15 and IM30 values. The IM30 parameter has been shown to be a better predictor of sediment yield than rainfall depth (Foster et

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

al., 1982). Even though IM30 is widely adopted in the empirical estimation of event soil loss (Bagarello et al., 2013; Kinnell, 2010), in our study, we found the maximum correlation between soil loss and IM10 values. This may result IM10 values to describe erosivity as a better parameter than IM30 for the region. IM15 values are medium correlated between soil loss and IM15 values, while runoff and IM30 parameters showed maximum correlation.

Table 4. *Correlation coefficients between soil loss, runoff, IM10, IM15 and IM 30*

Properties	IM10, mm h^{-1}	IM15, mm h^{-1}	IM30, mm h^{-1}
Soil loss, ton ha^{-1}	0.746^{**}	0.717^{**}	0.562^{**}
Runoff, mm	0.805^{**}	0.781^{**}	0.815^{**}

$^{**}p < 0.01$

Trend and slope analyses: Trend analysis gave appropriate data to describe trend and slope of some rainfall characteristics, MFI index, soil loss and runoff for the region. The Mann-Kendall test and Sen's slope estimator were used for this purpose. The Mann-Kendall statistics for trend and Sen's slope estimator is given in *Table 5* for Tokat city. The Mann-Kendall statistics showed significant trend for all parameters. The Sen's slope estimator had negative slope for research period for all parameters. The calculated p values are smaller than the critical p value from the table of the standard normal distribution. The Ho hypothesis related to no monotonic trend was rejected for all data sets.

Rainfall erosivity is the ability of rainfall to cause erosion due to the function of erosive characteristic of rainfall (Mikhailova et al., 1997). In Tokat region, some physical characteristics of rainfall which is related to rainfall erosivity showed decreasing trend whole study period. Likewise, similar trends exist for soil losses and runoff.

Table 5. *Trend and slope results for study area for study period (1978-1995)*

Parameters	z	p-value	Sen	CI for Sen
Daily rainfall, mm	-3.50	0.00	-0.063	(-0.085, -0.044)
Erosive rainfall, mm	-2.47	0.01	-0.013	(-0.019, -0.004)
Erosive rainfall duration, h	-3.78	0.00	-0.034	(-0.046, -0.025)
R factor, MJ mm ha^{-1} h^{-1}	-2.27	0.01	-0.010	(-0.023, -0.002)
MFI	-3.50	0.00	-0.063	(-0.085, -0.044)
IM10, mm h^{-1}	-3.11	0.00	-0.135	(-0.187, -0.060)
IM15, mm h^{-1}	-4.05	0.00	-0.070	(-0.092, -0.046)
IM30, mm h^{-1}	-2.82	0.00	-0.011	(-0.020, -0.005)
Runoff, mm	-3.50	0.00	-0.063	(-0.085, -0.044)
Soil loss, ton ha^{-1}	-2.47	0.01	-0.018	(-0.035, -0.004)

Comparison of R factor with Modified Fournier Index for the region: Computation of the erosion index (EI), which is basic for the determination of the R factor (Rain Factor) of Universal Soil Loss Equation (USLE) is tedious and time consuming and requires continuous records. Although there is pluviometer data in some rural areas but

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

pluviograph data is not available in most areas. To handle this handicap some researchers have introduced various indexes or models. The Modified Fournier Index (MFI) is one of them. Several studies (Renard and Freimund, 1994; Diodato and Bellocchi, 2007; Lee and Heo, 2011; Taguas et al., 2013; Yue et al., 2014) have shown a high correlation between the R factor and rainfall parameters such as the MFI. In this study we used Modified Fournier Index (MFI) and R factor of USLE to estimate and compare each method to describe erosivity. To have a better understanding of MFI performance, 216 monthly MFI values and R factor values were compared. A strong correlation between them (r = 0.516**) was obtained. Monthly correlation coefficients and some descriptive between R and MFI values are given in *Table 6*. Both methods presented good correlation. The best correlations and maximum coefficient of variance were found in summer session.

Table 6. *Monthly correlation coefficients and some descriptive between R and MFI values*

Month	Correlation coefficient	R values					MFI values				
		Min.	Max.	Mean	St. dev.	CV	Min.	Max.	Mean	St. dev.	CV
Jan	0.822**	0.00	5.22	1.86	1.61	86.56	0.35	17.16	5.72	5.31	92.83
Feb	0.707**	0.00	3.61	1.16	1.04	89.66	0.59	10.54	3.24	2.61	80.56
Mar	0.694**	0.00	5.03	1.22	1.43	117.21	0.01	23.86	4.03	5.49	136.23
Apr	0.632**	0.79	36.05	7.45	8.65	116.11	1.52	34.27	9.34	8.93	95.61
May	0.862**	1.34	83.79	13.63	21.00	154.07	1.40	67.18	12.21	15.05	123.26
Jun	0.846**	0.00	138.13	13.11	31.92	243.48	0.25	17.70	3.94	4.54	115.23
Jul	0.985**	0.00	31.39	2.76	7.44	269.57	0.00	10.33	1.00	2.43	243.00
Aug	0.903**	0.00	26.18	1.76	6.12	347.73	0.00	1.47	0.20	0.36	180.00
Sep	0.912**	0.00	58.85	8.41	17.29	205.59	0.00	4.79	1.11	1.50	135.14
Oct	0.689**	0.00	10.79	3.96	3.57	90.15	0.03	33.10	5.82	8.66	148.80
Nov	0.696**	0.00	8.31	2.70	2.43	90.00	0.01	27.32	8.18	7.96	97.31
Dec	0.646**	0.00	3.00	1.09	0.83	76.15	0.63	15.66	4.46	3.57	80.04

**$p < 0.01$

Conclusion

The rain gauge data from 1978 to 1995 were analyzed for Tokat, located in the middle Black Sea region in Turkey, in order to characterize rainfall erosivity. The annual average rainfall was 452 mm. The EI values of the erosive rainfalls during the research period generally were low. A significant portion of the erosive rainfalls is lower than 10 mm. Erosive rainfalls usually took place during April, May and June. The highest EI values observed during the daylight erosive rainfalls, however with the temperature increasing during the day soil presented lower erodibility. It is assumed that this situation is linked to the higher soil temperature observed during the daylight rainfalls increasing the infiltration and evaporation, causing lower levels of runoff. Taking this result into consideration, it is recommended to carry out rainfall erosivity studies considering the soil losses and runoff as well. Rainfall erosivity studies carried out supported by soil losses and runoff will provide more reliable results.

Night erosive rainfalls which presents highest soil loss and runoff, created only 19.57% of all the erosive rainfalls. Although they have lower EI value than the daylight rainfalls, night erosive rainfalls caused more soil loss and runoff. Night erosive rainfalls almost created twice the soil loss compared to daylight rainfalls.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

The Modified Fournier Index (MFI) was calculated using long term monthly data between 1978 and 1995. The MFI and R factor of USLE was compared and presented a strong correlation between them. This result shows MFI is useful an index to estimate rainfall erosivity which is not available pluviograph data for Tokat region.

According to trend analysis test results, the revealed findings indicated that the negative trends of all test parameters for the region. Considering to this result, a decrease tendency was determined in the erosion parameters of the region over time.

Tokat region rainfalls presented low erosivity. Mostly mix erosive rainfalls were occurred in study period. Mix erosive rainfalls have longer event duration but lower energy. Therefore, these rainfalls have lower erosion risk. Erosive rainfalls form almost 61% of the erosive rains. Although daylight and night erosive rainfalls have higher erosion risk, being low in ratio decreases the erosion risk. Soil losses can be taken under control by applying cultural practices on individual farmer basis, such as contour farming, no tillage, forage crop farming etc. Taking these precautions for sustainable farming does not create high levels of costs.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27192731

THE EFFECT OF DIFFERENT HARVEST AIDING CHEMICALS ON YIELD AND YIELD COMPONENTS OF COTTON (*GOSSYPIUM HIRSUTUM* L.)

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(Received 6th Dec 2018; accepted 4th Feb 2019)

Abstract. This study was conducted to determine the effect of different harvest aid chemicals on the yield and yield components of cotton in the experimental area of Osmanbey Campus in Harran University. The method employed in the study was the randomized complete block design with three replications in growing seasons in 2012 and 2013. The Stoneville-453 cotton cultivar was the subject of the study. Commercial names and compounds of the chemicals used in the experiment were as follows: Sonround (480 g/l Glyphosate), Efhun (Ethephon) (480 g/l Ethephon), Drop Ultra (120 g Thidiazuron + 60 g Diuron), Finish (480 g Etephon + 60 g Cyclanalide), and Appeal (54 g Fluthiacet-Methyl). Including the control plots, 10 treatments were performed when the bolls were 60% open. It was concluded that while the highest seed cotton yield was obtained from the Drop Ultra 600 cc ha^{-1} (5422.7 kg) and Appeal 75 ml ha^{-1} + Efhun 3000 ml ha^{-1} (5382.3 kg) applications in 2012, it was Sonround 3000 ml ha^{-1} (4150.7 kg) that produced the highest yield in 2013. On the other hand, the highest earliness ratio was obtained from the Drop Ultra 300 cc ha^{-1} + Efhun 3000 ml ha^{-1} treatment (96.30% and 96.30%) in both years. In the second year of the experiment, seed cotton yield and the number of bolls were lower when compared to the first year. The combination and single mixture treatments had different effects on examined properties.
Keywords: *Gossypium hirsutum L., defoliant, earliness ratio, boll weight, open boll percentage*

Introduction

Since cotton has a perennial and indeterminate growth property, it continues its vegetative growth as long as the conditions are appropriate. Thus, maturation is delayed (Stewart et al., 2000; Bondada and Oosterhuis, 2001). It is therefore important to harvest quality cotton on time. If a clean seed cotton harvest and higher harvest efficiency are desired, cotton must be defoliated before harvest either by hand or machine. Defoliation can shorten boll opening time by as much as 1-3 days, and provide an earliness ratio ranging between 1% and 20% in the first-hand seed cotton harvest (Oğlakçı and Gençer, 1992; Du et al., 2013).

Defoliation can be defined as the process of removing leaves when they become physiologically matured. Defoliation occurs when a release layer is formed at the point where the leafstalk (petiole) is attached to the plant stem (Görmüş, 2014). In order for the separation to come up in the leaves, certain conditions such as water stress (excess water or dehydration) need to be eliminated (Silvertooth, 2001). Separation of leaves, square, and bolls starts only when stress conditions are eliminated. However, separation can also take place within 24 hours after irrigation is applied to the dehydrated plants. In this case, defoliation takes place within 4-6 days (Osborne, 1974). For this reason, defoliation should not be delayed more than 4-5 weeks after the last irrigation.

Defoliants increase the synthesis of ethylene in the cotton plant. By creating an extra layer and encouraging the formation of a release layer at the point where the leafstalk joins the branch or main stem, they cause the leaf to break off. Meanwhile, the increase

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DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

of ethylene in plant tissues causes the rise of the auxin hormone, which promotes the opening of bolls (Morgan et al., 1977; Cathey, 1985; Suttle, 1988).

In addition to assisting the harvest, defoliation provides unspotted fiber, which results in better grades, faster and more efficient picking, faster drying of the dew, and thus early picks. It also helps delay the seed coat decay and encourage the boll opening (Edmisten, 1998). Therefore, it is necessary to use defoliants in order to accelerate the opening of the bolls before harvest, to increase harvesting efficiency, to reduce the seed cotton moisture, fiber contamination, and negative effects of pest and diseases populations (Oğlakçı, 1992). A plant, to which a defoliant or harvest-aid chemicals were applied, might have immature bolls on its top. As a general rule, the maturation of the first boll, placed at the 4^{th} or 5^{th} node downwards from the top boll on the plant to be collected, was used as a main determinant for the defoliant application time (Oğlakçı and Kaynak, 1992; Larson et al., 2005; Çopur et al., 2010). In defoliant applications, obtaining an optimum usage experience and desired result depends on when the farmers apply these defoliants rather than which chemicals they use (Edmisten, 1998). For this reason, the most suitable time of defoliation should be determined by considering genotype properties and region conditions (Çopur et al., 2010).

As a result of early defoliation, the yield is reduced and the fiber quality is negatively affected (Snipes and Baskın, 1994). Similarly, in the case of late defoliation, adverse weather conditions can be a problem (Kerby et al., 1992), and due to low temperature conditions, defoliation cannot be achieved sufficiently. However, late defoliations enable the development of immature bolls, thereby increases the yield. Defoliant timing has also a significant influence on the fiber quality. Too early or too late defoliation affects the fiber quality adversely (Samani et al., 1999; Wright et al., 2014). Early defoliation can be critical for maximum yield. On the other hand, the risk of yield loss as a result of the rain and early frost damages in winter season might be increased when defoliation is delayed (Bange and Milory, 2001).

Many researchers used defoliants and boll-opening chemicals that assist harvesting in their studies (Snipes and Baskın, 1994; Çopur et al., 2010; Du et al., 2013; Singh et al., 2015; Tashaev et al., 2016). Ethephon is a boll opener that is widely used as a chemical aid during the harvest. It increases the yield by increasing the number of opened bolls (Gwathmay and Hayes, 1997; Du et al., 2013; Singh et al., 2015).

While some researchers used a single chemical, others employed a mixture to achieve better results in harvest. If one or more defoliants and boll openers are mixed in the combinations, the activity and response of the defoliant increase positively (Snipes and Cathey, 1992; Gwathmay and Hayes, 1997; Du et al., 2013).

The joint use of defoliant and Etephon increases the rate of first-hand seed cotton yield significantly. But it does not have any effects on the boll weight, ginning outturn, and fiber properties (Du et al., 2013). Moreover, defoliant mixture and Ethephon (Thidiazuron+Ethephon and Thidiazuron-Diuron+Ethephon) can increase the number of opened bolls and enhance defoliation efficiency. They also increase the first-hand seed cotton yield more than a single defoliant and boll opener usage (Du et al., 2013).

Many studies have been carried out on defoliants and boll openers. In their study with different defoliants (Dropp Ultra and Round Up) and different application times (60, 75, and 90 days after flowering), Çopur et al. (2010) found out that Dropp Ultra treatment, applied 60 days after flowering, decreased the seed cotton yield, the number of bolls, boll weight and fiber index. However, they also pointed out that the delayed defoliant application increased the number of bolls, seed cotton yield and boll weight,

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DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

and that neither of the treatments had any effects on ginning outturn or fiber quality. On the other hand, Awan et al. (2012) reported that the defoliant treatments resulted in a twenty-five-day earlier harvest and provided a better seed cotton yield when compared to the control plots. In addition, they also argued that the treatments had significantly affected fiber fineness and uniformity, but had no significant effects on fiber strength. In a similar study, Singh et al. (2015) suggested that, after using the defoliants Dropp Ultra and Ethrel (Ethephon), the lowest yield was obtained from the highest dose (Dropp Ultra 225 ml ha^{-1}) while the highest yield was from the Dropp Ultra 200 ml ha^{-1} dose. Moreover, they claimed that the defoliants, applied 150 days after sowing, resulted in a more seed cotton yield when compared to the defoliants applied 140 days after sowing. They also stated that Dropp Ultra defoliant contributed better to the boll opening.

The Harran plain, where the study was conducted, is one of the most important cotton production centers in Turkey. The size of cotton sowing areas and the volume fibre production have increased significantly because of the increase in the number of irrigated lands following the GAP (Southeastern Anatolia Project).

However, in some years, the harvest is significantly delayed due to early autumn rainfalls. Seed cotton harvesting in this region is mostly performed with combine. Therefore, defoliation and boll opening are of great importance for increasing the combine harvest efficiency and a cleaner cotton harvest.

This study was carried out to determine the most appropriate dose or doses of different harvest-aid chemicals (single or mixture) and their effects on the yield and yield components of cotton.

Material and methods

The study was conducted in the experimental area of Osmanbey Campus in Harran University in accordance with randomized complete block design with three replications in 2012 and 2013 growing seasons. The experimental field is located in Harran Plain (altitude: 465 m; 37°08' North and 38°46' East) near the Turkish-Syrian border (*Figure 1*).

Figure 1. *The experimental field*

Stoneville-453 cotton cultivar was used as the study subject. ST-453 cotton cultivar Stonoville pedigreed seed co. was developed and registered in 1988 (Calhoun et al., 1997). In our region it was certificated in 1995 and has been widely used since then.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

Especially earliness at an intermediate level, high yield and 42% gin outturn were the main reasons for farmers' preferring this cotton cultivar (Harem, 2010).

In the experiment, each plot was established with a length of 12 m, 6 rows, 70 cm inter-rows and 15 cm intra-rows spaces (*Figure 2*). Sowing was done on 14th April in 2012, and on 22th April in 2013 with a pneumatic sowing machine. During the experiment, half of the pure nitrogen (N) and all of the pure phosphorus (P) were applied. The N and P were applied as 70 kg ha^{-1} N and 70 kg ha^{-1} P_2O_5 respectively from a 20.20.0 composed fertilizer at the sowing time. The remaining pure nitrogen was given with the lister tool as 90 kg ha^{-1} just before the first irrigation.

Figure 2. *A photography from the experimental field*

Total irrigation amount during growing seasons (surface irrigation) was about 1100 mm. The first irrigation was applied 3 weeks after sowing and the second one was 3 weeks after that. Thereafter, the plots were irrigated every 2 weeks until the end of the season, thus providing a total of ten irrigations.

The experimental area was a flat and nearly flat land, and its calcium content was 32%. All soil profiles had high clay content (60%) and pH was from 7.3 to 7.4. The organic and salinity level was 0.8% and 0.08%, respectively. It had a high cation exchange capacity of 57.1 meq/100 g. Na content in the 0-150 cm profile was 1.4. The soil belonged to Ikizce soil series and is classified as vertic calciorthid aridisol (Dinç et al., 1988).

The average temperature during the cotton growth period (from April to October in 2012 and 2013) ranged between 19.3-18.4°C in April, 22.4-22.9°C in May, 30.6-29°C in June, 33.3-32°C in July, 32.3-31.6°C in August, 28.4-26.6°C in September, and 21.0-19.3°C in October (*Table 1*). The total precipitation was between 0.2 and 42.3 mm with a monthly average of 18.17 mm in 2012 (April-October), and between 0 and 56.2 mm with a monthly average of 12.17 mm in 2013. In addition, during the experiment period in summer, there was no rain in June, July, August, September, and October in 2013 (Anonymous, 2013).

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Table 1. *The official record of Meteorology Directory, Sanliurfa, Turkey*

Months	2012			2013			1929-2013
	Monthly Average Temp. °C	Precipitation mm	Average Relative Humidity %	Monthly Average Temp. °C	Precipitation mm	Average Relative Humidity %	Annual Average Temp. °C
April	19.3	23.3	42.4	18.4	18.0	44.9	16.2
May	22.4	42.3	40.8	22.9	56.2	43.4	22.1
June	30.6	5.8	21.2	29.0	-	24.0	28.0
July	33.3	0.2	18.8	32.0	-	20.5	31.9
August	32.3	0.2	29.0	31.6	-	22.4	31.5
September	28.4	2.0	28.0	26.6	-	33.3	27.1
October	21.0	12.5	48.5	19.3	-	-	20.5
November	14.9	31.0	65.6	14.8	19.5	57.5	13.1

(Anonymous, 2013)

The compounds of the chemicals applied in the experiment are as follows:

1) Sonround (480 g/L Glyphosate),
2) Efhun (Ethephon) (480 g/L Ethephon),
3) Drop Ultra (120 g/L Thidiazuron + 60 g/L Diuron),
4) Finish (480 g/L Etephon + 60 g/L Cyclanalide),
5) Appeal (54 g/L Fluthiacet-Methyl).

In both years, chemicals were applied as defoliants in accordance with the recommendations by manufacturers. Both chemicals were mixed with water (300 L ha^{-1}) and applied by using a backpack sprayer with a pressure set to 4.22 kg cm^{-2}. The sprayers were calibrated for a 4.80 km h^{-1} walking speed before each treatment.

Ten defoliant treatments were tested as follows:

T_1 : Sonround (3000 ml ha^{-1})
T_2 : Efhun (3000 ml ha^{-1})
T_3 : Drop Ultra (600 cc ha^{-1})
T_4 : Drop Ultra (300 cc ha^{-1}) + Finish (2500 cc ha^{-1})
T_5 : Drop Ultra (300 cc ha^{-1}) + Efhun (3000 ml ha^{-1})
T_6 : Appeal (1500 ml ha^{-1})
T_7 : Appeal (750 ml ha^{-1}) + Efhun (3000 ml ha^{-1})
T_8 : Appeal (750 ml ha^{-1}) + Finish (2500 cc ha^{-1})
T_9 : Finish (2500 cc ha^{-1})
T_{10} : Water spray (control plot)

Harvest aiding chemical treatments were applied on September 13 in 2012, and on September 10 in 2013 (when the bolls were opened at 60% ratio) (Silvertooth, 2001; Edmisten, 2006; Mrunalini et al., 2018). Harvest was made 15 days after the treatments in an area that includes middle two rows of each plot (10 m x 1.4 m = 14 m^2), excluding the 1-meter length from both sides of the plots. The first-hand harvest was done on September 28 and the second-hand harvest on October 27 in 2012, whereas the first-hand harvest was done on September 24 and the second-hand harvest on October 27 in 2013. Twenty plants were randomly chosen from the central row of each plot to

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determine plant height, the number of opened bolls per plant, boll weight (g seed cotton boll^{-1}), seed cotton yield (g plant^{-1}), and plant height (cm). Earliness as percentage of the yield harvested in the first picking was calculated as follows: seed cotton yield in first picking was divided by the total seed cotton yield and multiplied by 100. Total seed cotton yield of each plot (including the twenty plant sub samples) was ginned on a laboratory roller gin stand to determine lint yield (kg lint he^{-1}), lint percent, seed index (g per 100 seeds), and lint index (g of lint per 100 seeds) (Sawan, 2016). Statistical analysis was performed by using the JMP 11 statistical program (SAS Inc.). Means were separated by Fisher's protected least significant differences (LSD) test and $p \leq 0.05$ was denoted as the significance level.

Results and Discussion

Seed Cotton Yield (kg ha^{-1})

Different results were obtained from different defoliant treatments in 2012 and 2013 years. The highest seed cotton yields (5422.7 and 5382.3 kg ha^{-1}) were obtained from the applications of T_3 (Drop Ultra 600 cc ha^{-1}) and T_7 (Appeal 75 ml ha^{-1} + Efhun 3000 ml ha^{-1}) in 2012, and T_1 (Sonround 3000 ml ha^{-1}) application provided the highest seed cotton yield (4150.7 kg ha^{-}1) in 2013 (*Table 2*).

Table 2. *Seed cotton yield, earliness ratio, and number of bolls in cotton with different harvest aid chemical treatments, and groups formed according to the LSD test*

Treatments	Seed Cotton Yield (kg ha^{-1})		Earliness Ratio (%)		Number of Open Bolls (no. plant^{-1})	
	2012	2013	2012	2013	2012	2013
T_1	4844.3 c	4150.7 a	92.23 e	92.23 d	20.07 a	11.50 cd
T_2	5042.7 b	3700.3 bc	93.90 d	93.90 c	20.20 a	12.73 b
T_3	5429.7 a	3765.3 ab	94.53 cd	94.47 c	18.00 c	10.63 e
T_4	5063.3 b	3313.0 c	92.57 e	92.23 d	16.87 d	12.43 b
T_5	5016.7 b	3543.3 bc	96.30 a	96.30 a	17.90 c	11.33 de
T_6	4576.0 d	3552.3 bc	94.30 cd	94.33 c	20.30 a	11.43 d
T_7	5382.3 a	3898.7 ab	95.23 b	95.23 b	19.20 b	11.53 cd
T_8	4933.3 bc	3751.7 ab	94.37 cd	94.37 c	17.90 c	12.23 bc
T_9	4805.7 c	3326.7 c	90.80 f	90.80 e	18.00 c	13.73 a
T_{10} (control)	5014.3 b	3676.0 bc	94.57 c	94.53 c	17.07 d	13.97 a
LSD (0.05)	16.74	41.22	0.64	0.66	0.62	0.75
% CV	1.95	6.55	0.61	0.41	1.94	3.61

* Means in each column followed by the same letter are not significantly different ($p<0.05$)

If one or more defoliants and boll openers are mixed in the combinations, the activity and response of the defoliant increase positively (Snipes and Cathey, 1992; Gwathmay and Hayes, 1997; Awan et al., 2012; Du et al., 2013). This idea is partially confirmed with an increase in treatments when compared to control plots. Thidiazuron accelerates boll dehiscence by increasing ethylene level in cotton leaves (Suttle, 1985). Light penetration is also improved by leaf removal. These crop conditions lead to early maturity and opening of bolls (Malik et al., 1991). Kerby (1988) stated that with stimulation of defoliation process, leaves transport most of their nutrients and metabolites to developing bolls. Results from this study also indicate that defoliants did not cause yield loss or deterioration in fibre quality in a physiologically matured crop.

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In the second year of the experiment, it was observed that there was a decrease in the seed cotton yield in all treatments, which can be associated with the environment and climate conditions. When the climate data of the year 2013 are examined, it is seen that during the growing period (April-October) the weather (*Table 1*) was very dry, evaporation was intensive, and precipitation was too low (Anonymous, 2013).

Earliness Ratio (%)

In both years of the trial, statistically significant differences have been seen among all treatments in terms of earliness ratio. Of all treatments, the best earliness ratio results were obtained from the T_5 subject (Drop Ultra 300 cc ha^{-1} + Efhun 3000 ml ha^{-1}) (96.30 and 96.30%, respectively) (*Table 2*) in both years. Our findings were compatible with the results of Du et al. (2013) who obtained the highest seed cotton yields in earliness ratio from the defoliant and boll opener Thidiazuron+Ethephon and Thidiazuron-Diuron+Ethephon treatments (83 and 87%). Meanwhile, the lowest earliness ratio was obtained from the T_1 (Sonround 3000 ml ha^{-1}) and T_4 (Drop Ultra 300 cc ha^{-1} + Finish 2500 cc ha^{-1}) in 2012, and T_9 (Finish 2500 cc ha^{-1}) in 2013 (*Table 2*).

Number of Bolls (no. plant^{-1})

Different groups were formed among all treatments in terms of the number of bolls in both years. As in seed cotton yield, more boll numbers were obtained in 2012 than in 2013. When the climate data of 2013 is analyzed, it is seen that during the growing period (April-October) the weather was very dry and evaporation was very intensive (Anonymous, 2013).

Whereas, in 2012, T_1 (Sonround 3000 ml ha^{-1}), T_2 (Efhun 300 ml ha^{-1}), and T_6 (Appeal 150 ml ha^{-1}) produced the highest number of bolls, in 2013, T_9 (Finish 2500 cc ha^{-1}) and T_{10} (Control) gave the highest number of bolls (*Table 2*). Similarly, while Çopur et al. (2010) reported that the highest number of bolls were obtained from the both late defoliant application and no defoliant application plots, Görmüş et al. (2017) suggested that the highest number of bolls were obtained from the control plots in the second year. These results are also compatible with our findings. The reason for this situation is that the leaves of the plants in the control plots continue to exist with their vitality and they keep producing photosynthesis on the plant.

Number of Open Bolls (no. plant^{-1})

In both years, statistically different groups were formed in terms of the number of open bolls. In the first year of the experiment, the highest number of open bolls (18.60 per plant) was taken from the T_2 subject (Efhun 3000 ml ha^{-1}) (*Table 3*). However, in the second year of the experiment, T_{10} (Control) formed the highest number of open bolls (12.40 per plant) in all treatments. When the climate data were taken into consideration (Anonymous, 2013), it can be said that since the summer was dry, the plants in the control plots were bent to open more bolls and to be matured.

Number of Unopened Bolls (no. plant^{-1})

As shown in *Table 3*, statistically different groups were formed in terms of the number of unopened bolls in both years. T_{10} (Control) formed a greater number of unopened bolls (1.83 no. $plant^{-1}$) than other treatments in 2012. On the other hand, T_5 (Drop Ultra 300 cc ha^{-1} + Efhun 3000 ml ha^{-1}) mixture yielded the best result

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(0.83 no. plant^{-1}). The result of Singh et al. (2015) illustrates that Dropp Ultra defoliant promotes the boll opening better. This is consistent with our study. For, T_9 Finish (2500 cc ha^{-1}) gave the highest number of unopened bolls (1.83 no. plant^{-1}) in 2013. The best results (0.80 no. plant^{-1}) was taken from the T_1 (Sonround 3000 ml ha^{-1}) application.

Table 3. *Number of open bolls, number of unopened bolls, and boll weight in cotton with different harvest aid chemical treatments, and groups formed according to the LSD test*

Treatments	Number of Open Bolls (no. plant^{-1})		Number of Unopened Bolls (no. plant^{-1})		Boll Weight (g)	
	2012	**2013**	**2012**	**2013**	**2012**	**2013**
T_1	18.47 ab	10.70 cd	1.53 b	0.80 e	6.73 a	5.97 e
T_2	18.60 a	11.63 b	1.60 b	1.07 d	6.30 cd	6.37 bc
T_3	18.87 cd	9.20 e	1.13 cd	1.37 bc	6.40 bc	6.20 cd
T_4	15.87 e	10.97 c	1.00 dc	1.40 bc	6.37 bc	6.67 a
T_5	17.27 c	10.23 d	0.83 e	1.03 de	6.57 abc	5.93 e
T_6	18.33 ab	10.23 d	1.63 ab	1.23 cd	6.57 abc	6.33 bc
T_7	18.00 b	10.27 d	1.20 cd	1.27 cd	6.67 ab	6.20 cd
T_8	16.77 d	10.90 c	1.13 cd	1.33 bc	6.47 abc	6.23 cd
T_9	16.70 d	11.90 ab	1.30 c	1.83 a	6.03 d	6.50 ab
T_{10} (control)	15.67 e	12.40 a	1.83 a	1.57 b	6.60 abc	6.10 de
LSD (0.05)	0.49	0.57	0.22	0.24	0.313	0.19
% CV	1.64	3.07	9.65	10.49	2.82	1.50

*Means in each column followed by the same letter are not significantly different ($p<0.05$)

Boll Weight (g)

The highest boll weight (6.73 g) was obtained from the T_1 (Sonround 3000 ml ha^{-1}) in 2012 and from the T_4 (Drop Ultra 300 cc ha^{-1} + Finish 250 cc ha^{-1}) (6.67 g) in 2013. Defoliant and boll-opening treatments gave better results when compared to the control plots (*Table 3*). This situation reveals that defoliant and boll openers increase boll weight. In parallel with our study, Awan et al. (2012) report that the defoliant and sulfur doses gave better results than control plots in terms of boll weight.

Boll Seed Cotton Weight (g)

Statistically significant different groups were formed among all treatments in terms of the boll seed cotton weight in both years (*Table 4*). The highest boll seed cotton weight (5.03 g) was obtained from the T_1 (Sonround 3000 ml ha^{-1}) in 2012 from the T_4 (Drop Ultra 300 cc ha^{-1} + Finish 250 cc ha^{-1}) (4.20 g) in 2013. Awan et al. (2012) claim that the defoliant and sulfur doses gave better results than control plots in terms of boll seed cotton weight. This is confirmed in our study, as well.

Ginning Outturn (%)

In terms of ginning outturn, there were statistically significant differences between the treatments only in 2012. The highest ginning outturn was obtained from the T_8 (Appeal 75 ml ha^{-1} + Finish 2500 cc ha^{-1}) (41.97%) in 2012 (*Table 4*). These differences between years may be resulting from differences in climate factors. However, in their studies, Denizdurduran and Efe (2009), Çopur et al. (2010), Du et al. (2013), and Tülemen (2016) state that the ginning outturn differences are not significant.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

Seed Index (g)

Different groups were formed among all treatments in terms of 100 seed weight in both years. In 2012, while the highest seed index weight (8.73 g) was obtained from the T_7 (Appeal 75 ml ha^{-1} + Efhun 300 ml ha^{-1}) and the lowest value was received from the T_{10} (Control) (7.83 g) treatment. All treatments formed more seed indexes than control plots (Awan et al., 2012). On the other hand, T_1, T_2, T_5, T_8, T_9 and T_{10} (control) had the highest seed indexes (*Table 4*) in 2013. This variability between years can be attributed to changing of climatic conditions (Anonymous, 2013).

Table 4. *Boll seed cotton weight, ginning outturn, and seed index in cotton with different harvest aid chemical treatments, and groups formed according to the LSD test*

Treatments	Boll Seed Cotton Weight (g)		Ginning Outturn (%)		Seed Index (g)	
	2012	2013	2010	2013	2012	2013
T_1	5.03 a	4.70 d	40.27 d	39.60	8.33 b	9.67 a
T_2	4.70 b	5.00 bc	40.60 cd	39.67	8.43 b	9.37 a
T_3	4.70 b	4.93 c	40.23 d	40.23	8.53 ab	9.03 c
T_4	4.70 b	5.20 a	41.67 ab	40.07	8.37 b	9.30 b
T_5	4.80 ab	4.70 d	41.53 ab	41.07	8.50 ab	9.87 a
T_6	4.90 ab	4.97 bc	40.53 cd	40.43	8.57 ab	9.33 b
T_7	4.93 ab	4.93 c	41.43 ab	40.43	8.73 a	8.80 d
T_8	4.80 ab	5.03 bc	41.97 a	39.77	8.33 b	9.73 a
T_9	4.40 c	5.10 ab	41.87 ab	39.93	8.47 ab	9.77 a
T_{10} (control)	4.83 ab	4.73 d	41.17 bc	40.67	7.93 c	9.87 a
LSD (0.05)	0.25	0.15	0.73	ns	0.89	0.22
% CV	3.03	1.82	1.04	1.16	1.97	1.33

*Means in each column followed by the same letter are not significantly different ($p<0.05$)

Conclusion

While the T_3 (Drop Ultra 600 cc ha^{-1}) (5422.7 kg) and T_7 (Appeal 750 ml ha^{-1} + Efhun 3000 ml ha^{-1}) subjects gave the highest seed cotton yield (5382.3 kg ha^{-1}) in 2012, T_1 (Sonround 3000 ml ha^{-1}) gave the highest seed cotton yield (4150.7 kg) in 2013. In both years, the best results were obtained from the T_5 (Drop Ultra 300 cc ha^{-1} + Efhun 3000 ml ha^{-1}) in terms of earliness ratio (96.30 and 96.30%, respectively). Furthermore, the seed cotton yield and the number of bolls were observed to be lower in the second year when compared to the first year. T_1 (Sonround 3000 ml ha^{-1}) (5.03 g) in 2012 and T_4 (Drop Ultra 300 cc ha^{-1} + Finish 250 cc ha^{-1}) (4.20 g) in 2013 provided the best results in terms of boll weight. Different groups were formed among all treatments in terms of seed index in both years. In the study, combination and single mixture treatments had shown different effects on the examined properties. In addition, according to the results of the present study, it was found that the increase in combine harvesting has contributed to a clean and timely picking of cotton in recent years. Therefore, it becomes compulsory to use harvest aiding chemicals. In conclusion, although defoliants and boll openers increase the yield slightly, we recommend the farmers to use them since they have no negative impacts on the plants.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27332743

COMPACTION PRESSURE AND DENSITY PROFILE IN PILE-TYPE SILOS

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(Received 11th Dec 2018; accepted 4th Feb 2019)

Abstract. The main aim of this study is to investigate the compaction pressure and density profile, and to determine the effectiveness of the compaction process applied to the pile-type silos. Chopped material was compacted into a pile-type silo. Pressure-sensing rubber globes were placed at the measuring points to determine the compaction pressure, during the silo filling. The compaction pressure and compaction time were acquired by the pressure measurement system. Data were stored by a recorder. The density measurements were made at pre-determined measurement points in each layer after the compaction process. The results showed that there was a significant relationship ($R^2 = 0.919$, $P < 0.01$) between the compaction pressure applied to silage and the density of silage under field condition. Pressure and density profile were found to be highly variable in the silo. The highest density (535.0 Kg m^{-3}) and pressure (0.46 bar) were at the bottom layer of the silo while the lowest density (206.6 Kg m^{-3}) and pressure (0.21 bar) were at the top layer of the silo.
Keywords: *silage, pile silo, pressure sensor, pressure measurement system, silage density*

Introduction

The compaction process is the most important process in silage making. This process is necessary to increase the density and remove oxygen from inside the silage as much as possible. Higher silage density and quality can be achieved by applying more compaction on the silage. It was indicated in the literature that the density of silage was variable in silage silos (Muck and Holmes, 2000; Roy et al., 2001; Tan et al., 2018). Latsch (2014) stated that the heterogeneous density occurring in silos was a major problem.

The efficiency of compaction is affected by the weight of equipment used (Darby and Jofriet, 1993; Muck et al., 2004), the layer thickness, the number of layers, the silo height (D'Amours and Savoie, 2005), the applied pressure (Savoie et al., 2004), the compression time (Roy et al., 2001), and the operator experience (Tan et al., 2018) while compaction pressure has an effect on silage fermentation (Tan et al., 2017). Toruk et al. (2010) reported that fermentation characteristics of the silage were positively affected by the increasing compaction pressure. Savoie et al. (2004) found that the density of corn silage was affected by pressure and layer thickness. In Turkey, the most common types of silos used for silage are the bunker silo and the pile-type silo. The use of pile-type silos is much more common in small livestock farms. However, determination of silage density and silage quality are significant problems for small livestock farms. Previous studies were generally conducted in laboratory conditions (Hoffman et al., 2013; Savoie et al., 2004), and studies to determine the compression

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

pressure in the silo under the field conditions are limited. Therefore, this study aimed to determine the relationship between density and compaction pressure in pile-type silo under field conditions.

Materials and methods

Experimental conditions

The second crop of maize (Pioneer ® P2948W) was harvested at 32% dry matter (DM) on November 2nd, 2017. The chopped material was ensiled in the pile-type silo within a day. The mean particle length of the chopped material was 12 mm. A single tractor (John Deere 6230) was used to compact the silage material. A mass of the compaction tractor was 4.6 tons. The tractor had a pressure of 2 bar at the front tire (380/85R24) and pressure of 2.3 bar at the rear tire (420/85R38). The transversal way has been used by the tractor to pack the silo. The compaction pressure applied during the packing of the pile-type silo in small livestock farm was also recorded. The densities of the material were calculated in the samples taken from predetermined measuring points. Before filling silo, a sample of fresh material was put aside to determine the dry matter content by oven drying at 103 °C during 24 h according to standard S358.2 (ASAE, 2002).

Pile silo and measurement points

The pile-type silo was 4.5 m wide, 12 m long, and 1.6 m high (theoretical volume of 86.4 m^3). The silo was divided vertically into three regions (north-N, center-C and south-S), and horizontally into three positions (1, 2 and 3) and three layers (top, middle, bottom). The height of layers in the silo was equal. The height of each layer is approximately 0.4 m. *Figure 1* shows the pressure and density measurement points and the location of pressure sensors.

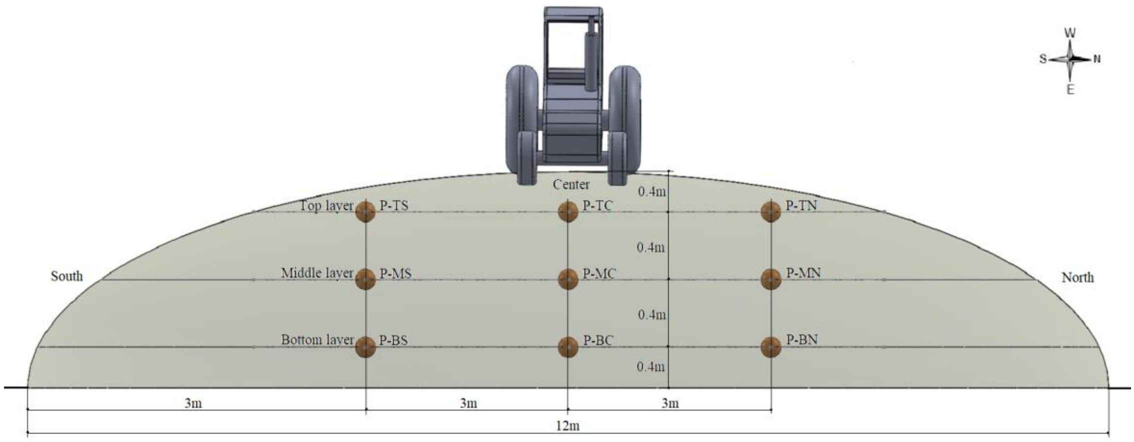

Figure 1. *Pressure measurement points in the pile silos*

Pressure measurement

The compaction pressure was acquired by the pressure measurement system. The measured pressure values were recorded by a data logger of the pressure measurement

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DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

system throughout the entire process. Pressure values were recorded as one data per second (1 value/second). The lowest pressure (Min.), maximum pressure (Max.) and residual pressure (RP) values measured during the compaction process were evaluated. The residual pressure was the total pressure remaining on the material after the compaction process.

The pressure measurement system has mainly three units (*Figure2*). These are data collection and recording (1), pressure sensors (2), and pressure-sensing rubber globes (3).

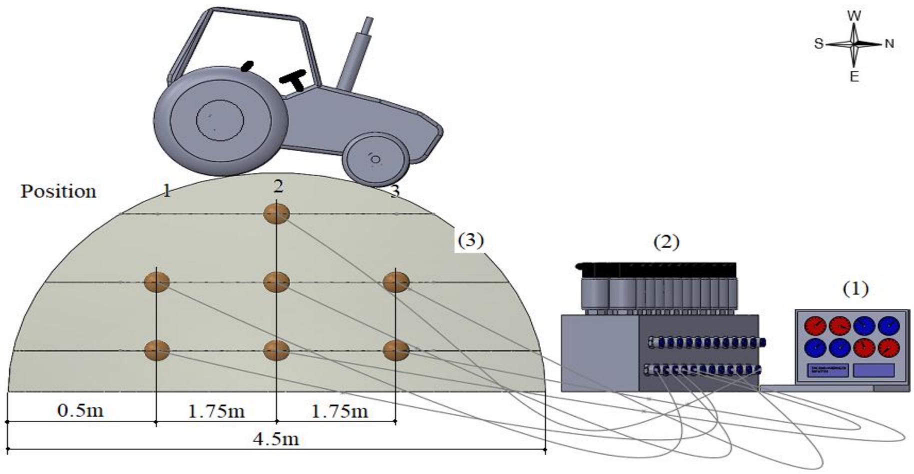

Figure 2. *Pressure measurement system; (1) data collection and recording, (2) pressure sensors, and (3) pressure sensing rubber globes*

The pressure-sensing rubber globes (PS) were installed into the silo to characterize the general condition of the silo and to measure the pressure during the ensiling stage. Pressure measurements were done by 27 PS. The pressure sensors were connected to the PSs via hydraulic hoses (Turner and Raper, 2001; Tan et al., 2018). The sensors (MPS500 series) used in the system had a measurement range of 0-25 bar, 4-20 mA output signal and a temperature range from -40 °C to +125 °C. The sensors were connected to NI DAQ measurement and storage system. The data (NI cDAQ-9184) were stored in an MS Excel file on the computer by using a user interface created with a NI Labview software (Tan et al., 2018). In order to determine whether the output values of the pressure sensors used in the measurement system were correct and reliable, especially under the dynamic conditions, two different calibration curves were created (Dalmıs, 2006; Akıncı, 1994). The pressure distribution was analyzed as a function of the three position factors (region, position, and layer).

Density measurement

The density measurements were made at each pressure measurement point after the compaction on each layer during the ensiling stage. The density distribution was also analyzed as a function of the three position factors (region, position and layer). The density of the silage was determined by taking the cylinder volume into consideration. The silage samples taken with the cylindrical container were then weighed (*Eq. 1*). The volume and weight of the silage materials were then used to calculate the density of the silage in kg m^{-3}:

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DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

$$\rho = \frac{m}{v} \quad \text{(Eq.1)}$$

where ρ is the ensiling material density, kg m^{-3}, m is the mass of the ensiling material filling, kg, and v is the cylinder volume, m^{-3} (Hoffmann et al., 2013; Wang, 2012).

Compaction time measurement

The compaction time was measured by the pressure measurement system. The measured time values were recorded by a data logger of the pressure measurement system throughout the entire process. The total compaction time measured during the compaction process was evaluated. The compaction time (hour/min) of packing tractor was recorded to estimate total compaction time on each layer.

Statistical analysis

In this study, to evaluate statistical significance between the compaction pressure and density in a pile-type silo, the data were analyzed by using the one-way ANOVA employing SPSS (version 18.0) software in a 3 x 3 x 3 factorial design. The minimum level of significance was 5%. Means were compared by the Tukey HDS test. A correlation test was performed among all parameters. The model statement included the effect of treatment, region, layer, position and interaction treatment (Eq.2).

The mathematical model used was;

$$y_{ijkl} = \mu + r_i + l_j + p_k + (rl)_{ij} + (rp)_{ik} + (lp)_{jk} + (rlp)_{ijk} + e_{ijkl} \quad \text{(Eq.2)}$$

in which y_{ijkl} = observed value of the variable that received the level of region i, layer j, position k, μ = overall mean; r_i = region effect, l_j = layer effect, p_k = position effect, interactions and e_{ijkl} = random error associated with each observation.

Results

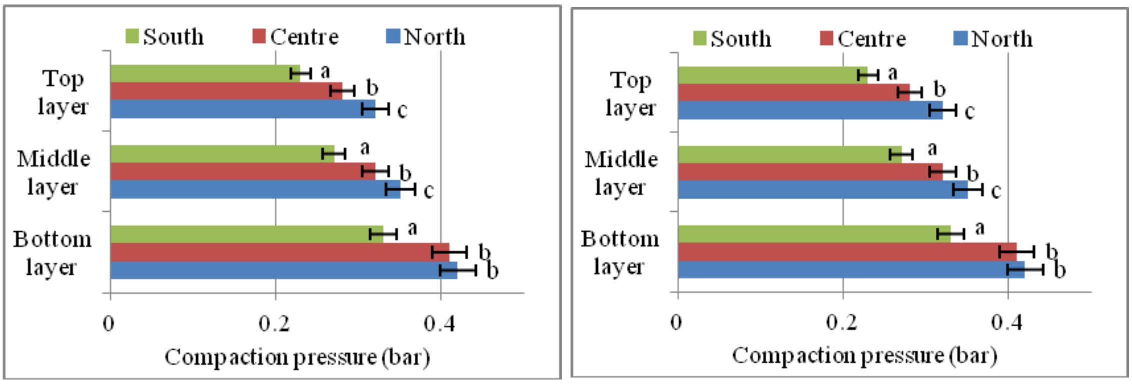
The compaction pressure (a) and the density (b) measured in different layers according to the north-south regions of the silo are given in *Figure 3*. Compaction pressure and density values were increasing from the top layer to the bottom layer in all regions of the silo. The average values of the compaction pressure (a) and the density (b) measured in different layers according to the position are given in *Figure 4*.

The density of the silage and the residual pressure, the minimum and maximum compaction pressure measured in different layers during the ensiling stage are given in *Table 1*. When the values of compaction pressure between three layers were compared to each other, the top layer data were lower than the middle and the bottom layers. The bottom layer had the highest average compaction pressure compared to the other layers. Different results were obtained for the pressure. Compared to the other points, at the B-N_3 (0.46 bar) point, residual pressure was the highest, and at the T-S_1 (0.21 bar) point the lowest residual pressure was measured.

The applied compaction pressure varied from the northern (N) region of the silo to the southern (S) region. The highest pressure was measured in the northern region of the silo. B-$N_{1,2,3}$ were 16.67% and 24.77% higher than the C-$N_{1,2,3}$ and S-$N_{1,2,3}$, respectively. This indicates that the operator spent more time in the northern region of the pile-type silo than other regions. The effects of the compaction pressure have been

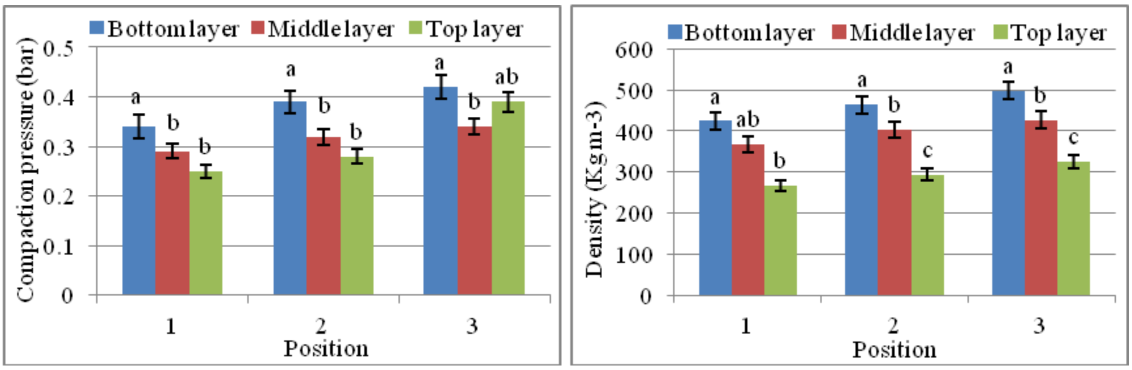
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

found statistically significant in the region and position ($P < 0.05$). The lowest mean compaction pressure was at position-1 (0.29 bar), and the highest compaction pressure was at position-3 (0.35 bar), which was 16.72% higher than the one at position-1.



a b

Figure 3. *The compression pressure (a) and density (b) measured in different layers according to the regions. Column with different letters within regions are statistically significant at P = 0.05 (n = 9)*



a b

Figure 4. *The compaction pressure measured (a) and density (b) in different layers according to the positions (positions 1, 2 and 3 have been shown in Figure 2). Column with different letters within regions are statistically significant at P = 0.05 (n = 9)*

The density of fresh matter increased from top to bottom in the pile-type silo. The bottom layer had the highest average fresh matter density compared to other layers. The average silage density of the top layer was the lowest and bottom layer had the highest silage density among the three layers. The compaction pressure applied to the silo is not equal which causes density differences. For this reason, the compaction time in the top layer of the silos should be higher than the other layers. The highest density was measured in the N-region of the silo. There was a difference among the regions according to the densities. B-$N_{1,2,3}$ were 15. 85% and 37.73% higher than the M-$N_{1,2,3}$ and S-$N_{1,2,3}$, respectively. The fresh matter density measured at position-3 of the silo in all layers was much higher than position-1 and position-2 of the silo. The effects of the density and the compaction pressure on the regions, layers, and positions are shown in *Table 2*.

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Table 1. *Compaction pressure values (min., max., mean and residual) and density measured during the ensiling stage according to layers, regions (north-N, center-C and south-S) and positions (1, 2 and 3)*

MP	**Min. (bar)**	**Max. (bar)**	**Ave. (bar)**	**RP (bar)**	**Density (Kg FM m^{-3})**
Bottom layer (B) Compacting time 3:42 (h/min)					
B-S_1	0.002	0.452	0.227	0.30	384.4
B-S_2	0.002	0.477	0.239	0.33	402.9
B-S_3	0.003	0.518	0.260	0.36	435.6
B-C_1	0.003	0.468	0.235	0.34	395.9
B-C_2	0.003	0.515	0.259	0.43	478.1
B-C_3	0.004	0.599	0.301	0.45	520.9
B-N_1	0.003	0.550	0.276	0.38	492.2
B-N_2	0.004	0.620	0.312	0.42	510.2
B-N_3	0.004	0.726	0.365	0.46	535.0
	0.0031	0.547	0.275	0.383	461.7
Middle layer (M) Compacting time 2:53 (h/min)					
M-S_1	0.001	0.387	0.194	0.26	322.6
M-S_2	0.001	0.410	0.205	0.28	351.1
M-S_3	0.001	0.435	0.218	0.29	389.1
M-C_1	0.001	0.410	0.205	0.29	370.8
M-C_2	0.002	0.435	0.218	0.32	419.8
M-C_3	0.002	0.461	0.231	0.36	444.2
M-N_1	0.003	0.480	0.242	0.32	408.9
M-N_2	0.003	0.513	0.258	0.36	436.4
M-N_3	0.003	0.657	0.330	0.38	448.5
	0.0018	0.465	0.233	0.317	399.0
Top layer (T) Compacting time 2:05 (h/min)					
T-S_1	0.001	0.358	0.179	0.21	206.6
T-S_2	0.001	0.366	0.183	0.24	245.6
T-S_3	0.001	0.370	0.185	0.25	297.8
T-C_1	0.001	0.392	0.196	0.26	290.9
T-C_2	0.001	0.410	0.205	0.28	324.0
T-C_3	0.001	0.435	0.218	0.30	330.3
T-N_1	0.001	0.420	0.210	0.29	302.3
T-N_2	0.002	0.482	0.242	0.32	310.0
T-N_3	0.002	0.568	0.285	0.34	345.1
	0.0012	0.422	0.212	0.279	294.7
Total compacting time 8:40 (hours/min)					

MP: the measurement point defined for density and pressure; Min.: the lowest pressure value measured at the specified point; Max.: the highest pressure value measured at the specified point; RP: the residual pressure on the material after compression

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The density was the lowest at T-S_1 position with 206.6 Kg m^{-3} and the highest at T-N_3 position with 345.1 Kg m^{-3}. The main reason for this is that there was a shelter near the right side of the silo. This was a problem for the quality of the compaction process. The density also increased with the increasing pressure. The densities obtained in our study were similar to several studies in the literature. In the present study, the density in the regions ranged from 337.32 Kg m^{-3} to 420.98 Kg m^{-3} (*Table 2*).

The highest density and pressure changes were calculated between the layers, which are 36.16% and 27.15%, respectively. The lowest density and pressure changes were calculated between the positions, which are 15.27% and 16.72%, respectively. The density and residual pressure in the northern and center region of the silos were higher than the southern region of the silos. The layers had significant effects on the density and pressure ($P < 0.05$). The highest values were measured at the bottom layer. In the present study, the density of the pile-type silo ranged from 294.77 Kg FM m^{-3} to 461.7 Kg FM m^{-3}. The compaction time was the lowest at the top layer and the highest at the bottom layer. The positions had also significant effects on the density and pressure (*Table 2*). The average residual compaction pressure on the material after compaction in the pile-type silo was calculated as 0.33 ± 0.62 bar, and the average density was calculated as 385.178 Kg m^{-3}.

Table 2. *The effects of density and compaction pressure on the regions, layers and positions*

Regions	D	RP	Layers	D	RP	Positions	D	RP
North	420.98^{a}	0.362^{a}	BL	461.71^{a}	0.383^{a}	1	352.77^{a}	0.294^{a}
Centre	397.22^{a}	0.338^{a}	ML	399.05^{b}	0.317^{b}	2	386.45^{ab}	0.331^{ab}
South	337.32^{b}	0.279^{b}	TL	294.77^{c}	0.279^{c}	3	416.30^{b}	0.353^{b}
RP				$R^2 = 0.989$				
Region*Layers				F = 9.412			$P < 0.05$	
Region*Position				F = 7.765			$P < 0.05$	
Layers*Positions				F = 17.353			$P < 0.05$	
Region*Layers*Positions				F = 5.397			$P < 0.05$	
D				$R^2 = 0.996$				
Region*Layers				F = 32.147			$P < 0.05$	
Region*Position				F = 31.291			$P < 0.05$	
Layers *Positions				F = 4.919			$P < 0.05$	
Region*Layers*Positions				F = 20.426			$P < 0.05$	

*Mean values in the same column with the same superscript do not differ significantly at ($P < 0.05$)
D: Density; RP: is the residual pressure on the material after compression

The correlations amongst all parameters are shown in *Table 3*. The correlation results showed that the fresh matter density of the silages and the compaction pressure were strongly ($R^2 = 0.919$, $P < 0.01$) correlated (*Table 3*).

The density of the silage in the pile-type silo was positively affected by the increasing compaction pressure. The pressure and density of the silage showed positive correlations with the regions and positions, whereas they were negatively correlated with the thickness of the increased layers. This indicates that the thickness of the layers, the compaction times reserved for the layers, and the movement of the compaction equipment is important in silo management.

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Table 3. *Correlations between all parameters*

	Density	Pressure	Region	Layers	Position
Density	1	0.919**	0.411**	-0.821**	0.312**
Pressure	0.919**	1	0.549**	-0.684**	0.388**

**Correlation is significant at the 0.01 level (2-tailed)

Discussion

In our study, the density and pressure values in some regions of the silo are higher than the other regions. However, according to Latsch (2014), there are no significant differences between the bulk density in compact areas. In position 1, compacting time was found a bit longer than in position 2 and 3. In the present study, compaction time was the lowest at the top layer and the highest at the bottom layer. This indicates that the operator spent more compaction time in some layers than the other ones. Similar results were reported by Latsch (2014), Tan et al. (2018), and Muck et al. (2004).

There is no literature on compression pressure measurement in the ensiling process performed under field conditions. Compaction efficiency has been described in relation to silage density in many studies. In this study, the compression pressures and silage density measured in the field conditions have been explained together. As a result of the study, it was observed that the densities related to the measured pressure values were consistent with the literature. As a result, it was seen that the compaction pressure applied in the pile-type silo meets the recommended density values in the literature.

In the present study, the density of the pile-type silo ranged from 294.77 Kg FM m^{-3} to 461.7 Kg FM m^{-3}, which are similar to those found by Muck and Holmes (2000) and Roy (2001). These values are higher than the ones (205.03-376.43 Kg m^{-3}) expressed by Norell et al. (2013) and (129-302 Kg m^{-3}) expressed by Oelberg et al. (2006). These results can be explained by the fact that they are not average value.

In our study, it was determined that the silo density and pressure increased from the top layer to the bottom one. This can be explained by the increase in the layer thickness and the weight of the mass. Huhnke (1995), Muck and Holmes (2000), D'Amours and Savoie (2005), and Oelberg et al. (2005) also observed that the density at the top of the silo was lower than the density measured at the bottom. However, the data about the compaction pressure were not determined in the literature. D'Amours and Savoie (2005) reported that the density in the silo was quite variable and was affected by the pressure. According to Tan et al. (2017), the highest compaction pressure measured in the bunker silo is 0.34 bar. In our study, the measured residual pressure was 0.46 bar, which was higher than the value reported by Tan et al. (2017). Although average residual pressure was 0.33 bar, the maximum pressure measured instantaneously was0.726 bar during the ensiling.

There was a strong and positive relationship between the silage density and the compaction pressure applied to the corn silage in a pile-type silo. The results showed a positive correlation with regions and position, whereas they were negatively correlated with increasing layers thickness. According to Tan et al. (2017), there was a relationship between the compaction pressure applied to silage and temperature. The relations between density and pressure were not reported in those studies conducted in field conditions in the previous studies.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

Conclusion

Pressure and density values in the silo were found to be quite variable for regions, positions and layers. The differences could be largely explained by different compaction times and tractor mass for regions, the way used by the tractor to pack the silo for positions and the increasing material mass for layers. At small farms, very heavy compaction equipment is not available. For this reason, it may be advisable to make more compaction time for packing or more passages on a thinner layer to achieve high density in these farms.

Results indicated that it is important to select areas that will not restrict tractor movements when locating the silo. This situation causes differences in silo compaction pressures due to restrictions on tractor movements. The effects of measured exact regional compaction time on the quality of silage and silage compaction pressure can be worked for future works.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27452754

DETERMINATION OF HEAVY METAL ACCUMULATION IN AIR THROUGH ANNUAL RINGS: THE CASE OF *MALUS FLORIBUNDA* SPECIES

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(Received 6th Dec 2018; accepted 4th Feb 2019)

Abstract. Air pollutants can be observed all over the world. Sulfur dioxide and nitrogen oxides are the most worrying ones among them. The growth rates of many forested areas in the world decrease at high altitudes, and air pollutants are more abundant as the cause of this. The leaf surface constitutes the interface between plants and the worsening atmospheric environment. Therefore, it is the first point of contact between plants and air pollutants and constitutes an effective barrier to the pollutant input. The outer surfaces of leaves are covered with a thin membrane called the cuticle. This membrane layer has many basic functions, such as preventing the excessive water loss of the plant, regulating the intake of dissolved substance and protecting photosynthetic tissues, preventing of harmful irradiation, such as improved UV-B caused by stratospheric ozone depletion. The wood structure also provides us with information on the pollutants to which they are exposed to and their levels due to the fact that trees are stationary. In this study, the accumulations of Co, Cu, Cd, Pb, Ni, Cr, Mn, Fe, Al, Zn, Na, Ca, Ba, Mg, and As elements, accumulated on the annual rings of *Malus floribunda* species in Ankara-Yenimahalle between 1962 and 2017, by years were determined using the GBC Integra XL–SDS-270 ICP-OES device. In conclusion, it is observed that amounts of all elements in the wood in different age ranges are statistically different at a confidence level of at least 99.9%.
Keywords: *wood, air pollutant, tree species, bioindicator, elements*

Introduction

Toxic pollutants have increased considerably in the last century and adversely affect the lives of most living beings around them (Wolz et al., 2003; Tang et al., 2013). Trees in the settlements contribute to preventing air pollution; reducing noise, wind, dust and greenhouse effect; balancing temperature; providing energy savings and moisture; and creating habitats for fauna and flora (Cetin, 2018a, b, c, d). Environmental pollution includes the mixture of organic and inorganic compounds. Although organic components decompose harmlessly and as fully mineralized, the separation of inorganic compounds from the medium is technically difficult since do not change with a physical process (Meagher, 2000). Inorganic pollutants are basically heavy metals, such as As, Ag, Hg, Sb, Cd and Pb, containing the elements with biological functions (such as Fe, Mo, Mn, Zn, Ni, Cu and Co), which are harmful when they are present in excessive amounts and which do not play a known role in living organisms (DalCorso et al., 2013). Inorganic pollutants may arise from natural conditions such as the exposure of rocks to weather conditions, soil erosion, and volcanic activities or human-induced factors such as industrial or agricultural activities (Fasani et al., 2018).

Human-induced factors can be defined by the high concentration of primary pollutants such as SO_2 in the air and initiate local pollution. These activities can be the cause of tree deaths, and the high level of air pollutants in neighboring areas (Garrec, 1993).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

Secondary pollutants are characterized by physiological effects, such as the yellowing of leaves and a decrease in the viability of trees as a result of the interaction of secondary pollutants with trees. These pollutants, usually along with climatic stress, initiate forest decline with a very irregular localization on a regional scale. It is necessary to consider the nature and level of these pollutants to determine the plant stress caused by air pollution. As an example of this, a summary of the scientific literature on the effects of air pollutants on epidermis characteristics during two periods was created (*Table 1*) (Garrec, 1993).

Table 1. *The effects of air pollutants on epidermis characteristics from the scientific literature*

Air pollutant	**Cuticular characteristics**				
	Wax erosion	**No wax erosion**	**Increasing wax amount**	**Decreasing wax amount**	**No effect on wax amount**
SO2	*******	****	**	**	**
O3	*****	*****		**	**
Acid rain	******	***	*	*	**
Acid mist	**	**			**
NH3	**	**			

*Shows the density of pollutants

The data presented in *Table 1* show the variability of the response of the plant surface and/or the inhomogeneity of the action of these pollutants. Nevertheless, based on these data, primary air pollutants such as SO_2 are likely to have a detrimental effect on the epidermis. Unlike the concentrated primary pollutants, secondary air pollutants such as O_3 or acid deposits seem to have a less significant effect on the cuticle layer. In fact, most of the results show that the effect of these less concentrated secondary pollutants is similar to the aging process and does not have a polluting characteristic (Gunthardt-Goerg and Keller, 1987; Lutz et al., 1990).

Trees are regarded as the sensors that record environmental disturbances since they live for a long time, that remain in the same place throughout their lives and thus display chronology, and that are geographically common (Kord et al., 2009; Balouet et al., 2007). The fact that trees accumulate the heavy metals they receive from the air with the components in and above the soil makes them good indicators. Therefore, various organelles such as trunks, branches, and leaves of trees have been used for many years to determine the concentration of heavy metals in them. Leaves are the most commonly used organelles. The main reasons for this can be listed as the fact that leaves incorporate heavy metals through their stomata during photosynthesis and accumulate them, the fact that the collection of leaves does not cause permanent damage to the tree, and the fact that how long the accumulated heavy metal accumulates is known since the ages of leaves are known. Furthermore, herbarium collections (Herpin et al., 1997), the annual growth rings of trees (Watmough, 1999) also provide historical examples for bio-analysis. In fact, trees are not better indicators compared to fungi, algae, and moss. However, the use of trees throughout cities and the fact that they live longer compared to other indicator plants provide more information to scientists about the increase in heavy metal pollution in the air from past to present in the studies carried out on them (Sawidis et al., 2011; Turkyilmaz et al., 2018a; Shahid et al., 2017; Shcerbenko et al.,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

2008). In recent years, many authors have reported the temporal and spatial reconstructions of heavy metals recorded on tree rings of different tree species (Aznar et al., 2008; Lageard et al., 2008; Nabais et al., 1999; Orlandi et al., 2002; Vives et al., 2005; Türkyılmaz, 2018b).

Materials and methods

The study was carried out on the wood materials obtained from *Malus floribunda* tree located in Yenimahalle region, which is one of the regions of Ankara province with the heaviest traffic. Ankara province has an area of 26.897 km^2 and is located between 39.57 N latitude and 32.53 E longitudes. Its altitude above sea level is approximately 890 m. Ankara province is located in B4 square (Grid) according to P. H. Davis's Turkey map grid system (Davis, 1965-1985). In terms of biogeography, it is located in the Irano-Turanian floristic region. The Irano-Turanian floristic region covers the central and eastern Anatolia regions of Turkey. These regions are cold and snowy in winters and usually dry and without rain in summers (Atalay and Efe, 2014; Saya and Güney, 2014). The average temperature on a provincial scale is 11.7 °C, and the annual average precipitation is 389.1 mm. The highest and lowest temperature values were determined to be 40.8 °C and -24.9 °C, respectively. Upon examining the provincial center and the wind status of stations in general, the dominant wind is observed to vary depending on the land structure. Accordingly, the dominant wind blows in the northeast direction in Ankara (center), Esenboğa and Yenimahalle districts where the study area is located. The highest wind speed determined in Ankara is 29.2 m/s (URL 1) (*Fig. 1*).

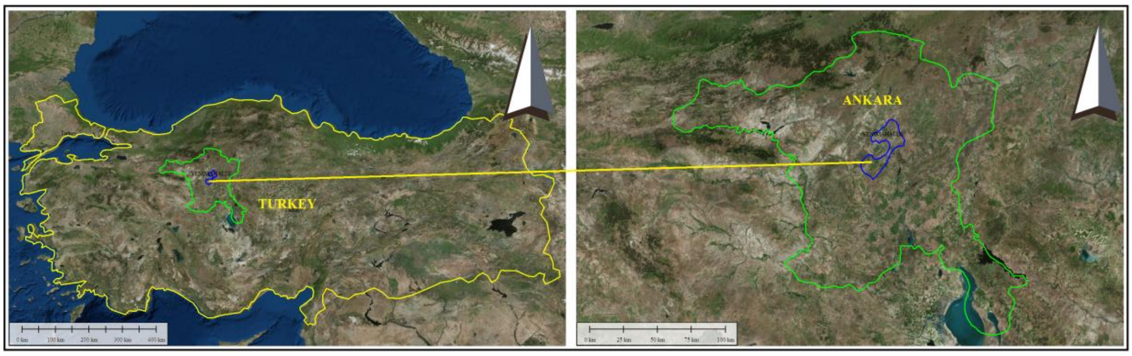

Figure 1. *A map of Ankara*

The samples were cut from *Malus floribunda* in Ankara-Yenimahalle (Turkey) in December, when the vegetation season ends, in 2017. The cut samples were brought to the laboratory and separated into pieces in the form of 1 cm-thick discs in the laboratory environment. The sectional surfaces were made smooth by sandpapering so that annual rings could be observed clearly. During the macroscopic observation, it was determined that the tree was 55 years old, and it was decided that it was appropriate to perform grouping into five age ranges by considering the widths of annual rings. The annual rings were divided into five-year sections, and the samples taken from two opposite sides of the tree were classified. Thus, 11 samples including five-year annual rings and barks were obtained.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

The wood samples were shredded and turned into sawdust. During these processes, attention was paid to not using any tool made of the elements discussed in the study. The wood samples were first kept for 30 days until they became dry. Then, they were dried in the drying oven at 50 °C for one week. 0.5 g of the dried samples were taken, 6 ml of 65% HNO_3 and 2 ml of 30% H_2O_2 were added to them, and they were placed in a microwave oven. The program of the microwave device was set to 15 min at 200 °C. After burning the samples in the microwave oven, the samples which were dissolved into solution were taken into balloons and completed to 50 ml with ultrapure water and made ready for Fe, Co, Ni, Zn, Cd, Hg and Pb analyses with the GBC Integra XL–SDS-270 ICP-OES device (GBC Scientific Equipment Pty Ltd., Melbourne, Australia) For the analysis of the samples, the plasma of the ICP device was burned, and ultra-pure water was passed through the system for 15 min to equilibrate. The calibration chart was created by preparing standard solutions according to the elements to be analyzed. After constructing the calibration chart, the samples were given to the system and read. Since the sample was taken by 0.5 g and completed to 50 g with water, the analysis results were multiplied by 100. Different calibration charts were created at the ppm or ppb level according to the analysis results that did not fall into the calibration chart, and reading was performed again. The detection limits for the GBC device were determined to be (Pb---> 0.377 ppb, Cu---> 0.639 ppb, Ca---> 0.00208 ppm, Mg--->0.00758 ppm, Cd---> 0.063 ppb, Cr---> 0.311 ppb, Ni---> 0.171 ppb, Fe---> 0.00068 ppm, Mn---> 0.00015 ppm, Zn---> 0.00634 ppm). In the study, all measurements were performed in three replicates. Then, the data were evaluated by the analysis of variance, Duncan's test, and correlation analysis using SPSS package program (Anonim, 1998; Eymen, 2007).

Results

A total of 15 elements were discussed for the determination of heavy metal accumulations in the wood and bark. The analysis of variance and Duncan's test were applied to the results obtained in terms of the studied elements. The average values obtained for wood and bark and the F values calculated as a result of the analysis of variance are presented in *Table 2*.

Based on the results summarized in *Table 2,* it was determined that there was no significant difference among Cu, Fe, Ca and As values at a confidence level of at least 95%. For the rest of the elements, statistically significant differences were found between the wood and bark. According to the F values calculated, the values obtained for bark and wood were significantly different at a confidence level of 99.9% for all elements (*Table 2*). However, Duncan's test was not applied to determine the homogeneous group since it was performed on 2 characters including bark and wood.

The analysis of variance was performed using SPSS according to all the elements studied on annual rings in the wood sample, and the analysis result, F value, and Duncan's test results are presented in *Table 3*.

Upon examining *Table 3,* it was observed that there were significant differences at a confidence level of at least 99.9% in terms of all the characters subjected to the analysis of variance. It was determined that Cu value was divided into 9 different homogenous groups and that the highest value (26674.13) was reached between the years 1967-1972. Similarly, it was determined that Ni (1907.5), Al (2327.9), Mg (907.56) and Zn (14033.33) also had the highest values between those years. It was determined that As

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

value was divided into 2 different homogenous groups and that Na, Ba, and B values were divided into 10 different homogenous groups. Na value was determined to be the highest (2016.6) between the years 2000 and 2005. It was determined that Mg element reached its highest value between 1955 and 1960.

Table 2. *Average values obtained for wood and bark and the analysis of variance results*

Elements	Wood	Bark	F value
Co ppb	**529.6**	1229.4	21.807***
Cu ppb	**7779.2**	11767.2	0.728 ns
Cd ppb	**176**	330.5	63.992***
Pb ppb	**2077.4**	4532.9	48.949***
Ni ppb	**1132.8**	2977.7	86.53***
Cr ppb	**453.2**	2154.3	623.793***
Mn ppb	**1058**	30294.2	4179.785***
Fe ppb	**18442.2**	29312.2	0.595 ns
Al ppb	**770.4**	28813.1	4381.278***
Zn ppb	**3763**	8640.8	2.648***
Na ppm	**912.1**	511.3	1.661***
Ca ppm	**2097.5**	3218.9	4.385 ns
Ba ppm	**9.1**	56	138.572***
Mg ppm	**417.2**	1767.5	44.028***
As ppm	**1.6**	2	11.656 ns

ns: Not significant
***Significant at 0.001 level

Table 3. *Average values by age in wood samples and the analysis of variance results and F value*

Ages / Elements	1955-1960	1961-1966	1967-1972	1973-1978	1979-1984	1985-1987	1988-1993	1994-1999	2000-2005	2006-2011	2012-2017	f
Co ppb	800.67c	775.23c	1149.4d	412.67b	387.07ab	377.87ab	429.2b	419.07b	325.2a	356.93ab	392.3ab	127.727
Cu ppb	15719.4g	16152.93h	26674.13ı	8459.5f	4631.2e	2409.57c	2168.8b	3273.4d	1561.03a	2172.9b	2348.63c	35580.948
Cd ppb	204.33d	203.27d	245.23e	193.47d	175.63c	167.17c	166.13c	163.2c	148.77c	137.2ab	131.63a	63.162
Pb ppb	2769.13c	2814.73c	3208.7d	2037.67b	1965.03b	1817.43b	1943.1b	1874.1b	1516.43a	1448.97a	1456.33a	50.877
Ni ppb	1486.5h	1419.1g	1907.5ı	1019.9d	1141.4e	1200.57f	865.23b	956.2c	791.8a	846.9b	825.5ab	563.292
Cr ppb	523.83e	657.57f	628.2f	485.47de	463.3cde	404.73bc	432.43cd	406.47bc	330.17ab	314.83a	338.2ab	21.291
Mn ppb	2925.93ı	1069.3f	2079.73h	663.77d	511.8c	278.47a	515.03c	1276.5g	423.57b	957.6e	936.47e	3790.982
Fe ppb	7350.6c	33867f	78524h	40929.83g	27887.5e	33867d	3710.66b	41.37a	386.06a	226.26a	254.36a	46162.103
Al ppb	1837.1h	1284.73g	2327.9ı	602.03e	865.23f	461.53d	87.83a	349.47c	58a	421.17d	179.1b	1357.294
Zn ppb	13135.63h	6938.7g	14033.33ı	3663.83f	1992.53e	371.3c	61.03a	653.76d	375.2c	147.6b	20.1a	75236.672
Na ppm	521.06c	422.23b	868.5f	841.33e	1016.8g	1198.26h	1716.03ı	360.36a	2016.6j	719.6d	352.3a	28471.922
Ca ppm	3250.4g	2914.96e	3136.86f	1955.93d	1956.7d	1982d	1974.83d	3247.1g	1073.93c	823.36b	756.7a	12064.255
Ba ppm	25.9j	10.93h	17.7ı	10.2g	7.8f	4.66d	6.13e	6.033e	4.4c	3.36b	2.8a	21305.217
Mg ppm	1227.3k	499ı	907.56j	174.4h	466g	220.4f	174.4d	186.33e	119.03a	144.7b	160.2c	54894.807
As ppm	1.9b	1.83b	1.83b	1.5a	1.53a	1.46a	1.46a	1.53a	1.5a	1.46a	1.53a	9.745

The graphics, which were prepared to make the changes associated with heavy metals according to the ages more clearly understandable, are presented in *Figure 2*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

Figure 2. *Annual changes in heavy metal concentrations over years (A changes in Co concentrations, B changes in Cu concentrations, C changes in Cd concentrations, D changes in Pb concentrations, E changes in Ni concentrations, F changes in Cr concentrations, G changes in Mn concentrations, H changes in Fe concentrations, I changes in Al concentrations, J changes in Zn concentrations, K changes in Na concentrations, L changes in CA concentrations, M changes in Ba concentrations, N changes in Mg concentrations, O changes in As concentrations*

As seen in *Figure 2,* especially Pb, Cu, Co, Mg and Ba elements were found to decrease after 1979-1984 years. One of the reasons for this could be the use of natural gas in Ankara since the 1980s.

As a result of the study, correlation analysis was performed to determine the relationships between the elements and the results are presented in *Table 4*. The studied relationship that was not measured in the correlation analysis is related to the linear part of the relationship between the variables. The correlation coefficient calculated as a result of the correlation analysis is indicated with r and takes values between –1 and +1. The fact that the coefficient is close to +1 indicates that there is a good correlation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

between the two variables, and the fact that it is close to -1 indicates that there is a good but inverse correlation; in other words, one of the variables increases while the other one decreases. Upon evaluating the results in this respect, it is observed that the level of relationship between some elements is really high.

Table 4. *Correlation analysis results*

	Cu ppb	Cd ppb	Pb ppb	Ni ppb	Cr ppb	Mn ppb	Fe ppb	Al ppb	Zn ppb	Na ppm	Ca ppm	Ba ppm	Mg ppm	As ppm
Co ppb	.839**	.906**	.940**	.914**	.754**	.675**	.649**	.686**	.867**	-.365*	.714**	.830**	.877**	.867**
Cu ppb		.648**	.691**	.615**	.330*	.208	.826**	.227	.945**	-.345*	.681**	.482**	.649**	.744**
Cd ppb			.973**	.968**	.904**	.835**	.597**	.850**	.710**	-.266	.701**	.929**	.917**	.755**
Pb ppb				.957**	.881**	.803**	.556**	.815**	.755**	-.334*	.735**	.917**	.919**	.846**
Ni ppb					.924**	.875**	.523**	.889**	.692**	-.332*	.650**	.943**	.925**	.769**
Cr ppb						.979**	.294	.986**	.428**	-.273	.486**	.939**	.833**	.635**
Mn ppb							.154	.998**	.341*	-.259	.389*	.927**	.799**	.560**
Fe ppb								.195	.673**	-.148	.463**	.322	.452**	.448**
Al ppb									.349*	-.246	.394*	.926**	.801**	.563**
Zn ppb										-.350*	.720**	.642**	.805**	.818**
Na ppm											-.392*	-.312	-.370*	-.429**
Ca ppm												.603**	.671**	.674**
Ba ppm													.961**	.745**
Mg ppm														.815**

Upon examining *Table 4,* it is observed that there is a positive correlation in general with respect to the measured characters. When the results are examined in this context, the relationship between some elements is observed to be quite high. For example, the correlation coefficients between Cr and Al (0.986), Mn and Al (0.998) and Cr and Mn (0.979) are quite high. Similarly, the correlation coefficient calculated between Na and As (-0.429) is negative but very strong. Furthermore, very strong relationships were observed among many elements (*Table 4*).

Discussion and conclusion

The results of the study reveal that the concentration of all heavy metals that adversely affect human health increases with age. It can be said that the increase in the amount of heavy metals in tree rings is due to the morphological structure of the tree and ecological impacts, which does not exhibit a linear increase, high in some years and low in other years. The morphological structure of the epidermis layer varies by age and polluted air (Huttunen and Laine, 1983; Mengel et al., 1989; Turunen and Huttunen, 1990). Similar results were also obtained in the studies carried out on different species (Beramendi-Orosco et al., 2013; Türkyılmaz et al., 2018c, 2019). In the study carried out by Beramendi-Orosco (2013): on *Prosopis julifora* annual rings, the researchers ported that the amount of Cu, which was 1.09 ppm during 1988-1992, was 1.27 ppm during 2003-2007 and that the amount of Pb, which was 0.35 ppm during 1998–2002, was 0.46 ppm during 1993-1997. Similar results were also obtained in the studies in which changes were determined by months (Norouzi et al., 2015).

When tree annual rings were examined, it was determined that heavy metal accumulation was intense in the early years in elements such as Cd, Pb, Al, Zn, Mg, Ca and B. However, there is no clear evidence on whether this intensity was due to the accumulation during the first years of the tree or the accumulation of all years. As seen

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DOI: http://dx.doi.org/10.15666/aeer/1702_27552764

in *Figure 2,* especially Pb, Cu, Co, Mg and Ba elements were found to decrease after 1979-1984. One of the reasons for this could be the use of natural gas in Ankara since the 1980s.

Although some elements such as K, S, Mg, CI, and P can be transported to the plant's metabolic activities, the transport of certain elements in the phloem (B, Ba, Ca, Cu, Fe, Li, Mn, Mo, and Zn) is more limited (Perone et al., 2018). In recent years, the nature and intensity of the concentration of elements in nature have increased due to the increase in human effects and vehicle traffic. Some data indicate that climate change is affected not only by the traces of pollution but also by plant metabolism and by the distribution of elements in plants (Cui et al., 2013; Li et al., 2004). Nowadays, in the world, motor vehicles exhausts, industrial activities, mineral deposits and enterprises, use of urban wastes as fertilizers, chemical fertilizers and pesticide applications, and reach a significant amount of heavy metal soil. The accumulation of heavy metals in the soil not only affects soil fertility and ecosystem activities, but also affects many metabolic events such as photosynthesis, respiration, growth and development within the plant and therefore affects animal health and human health significantly due to deteriorated food chain. In the soils where heavy metal accumulation is observed, heavy metals reach the human body through the skin contact of people, breathing, growing vegetables and fruits. For this reason, heavy metal that can be harmful to human health, etc. the means of transmission and prevention of all kinds of substances should be investigated.

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A NEW ETHNOBIOLOGICAL SIMILARITY INDEX FOR THE EVALUATION OF NOVEL USE REPORTS

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(Received 6th Aug 2018; accepted 7th Nov 2018)

Abstract. Similarity Indices are widely applied in the field of ecology to measure species diversity as well as to map patterns of conservation and monitor threats to biodiversity. Among the known, Jaccard's and Sorensen's indices are the most frequently employed similarity Indices. Here, we propose a new and efficient statistical approach in the field of ethnobiology and validate its efficacy by comparing the results with predefined similarity Indices used in previous studies. The core objective was to propose a new index for quantitative ethnobiological analyses and to find out solutions for sorting the plants having similar ethnobiological uses in allied, aligned, national and global regions; as the pre-existing indices like Jaccard's and Sorensen's indices provides best estimates in the field of ecology but not in ethnobiological studies. In comparative ethnobiological studies, ethnobiologists use conventional ecological tools for evaluation of similarities and dissimilarities. Our proposed similarity index is based on the quantification of similar uses of common medicinal plants via comparing present study with previously published reports from various areas where, the author(s) have used the Sorensen's index and/or Jaccard's index. To assess the significance and validity of this newly developed index, similarities and differences in ethnomedicinal studies on medicinal plants in different regions were evaluated. Data regarding medicinal plants usage here was compared with 20 previously published studies and then analyzed through pre-existing indices as well as Rahman's index to examine the novelty in the study. Our preliminary results revealed noteworthy coherence with the existing similarity indices, albeit, the new index was more efficient than the previous. Our comparison revealed, that as far as common vegetation and floral levels are concerned, the existing ecological coefficients of similarity are efficient and precise; but for similarities in the field of medicinal plant studies certain constraints are overcome by the proposed similarity index. Inferences derived from Rahman's similarity index (RSI) are as reliable as the previously known and well-established similarity indices. Further, RSI specifically targets the ethnobiological similarities, a limitation in Jaccard's and Sorensen's indices. Thus, RSI would be a useful tool/index in the assessment of rigorous quantitative ethnobiological data.

Keywords: *similarity index, novel uses assessment, quantitative ethnobiology, ethnomedicine, cultural use similarities*

List of abbreviations: RSI: Rahman's similarity index; JI: Jaccard's similarity index; QS: Sorensen's similarity index; AJK: Azad Jammu and Kashmir

Introduction

What are quantitative similarity indices?

For comparing two populations, a similarity index provides a quantitatively based measurement, analogous to the application of similarity for DNA-fingerprinting (Lynch, 1990; Chuang, 2012). Similarity indices are also been widely used in ecology (Hubalek, 1982; Chao et al., 2006). Johnston (1976) investigated the characteristics of 25 similarity indices. Among these indices, the Jaccard's index (Jaccard, 1902) and Sorensen index (Sorensen, 1957) are often applied in the ecological studies. These indices are used to note the species-diversity for nature and natural protection (Higgs and Usher, 1980; Legendre and Legendre, 1998). The Jaccard's index is defined as the number of shared species divided by a total number of distinct species in two communities. The Sorensen index is the ratio of the number of shared species to the average number of total species in two communities. The definitions of the Jaccard's and Sorensen's indices are constructed on the numbers of species in two populations (Chuang, 2012).

Why is an ethnobiological similarity index needed?

It is desirable to make comparisons between floral or faunal samples taken at different times, different places, or by different techniques. Such comparisons seem profitable and take advantage of the existence of similarity indices, many of which have been developed earlier in this century. Some of these indices merely take into account the presence or absence of species in the samples, while others integrate information on the relative abundance of the species. The desirable index depends on the questions asked and the kind of data available in a given case (Wolda, 1981). In ethnobotanical studies, researchers also may wish to integrate information about the reported uses of a species. The calculation of diversity indices is a very useful tool for ethnobotanical studies, which helps researchers to ask questions and analyze data obtained through this method, besides permitting comparisons among different communities in different or similar environments (Höft et al., 1999).

Jaccard's and Sorensen's similarity indices are widely applied in the ethnobotanical studies from national and global areas, i.e. Alpine and Sub-alpine regions of Pakistan (Kayani et al., 2015), Thar Desert (Sindh), Pakistan (Yaseen et al., 2015), Abbottabad, Pakistan (Ijaz et al., 2016), Mansehra, Pakistan (Rahman et al., 2016a, b, c), Azad Kashmir, Pakistan (Ahmad et al., 2017; Amjad et al., 2017), Dindigul district, Tamilnadu, India (Faruque et al., 2018), Bandarban District of Bangladesh (Sivasankari et al., 2014), central East Shewa of Ethiopia (Feyssa, 2012), Republic of Benin (Laleye et al., 2015), Kembatta Tembaro (KT) Zone, Southern Ethiopia (Maryo et al., 2015), Brazilian Pampa Teixeira et al., 2016), but they are not amenable to comparative Ethnopharmacology (Weckerle et al., 2018).

Here, we propose a new and efficient statistical approach in the field of ethnobiology and validate its efficacy by comparing the results with predefined similarity indices used in previous studies.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

Material and methods

Study area

Manoor Valley is remote area of District Mansehra (Rahman et al., 2016a), situated on north side about 50 km from main Kaghan road at ‘Mahandri’ in the Lesser Himalayas of Pakistan (*Fig. 1*).

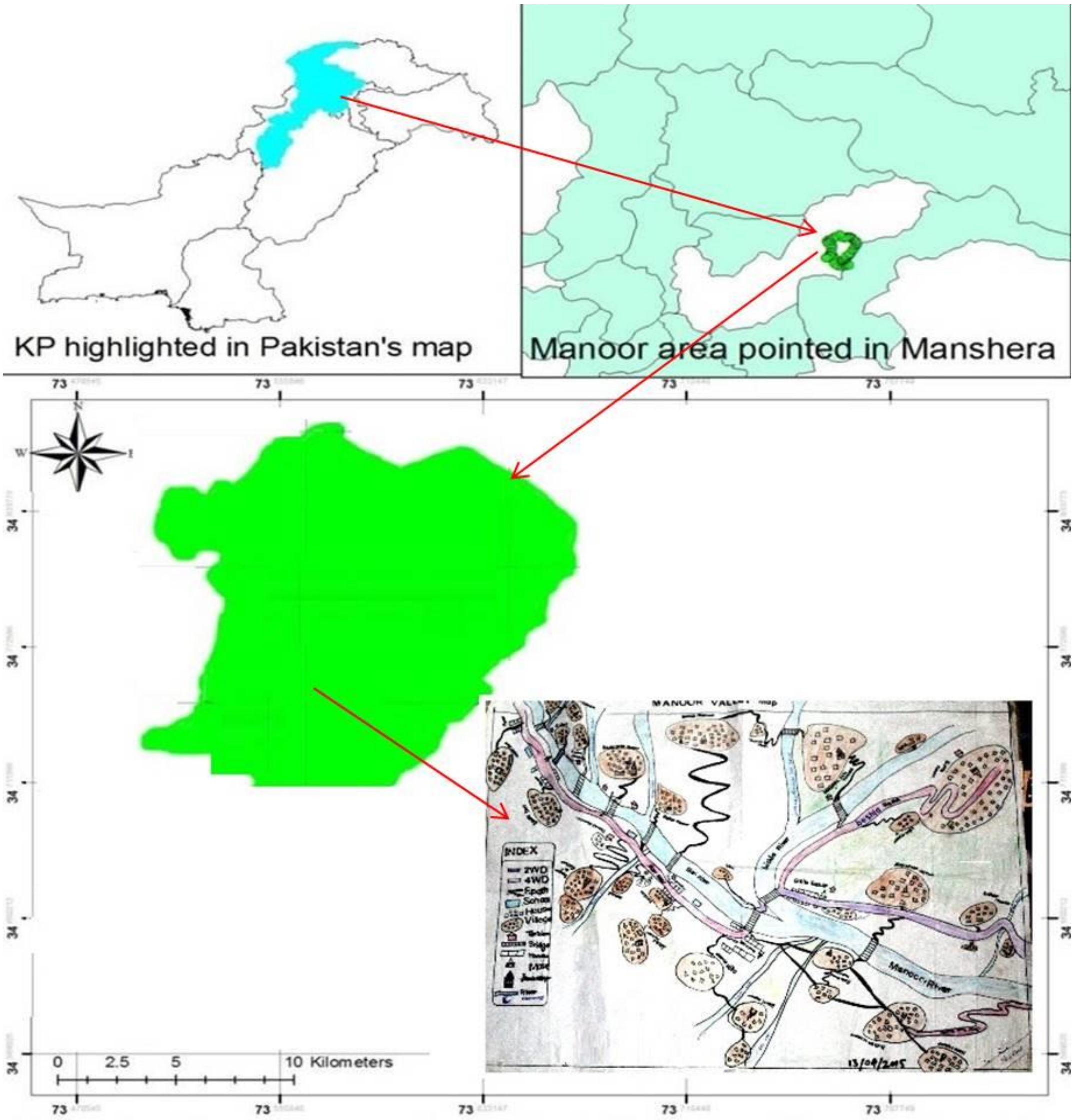

Figure 1. *Map of the study area*

Collection and identification of medicinal plants

Frequent field surveys were undertaken during the early, mid and late summer season of 2016. Plant specimens were collected, tagged and pressed. For botanical information, local informants and traditional healers were interviewed for cultural uses for various diseases. The plant specimens were identified with the help of available literature (Nasir and Ali, 1971-1994; Ali and Qaiser, 1995-2004) and submitted to the Herbarium, Department of Botany, Hazara University Mansehra, Pakistan (HUP).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

Similarity indices

There are a number of indices that calculate similarities in plant species and used in the field of ecology such as; Jaccard's and Sorensen's similarity indices, but no index to date has been designed for evaluation of ethnobiological similarities. So, in comparative ethnobiological studies, the ethnobiologists mostly use conventional ecological tools for evaluation of similarities and dissimilarities. We propose a similarity index named as Rahman's similarity index (RSI), based on the quantification of similar uses of common medicinal plants by comparing current study with previously published documentations from various areas. To evaluate the significance of this newly developed index and its validity in similarities and differences in ethnobiological studies of different regions, the data regarding medicinal plants usage from the current project was taken and compared with 20 previously published studies. Further, we then analyzed this data set through pre-existing indices and Rahman's index to examine the novelty in the study.

Jaccard's similarity index (JI%)

Jaccard's similarity index (JI%) is calculated by comparison of previously published studies from aligned, regional and at global countries by analyzing the percentages of quoted species and their medicinal uses by using the following formula:

$$\mathrm{JI} = c \times \frac{100}{a + b - c}$$

where, a = number of species unique in site A, b = number of species unique in site B. c = number of species common to A and B (Jaccard, 1902; Kayani et al., 2015).

Sorensen's similarity index (QS%)

Sorensen's similarity index (QS%) was developed by a botanist Thorvald Sorensen and published in 1948. The comparison with previously published data collected from different regions was performed by evaluating percentages of the quoted species and their medicinal uses by applying Sorensen similarity index formula (Sorensen, 1948; Wolda, 1981).

$$\mathrm{QS} = \frac{2c}{a + b} \times 100$$

where, a = number of species unique in an area A, b = number of species unique in an area B and c = number of species common to area A and B.

New ethnobiological similarity index

Rahman's similarity index (RSI)

Rahman's similarity index (RSI) is proposed by Inayat Ur Rahman and Farhana Ijaz. RSI is calculated as "by comparison of the present study with the studies previously published from allied, regional, national and global level through the percentages of plant species analyzed and commonly cited with same cultural medicinal uses". The formula used as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

$$RSI = \frac{d}{a + b + c - d}$$

where, “a” is the number of species unique in an area A, “b” is the number of species unique in an area B, “c” is the number of common species in both A and B areas and “d” is the number of common species used for similar ailment in both A and B areas. While a & b ≠ 0 and c & d ≥ 0.

To find out the percentage of common uses between two areas, the formula can be written as

$$RSI = \frac{d}{a + b + c - d} \times 100$$

The probability was calculated (number of events divided by number of possible outcomes) by using the discrete random variables; a = number of common species used for similar ailments in data set A, b = total number of possible species in data set A, a’ = number of common species used for similar ailments in data set B, b’ = total number of possible species in data set B). To quantify the strength of evidence, we advocated 5% significance as a standard level for concluding that there is evidence against the hypothesis tested (Dahiru, 2008).

Results

In present study, the local inhabitants and traditional healers were using 27 medicinal plant species belonging to 19 families for treating 42 different diseases. Traditional medicinal uses of plants mentioned in *Table 1* are compared with 20 published ethnomedicinal documentations of allied, regional, national and at global level *Table 2*.

Critical comparison of JI and QS with RSI

Comparative analysis of the present study and previously published investigations reveals the similarity index of 27 reported medicinal plants ranging from 0% to 15.69% (JI%), 0% to 27.12% (QS%) and 0% to 6.78% (RSI%) as shown in *Table 2*. The analytical approach of Jaccard’s and Sorensen’s similarity indices both determines only the common floral similarity by comparison of a case study with previous documentations. They don’t address the common plants with similar use(s) (*Table 2*). The Jaccard’s index derives similarity of community ecology but now it is frequently used for assessing the similarity of pharmacopoeias and medical floras. Imagine two datasets (medicinal flora) with sample a = 100 and sample b = 100 and an overlap of similar plant species is c = 50; out of these, 25 plants are with similar usage. While JI delivers a similarity index of 20%, the actual overlap is 25% (plant species with similar usage). When we employ QS on the same datasets, it delivers a similarity index of 33%, the actual overlap is 25% (plant species with similar usage). While, the proposed similarity index (RSI) shows the cultural similarities between ethnic communities of different areas by calculating particular plant species, same medicinal usage. Imagine same datasets (medicinal flora) with sample a = 100 and sample b = 100 and an overlap of plant species is c = 50 but out of these, 25 plants are with similar usage d = 25 for RSI. It delivers a similarity index of 25% which is the actual overlap (plant species with similar usage). Upon comparison with pre-existing similarity indices (JI and QS), new medicinal use reports of plant species are more accurately determined by the proposed similarity index (RSI).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

Cross-cultural analysis of the reported species assessed through the newly developed similarity index (RSI) revealed new medicinal uses for *Ajuga integrifolia, Dysphania ambrosioides, Cichorium intybus, Convolvulus arvensis, Indigofera heterantha, Malva parviflora, Plantago major, Medicago sativa*, *Portulaca oleracea, Punica granatum, Taraxacum officinale, Trachyspermum amii, Trifolium repens, Xanthium strumarium* and *Zanthoxylum armatum* for the first time, but not indicated by the JI or QS indices. These results indicate that as far as common vegetation and the floral levels are concerned, these ecological similarity coefficients are accurate and precise but for medicinal similarities, both pose limitations. To overcome these limitations, we propose this new index, which could address the similarity between two regions/sites on the basis of common plant/animal species with respect to their uses.

Table 1. *Randomly selected medicinal plants from the first author PhD study, a supposition for comparison with other documentations from different regions for similarities to show differences in results of two well-known indices with our newly developed one, documentations of shared species are mentioned in the column 'literature comparison'*

S. No	Botanical name	Medicinal uses	Literature comparison
1	*Ajuga integrifolia* Buch.-Ham. ex D. Don.	Diabetes	5♣, 7♣, 11♣, 12♣
2	*Bauhinia variegata* L.	Fatness	4♣, 8♣, 14♣, 18♣
3	*Cannabis sativa* L.	Warmness, insomnia	3♣, 5♣, 7♣, 12♥, 17♣, 18♣
4	*Dysphania ambrosioides* (L.) Mosyakin & Clemants	Fever	5♣
5	*Cichorium intybus* L.	Typhoid fever	3♣, 12♥
6	*Convolvulus arvensis* L.	Diarrhoea, dysentery	2♣, 3♣, 5♣, 7♣, 12♣, 17♣,
7	*Indigofera heterantha* Wall. ex Brandis	Diuretic	2♣, 4♣, 12♣, 14♣
8	*Justicia adhatoda* L.	Throat infection, cough	3♥, 4♥, 14♥, 20♥
9	*Malva parviflora* L.	Gas trouble	17♣
10	*Medicago sativa* L.	Gas trouble	
11	*Mentha longifolia* (L.) Huds.	Abdominal pain, gas trouble	2♥, 5♣, 6♥, 19♥
12	*Mentha royleana* Benth.	Diarrhoea, vomiting	2♥, 12♥
13	*Oxalis corniculata* L.	Vitamin C deficiency, mouth smell	3♣, 5♣, 12♣, 14♥, 16♣
14	*Plantago major* L.	Diarrhoea, fatness	2♣, 5♣, 6◙, 7♣, 12♣,
15	*Polygonum plebeium* R. Br.	Cough	2♣, 5♣, 14♥
16	*Portulaca oleracea* L.	Diuretic	12♣, 14♣
17	*Punica granatum* L.	Gas troubles, indigestion	7♣, 9♣, 12♣, 13♣, 14♣, 16♣, 17♣, 18♣
18	*Ricinus communis* L.	Constipation	1♥, 3♥, 5♥, 7♥, 12♣, 13♣, 17♣, 18♣, 20♣
19	*Salvia moorcroftiana* Wall. ex Benth.	Cough, diarrhoea	2♣, 12♣, 16♣
20	*Silybum marianum* (L.) Gaertn.	Liver problems	1♥, 3♥
21	*Taraxacum officinale* F.H. Wigg.	Diabetes	1♣, 2♣, 6♣
22	*Trachyspermum ammii* (L.) Sprague	Diuretic, kidney stone removal	7♣
23	*Trifolium repens* L.	Fever	2♣
24	*Verbascum thapsus* L.	Diarrhoea	1♣, 2♣, 3♥, 5♥, 7♥, 14♥
25	*Vitex negundo* L.	Indigestion, stomach-ache, gas troubles	9♣, 13♣, 14♥, 20♣
26	*Xanthium strumarium* L.	Diuretic, kidney stone removal	5♣, 12♣, 14♣
27	*Zanthoxylum armatum* DC.	Abdominal pain, indigestion	7♣, 8♣, 12♣, 13♣

♥ = Similar uses reported and ♣ = Dissimilar uses reported
1 = Jamal et al. (2012); 2 = Khan et al. (2013); 3 = Qureshi et al. (2008); 4 = Jan et al. (2011); 5 = Matin et al. (2001); 6 = Ume-Ummara et al. (2013); 7 = Abbasi et al. (2013); 8 = Ahmad et al. (2012); 9 = Ajaib and Khan (2014); 10 = Ahmad et al. (2009); 11 = Tariq et al. (2014); 12 = Akhtar et al. (2013); 13 = Rashid et al. (2015); 14 = Ijaz et al. (2016); 15 = Bano et al. (2014); 16 = Ahmad and Pieroni (2016); 17 = Ullah et al. (2014); 18 = Kichu et al. (2015); 19 = Ozdemir and Alpinar (2015); 20 = Kadir et al. (2014)

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DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

Discussion

In present study, 27 plant species belonging to 19 families were reported by the local informants for 42 various health issues. The ethnomedicinal uses reported in the present study (*Table 1*) were compared with 20 published ethnomedicinal studies from allied, regional, national as well as at global level (*Table 2*). Comparative analysis of present study with previous documentations revealed that the similarity index of 27 reported medicinal plants ranged from 0% to 15.59% (JI) and 0% to 27.12% (QS) (*Fig. 2*). Highest degree of similarity index was found with studies conducted by Qureshi et al. (2008); Akhtar et al. (2013); Ijaz et al. (2016), Matin et al. (2001) and Khan et al. (2013) with JI (15.69%, 15.22%, 13.89%, 10.78% and 10.10%) respectively and QS (27.12%, 26.42%, 24.39%, 19.47% and 18.35%) respectively. Furthermore, three more international documentations having the common species with the present study, due to which JI and QS shows similarity percentage with the study area, but interesting thing is that all of them were used for different medicinal purposes which clearly means that Jaccard's and Sorensen's similarity indices both targets only the common medicinal plant species in both areas but not its common medicinal uses (*Table 1*). *Table 1* shows three different values which are of species enlisted only in the study area, common species with similar uses and common species with dissimilar uses. But JI and QS shows the results by combining both common species with similar uses and common species with dissimilar uses in similarity. This simply reveals that either the common plant species is similar or different in medicinal use but it is in similarity by JI and QS. In comparison, we found Jaccard's similarity index and Sorensen's similarity index value 0% with 3 studies from Siran Valley, (Mansehra), Pakistan (Ahmad et al., 2009), Deosai Plateau, Gilgit Baltistan, Pakistan (Bano et al., 2014) and Aladaglar, Nigde-Turkey (Ozdemir and Alpınar, 2015). But in this case, no single common plant species has been found that is why JI and QS also showed 0% similarity (*Fig. 2*).

Table 2. *Comparison of the present study with previous studies at regional, neighboring and global level*

Study area	NRSAA	TSCBA	SEOAA	SEOOA	CSSU	CSDU	JI%	QS%	RSI%	Citation	Sign. (5%)
Kaghan Valley, Pakistan	30	4	26	23	2	2	8.70	16.00	4.00	Jamal et al. (2012)	0.49
Naran Valley, Pakistan	101	10	91	17	2	8	10.10	18.35	1.83	Khan et al. (2013)	0.15
Abbottabad, Pakistan	47	8	39	19	4	4	15.69	27.12	6.78	Qureshi et al. (2008)	1.26
Kaghan Valley, Pakistan	75	3	72	24	1	2	3.19	6.19	1.03	Jan et al. (2011)	0.05
Shogran Valley, Pakistan	107	11	96	16	2	9	10.78	19.47	1.77	Matin et al. (2001)	0.14
Shogran Valley, Pakistan	50	2	48	25	1	1	2.78	5.41	1.35	Ume-Ummara et al. (2013)	0.07
Himalaya, Pakistan	89	9	80	18	2	7	10.00	18.18	2.02	Abbasi et al. (2013)	0.17
Kotli, AJK, Pakistan	112	2	110	25	0	2	1.49	2.94	0.00	Ahmad et al. (2012)	0.00
Kotli, AJK, Pakistan	50	2	48	25	0	2	2.78	5.41	0.00	Ajaib and Khan (2014)	0.00

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Siran Valley, Pakistan	143	0	143	27	0	0	0.00	0.00	0.00	Ahmad et al. (2009)	0.00
Nathiagali, Pakistan	31	1	30	26	0	1	1.79	3.51	0.00	Tariq et al. (2014)	0.00
Swat, North Pakistan	106	14	92	13	3	11	15.22	26.42	2.83	Akhtar et al. (2013)	0.31
AJK, Pakistan	73	4	69	23	0	4	4.49	8.60	0.00	Rashid et al. (2015)	0.00
Sarban Hills, Abbottabad, Pakistan	74	10	64	17	5	5	13.89	24.39	6.10	Ijaz et al. (2016)	1.25
Deosai Plateau, Gilgit Baltistan, Pakistan	50	0	50	27	0	0	0.00	0.00	0.00	Bano et al. (2014)	0.00
Thakt-e-Sulaiman Hills, Pakistan	51	3	48	24	0	3	4.29	8.22	0.00	Ahmad and Pieroni (2016)	0.00
Lakki Marwat, Pakistan	72	5	67	22	0	5	5.88	11.11	0.00	Ullah et al. (2014)	0.00
Chungtia village, Nagaland, India	135	3	132	24	0	3	2.65	5.16	0.00	Kichu et al. (2015)	0.00
Aladaglar, Nigde-Turkey	110	0	110	27	0	0	0.00	0.00	0.00	Ozdemir and Alpinar (2015)	0.00
Thanchi, Bandarban Hill, Bangladesh	84	3	81	24	1	2	2.91	5.66	0.94	Kadir et al. (2014)	0.04

NRSAA: Number of recorded plants species of aligned areas, **TSCBA:** Total species common in both area, **SEOAA:** Species enlisted only in aligned areas, **SEOOA:** Species enlisted only in our study area, **CSSU:** Common species with similar uses, **CSDU:** Common species with dissimilar uses, **JI:** Jaccard's similarity index, **QS:** Sorrenson's similarity index, **RSI:** Rahman's similarity index, **Sign.:** Significance level

Applications of Rahman's similarity index (RSI)

The similarities and differences in ethnomedicinal studies seem to target the importance of traditional knowledge on medicinal plants in different regions (Ijaz et al., 2016). The proposed similarity index (RSI) shows the cultural similarities between ethnic communities of different areas by calculating particular plant species, same medicinal usage. Traditional medicinal uses of plants mentioned in *Table 1* are compared with 20 published ethnomedicinal documentations of allied, regional, national and at global level (*Table 2*). Review of the literature indicates the medicinal similarity index uses ranges from 0% (Ahmad et al., 2009, 2012; Ajaib and Khan, 2014; Bano et al., 2014; Tariq et al., 2014; Ullah et al., 2014; Ozdemir and Alpınar, 2015; Rashid et al., 2015; Kichu et al., 2015; Ahmad and Pieroni, 2016) to 6.78% (Qureshi et al., 2008). The highest degree of similarity index of the present study was found with a study conducted in Abbottabad, Pakistan by Qureshi et al. (2008) with RSI = 6.78% (*Fig. 2*). In comparison, we found RSI value 0% with 10 previous studies and out of these seven studies had common plant species but no single common plant species has been cited for common medicinal use(s) that is why RSI showed 0% similarity (*Fig. 2*). These results indicate that the new index (RSI) could address the similarity between two regions/sites on the basis of common plant/animal species with respect to their uses. Medicinal uses comparative analysis reveals that maximum variation in RSI might be due to cultural/ethnic or traditional differences between the current study area and previously documented studies in allied, regional, national and global levels. Distance

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DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

between study area and other regions also support the variation in results as it directly correlates the vegetation of an area due to the differences in their edaphic factors (Coughenour and Ellis, 1993; Witkowski and O'Connor, 1996) and physiographic because each area has specific surface features and their form (Barnes et al., 1998). RSI shows the cultural similarities between ethnic communities of different areas by calculating particular plant species similar medicinal usage. RSI focuses on similar uses of common medicinal plants (*Table 2* and *Fig. 2*). Upon comparison with both the similarity indices, new medicinal use reports of plant species are more accurately determined by the proposed similarity index (*Fig. 2*).

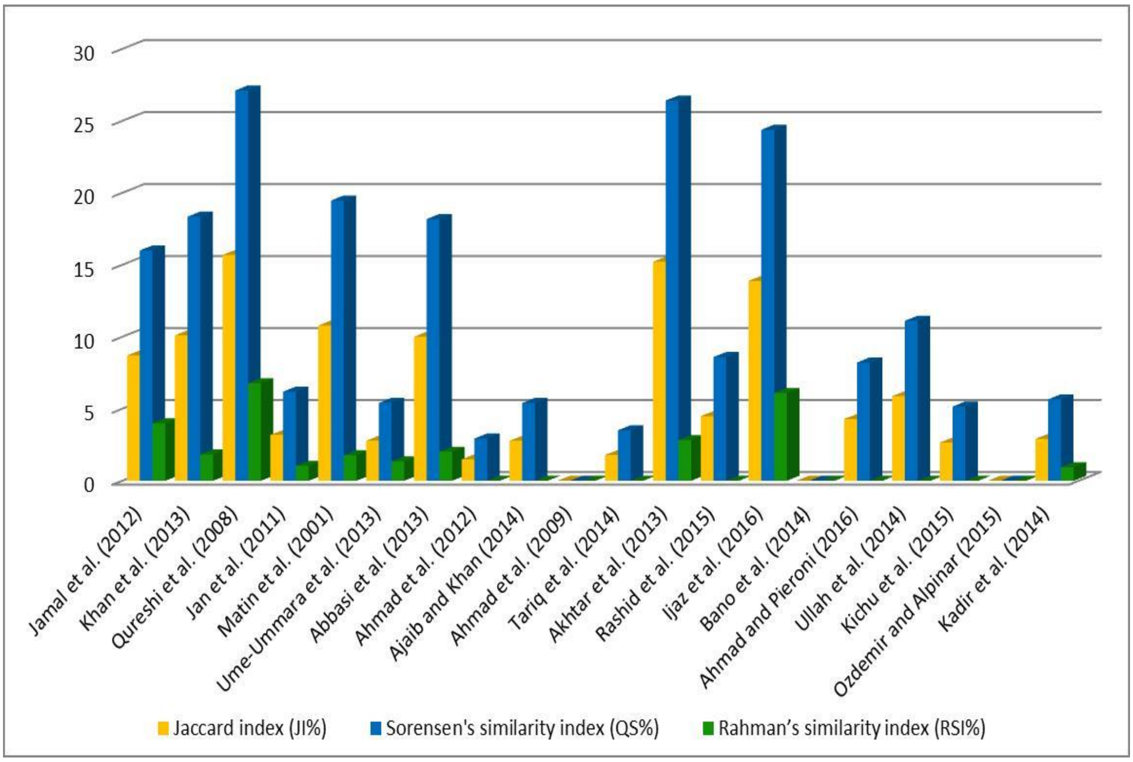

Figure 2. *Comparison of the case study with previous studies at regional, national and global level through Jaccard's, Sorensen's, Rahman's similarity indices*

Similarity indices and significance level

Comparative analyses of the present study with Matin et al. (2001) revealed that in total 27 reported medicinal plants; two species were common in medicinal usage to both areas. The JI is consequently 10.78% and QS is 19.47%, but newly proposed index (RSI) showed 1.77% similarity. Whereas the results of RSI are also supported by significance test (5%), as the level of significance between present study and Matin et al. (2001) indicating the significance value of 0.13. We found RSI value of 0% while comparing with previously done 10 ethnobiological studies (Ahmad et al., 2009, 2012; Ajaib and Khan, 2014; Bano et al., 2014; Tariq et al., 2014; Ullah et al., 2014; Ozdemir and Alpınar, 2015; Rashid et al., 2015; Kichu et al., 2015; Ahmad and Pieroni, 2016), and out of these 7 studies had common plant species but no single common plant species has been cited for common medicinal use(s) that is why RSI showed 0%

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similarity. The level of significance also strongly supports these results by indicating the highest level of significance (0.00%) (*Table 2*). The threshold level of significance for RSI is >1% (more than 1), as this is the border line of significance level at 5% ($p = 0.05$). Furthermore, with 2 previous studies (Ijaz et al., 2016; Qureshi et al., 2008) maximum RSI (6.1% and 6.78%) was found and following these results the significance level was also increased (1.25% and 1.26%) respectively in comparison with other studies. Our results are in accordance with Goodall (1966) who reported that a lower value of the similarity index may often correspond with a less probable degree of similarity and vice versa.

Conclusion, novelty and future impact

After analytical comparison, some new medicinal uses of *Ajuga integrifolia, Bauhinia variegata, Dysphania ambrosioides, Convolvulus arvensis, Indigofera heterantha, Malva parviflora, Plantago major, Salvia moorcroftiana, Taraxacum officinale, Xanthium strumarium, Medicago sativa, Portulaca oleracea, Punica granatum, Trachyspermum ammii, Trifolium repens* and *Zanthoxylum armatum* were recorded for the first time for the current reported medicinal uses from the study area. These novel/new medicinal use reports of plant species were pinpointed by the newly developed similarity index (RSI) but not indicated by the JI or QS indices. Inferences derived from RSI are reliable and upon comparison can lead to novel findings and new medicinal use reports of plant species more accurately. Further, RSI specifically targets the ethnobiological similarities and would be a useful tool/index for future studies in the assessment of rigorous quantitative ethnobiological data.

Acknowledgements. First author would like to thank Higher Education Commission (HEC), Pakistan for granting scholarship under International Research Support Initiative Program (IRSIP) to conduct a research work at Missouri Botanical Garden, USA. The authors gratefully acknowledge Dr. Robbie Hart (Missouri Botanical Garden, P. O. Box 299, St. Louis, MO 63166-0299, USA) for his confirmatory input in the index. We are thankful to Dr. Shahzad, Head Department of Mathematics, Hazara University Mansehra, Pakistan as well for. helping us in statistical work. The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding to the Research Group number (RG-1435-014).

Author contributions. IUR designed and conceived the study. IUR and FI developed the formula, gathered relevant literature and analyzed the data. IUR wrote the manuscript and NA helped in writing. AA, ZI and RH supervised the work, RH and SMK helped in the data analysis, RBU, EFA and AAA critically reviewed the manuscript. IUR and NA revised the manuscript, MSA helped in revision. All the authors have read and approved the final manuscript.

Ethical approval. This ethnomedicinal study was approved by the “Advanced Studies Research Board, Hazara University Mansehra, Pakistan”. A semi-structured questionnaire (written consent) was developed and filled during interviews with local informants and traditional healers.

Conflict of interests. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_27652777

EFFECT OF SELECTED PLANT PREPARATIONS CONTAINING BIOLOGICALLY ACTIVE COMPOUNDS ON WINTER RAPE (*BRASSICA NAPUS* L.) YIELDING

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(Received 9th Sep 2018; accepted 22nd Nov 2018)

Abstract. A field experiment was carried out in 2013-2016 in the Agricultural Experimental Station – Zawady of the University of Natural Sciences and Humanities in Siedlce, Poland. The examined factors included: I – three morphotypes of winter rape: population cultivar (Monolit), hybrid semi-dwarf (PR44D06) cultivar, restored hybrid cultivar of conventional growth type (PT205); II – two sowing methods: row spacing of 22.5 cm, row spacing of 45.0 cm; III – four types of applied biostimulators: control variant (without application of biostimulators), Tytanit® biostimulator, Asahi®SL biostimulator and Silvit® biostimulator. The aim of the research was to determine the effect of natural plant preparation on the yield of four cultivars of winter rape. It was found that biostimulators applied in the experiments increased the thousand-seed weight on average from 0.03 to 0.1 g in comparison to the control variant. The highest value of this feature was obtained in the variant with Asahi®SL treatment and significantly lower in plots treated with Silvit® and Tytanit® bioregulators. A significant increase in seed yielding was observed as the effect of natural growth stimulators. The highest value of this feature was observed in all cultivars after the application of Asahi®SL and Silvit®.
Keywords: *Brassica napus, biostimulator, morphotype, seed yield, straw yield*

Introduction

Unfavourable soil conditions and stress related to drought are one of numerous factors affecting a decrease in the quantity and quality of yield. Strong stress causes a disturbance of the cell structure and metabolism and, as a result, results in stopping the photosynthesis and disturbing plant metabolism. In the opinion of Sharm et al. (2014), the application of natural growth stimulators increases plant resistance to the unfavourable effects of various stress factors, including pathogens. Van Oosten et al. (2017) believe that biostimulators have a beneficial effect on the growth and productivity of plants, which is why they are gradually becoming elements of standard agrotechnics in the field cultivation. The composition of biostimulators usually include one or more biologically active organic compounds, such as: amino acids, vitamins, enzymes, plant hormones and macro and microelements, while a separate group consists of biopreparations produced with the participation of live microorganisms containing various types of bacteria and fungi.

Asahi SL, also known as Atonik or Chaperone, is the most often applied biostimulators in farming and horticulture. Asahi SL contains phenolic compounds: sodium o-nitrophenolate and sodium p-nitrophenolate and sodium 5-nitroguaiacolate, which naturally occur in plant cells and take part in biochemical and physiological plant processes. Phenols increase plant tolerance to all harmful stimuli from the environment, especially drought. Additionally, they reduce the decrease in auxins concentration in plants caused by water shortage, and control plant growth and development (Matysiak et al., 2011). Titanite is a mineral growth stimulant containing titanium ions that are organically complexed and are well absorbed by plants. Titanium is considered an essential element for the growth and development of plants. The application of even a small amount of titanium affects the growth of plants and increases their resistance to stress factors. It increases the activity of enzymes and thus increases the durability of mineral uptake in the soil.

Silvit is a natural growth stimulant containing the active silicon that is fully absorbed by plants. This bioregulator limits the physical penetration of tissues by pests, because cell walls become more resistant to enzymes produced by pathogens (fungistatic effect), increases plant tolerance to low temperatures, reduces transpiration during drought, intensifies photosynthesis in low light conditions, reduces the impact of high salinity for plant growth and phosphorus deficiency by regulating its uptake and eliminating toxic effects of aluminium on root growth.

The study assumed the research hypothesis that the application of natural plant preparations can contribute to an increase in winter rape (*Brassica napus* L.) yielding. Due to scarce research concerning the effect of this factor, a study was undertaken to determine the effect of the Tytanit®, Asahi®SL and Silvit® biostimulator on the thousand-seed weight, seed yield, straw yield and harvest index of three winter rape cultivars.

Materials and Methods

Field experiments were conducted in three growing seasons: I – from 13.08.2013 to 11.07.2014; II - 11.08.2014 to 17.07.2015; III - 14.08.2015 to 14.07.2016 in the Agricultural Experimental Station – Zawady (52º03'N and 22º33'E) of the University of Natural Sciences and Humanities in Siedlce, Poland. The experiment was established at the random sub-blocks design (split – split – plot), in three replications. The surface of one plot for harvest was 18 m^{-2}. It involved examination of the following factors: rape cultivars, sowing methods and types of biostimulators applied. With regard to the main experimental factor, three cultivars were sown, representing three cultivation types: population cultivar – Monolit, restored hybrid cultivar of a semi-dwarf growth type –

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27792789

PR44D06, restored hybrid cultivar of a conventional growth type – PT205. The secondary factor included two sowing methods: row spacing of 22.5 cm (row sowing – density sowing of 60 seeds per 1 m^{-2}), 45.0 cm planting space (precision sowing – sowing density 40 seeds per 1 m^{-2}). With regard to the tertiary factor, three types of biostimulators were applied against the control variant, cultivated without biostimulator application. Tytanit® biostimulator (active substance – titanium) was applied in autumn at the 4-8 leaves unfolded stage (BBCH 14-18) at the dose of 0.20 $dm^3 \cdot ha^{-1}$ (date I), in spring after vegetation started: beginning of side shoot development at the dose of 0.20 $dm^3 \cdot ha^{-1}$ (date II), at the stage of flower bud development (inflorescence emergence) – beginning of flowering at 0.20 $dm^3 \cdot ha^{-1}$ (date III). Asahi®SL biostimulator was applied on three dates: in autumn at the BBCH 13-15 stage at the dose of 0.60 $dm^3 \cdot ha^{-1}$, in spring, after vegetation started at the dose of 0.60 $dm^3 \cdot ha^{-1}$, two weeks after performing the second procedure (0.60 $dm^3 \cdot ha^{-1}$). Silvit® biostimulators containing active silicon were applied three weeks after emergence at the dose of 0.20 $dm^3 \cdot ha^{-1}$, in spring, after vegetation started at the dose of 0.20 $dm^3 \cdot ha^{-1}$ and two weeks after the second procedure, at the dose of 0.20 $dm^3 \cdot ha^{-1}$.

The experiment was carried out on soil classified in the order of soils with clay translocation, type – Luvisols, subtype – Albic Luvisols. This soil was classified as IVa bonitation class soil, of very good rye complex for agricultural use. During the years of the experiment, soil pH (in 1n KCl) was slightly acidic, ranging from 5.68 to 5.75. The soil was characterized by a low total content of nitrogen (on average 0.85 $g \cdot kg^{-1}$), phosphorus (on average 0.44 $g \cdot kg^{-1}$), potassium (on average 0.65 $g \cdot kg^{-1}$) and calcium (on average 0.83 $g \cdot kg^{-1}$) and the medium content of magnesium (on average 0.42 $g \cdot kg^{-1}$) and sulphur (on average 0.13 $g \cdot kg^{-1}$). It demonstrated low content of available phosphorus forms (on average from 75 to 80 $mg \cdot kg^{-1}$) and medium content of available forms of potassium (on average from 200 to 205 $mg \cdot kg^{-1}$) and magnesium (on average from 59 to 61 $mg \cdot kg^{-1}$). The forecrop for winter rape in the first year of research was spring wheat and in subsequent years it was winter triticale. After forecrop harvest, a set of post-harvest procedures was carried out using the ploughing aggregate + open cage roller, and then two weeks after the first procedure, pre-sow ploughing at the depth of 20.0 cm was carried out, using a ring roller at the same time. To prepare the soil for sowing and to mix fertilizers, a complex soil tillage unit was used. Before sowing, phosphorus-potassium fertilization was applied at the dose of 40 kg $P \cdot ha^{-1}$ and 110 kg $K \cdot ha^{-1}$ and the first dose of 40 kg $N \cdot ha^{-1}$. Fertilization was applied in the form of Lubofos at the dose of 600 kg. Fertilizing doses were supplemented with 55.9 $kg \cdot ha^{-1}$ of ammonium nitrate (19 kg $N \cdot ha^{-1}$), 29.6 $kg \cdot ha^{-1}$ triple superphosphate (13.6 kg $P \cdot ha^{-1}$) and 29 $kg \cdot ha^{-1}$ potassium salt (17.9 kg $K \cdot ha^{-1}$). The second dose of nitrogen in the amount of 100 $kg \cdot ha^{-1}$ was applied in spring, before vegetation started (BBCH 28-30) applying ammonium nitrate at the dose of 255.5 $kg \cdot ha^{-1}$ (86.9 kg $N \cdot ha^{-1}$) and ammonium sulphate at the dose of 62.5 $kg \cdot ha^{-1}$. The third dose of ammonium 60 $kg \cdot ha^{-1}$ was applied at the inflorescence emergence (BBCH 50), by applying ammonium nitrate at the dose of 176.5 $kg \cdot ha^{-1}$ (60 kg $N \cdot ha^{-1}$). Winter rape was sown in two row spacing widths: 22.5 cm and 45.0 cm, maintaining the density of 60 $pieces \cdot m^{-2}$ and 40 $pieces \cdot m^{-2}$, respectively. The sowing was done at the optimal time recommended for this region (in 2013 – August 13, 2014 – August 11, and in 2015 – August 14). Directly after sowing, herbicide Command 480 EC at the dose of 0.25 $dm^3 \cdot ha^{-1}$ was applied to carefully cultivated soil. Next, at the stage of BBCH 13-14, Fusilade Forte 150 EG at the dose of 2.0 $dm^3 \cdot ha^{-1}$ was applied. At the 4-8 leaves unfolded stage (BBCH 14-18)

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fungicide Horizon 250 EW was applied (0.75 $dm^3 \cdot ha^{-1}$). At the stem elongation stage (BBCH 30), flower bud emergence stage (BBCH 50-58), flowering (BBCH 60-69) – Proteus 110 OD insecticide was applied at the dose of 0.6 $dm^3 \cdot ha^{-1}$. At the beginning of flowering (BBCH 61) – Propulse 250 SE fungicide was applied at the dose of 1.0 $dm^3 \cdot ha^{-1}$. At the stage of first petals falling – Mondatak 450 EC was applied at the dose of 1.0 $dm^3 \cdot ha^{-1}$. Rape was collected in two stages, in the first and second decade of July (I year: 11.07.2014; II year: 17.07.2015; III year: 14.07.2016).

The thousand-seed weight was specified for two samples of 500 seeds, collected from purified yield from each plot. The volume of seed yield from each plot was determined by weight after threshing, and then, after adjusting to the normative humidity (9.0% of water content in seeds), it was converted to the area of 1 ha.

The value of harvest index was calculated according to the formula:

$$HI = \frac{Pn}{Pn + Ps} * 100\% \qquad \text{(Eq.1)}$$

where:
HI – harvest index (%)
Pn – seed yield ($Mg \cdot ha^{-1}$)
Ps – straw yield ($Mg \cdot ha^{-1}$).

The results of the study were statistically analysed with the use of the analysis of variance. The significance of variation sources was tested with the "F" Fischer-Snedecor test, and the assessment of significance at the significance level of p=0.05 between compared means – with Tukey's range test.

Weather conditions were diversified in the years of the research (*Table 1*).

Table 1. *Characteristics of weather conditions in the years 2013-2016 (Zawady Meteorological Station, Poland)*

Months	Rainfalls (mm)				Air temperature (°C)			
	Multiyear sum	Monthly sum			Multiyear mean	Monthly means		
	1996-2010	2013-2014	2014-2015	2015-2016	1996-2010	2013-2014	2014-2015	2015-2016
VIII	59.9	15.0	105.7	11.9	18.5	18.8	18.1	21.0
IX	42.3	94.3	26.3	47.1	13.5	11.7	14.1	14.5
X	24.2	32.8	3.0	37.0	7.9	9.3	8.5	6.5
XI	20.2	34.7	32.5	42.2	4.0	5.1	3.4	4.7
XII	18.6	15.4	90.4	16.5	-0.1	1.2	0.1	3.7
I	19.0	28.6	51.4	10.9	-3.2	-4.5	0.6	-4.5
II	16.0	34.0	0.7	29.0	-2.3	0.7	0.7	2.5
III	18.3	29.6	53.1	33.5	2.4	5.8	4.6	3.5
IV	33.6	45.0	30.0	28.7	8.0	9.8	8.2	9.1
V	58.3	92.7	100.2	54.8	13.5	13.5	12.3	15.1
VI	59.6	55.4	43.3	36.9	17.0	15.4	16.5	18.4
VII	57.5	10.0	62.6	35.2	19.7	20.8	18.7	19.1
VIII-VII	**427.5**	**487.5**	**599.2**	**383.7**	**8.2**	**9.0**	**8.8**	**9.5**

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In the growing season 2013-2014 the total annual precipitation was by 60.0 mm higher in comparison to the average total precipitation from the multiannual period, and the mean air temperature was by 0.8°C higher. The highest annual total precipitation (on average 599.2 mm) and the lowest mean annual air temperature (on average 8.8°C) were recorded in the vegetation season of 2014-2015. The total annual precipitation in this period was higher by 171.7 mm in comparison with the total precipitation from the multiannual period. The 2015-2016 growing season was the warmest and most dry. The total annual precipitation was 43.8 mm lower than the multiannual average, and the average annual air temperature was 1.3°C higher in comparison to the mean of 1996-2010.

Results and discussion

The research demonstrated that Asahi SL, Silvit and Tytanit biostimulators significantly increased the thousand-seed weight on average from 0.03 to 0.1 g in comparison to the control variant (*Table 2*). The highest value of this feature was obtained in the variants where Asahi®SL was applied – on average 5.64 g, and significantly lower in plots treated with Silvit® and Tytanit® bioregulators (on average 5.58 g and 5.57 g). This is consistent with previous research by Harasimowicz-Hermann and Borowska (2006), who found that the thousand-seed weight was also higher by 18.0% in comparison to the control variant as a result of applying Asahi SL biostimulator.

Table 2. *The weight of one thousand seeds (g) depending on factors of experience*

Year	Cultivars			Ways of sowing		Types of biostimulators used				Mean
	2013-2014	2014-2015	2015-2016	45.0	22.5	Control variant	Tytanit®	Asahi®SL	Silvit®	
Monolit	5.47	5.33	5.51	5.46	5.41	5.39	5.42	5.51	5.42	**5.43**
PR 44D06	5.85	5.71	5.61	5.71	5.73	5.68	5.70	5.77	5.73	**5.72**
PT 205	5.72	5.60	5.48	5.60	5.60	5.55	5.59	5.65	5.60	**5.60**
Mean	**5.68**	**5.54**	**5.53**	**5.59**	**5.58**	**5.54**	**5.57**	**5.64**	**5.58**	**-**

$LSD_{0.05}$ for: years - 0.01; cultivars - 0.01; types of biostimulators used - 0.02; ways of sowing - 0.01 interaction: years x cultivars - 0.02; cultivars x types of biostimulators used - 0.03; ways of sowing x cultivars - 0.02

On the other hand, Malarz et al. (2008) and Słowiński and Jarosz (2008) did not demonstrate a significant effect of biostimulators on thousand-seed weight. Matysiak et al. (2011) after the application of Asahi SL at the BBCH 28-30 stage obtained only a slight increase in thousand-seed weight and only in one research season, while Budzyński et al. (2008) after applying the same bioregulator, demonstrated only a trend towards an increase in the value of this feature. The thousand-seed weight of the semi-dwarf cultivar PR44D06 was on average higher by 0.12 g than for the conventional form PT 205, while the lowest (on average by 5.43 g) was found for the line cultivar (Monolit). A similar trend was reported by Mekki (2013) and Czarnik et al. (2015). The authors obtained higher thousand-seed weights in heterotic cultivars than in population cultivars. On the other hand, Jankowski and Budziński (2007) recorded lower thousand-

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seed weight, on average by 8.0%, in heterotic cultivars Kaszub and Kronos, as compared to population cultivar Contact. The thousand-seed weight was not significantly diversified in relation to the sowing method. The absence of a significant effect of this factor was also demonstrated by Ozera (2003), Shahin and Valiollah (2009), Uzun et al. (2012) and Krcek et al. (2014). On the other hand, Różyło and Pałys (2011) reported a slight trend to increase the thousand-seed weight for the 33.0 cm spacing in comparison to 25.0 cm spacing. The thousand-seed weight of the examined cultivars significantly depended on weather conditions in individual years of the experiment. Restored hybrids PR44D06 and PT205 demonstrated the highest thousand-seed weight in the first year of the research, characterized by excessive precipitation in June and in the first decade of July, and lower values were obtained in the third year of research, with precipitation shortage occurring in May and June, and the average temperature in those months higher than the multiannual average. On the other hand, in that season, the population cultivar Monolit demonstrated the highest value of this feature in comparison to the initial years of the research. The types of applied biostimulators significantly affected the seed yield in the examined cultivars (*Table 3*).

Table 3. *Seed yield (Mg·ha^{-1}) depending on factors of experience*

Year	Cultivars			Ways of sowing		Types of biostimulators used				Mean
	2013-2014	**2014-2015**	**2015-2016**	**45.0**	**22.5**	**Control variant**	**Tytanit®**	**Asahi®SL**	**Silvit®**	
Monolit	4.38	3.86	2.57	3.13	4.08	3.48	3.55	3.79	3.60	**3.61**
PR 44D06	4.93	4.45	2.74	3.66	4.42	3.84	3.99	4.24	4.09	**4.04**
PT 205	5.20	4.68	2.73	3.89	4.52	4.07	4.19	4.37	4.17	**4.20**
Mean	**4.84**	**4.33**	**2.68**	**3.56**	**4.34**	**3.80**	**3.91**	**4.13**	**3.96**	**-**

$LSD_{0.05}$ for: years - 0.02; cultivars - 0.02; types of biostimulators used - 0.03; ways of sowing - 0.01
interaction: years x cultivars - 0.03; cultivars x types of biostimulators used – n.s.; ways of sowing x cultivars – n.s.

The highest value of this feature in all cultivars was observed after the application of Asahi®SL – on average 4.13 Mg·ha^{-1} and Silvit® – on average 3.96 Mg·ha-1. Similarly, Matysiak et al. (2011, 2012) found that Kelpak and Asahi SL bioregulators increased the volume of the seed yield on average by about 25%. Harasimowicz-Hermann and Borowska (2006) depending on the vegetation season, found an increased yield in the range of 9.0 to 19.0% in comparison to the variant without natural growth stimulators. Budzyński et al. (2008) and Matysiak and Kaczmarek (2008) obtained the best yield producing effect in the variants where a single application was used at the BBCH 30 stage or later (BBCH 50). The authors found that single application resulted in a yield increase on average by 3.0% (140 kg), and double application (on average by 5.0% (220 kg)). The effect of the genetic factor on seed yield was demonstrated by Zhang et al. (2011), Mekki (2013) and Nowosad (2016). In own research, the highest seed yield (on average 4.20 Mg·ha^{-1}) was found for the long-stem cultivar PT205, significantly lower (on average 4.04 Mg·ha^{-1}) for PR44D06, and the lowest (on average 3.61 Mg· ha^{-1}) for the population cultivar Monolit. This is in line with the research results obtained by Jankowski and Budzyński (2007), El-Habbasha and El-Salama (2010) and Wójtowicz and Jajor (2010), who also emphasized that heterotic varieties demonstrated higher

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yielding potential than conventional ones. A higher seed yield in restored hybrid (DK Exquisite) of a conventional growth type was documented in Krcek et al. (2014). Jankowski et al. (2016) also recorded higher seed yields (on average 4.73 $Mg \cdot ha^{-1}$) in the conventional morphotype of the Visby cultivar in comparison to semi-dwarf cultivars PR445D03 and Avenir. On the other hand, Kotecki et al. (2004) obtained the highest value of the analysed feature in the population morphotype (Lirajet and Lisek). The statistical calculations did not show any significant interaction between the varieties of biostimulators used. Increasing sowing density from 40 to 60 seeds per 1 m^{-2} significantly affected an increase in seed yield (on average by 0.78 $Mg \cdot ha^{-1}$). No interaction was proven between cultivars and methods of sowing. This shows that similar cultivars responded in a similar way to row spacing and sowing density. Similar results were obtained by Cheema et al. (2001), Ozera (2003) and Champiri and Bagheri (2013). Those authors found a higher value of the discussed feature in narrow row spacing in comparison to cultivation at 30.0 and 45.0 cm. This regularity was also confirmed by Uzun et al. (2012), Różydło and Pałys (2014) and Kutcher et al. (2015). Different research results were obtained by Krcek et al. (2014) who, based on one-year studies, did not reveal significant differences in the seed yield for narrow (12.5; 25.0 cm) and wide (35.0; 45.0 cm) row spacing. The analysis of variance did not show any significant interaction of varieties with the sowing methods, which indicates that the studied varieties reacted in the same way to changing the inter-row spacing. Yana and Hunta (1998) provide that humidity and thermal conditions in 80% affect the yield. In own research, the highest precipitation in September at the level of 94.3 mm, more than twice as high as the multiannual average, occurred in the first year of the research. In that season, characterized by an annual total precipitation at the level of 487.5, the highest seed yield was recorded. Similar research results were obtained by Kotecki et al. (2004), who found the highest value of the analysed feature in the years of the total annual precipitation of 520 mm. Our own research demonstrated that cultivars were characterized by varied seed yield in individual years of the research. In the season characterized by the highest average air temperature and the lowest total precipitation, significantly the highest value of the analysed feature was recorded in restored hybrid morphotypes. Gehringer et al. (2007) found that heterotic cultivars also demonstrated a higher ability to adapt to unfavourable weather conditions. The types of applied biostimulators significantly affected the straw yield and harvest index (*Tables 4, 5*). The highest values of those features were obtained in the variant treated with Asahi SL biostimulator (on average 6.41 $Mg \cdot ha^{-1}$, on average 38.8%). Other biostimulators increased the straw yield (on average by 0.10 $Mg \cdot ha^{-1}$) in comparison to the control variant and harvest index (on average by 3.8%). The straw yield of semi-dwarf cultivar PR44D06 was lower on average by about 4.0% in comparison to other winter rape cultivars. The by-product yield was not conditioned by weather conditions in the years of the experiment. The statistical calculations did not show any significant interaction of the studied varieties with the climatic conditions prevailing in the years of conducting the experiment, which means that varieties reacted in the same way to weather conditions in individual years of research. The seed yield to the biomass yield ratio (harvest index – HI) was more favourable in heterotic cultivars than in the population cultivar Monolit. Different results were obtained by Zając et al. (2013). Those authors statistically found insignificant differences between the population morphotype Poznaniak and the restored hybrid Adam. Our own research demonstrated that the by-product yield of the analysed cultivars increased significantly along with an increase in

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sowing density. Statistical calculations did not show any significant interaction between the studied varieties and the sowing methods. Similarly, the harvest index assumed more favourable values in conditions of reduced row spacing. The highest by-product yield in the three-year research cycle (on average 7.19 $Mg \cdot ha^{-1}$) was obtained in the vegetation period of 2014-2015, which was characterized by varied humidity and thermal conditions during the phase of plant growth and development, significantly lower – (on average 6.89 $Mg \cdot ha^{-1}$ in 2013-2014) was characterized by optimum weather conditions during the vegetation season and winter rest and the lowest (on average 4.86 $Mg \cdot ha^{-1}$) was in 2015-2016, where significant frost damage to plants occurred and a precipitation shortage was recorded in April, May and June. In the first year of the research, characterized by average precipitation (487.5 mm), the highest value of harvest index was obtained and the lowest (on average 35.4%) was obtained in the vegetation season of 2015-2016 with the significantly lowest precipitation (383.7 mm).

Table 4. *Straw yield ($Mg \cdot ha^{-1}$) depending on factors of experience*

Year	Cultivars			Ways of sowing		Types of biostimulators used				Mean
	2013-2014	2014-2015	2015-2016	45.0	22.5	Control variant	Tytanit®	Asahi®SL	Silvit®	
Monolit	6.92	7.23	4.87	6.15	6.53	6.18	6.35	6.47	6.36	**6.34**
PR 44D06	6.75	7.04	4.74	6.09	6.25	6.15	6.17	6.23	6.14	**6.17**
PT 205	7.00	7.31	4.97	6.29	6.57	6.31	6.40	6.52	6.47	**6.43**
Mean	**6.89**	**7.19**	**4.86**	**6.18**	**6.45**	**6.21**	**6.31**	**6.41**	**6.32**	**-**

$LSD_{0.05}$ for: years - 0.03; years - 0.03; types of biostimulators used - 0.03; ways of sowing - 0.02
interaction: years x cultivars – n.s.; cultivars x types of biostimulators used – 0.06; ways of sowing x cultivars – n.s.

Table 5. *Harvesting index (HI)(%) depending on factors of experience*

Year	Cultivars			Ways of sowing		Types of biostimulators used				Mean
	2013-2014	2014-2015	2015-2016	45.0	22.5	Control variant	Tytanit®	Asahi®SL	Silvit®	
Monolit	37.6	35.7	34.3	33.4	38.3	35.6	35.5	36.6	35.8	**35.9**
PR 44D06	41.1	39.7	36.5	37.0	41.1	37.8	38.8	40.1	39.6	**39.1**
PT 205	41.5	40.0	35.4	37.6	40.3	38.6	39.0	39.6	38.6	**39.0**
Mean	**40.1**	**38.5**	**35.4**	**36.0**	**39.9**	**37.4**	**37.8**	**38.8**	**38.0**	**-**

$LSD_{0.05}$ for: years - 0.1; years - 0.1; types of biostimulators used - 0.2; ways of sowing - 0.1
interaction: years x cultivars – n.s.; cultivars x types of biostimulators used – 0.3; ways of sowing x cultivars – 0.2

Conclusions

1. Biostimulators applied in the experiment had a significant effect on the thousand-seed weight, the main yield, the by-product yield and the seed yield to biomass yield ratio (HI) of the examined winter rape morphotypes. The strongest stimulating effect was found after the application of Asahi SL biostimulator.

2. The highest yielding was found for restored hybrids PT205 and PR44D06. It was demonstrated that their yield was on average 43 to 59 Mg·ha^{-1} higher in comparison to the conventional cultivar.
3. In the examined winter rape morphotypes, under the effect of increased row spacing and decreased sowing density, a reduction of seed yield was found, on average from 0.63 to 0.95 Mg·ha^{-1}. The lowest main yield resulting from a factor change was demonstrated in the line cultivar Monolit.
4. Weather conditions in the year of the experiment had a significant effect on the value of the examined parameters. The highest seed yield was observed in the vegetation season of 2013-2014, characterized by the best pluvio-thermal conditions during the spring-summer development of winter rape.

Acknowledgements. The results of the research carried out under the research theme No. 363/S/13 were financed from the science grant granted by the Ministry of Science and Higher Education.

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SACCHARIDE CONTENT IN POTATO TUBERS TREATED WITH BIOSTIMULATORS

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(Received 18th Sep 2018; accepted 22nd Nov 2018)

Abstract. Field research was carried out in 2015-2017 on an individual farm in Międzyrzec Podlaski (51°59' N and 22°47' E) in Poland. The aim of the study was to determine the effect of the type of biostimulators used (Kelpak SL, Titanit, GreenOk, BrunatneBio Złoto Cytokininy) on the saccharides content in tubers of three edible potato varieties (Honorata, Jelly, Tajfun). Potato plants were treated three times with biostimulators (starting from the BBCH 39 phase, every 10-14 days) with the potato plants sprayed with distilled water as the control object. The experiment was based on the split-plot method. A large amount of precipitation –325.4 mm and the lowest average air temperature –14.6 °C in 2017 contributed to the increase in the content of reducing sugars in potato tubers. Tubers of plants treated with the GreenOk preparation were characterized by the highest total sugar content compared to plants from the control object. The content of saccharides in tubers was significantly differentiated by the genotype of the variety. The largest amount of sugars was collected by the Honorata variety, and the least of the Tajfun variety.
Keywords: *Solanum tuberosum L., varieties, total sugars, reducing sugars, sucrose, types of bioregulators*

Introduction

One of the important characteristics of potato (*Solanum tuberosum* L.) determining the suitability of varieties for direct consumption and for processing is the content of reducing sugars, called monosaccharides (glucose + fructose) and the sums of sugars, or the total sugar (reducing sugars + sucrose). The content of reducing sugars in potato tubers is about 0.3%, and the total sugar to 1.0% in fresh tuber mass. Higher concentration of monosaccharides increases the intensity of Maillard reactions (Copp et al., 2000; Edwards et al., 2002), and causes darkening of the pulp during thermal processing (such as frying and drying) and deterioration of taste and smell, and in the final frying phase the formation of acrylamides (Hebeisen et al., 2005; Saraiva and Rodrigues, 2011; Shepherd et al., 2010). Aspargins and acrylamides derived from reducing sugars are dangerous to health and have been classified by the World Health Organization (WHO) as carcinogens for humans and animals (Keijbets, 2006; Tajner-Czopek, 2011). The use of raw material with low (0.1–0.2%) concentration of reducing sugars is considered the basic factor inhibiting the formation of acrylamide (Biedermann-Brem et al., 2003; De Wilde et al., 2006). In limiting the amount of acrylamide formed it may be helpful to soak and blanch French fried before frying, which reduces the content of reducing sugars in the fries (Pedreschi et al., 2007). The high sugar content (above 1% in fresh mass) decreases the taste properties, as tubers become sweetish and darken the pulp (Boguszewska, 2007; Kraska, 2002; Leszczyński, 2000; Zgórska et al., 2006a). Biostimulators control mineral metabolism, increase plant resistance to stressful environmental conditions (Sawicka and Mikos-Bielak, 2002; Wierzbowska et al., 2015), affect chemical composition, as well as increase resistance to attacks from diseases and pests (Grzyś, 2012). The content of reducing sugars and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27912797

sucrose in the potato is influenced by the genetic factor, types of biostimulators and weather conditions.

Due to the few studies on the beneficial effects of biostimulators on the content of sugars, reducing sugars and sucrose, studies were undertaken to determine the effect of biostimulators (Kelpak SL, Titanit, GreenOk, BrunatneBio Złoto Cytokininy) on the saccharides content in tubers of three edible potato varieties (Honorata, Jelly, Tajfun).

Materials and methods

The experimental site

The potato tubers from a three-year field experiment carried out in 2015-2017 on an individual farm in Międzyrzec Podlaski (51°59’ N i 22°47’ E), Biała Podlaska county, Lublin voivodeship, in Poland were the material for studies. The experiment was established in three replications using the split-plot method, on the soil included in the very good rye complex, class IVa.

In individual years of research, soils differed in the content of organic matter and absorbable macro-elements. In 2015 and 2016, the soil was characterized by slightly acidic reaction, and in the last year of research, alkaline. The content of organic matter ranged from 15.0 to 18.7 $g \cdot kg^{-1}$. The content of absorbable phosphorus (P) was from high to very high, potassium (K) from medium to very high, and magnesium (Mg) was high. The first factor were three moderately early varieties of edible potato: Honorata, Jelly and Tajfun, and the second one, four types of biostimulators used in three dates (beginning of flowering, fully flowering and after flowering of plants):

1. Control object – without the use of biostimulators spraying with distilled water
2. Biostimulator Kelpak®SL (active substance - *Ecklonia maxima* algae extract), containing plant hormones: auxin – 11 $mg \cdot l^{-1}$ and cytokinin – 0.031 $mg \cdot l^{-1}$, at a dose of 0.20 $l \cdot ha^{-1}$
3. Biostimulator Tytanit® (active substance – titanium) at a dose of 0.20 $l \cdot ha^{-1}$,
4. Biostimulator GreenOk® (active substance – humus substances 20 $g \cdot l^{-1}$) at a dose of 0.20 $l \cdot ha^{-1}$
5. Biostimulator BrunatneBio Złoto (active substances – plant hormones: auxin – 0.06 $mg \cdot l^{-1}$ and cytokinin – 12 $mg \cdot l^{-1}$) at a dose of 0.20 $l \cdot ha^{-1}$

The forecrop for potato in particular years of research was winter wheat. After harvesting the forecrop, a team of post-harvest crops was made. In autumn, each year preceding planting, natural fertilization in the form of manure in the amount of 25.0 $t \cdot ha^{-1}$ and mineral fertilization with phosphorus-potassium in the amount of P – 44.0 (100 $P_2O_5 \cdot 0.44$) $kg \cdot ha^{-1}$ (lubofos for potatoes 7%) and K – 124.5 (150 $K_2O \cdot 0.83$) $kg \cdot ha^{-1}$ (lubofos for potatoes 25%) was applied. These fertilizers were plowed pre-season plowing. Nitrogen fertilizers were sown in the spring in an amount of N 100 kg/ha (nitro-chalk 27%) and mixed with the soil using a cultivator. Potatoes were planted manually under the marker at a spacing of 67.5 × 37 cm, in the third decade of April (24 April 2015, 30 April 2016, 22 April 2017). Each plot consisted of five ridges. Cultivation and care treatments were carried out in accordance with the requirements of correct agrotechnics and methodological assumptions of the experiment. During the growing season, observations of the most important phases of potato development were carried out on each object: emergence, flowering buds, flowering, maturation of plants. Potato plants were treated three times with biostimulators (starting from the BBCH 39

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27912797

phase, every 10-14 days) with the potato plants sprayed with distilled water as the control object. Prior to harvest, random samples of tubers were collected from ten potato plants (excluding the marginal crops) each plot and used for chemical analysis and assessment of consumption-related characteristics of the tubers. The harvest was made during the technological maturity of tubers, in the first decade of September (02 September 2015, 03 September 2016, 05 September 2017).

Chemical analysis methods

For the tests, 5 kg of potato samples were taken from each variety and combination. The tubers were homogenized by in-depth mixing of the pulp and samples were taken for laboratory determinations for the content of reducing sugars and total. The content of reducing sugars and total sugar was determined in fresh weight of unpeeled tubers with the Luff-Schoorl method. This method is based on the reduction reaction in the alkaline medium at the boiling temperature of the copper salts in the Luff solution by the reducing sugars contained in the solution (Krełowska-Kułas, 1993). The sucrose content was calculated from the difference of the total sugar after hydrolysis and reducing sugars × 0.95.

Meteorological conditions

The weather conditions in the potato growing years were shown by means of precipitation sums and average air temperatures in *Table 1*. The growing season of 2015 had the average air temperature of 15.2 °C, higher by 0.2 °C than the long-term mean and rainfall at 295.1 mm, but unevenly distributed throughout the growing season. The highest average air temperature was recorded in 2016 and amounted to 15.8 °C, it was higher than the long-term average by 0.8 °C, while this year was characterized by the lowest amount of precipitation – 200.9 mm, lower by 134.5 mm from the long-term sum, was one of the least favourable years, with a high rainfall deficit in the period of June-August, decisive for yield accumulation. The highest number of rainfall was recorded in the growing season 2017 –325.4 mm and the lowest average air temperature –14.6 °C (*Table 1*).

Table 1. *Air temperature and rainfall potato growing seasons according*

Months	**Air temperature (°C)**				**Rainfall (mm)**			
	Multi-year mean	**Monthly means**			**Multi-year mean**	**Monthly sums**		
	1996-2010	2015	2016	2017	1996-2010	2015	2016	2017
April	8.0	8.2	9.1	6.9	33.6	30.0	28.7	59.6
May	13.5	12.3	15.1	13.9	58.3	100.2	54.8	49.5
June	17.0	16.5	18.4	17.8	59.6	43.3	36.9	57.9
July	19.7	18.7	19.1	16.9	57.5	62.6	35.2	23.6
August	18.5	21.0	18.0	18.4	59.9	11.9	31.7	54.7
September	13.5	14.5	14.9	13.9	42.3	47.1	13.6	80.1
April–September	15.0	15.2	15.8	14.6	335.4	295.1	200.9	335.4

Statistical analysis

Results of the study were analysed by ANOVA. Significance of sources of variation was checked with the Fisher-Snedecor test and the significance of differences between

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27912797

means was tested using the multiple comparison Tukey's test at the significance level of P = 0.05. Statistical calculations were performed in Excel using the authors' own algorithm based on the split-plot mathematical model.

Results and discussion

Based on a three-year study, the level of total sugars, reducing sugars and sucrose was determined in three varieties of edible potato tubers, and it was up to 1% in the fresh weight of tubers. These results were confirmed by the studies of Zgórska et al. (2006a). Chemical analyses have shown, and statistical calculations have confirmed the significant impact of varieties, types of biostimulators, as well as weather conditions in the years of research on the saccharides content in potato tubers. In own studies, the average content of total sugars in edible potato tubers was 6.72 $g{\cdot}kg^{-1}$, reducing sugars 3.25 $g{\cdot}kg^{-1}$, sucrose 3.30 $g{\cdot}kg^{-1}$ (*Tables 2, 3* and *4*). The research presented shows that the genetic factor had an effect on the sum of sugars, reducing sugars and sucrose in potato tubers. The variety that contained the most sugars in total was Honorata, then Jelly and Tajfun. The varieties compared were different in terms of the accumulation of the component in question. The highest amount of sugars was accumulated by the Honorata variety – on average 6.94 $g{\cdot}kg^{-1}$, Jelly – on average 6.70 $g{\cdot}kg^{-1}$, and the least by Tajfun – on average 6.54 $g{\cdot}kg^{-1\cdot}$ Among the tested varieties, the most reducing sugars in tubers was accumulated by the Tajfun variety, significantly lower content was found in Jelly tubers, while the smallest amounts were found in the tubers of the Honorata variety. In reference to sucrose, it was found that significantly most of this saccharide was found in tubers of the Honorata variety, and significantly least was found in the tubers of the Tajfun variety. In this study, varietal differences in the content of reducing sugars and sucrose in potato tubers have been demonstrated, which was the subject of research by Grudzińska and Zgórska (2008). These results were confirmed by Gugała et al. (2013), Sawicka and Pszczółkowski (2005). According to these authors, the content of reducing sugars and sucrose is a feature directly related to the genotype of a given variety. Grudzińska et al. (2016), Zgórska and Grudzińska (2012) showed a significant effect of the genetic factor on the content of reducing sugars in edible potato tubers. It was observed that the biostimulators had a significant influence on the content of reducing sugars. Under the influence of biostimulators used, there was a slight increase in the saccharides content compared to tubers from the control object – on average from 0.4 to 1.3%. Tubers of plants treated with the GreenOk preparation were characterized by the highest content of sugars - 6.78 $g{\cdot}kg^{-1}$, in comparison to plants from the control object, while reducing sugars from 2.71 $g{\cdot}kg^{-1}$ to 3.51 $g{\cdot}kg^{-1}$, respectively. With regard to sucrose, the minimum content was from 3.2 $g{\cdot}kg^{-1}$ on the object, where Kelpak SL was used, and the largest on the object where the BrunatneBio Złoto biostimulator 3.5 $g{\cdot}kg^{-1}$ was used. However, not all biostimulators have been statistically proven. In studies by Czeczko and Mikos-Bielak (2004), the Asahi SL biostimulator used increased the concentration of sucrose, but these changes have not been statistically proven. The random factor, i.e. the conditions prevailing in the years of research, resulted in the diversification of the sum of sugars in potato tubers. The highest content of total sugars and sucrose was determined by tubers harvested in 2015 and 2016, which were characterized by high air temperature of 15.2 °C and 15.8 °C and precipitation of 295.1 mm, and the lowest total sugars and sucrose level was accumulated by tubers harvested in 2017, which was distinguished by favourable weather conditions during the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_27912797

growing season (*Table 1*). Grudzińska (2012) also confirmed the significant impact of weather conditions on the level of total sugars in edible potato tubers. The highest content of reducing sugars 3.46 g·kg^{-1} was characteristic of tubers harvested in `hich was characterized by the highest rainfall and the lowest average air temperature – 14.6 °C and longer vegetation period. The prolongation of the growing season resulted in the accumulation if simple sugars, but in a quantity not lowering the technological value of potato tubers. Variable atmospheric conditions in the years of conducting the experiment resulted in significant interactions: years × variety, years × types of biostimulators and years × varieties × types of biostimulators. The proven interaction between the studied factors documents the varied response of cultivated varieties and their combination with plant growth biostimulators. Mazurczyk and Lis (2001) and Zgórska and Frydecka-Mazurczyk (2002) found that high temperature and low soil moisture limited the accumulation of sugars regardless of the variety. In contrast, Antonious et al. (2001) found the effect of weather on the concentration of reducing sugars. The prolongation of the growing season resulted in the accumulation of simple sugars but in the amount not lowering the technological value of potato tubers. The highest content of sucrose was characterized by tubers harvested in the growing season with low temperature at harvest time and with a lot of rainfall. Such climatic conditions could have caused secondary tuber growth and subsequent maturation. At high temperatures and a very small sum of precipitation, all tested varieties were characterized by the lowest content of

Table 2. *Content of total sugars in potato tubers, g·kg^{-1} fresh matter*

Objects	Cultivars			Years			Mean
	Honorata	Jelly	Tajfun	2015	2016	2017	
1. Control object	6.93	6.50	6.52	6.59	6.82	6.54	6.65
2. Kelpak SL	6.98	6.60	6.49	6.77	6.77	6.54	6.69
3. Tytanit	6.93	6.75	6.47	6.89	6.76	6.50	6.72
4. GreenOk	6.92	6.79	6.63	6.89	6.90	6.56	6.78
5. BrunatneBio Złoto	6.93	6.80	6.57	6.96	6.83	6.52	6.77
Mean	6.94	6.70	6.54	6.82	6.82	6.53	-

$LSD_{0,05}$ for: cultivars – 0.014; objects – 0.022; years – 0.014; interaction: objects × years – 0.038; cultivars × years – 0.024; cultivars × objects – 0.032; cultivars × objects × years – 0.056

Table 3. *Content of reducing sugars in potato tubers, g·kg^{-1} fresh matter*

Objects	Cultivars			Years			Mean
	Honorata	Jelly	Tajfun	2015	2016	2017	
1. Control object	3.06	3.48	3.48	3.40	3.16	3.46	3.34
2. Kelpak SL	3.07	3.38	3.51	3.23	3.22	3.45	3.32
3. Tytanit	3.07	3.25	3.48	3.10	3.24	3.50	3.28
4. GreenOk	3.08	3.21	3.35	3.11	3.09	3.43	3.21
5. BrunatneBio Złoto	2.71	3.19	3.42	3.03	2.81	3.48	3.11
Mean	3.00	3.30	3.45	3.17	3.10	3.46	-

$LSD_{0,05}$ for: cultivars – 0.157; objects – 0.211; years – 0.157; interaction: objects × years – 0.366; cultivars × years – 0.272; cultivars × objects – 0.312; cultivars × objects × years – 0.541

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DOI: http://dx.doi.org/10.15666/aeer/1702_27912797

Table 4. *Content of sacharose in potato tubers, g·kg^{-1} fresh matter*

Objects	Cultivars			Years			Mean
	Honorata	Jelly	Tajfun	2015	2016	2017	
1. Control object	3.67	2.87	2.80	3.03	3.47	2.90	3.14
2. Kelpak SL	3.71	3.05	2.80	3.36	3.37	2.90	3.20
3. Tytanit	3.67	3.30	2.84	3.60	3.34	2.85	3.30
4. GreenOk	3.65	3.40	3.12	3.60	3.62	2.90	3.40
5. BrunatneBio Złoto	4.00	3.40	2.99	3.70	3.82	2.88	3.50
Mean	3.70	3.20	2.90	3.65	3.50	2.90	-

$LSD_{0,05}$ for: cultivars – 0.151; objects – 0.211; years – 0.151; interaction: objects × years – 0.344; cultivars × years – 0.252; cultivars × objects – 0.332; cultivars × objects × years – 0.521

Conclusions

Under the influence of the biostimulators used, there was a slight increase in the total sugar content and sucrose, a reduction in reducing sugars compared to tubers from the control object. Tubers of plants treated with the BrunatneBio Złoto Cytokiny preparation were characterized by the highest content of sucrose and the smallest content of reducing sugars. While tubers of plants treated with the GreenOk biostimulator were characterized by the highest content of total sugars, in comparison to plants from the control object.

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ETHNO-BOTANICAL EVALUATION OF INDIGENOUS FLORA FROM THE COMMUNITIES OF RAJH MEHAL AND GOI UNION COUNCILS OF DISTRICT KOTLI, AZAD JAMMU KASHMIR PAKISTAN

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(Received 27th Sep 2018; accepted 14th Nov 2018)

Abstract. The present study reports ethnobotanical information about plants, which were used by indigenous communities of two union councils of district Kotli AJK. The present study is first report of quantitative ethnobotany from study area. Rajh Mehal and Goi are representative union councils of district Kotli, Azad Jammu and Kashmir having large ranges diversity in habitat and climatic conditions. This district is naturally blessed with highly medicinal plants but unfortunately ethno-botanical information is sporadically known. The study area was surveyed for two consecutive years during moon soon period and ethnobotanical data was collected from 112 local inhabitants using a semi-structured, open ended questionnaire and by free listing method. Several quantitative indices including Informant Consensus Factor, Use Value, Frequency of Citation, Relative Frequency of Citation and Relative Importance Index were used to determine relative usage, benefits and coverage of ethno-medicine. The agreement of homogeneity between the present and previous studies and among the indigenous communities was evaluated using the Jaccard Index. The summary statistics and correlation between use value and relative frequency of citation was calculated in SPSS v 16. The data from free listing method was analyzed by using ANTHROPAC package in R software. Comparison of indigenous knowledge among different genders and age groups was made using Spearman correlation test, the result showed that men had higher information (14.05) about plant uses than females (8.55) in the study area. Male informants reported 63.8% (±10.18) of total recorded species and 76% (±4.13) uses while female informants reported 38.8% (±6.06) species and 68% (±3.65) uses. Smith's Salience analysis indicates that most of the plants in the study area were useful against abdominal pain and worms, constipation and pain. Diseases were classified into twelve different disease categories based on International Classification of Diseases (ICD) classification, with Skin and related symptoms (S00-T98) had highest value (0.91) of Informant's Consensus Factor (IC) followed by Circulatory problems (I00-I99) and Diabetes (E10-E14) each having IC value of 0.90. Aerial parts of 21 plant species were utilized in herbal preparations followed by leaves (20 spp.) and oral mode of admiration was most common among inhabitants. Among all the studied species *Zanthoxylum alatum* was found highly important with a relative importance value of 93.75 followed by *Adhatoda zeylanica* with an importance value of 91.67. High dependency of local inhabitants on traditional medicines was confirmed by higher values of informant consensus factor, which showed that people in study area still use herbal medicines as a primary source of their health care. The present study indicated that area was rich in medicinal plant knowledge and there is a need to exploit this information for drug development and pharmacological activities in addition of conservation and management of this valuable plant resource of the area.
Keywords: *ANTHROPAC, frequency of citation, relative importance, informants consensus factor, medicinal importance*

Abbreviations: prior informed consent (PIC), informant consensus (IC), use value index (UV), relative frequency of citation (RFC), medicinal importance (MI), number of use-reports (Nur), number of species cited (Nt), frequency of citation (FC), international plant name index (IPNI), international classification of diseases (ICD), relative pharmacological properties (PH), relative body system treated (BS), relative importance (RI), oral (Or), tropical (Tr)

Background

One of survey by World Health Organization estimates that about 80 percent of world population depends upon herbal medicines made from approximately 50,000 flowering plants of total world flora (Govaerts, 2001; Schipmann et al., 2002). Effectiveness of plants to cure various aliments is known to humans right from their origin and many herbal drugs are still being utilized in current day herbal system effective against various diseases (Goyal et al., 2014). Indigenous knowledge about plants is an important asset that is gathered from countless activities involving empirical observation, 'trial and error' by the indigenous peoples seeking a cure for an ailment (Locher, 2013). Traditionally, ethnobotanists are interested in exploring the effect of different demographic factors, i.e. age, gender, ethnicity and other attributes of indigenous knowledge coupled with quantitative ethnomedicinal documentation of traditional knowledge by employing different statistical tools (Ullah et al., 2013).

Ethnobotanical knowledge is considered to be an integral part of the knowledge for the drug development. It either helps in identifying new drugs or refocusing on the plants earlier reported to have bioactive components (Mahwasane et al., 2013). The indigenous communities residing in Pakistan mostly use herbal therapies to cure their ailments. Resultantly various studies significantly contributed ethno-pharmacological inventories. With reference to Azad Jammu and Kashmir, some ethnomedical studies have been reported from various areas such as Neelum (Mahmood et al., 2011), Poonch valley (Khan et al., 2010, 2012) and Sudhnoti (Ishtiaq et al., 2015). Few papers have been written on ethnobotanical aspects form the District Kotli (Ajaib et al., 2010; Amjad et al., 2015; Amjad and Arshad, 2014); however, these studies are superficial and lack of novelty in terms of quantitative data in addition to traditional phytotherapies. Therefore, the present study was aimed to:

1. Record and discuss knowledge on medicinal plant uses in the local traditional practices including method of preparation and use of plants as herbal medicine
2. The differential distribution of knowledge about plants and medical properties among sectors of the society
3. Identifying novel uses of plants compared to previously reported work
4. Analysis of ethnobotanical data by using different quantitative indices

Methods

The study was organized into three stages (*Fig. 1*).

a) Field work

The study area lies near line of control and is diversity hotspot due to frequent rain and pleasant weather conditions. Being remote and having less literacy rate people in area still depend upon herbal medicines but documentation of this traditional knowledge is very rare. The present study was conducted by interviewing 112 local inhabitants

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DOI: http://dx.doi.org/10.15666/aeer/1702_27992829

with age ranges between 40 to 75 years, including herbalists, midwives (*Daai*) and local peoples (male, female). Legal permission was taken from representatives of the municipality for conducting interviews and collection of ethnobotanical data. All respondents were asked to sign a prior informed- consent (PIC) form after the objectives and possible consequences of the study had been explained. The prior informed consent (PIC) form was translated into the local *Pahari* language. The study area was explored for three years (July 2015–September 2016), plant samples were collected and information was gathered by group discussions, interviews and field visits. Sample size was determined by using method developed by Kadam and Bhalerao (2010).

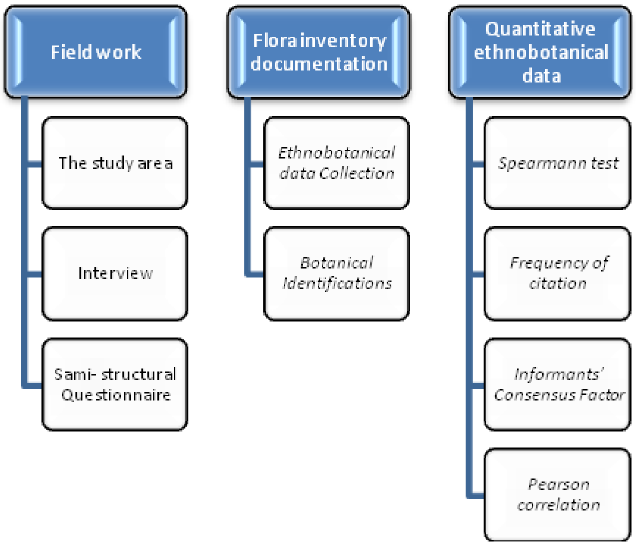

Figure 1. *Experimental design for the collection of data*

b) Floral inventory documentation

Ethnobotanical information collected through interviews was recorded in a semi-structured questionnaire. Information obtained was cross-checked and compared with existing available literature. Furthermore, the indigenous learning about the plant assets, religious and social perspective, for example, populace density was additionally recorded. The Minimum Standards of Reporting Checklist contains details of the experimental design, and statistics, and resources used in this study. During field surveys, the local/common names of plants were recorded with the help of a local guide or local informants. For taxonomic confirmation of plant was done using different Floras and monographs of the different areas of Pakistan, whereas the International Plant Name Index (IPNI) (www.ipni.org) was also used to obtain the correct botanical name confirmation.

c) Quantitative analyses of ethnobotanical data

Frequency of citation (FC)

The result of FC presented as %age and proportions. Each plant reported by informants with respect to the native use was calculated as frequency of citation (FC). The collected ethnomedicinal information was quantitatively analyzed using relative frequency citation index (RFC) after Vijaykumar et al. (2015) as follows:

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DOI: http://dx.doi.org/10.15666/aeer/1702_27992829

$$RFC = FC / N$$

where FC is frequency of citation N is number of informants.

Informants' consensus factor (F_{IC})

IC is utilized to build up the consistency of learning among the sources for each sickness treated and diverse illness categories (Heinrich et al., 1998). Informant consensus factor was drawn to search agreements of the informants against the cures for each diseases category. For this purpose, following formula was used:

$$F_{IC} = \frac{Nur - Nt}{Nur - 1}$$

where *Nur* is the number of use-reports in each group of disease; *Nt* is the number of species cited for the particular disease by all the informants. The F_{IC} values are ranged between 0 and 1, in which '1' shows the maximum level of consensus. Consequently, high F_{IC} can be used in identifying important plants for searching novel bioactive molecules (Carrió and Vallès, 2012).

Medicinal importance (MI)

The MI was figured out by utilizing following equation after Baydoun et al. (2015).

$$MI = \frac{\text{No. of use reports for disease category}}{\text{No. of species used}}$$

Use value index (UV)

The use value was calculated by using following formula (Vijayakumar et al., 2015):

$$UV = \sum Ui / N$$

Ui is the number of uses stated by each informant for a given species and N is the total number of sources (informants).

Relative importance (RI)

RI was determined by the formula

$$RI = \frac{\text{Rel PH} + \text{Rel BS x 100}}{2}$$

$$\text{Rel PH} = \frac{\text{PH of a given plant}}{\text{Maximum PH of all reported plant species}}$$

where PH is pharmacological attributes of the each provided plant and Rel PH is the relative number of pharmacological properties for an individual plant.

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$$BS = \frac{\text{BS of a given Plant}}{\text{Maxi. BS of all reported plant species S}}$$

BS is the number of body system healed by single species and Rel PH is relative number of body system treated by a single species.

Smith's salience analysis (SS)

The analysis of agreement and variation in free-lists using cultural domain analysis is described and justified in detail by Puri (2010), Quinlan (2005), Weller and Baer (2002), and several statistical software packages, including ANTHROPAC, can manage and analyze data in this way. The cultural domain analysis using ANTHROPAC was performed using Borgatti's (1996) Methods Manual. The main steps can be briefly summarized free-lists are compiled in an informant by disease matrix, which indicate both the rank order and the presence or absence of items in each informant's list from this, the frequency of items, their average rank in the list, and a measure of salience (based on Smith, 1993) can be calculated. This is essentially a weighted average of the (inverse) rank of an item across multiple free-lists, where the number of items in the list weights each list.

Pearson correlation

Pearson correlation analysis was calculated in order to access the linear relationship b/w the RFC and Use value index (UV) by using SPSS ver. (16, the r^2) also estimated to measure cross species variability in RFC that was described by variance in UV. The r^2 was computed simply taking square of r (Amjad et al., 2017).

Spearman test

To decide if a statistically significant correlation exists between the number of plants mentioned and the age of informant, we used the Spearman test. The Mann-Whiney U and Kruskal-Wallis tests were used to find significant differences among two and 5 groups related groups, respectively, all values set at 0.5 alpha level of significance (Bruschi et al., 2011; Shaheen et al., 2017a).

Results

Demographic data

Ten field visits (to complete regular varieties) were done to accumulate ethnobotanical information related to use of medicinal plants. The aggregate length of the field work was roughly about 3 years i.e. from July 2015 to September 2016 and data was recorded form 112 informants (male 67 and females 45). The informants were divided in to four categories based on their gender, age group, education and profession. Mostly herbalists were present in the age group range between 51-60 while least number of informants had age more than 60 years. Literacy rate of study area was very low as most of informants (about 26.7%) were uneducated and only 5 informants which is 4.46% of total informants had higher education. On average male informants had more information about the plant uses than female informants, male informants on average know 14.05 species than female who know 8.55 species. Similarly uses known

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by male and female were different, on average males report 6.12 uses of plant while female reports 5.82 uses of the plant (*Table 1*). Informants of age between 41 and 50 know most plants than other age groups but information about plant use was provided most by informants with age between 51-60 years and more than 60 years as they on average report 6.25 and 9.91 uses of plants. Among educated informants, those having middle (secondary school) education know most of the plant species (20.38) and people with intermediate education (Higher secondary school) know most uses of plant species (7.30) among different professionals' traditional health practitioners know most of the species (23.08 on average) and also reported multiple uses (on average 9.60) of recorded species (*Table 1*). Many examinations have demonstrated that age and sex are two vital variables to be considered while assessing the appropriation of information inside a gathering of sources (Torres-Avilez et al., 2016; Albuquerque et al., 2011). In any case, not many investigations have broken down the impact of these factors on the appropriation of ethno-medicinal information in Pakistan and none of them utilized factual examinations for approval of the gathered information (Ahmad et al., 2014). Spearman's correlation analysis showed significant positive differences between the age and the number of both mentioned species ($rs = 0.24$; $p < 0.05$) and different uses ($rs = 0.35$; $p < 0.05$), indicating that there is a trend of older people being more knowledgeable than younger people.

Table 1. *Demographic data of informants and knowledge about TAB. F = female; M = male*

Informants	#		# Known species		# Cited different uses	
	F	M	F	M	F	M
Total	45	67	8.55±6.06	14.05±10.18	5.82±3.65	6.12±4.13
Age class						
18-30	17		5.50±4.68		5.35±2.64	
31-40	27		10.16±8.55		4.52±2.41	
41-50	23		15.57±12.21		4.52±3.38	
51-60	30		12.32±7.94		6.25±4.91	
> 61	15		12.91±5.16		9.91±3.70	
Education level						
Uneducated	30		7.32±5.09		4.50±2.68	
Intermediate	25		12.39±6.84		7.30±4.86	
Primary	25		12.65±9.50		7.13±4.51	
Graduate	12		7.60±3.69		5.50±3.78	
Middle	15		20.38±13.49		5.23±2.42	
Post graduate	5		12.00±10.58		6.00±2.00	
Profession						
Worker	6		9.40±7.40		7.40±2.30	
Traditional health practitioner	26		23.08±8.86		9.60±4.63	
Retired	10		10.28±2.69		5.71±3.15	
House wife	15		6.15±3.84		3.84±2.30	
Midwifery	9		14.87±5.54		8.00±3.16	
Labour	5		6.00±1.82		3.50±1.91	
Teacher	20		6.29±2.86		3.70±2.05	
Agriculturist	15		6.73±3.45		4.40±2.58	
Student	7		11.58±8.96		5.99±3.90	

Ethno floral diversity

During the ethnobotanical survey, 80 plants were explored, which belonged to 69 genera and 50 families. The detailed inventory of medicinal plants along with related information is presented in *Table 2*. Family Asteraceae contributed maximum to ethno-flora with 9 species, followed by Lamiacea, Rosaceae and Solanaceae (4 species each) (*Fig. 2*). The greater diversity of a family from study area was related to high occurrence of its members, familiarity of local people with its species and presence of active ingredients in its members which are being utilized to treat various ailments (Arnold, 2015; Mouterde, 1966, 1978, 1983). These results are in accordance with the different studies carried out in different regions of Pakistan and world (Bibi et al., 2014; Khan et al., 2014; Lulekal et al., 2013).

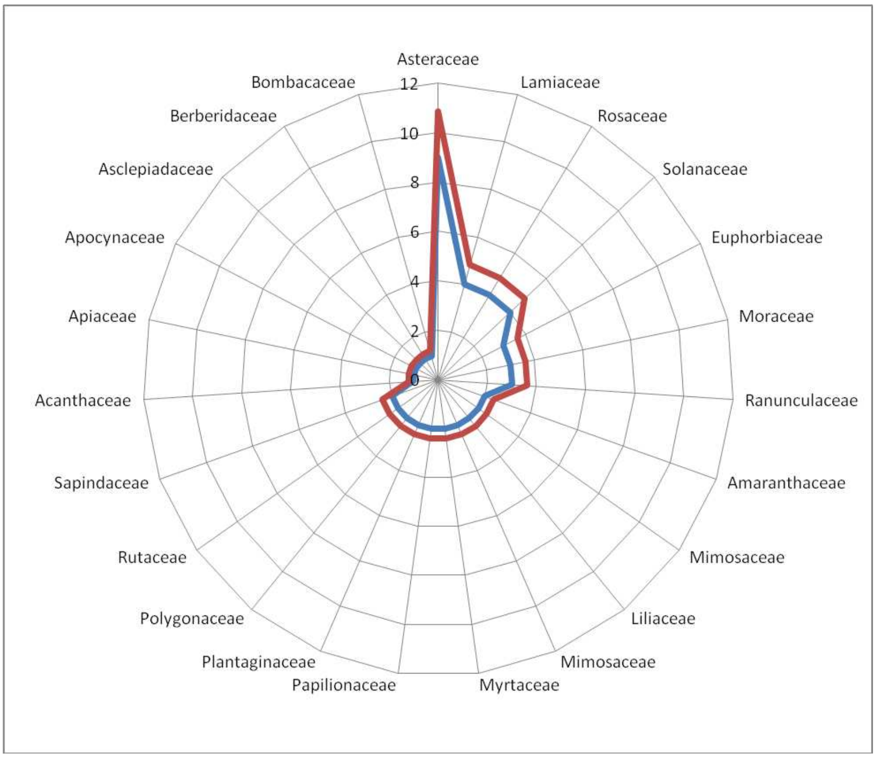

Figure 2. *Contribution of major families in the ethnoflora of the study area*

Medicinal usage

In this survey, fifty-three diseases/ailments were treated by 80 plant species with maximum 16 species (30% of total) were used abdominal pain and constipation which were common and prevailing problems of the area, followed by pain (11 spp., 20.1%), sexual dysfunction (10 spp., 18.84%), tooth problems and wounds (9 spp., 16.9% each) (*Fig. 3*). The documented uses of ethnomedicinal plants were compared with recently published literature (Ishtiaq et al., 2015; Amjad et al., 2017; Shaheen et al., 2017b) to seek the novelty of uses. Most of the documented species possessed similar uses; however, 16 species possessed novel uses (*Fig. 4*), which were not previously reported in literature. The main reason for the similarity among these studies was the same climatic conditions which resulted into the same floristic composition as well as the dissemination of information related to the use of plants among the informants of

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different areas. The reason for less similarity among the studies may be due to the existence of different cultural values in communities inhabiting the areas so they use plants differently for their healthcare needs. (Baydoun et al., 2015; Panyaphu et al., 2011). However, the informants were unaware of such concern and they informed only beneficial effects of plants without pointing out any side effects. Different parts were used for preparing herbal recipes. Leaves (31%) were the most used plant part, followed by aerial parts (17%), fruit (14%), roots (12%), bark (8%), flower (6%) and bulb, cone branches, pulp, resins (1% each) (*Fig. 5*). The preference of leaves in preparing herbal preparations may be due to storage of most of the active ingredients in leaves in order to attain resistance from the herbicides and insects. Furthermore, they are accessible and easy to collect (Panyaphy et al., 2011; Mesfin et al., 2013; Ahmed et al., 2013).

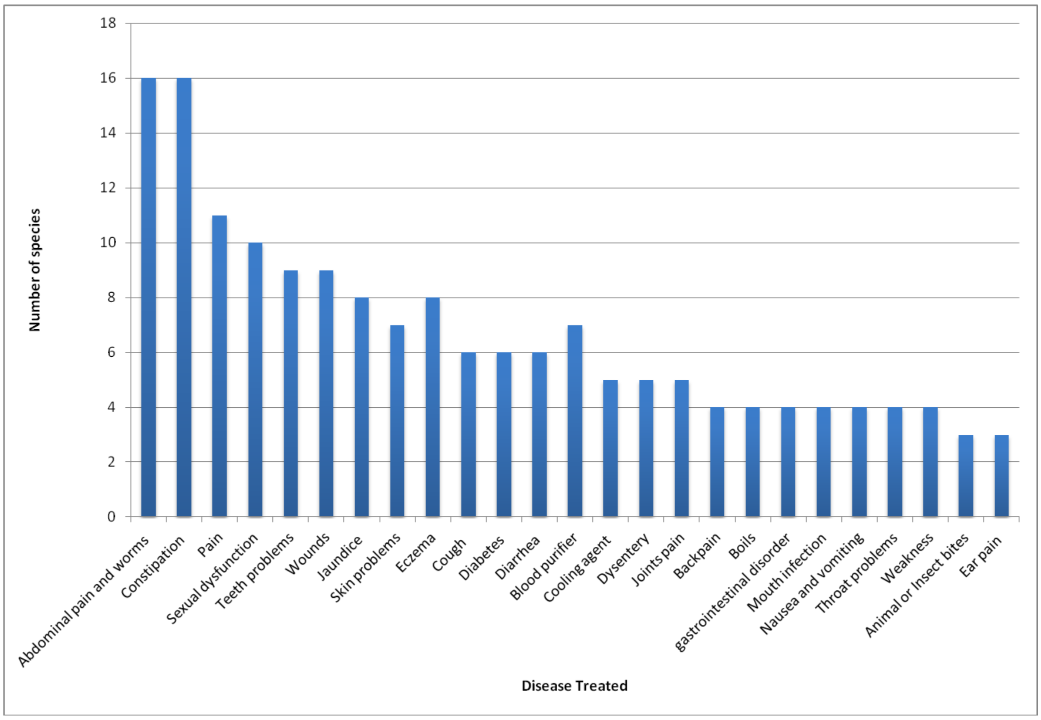

***Figure 3.** Contribution of ethnoflora for treating different ailments in the study area*

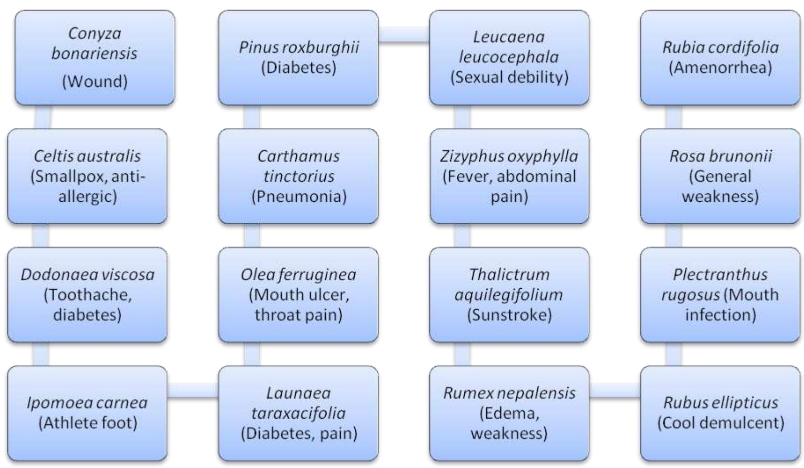

***Figure 4.** Sixteen plants spp., which have novel uses in the study area*

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Table 2. *Inventory of medicinal plants including families, plant species, specimen numbers, local names, part used, preparation(s), application, properties/ailment treated, quantitative indices and literature*

Sr.#	Family	Plant species/ Specimen number	Local name	[A]Part used	[B]Preparation	[C]Application	[D]Properties/Aliment treated	[E]FC	[F]RFC	[G]Ui	[H]UV	[I]Rel. PH	[J]Rel. BS	[K]RI	Literature
1	Acanthaceae	*Adhatoda zeylanica* Medic./KD-24	Baiker		Powder	Or	Abdominal pain (NI 14), asthma (NI 12), cough (NI 9), rheumatic pain (NI 6), ear infection (NI 13), *insect repellent (NI 5)	59	0.79	83	1.11	0.75	0.83	79.17	[10, 45]
2	Amaranthaceae	*Achyranthes aspera* var. *perphyristachya* Hook.f. /KD-47	Puthkanda	 	Paste, tea	Tr	*Pain and inflammation (NI 7), kidney stone (NI 6), insect bite (NI 2), bleeding (NI 3), fever (NI 5)	23	0.31	59	0.79	0.63	0.67	64.58	[46-49]
3	Amaranthaceae	*Amaranthus viridis* L./ KD-97	Ghanar	 	Powder	Tr	*Hot tonic (NI: 14) , piles (NI: 6), gonorrhea (NI: 7), Stomach problems (NI: 9)	36	0.48	63	0.84	0.63	0.50	56.25	[11, 49, 50]
4	Apiaceae	*Daucus carota* L./KD-58	Gajar	  	Juice, extract	Or	Weak eye sight (NI: 17), stimulants of the uterus (NI 4), anemia (NI: 10), blood purifier (NI: 8)	39	0.52	48	0.64	0.38	0.50	43.75	[50, 51]
5	Apocynaceae	*Carissa opaca* Stapf. ex Haines/KD-43	Garanda		Exude	Tr	*Pain and inflammation (NI 15)	53	0.71	78	1.04	0.75	1.00	87.50	[52, 53]
				 	Powder	Or	*Abdominal pain (NI 13), jaundice (NI 9), sexual debility (NI 4), skin infection (NI 2), earache (Ni 10)								
6	Asclepiadaceae	*Calotropis procera* (Aiton) W.T. Aiton/ KD-62	Ak		Latex	Tr	*Dog-bite (NI: 9), back pain (NI: 6), infection (NI: 9) , *sexual problems (NI: 5), ring worm (NI: 2), asthma (NI: 7), insect bite (NI: 4)	42	0.56	61	0.81	0.88	0.67	77.08	[48, 49]
7	Asteraceae	*Artemisia absinthium* L.	Babuna	 	Decoction, powder	Or	Stomach pain (NI: 6), earache (NI: 4), kill worms (NI: 2), inflammation (NI: 5)	21	0.28	36	0.48	0.63	0.67	33.33	[47]
							*Insect repellent (NI: 4)								

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Sr.#	Family	Plant species/ Specimen number	Local name	[A]Part used	[B]Preparation	[C]Application	[D]Properties/Aliment treated	[E]FC	[F]RFC	[G]Ui	[H]UV	[I]Rel. PH	[J]Rel. BS	[K]RI	Literature
8	Asteraceae	*Carthamus tinctorius* L./ KD-93	Kasumba (zafran)		Boiled in oil	Tr	*Pneumonia (NI: 8), fever (NI: 12), cough (NI: 5), throat problems (NI: 6)	31	0.41	52	0.69	0.50	0.50	50.00	[54]
9	Asteraceae	*Cichorium intybus* L./ KD-61	Kahsni		Juice	Or	Fever (NI 5), abdominal disorders (NI 3), indigestion (NI 4)	12	0.16	26	0.35	0.38	0.33	35.42	[8, 47]
10	Asteraceae	*Conyza bonariensis* (L.) Cronquist/ KD-152			Powder	Tr	Wound (NI 4), bleeding piles (NI 4), painful menstruation (NI 2)	10	0.13	15	0.20	0.38	0.50	43.75	[55]
11	Asteraceae	*Launaea taraxacifolia* (Willd.) Amin/ KD-89	Hand		Potherb	Or	*Diabetes (NI: 9), pain (NI 6)	15	0.20	25	0.33	0.25	0.33	29.17	[56]
12	Asteraceae	*Sonchus asper* (L.) Hill./ KD-81	Hundh		Powder	Or	*Diabetes (NI 7) ,* body pain (NI 5)	12	0.16	18	0.24	0.25	0.33	29.17	[57]
13	Asteraceae	*Tagetes minuta* L.	Sadberga		Extract	Or	Kidney pain (NI 5), expel kidney stones (NI 4)	9	0.12	9	0.12	0.25	0.17	20.83	[58, 59]
14	Asteraceae	*Taraxacum officinale* F.H. Wigg/ KD-26	Khali Hand		Potherb, paste	Or	*Fever (NI 8), jaundice (NI 3) , swelling (NI 11), joints pain (NI 5)	27	0.36	64	0.85	0.50	0.50	50.00	[49]
15	Berberidaceae	*Berberis lycium* DC./ KD-74	Sumbalu	 	Chewed, paste, extract	Tr	*Mouth inflammation (NI 11), jaundice (NI 9), injury (NI 3), wounds (NI 6), tonic (NI 5), diarrhea (NI 9), rheumatism (NI 7), diabetes (NI 8),	58	0.77	89	1.19	1.00	0.67	83.33	[46, 48, 60] [10]
16	Bombacaceae	*Bombax ceiba* L./ KD-136	Simble		Powder	Or	Weakness (NI 11), dysentery (NI 7), diarrhea (NI13), impotence (NI 8)	39	0.52	49	0.65	0.50	0.50	50.00	[61, 62]
17	Boraginaceae	*Trichodesma indicum* (L.) R. Br./KD-125	Handusi	 	Cooked with sugar, Infusion	Or	*Back pain (NI 14), *weakness (NI 9), swellings (NI 3), *urinary diseases (NI 7), dysentery (NI 8)	41	0.55	62	0.83	0.50	0.67	58.33	[63-65]
18	Brassicaceae	*Lepidium sativum* L./ KD-28	Haleon		Seeds are boiled in milk	Or	Backache (NI 5), pain (NI 2), abortion (NI 1) *eye cleaner (NI 4)	13	0.17	22	0.29	0.50	0.50	50.00	[66]
19	Cactaceae	*Opuntia dillenii* Haw./ KD-15	Thor		leaf made into a pulp /Paste	Tr	Ophthalmia (NI 8) *joint pain (NI 10)	18	0.24	35	0.47	0.25	0.33	29.17	[8]
20	Caesalpiniaceae	*Cassia fistula* L./ KD-09	Amaltas	 	Juice, decoction	Or	Abdominal pain (NI 12), constipation (NI 7), joint pain (NI 9) respiratory problems (NI10), jaundice (NI 6), piles (NI 5)	49	0.65	79	1.05	0.75	0.83	79.17	[46, 67] [11]

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Sr.#	Family	Plant species/ Specimen number	Local name	[A]Part used	[B]Preparation	[C]Application	[D]Properties/Aliment treated	[E]FC	[F]RFC	[G]Ui	[H]UV	[I]Rel. PH	[J]Rel. BS	[K]RI	Literature
21	Cannabinaceae	*Cannabis sativa* L./ KD-73	Bahng		Paste made with turmeric and rape seed/ smoked	Tr	Swellings NI 4, sedative (NI 7), relief pain (NI 2).	13	0.17	25	0.33	0.38	0.50	43.75	[46, 47, 68, 69]
22	Chenopodiaceae	*Chenopodium album* L./ KD-119	Ghanari	  	Potherb, decoction, infusion	Or	Constipation (NI 9), pain in limbs (NI 5), intestinal problems (NI 12), jaundice (NI 8), pile (NI 3), throat and eye problems (NI 9)	46	0.61	56	0.75	0.75	0.83	79.17	[61, 47, 50]
23	Convolvulaceae	*Ipomoea carnea* Jac./ KD-02	Jungli bakhir		Paste, decoction	Tr	*Athlete foot (NI 5) , loose stools (NI 23)	27	0.36	24	0.32	0.25	0.33	29.17	[50, 53]
24	Cucurbitaceae	*Momordica charantia* L./ KD-22	Khrella		Juice	Or	Diabetes (NI 6), jaundice (NI 4), piles (NI 2)	15	0.20	21	0.28	0.50	0.50	25.00	[11, 70]
					Leaves coated with oil warmed	Tr	Pain/swelling (NI 1)								
25	Cuscutaceae	*Cuscuta reflexa* Roxb./ KD-114	Neeladari	 	Decoction, juice	Or	Jaundice (NI 6), blood purifier (NI 9), Hair fall (NI 11) , skin disease (NI 4)	38	0.51	62	0.83	0.63	0.67	64.58	[48]
					Paste	Tr	Fractured bones (NI 8)								
26	Euphorbiaceae	*Ricinus communis* L./ KD-10	Arind		Leaves coated with mustard oil and warmed/ rapped on abdomen of kids, powder, poultice	Tr	Wounds (NI 7), boils (NI 5), swellings (NI 3), Joints pain/inflammation (NI 14), flatulence (NI 9) Increase milk production in females (NI 4)	45	0.60	58	0.77	0.88	0.67	33.33	[48, 50]
					Given with milk	Or	Constipation (NI 3)								
27	Euphorbiaceae	*Euphorbia helioscopia* L./ KD-42	Dodak		Paste	Tr	Boils (NI 19), female problems (NI 7)	26	0.35	30	0.40	0.25	0.33	29.17	[13]
28	Euphorbiaceae	*Mallotus philippensis* (Lam.) Muell./ KD-163	Kamilla		Powder mixed with mustard oil	Tr	Hair tonic (NI 4), anti-lice (NI 5), measles mumps (NI 2)	29	0.39	47	0.63	0.63	0.50	25.00	[45, 71]

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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					Powder	Or	*Cough (NI 10), intestinal worms (NI 8)								
29	Gentianaceae	*Swertia chirata* Ham./ KD-98	Chiraeta		Powder	Or	Nausea (NI 5) and vomiting (NI 8)	13	0.17	29	0.39	0.25	0.17	20.83	[46, 48]
30	Juglandaceae	*Juglans regia* L./ KD-90	Khor		Paste	Tr	Dermatitis (NI 7)	56	0.75	58	0.77	0.63	0.83	41.67	[10, 61, 67]
					Mixed with corn and crushed in water with sugar	Or	Nerve tonic (NI 11), tooth discoloration (NI 16), kidney stone (NI 12), constipation (NI 10)								
31	Lamiaceae	*Otostegia limbata* (Benth.) Benth. ex Hook. f./ KD-52	Chitta kanda		Chewed, extract	Tr	Mouth ulcer (NI 15), throat pain (NI 11), asthma (NI 13)	39	0.52	63	0.84	0.38	0.50	43.75	[13, 67, 72]
32	Lamiaceae	*Plectranthus rugosus* Wall./ KD-92	Safed Manja		Chewed	Tr	*Mouth infection (NI 21)	21	0.28	34	0.45	0.13	0.17	14.58	[14, 60]
33	Lamiaceae	*Mentha arvensis* L./KD-72	Podina		Decoction	Or	Diarrhea (NI 5), nausea (NI 8), vomiting(NI 5), abdominal pain (NI 18)	36	0.48	64	0.85	0.50	0.33	41.67	[10, 68]
34	Lamiaceae	*Mentha longifolia* (L.) Huds./ KD-28	Jungli podina		Decoction	Or	Diarrhea (NI17), dysentery (NI 22)	39	0.52	57	0.76	0.25	0.17	20.83	[53, 73]
35	Liliaceae	*Allium cepa* L./ KD-85	Piaz		Boiled in rapeseed oil, paste, juice	Tr	Earache (NI 19), boils (NI16), Cough (NI 9), infection (NI 3)	47	0.63	48	0.64	0.50	0.67	58.33	[10, 48]
36	Liliaceae	*Allium sativum* L./ KD-61	Thoom		Juice mixed with equal quantity of vinegar and honey	Or	Blood pressure (NI 14), ear pain (NI 8), infection (NI 7)	29	0.39	34	0.45	0.38	0.50	43.75	[74]
37	Meliaceae	*Melia azedarach* Him./ KD-265	Dharek	 	Powder	Or	Diabetes (NI 16), jaundice (NI 8)	32	0.43	54	0.72	0.50	0.50	50.00	[10, 51, 61]
				 	Paste	Tr	*Hair fall (NI 6), small pox (NI 2)								

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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38	Mimosaceae	*Albizia lebbeck* (L.) Benth./ KD-89	Shrin		Paste	Tr, Miswak	Inflammations (NI 10) , burns (NI 9), toothache (NI 14), strengthens the gums and teeth (NI 4),			73	0.97	0.63	0.50	56.25	[75]
					Powder mixed with mint leaves,	Or	*Sexual debility (11),	48	0.64						
39	Mimosaceae	*Leucaena leucocephala* (Lam.) de Wit/ KD-118	Kikar		Equal quantity of seeds mixed with *Albizia lebbeck*and made into powdered, oil, decoction	Or	*Sexual debility (NI 14), bacterial infection (NI 7), contraceptive (NI 13)	34	0.45	49	0.65	0.38	0.50	43.75	[76]
40	Moraceae	*Ficus palmata* Forssk./ KD-168	Phagwara		Thin layer wrapped/ eaten	Or	*Wounds (NI 11), constipation((NI 9), extraction of spines from flash (NI 15)	35	0.47	59	0.79	0.38	0.33	35.42	[73, 77]
41	Moraceae	*Ficus benghalensis* L./ KD-260	Bhohar			Tr	Heel cracks (NI 5)	31	0.41	25	0.33	0.50	0.50	50.00	[11]
					Poured into candy (*Batasha*)	Or	*Sexual debility (NI 17), premature ejaculation (NI 8), Scurvy (NI 1)								
42	Moraceae	*Morus nigra* L./ KD-81	Kala toot		Juice is made into syrup, Infusion	Or	Cough (NI 13), throat pain (NI 12), inflammation (NI 9), diarrhea (NI 4), Pain (NI 3), diabetes (NI 5),	46	0.61	64	0.85	0.75	0.83	79.17	[10, 61] [63]
43	Myrtaceae	*Eucalyptus camaldulensis* Dehnh./ KD- 99			Rubbed and smelled	Tr	Cold (15), cough(12), *condiment (6)	33	0.44	25	0.33	0.38	0.33	35.42	[78]
44	Myrtaceae	*Psidium guvajva* L./ KD-110	Amrood		Kept in warm ash for few minutes, chewing, decoction	Or	Flu (NI 9), cough (5), anthelmintic (14), bleeding gums (NI 5), bad breath (NI 4), wounds (2), vaginal irritation and discharges (3)	42	0.56	61	0.81	0.88	0.67	77.08	[67, 79];
45	Nyctaginaceae	*Boerhavia procumben* Banks ex Roxb./ KD-235	Budh bail		Decoction	Or	*Refrigerant (NI 6), demulcent drink (NI 6)	12	0.16	31	0.41	0.25	0.17	20.83	[47]
46	Oleaceae	*Olea ferruginea* Royle/KD-21	Kohu		Chewed, toothbrush	Tr	*Mouth ulcer (NI 17), throat pain (NI 5) , diabetes (NI 4), toothache (NI 8)	53	0.71	64	0.85	0.63	0.67	64.58	[57, 80]

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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					Oil	Tr	Pain (NI 10), inflammation (NI 9)								
47	Oxalidaceae	*Oxalis corniculata* L/ KD-193	Jandouro		Crushed with red chilies (*Chatni*), paste	Or	Lack of appetite (NI 15)	51	0.68	49	0.65	0.63	0.50	25.00	[10, 80]
			Jandouro		Paste, rubbed	Tr	*Pain (NI 12), *pus formation (NI 6), Snake bite (NI 8), wounds (NI 10)								
48	Papilionaceae	*Medicago polymorpha* Willd./ KD-73	Siree		Potherb	Or	Heart tonic (NI 4), constipation (NI 8), wounds (NI 3)	15	0.20	25	0.33	0.38	0.50	43.75	[49, 57]
49	Papilionaceae	*Dalbergia sissoo* Roxb. / KD-37	Tali		Oil, Wrapping	Tr	Scabies (NI 3), swelling of the breast (NI 7)	35	0.47	34	0.45	0.75	0.83	79.17	[67, 71] [81]
				  	Decoction, Juice, powder mixed with oil	Or	*Cooling agent (NI 10), diarrhea (NI 4), dandruff (NI 5), gonorrhea (NI 6),								
50	Papaveraceae	*Papaver somniferum* L./ KD-187	Post		Roasted fruit ground with *Terminalia chebula, Foeniculum vulgare* and black salt	Or	Abdominal pain (NI 11), sleeplessness (NI 6)	17	0.23	21	0.28	0.25	0.33	29.17	[47, 68]
51	Pinaceae	*Pinus roxburghii* Sargent./ KD-203	Chir		Plasters, herbal steam baths	Tr	Ringworm (NI 5), boils (NI 7), rheumatism (NI 6), cough (NI 10), cold (NI 2)	45	0.60	56	0.75	0.88	0.67	77.08	[10, 46, 68]
					Soaked overnight in water (Juice)	Or	*Diabetes (NI 12), kidney and bladder problems (NI 3)								
52	Plantaginaceae	*Plantago major* L./ KD-109	Chamchapatar	 	Mixed with curd, Infusion	Or	Dysentery (NI 6), haemoptysis (NI 7)	13	0.17	23	0.31	0.25	0.33	29.17	[61]
53	Plantaginaceae	*Plantago ovata* Forssk./ KD-16	Ismaghol	 	Heated leaves	Tr	Wounds (NI 2), toothache (NI 3)	10	0.13	15	0.20	0.38	0.50	18.75	[68]

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					Mixed with curd	Or	Dysentery (NI 5)								
54	Poaceae	*Cynodon dactylon* (L.) Pers./ KD-11	Khabal		Infusion along milk, juice,	Or	Piles (NI 8), bleeding from nose (NI 5)	25	0.33	44	0.59	0.50	0.50	50.00	[68, 75]
					Paste, poultice	Tr	Cuts (NI 10), wounds (NI 2)								
55	Polygonaceae	*Rumex hastatus* D. Don/ KD-121	Ambhi		Potherb, Juice	Or	Headache (NI 9), dyspepsia (NI 5), *flatulence in animals (NI 6), constipation (NI 9), dysentery with blood (NI 8)	37	0.49	33	0.44	0.63	0.33	47.92	[61, 68, 79]
56	Polygonaceae	*Rumex nepalensis* L./ KD-22	Halfari	 	Leaves mixed with *Nasturtium officinale*(Potherb), decoction, paste	Or	*Edema (NI 5), weakness (NI 3), Body pain (NI 2), swollen gums (NI 2)	12	0.16	15	0.20	0.50	0.33	41.67	[82]
57	Punicaeae	*Punica granatum* var. *nana* L./ KD-61	Darunna		Powder	Or	* Leucorrhoea (NI 5)	55	0.73	58	0.77	1.00	1.00	100	[13] [83]
				  	Powder	Or	Diarrhea (NI 9), dysentery (NI 7)								
					Soaked in water overnight (Juice)	Or	Jaundice (NI 12), heart disease (NI 7), blood pressure (NI 6), bleeding from nose (NI 4) , expel worms (NI 5)								
58	Ranunculaceae	*Thalictrum aquilegifolium* L./ KD-230	Beeni		Crushed in water (Juice)	Or	Sunstroke (NI 9), jaundice (NI 5)	14	0.19	25	0.33	0.25	0.33	29.17	[84, 85]
59	Ranunculaceae	*Ranunculus arvensis* L./ KD-198	Chichumbha		Potherb	Or	Indigestion (NI 22)	43	0.57	67	0.89	0.38	0.33	18.75	[10, 68, 83]
					Boiled in rapeseed oil and ground	Tr	*Boils (NI 18), pimples (NI 3)								
60	Ranunculaceae	*Rananculus scleratus* L./ KD-398	Jal dhania		Juice	Or	*Blood purifier (NI 15)	15	0.20	34	0.45	0.13	0.17	14.58	

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61	Rhamnaceae	*Zizyphus oxyphylla* Edgew./KD-312	Ber	  	Root barks and *Achyranthes aspera* (Powder)	Or	*Fever (NI 11),* abdominal pain (NI 5), Diabetes (NI 10), pain (NI 2)	28	0.37	32	0.43	0.50	0.67	58.33	[86]
62	Rosaceae	*Prunus persica* (L.) Batsch./ KD-291	Arwaari		Cooked with rice	Or	Constipation (NI 13), edema (NI 3)	21	0.28	51	0.68	0.38	0.50	43.75	[57, 60, 70, 86]
					powder	Tr	Wounds (NI 4)								
63	Rosaceae	*Rosa indica* L./ KD-102	Gulab		Soaked in water overnight (Infusion/Arq-e-Guhlab)	Tr/Or	Eye cleaner (NI 12), heart tonic (NI 7)	29	0.39	46	0.61	0.38	0.50	43.75	[61, 72]
					Crushed in sugar and kept few days under moonlight	Or	Constipation (NI 10)								
64	Rosaceae	*Rosa brunonii* Lindl./ KD- 173	Tarnari		Powder, decoction	Tr	Wounds (NI 7), infections(NI 11), constipation (NI 9)	35	0.47	56	0.75	0.50	0.33	25.00	[60, 67, 87]
					Powder	Or	*General weakness (NI 8)								
65	Rosaceae	*Rubus ellipticus* Smith./ KD-124	Aakhra		Paste	Tr	Wounds (NI 8)	45	0.60	53	0.71	0.88	1.00	93.75	[57, 61, 70] [72]
				  	Crushed in water (Juice),	Or	*Cool demulcent (NI 9), vaginal/seminal discharge (NI 6), fever (NI 3), colic (NI 8), coughs (NI 6), sore throat (NI 5).								
66	Rubiaceae	*Rubia codifolia*Roxb. ex Desv./ KD-109			Crushed in water (Juice)	Or	*Amenorrhea (NI 12), dysentery (5) intestinal worms in animals (2)	19	0.25	32	0.43	0.38	0.50	43.75	[53]

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67	Rutaceae	*Zanthoxylum alatum* Roxb./ KD-356	Timmber		Kept on hand (*Asa*), powder mixed with *Mentha* spp. and table salt eaten with boiled egg, decoction	Tr	Piles (NI 9), chest pain (NI 7), *digestive problems (NI 9), fever (NI 5), dyspepsia (NI 6), cholera (NI 8), toothache (NI 6), rheumatism (NI 4)	54	0.72	64	0.85	1.00	0.67	83.33	[10, 61, 68, 72]
68	Rutaceae	*Citrus medica* var. *acida* Brandis/ KD-246	Girgal		Juice mixed with the infusion of *Rosa indica* and glycerin	Or	Softening of skin (NI 15) , pain (NI 7), heart tonic (NI 5), dysentery (NI 2), antibiotic (NI 4)	33	0.44	35	0.47	0.63	0.83	72.92	[61, 88]
69	Sapindaceae	*Dodonaea viscosa*(L.) Jacq./ KD-100	Sanatha		Exude, extracts	Tr	Sore throat (NI 8), *diabetes (NI 11), pain (NI 9), toothache (NI 7), wound (NI 8)	43	0.57	58	0.77	0.63	0.67	64.58	[10, 48, 60, 61]
70	Sapindaceae	*Sapindus mukorossi* Gaertn. de Fruct./ KD-132	Raintha		Paste	Tr	*Snake/ scorpion bites (NI15), allergy (NI 7), skin freckles (NI 4), inflammation (NI 6), anti-lice (NI 8), dandruff (NI 9)	49	0.65	55	0.73	0.75	0.33	54.17	[10, 70, 79]
71	Scrophulariaceae	*Verbascum thapsus* L./ KD-200	Giddar tammako		Paste, smoked	Tr	*Joint pain (NI 5), chest complaints (NI 4), asthma (NI 2)	11	0.15	22	0.29	0.38	0.33	35.42	[46, 72, 89]
72	Solanaceae	*Capsicum frutescens* var. *acuminate* Fingerh/ KD-301	Mirch		Eaten with bread	Or	*Abdominal worms (NI 5), stomach pain (NI 9), back pain (NI 7)	21	0.28	43	0.57	0.38	0.33	35.42	[89]
73	Solanaceae	*Solanum incanum* L./KD-26	Mohkari		Paste/ Smoked	Tr	Toothache (NI 6), snake bite (NI 4)	17	0.23	38	0.53	0.50	0.67	58.33	[51]
					Juice	Or	Pneumonia (NI 4), sore throat (NI 3)								
74	Solanaceae	*Solanum nigrum* L. / KD-69	Kachach		Potherb/ juice	Or	Heart disease (NI 15), fever (NI 8), pain (NI 3)	26	0.35	35	0.47	0.38	0.50	43.75	[47, 50]

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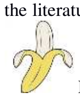
75	Solanaceae	*Solanum surattense* Shord Wendl. / KD-37	Mohkari		Paste, smoke	Tr	Pain (NI 9), inflammation (NI 7), piles (NI 8)	53	0.71	39	0.52	0.50	0.50	50.00	[47]
					Cooked in milk (Curry), Decoction with the stem of *Tinospora cordifolia*	Or	Backache (NI 11), fever (NI 12), phlegmatic Cough (NI 6)								
76	Ulmaceae	*Celtis australis* L./KD-67	Khirk		Paste, decoction	Tr	*Smallpox (NI 15), anti-allergic (NI 10), dysentery (NI 12), inter menstrual bleeding (NI 7), bacterial infection (NI5).	49	0.65	61	0.81	0.75	1.00	87.50	[60, 69]
77	Urticaceae	*Debregeasia salicifolia* (D. Don.) Rendle./KD-96	Sindari		Exude/paste mixed with mustard oil	Tr	*Toothache (NI 7), skin rashes (NI 4), infection (NI 10), eczema (NI 9)	30	0.40	43	0.57	0.50	0.33	41.67	[60, 69, 79]
78	Verbenaceae	*Vitex negundo* Haussk./ KD-59	Banah		Powder, decoction	Or	Abdominal pain (NI 8), dispersing swellings of the joints from acute rheumatism (NI 2), *toothache (NI 12) , ulcers (NI 4), flatulence,(NI 3) fever (NI 3)	32	0.43	53	0.71	0.75	0.50	62.50	[90, 91]
79	Violaceae	*Viola canescens*Wall. Ex Roxb./ KD-43	Banakhshah		Decoction	Or	Jaundice (NI 24), fever (NI 13), gastrointestinal disorder (NI 8), cough (NI 9)	52	0.69	47	0.63	0.50	0.50	50.00	[10, 82]
80	Xanthorrhoeaceae	*Aloe vera* (L.) Burm.f./ KD-77	Kunwaargandal		Potherb	Or	*Pain (NI 5),* inflammation (NI 8), wound healing (NI 3),	16	0.21	33	0.44	0.38	0.17	27.08	[60, 79, 92]

FC = frequency of citation, RFC = relative frequency of citation, Rel. PH = relative pharmacological properties, Rel. BS = relative body system treated, RI = relative importance, UV = use value index, Or = oral, Tr= tropical. *Novel use not recorded in the literature.  Leaves,  Roots,  Seeds,  Tuber,  Areal parts,  Stem and Branches,  Flowers,  Bark,  Pulp,  Fruit,  Whole Plant,  Petal,  Bulb,  Fruit peelings,  Unripen fruit,  Latex,  Peel oil,  Husk,  Cone,  Fresh Fruit,  Resin

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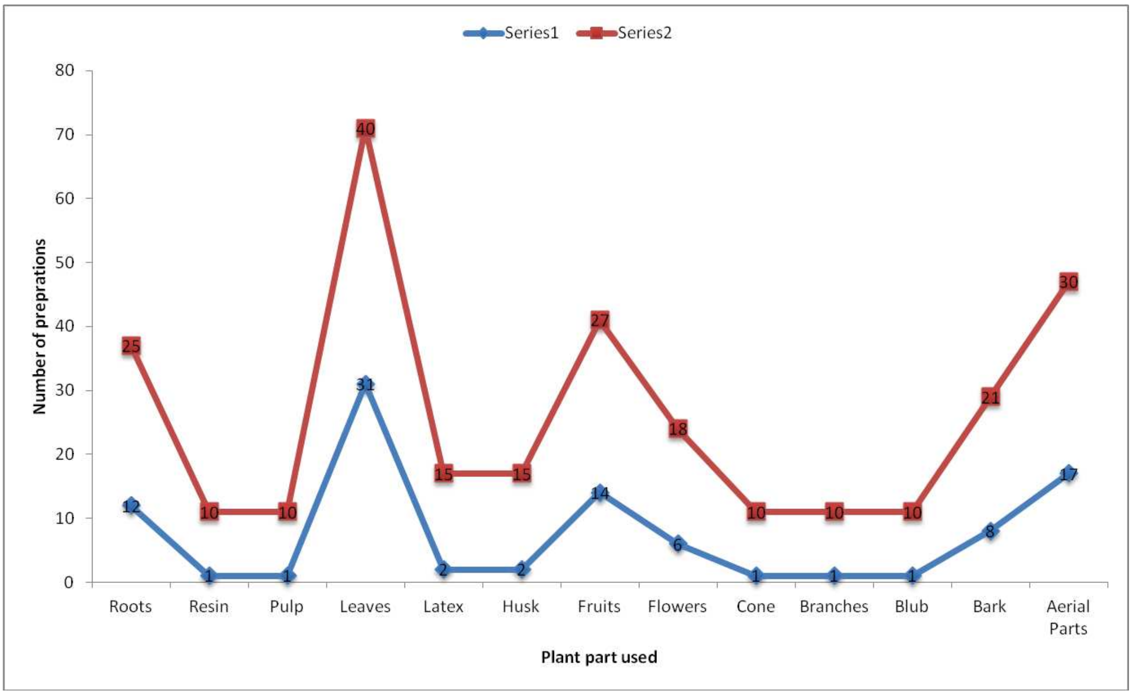

Figure 5. *Different plant parts used in herbal preparations*

Methods of preparation and administration

Different herbal recipes were prepared from the medicinal plants and method of preparation and application vary based on type of disease treated and actual site of application (*Fig. 6*). The most herbal recipes were prepared by grounding plant parts into powder about 16.4%. Other methods of preparation and administration include: juice (15%), paste (14%), decoction (11%), chewing (6.3%), potherb (5.7%), infusion (3.8%), extract (3.16%), boiling, smoking, poultice, latex and wrapping (2.53% each), cooking and oil (1.9% each), rubbing (1.27%), herbal bath, smelling and tea (0.63% each). Although most of the preparations were made from a single plant few were made from mixture of more than one plants, for example, seeds of *Leucaena leucocephala* were grounded and mixed with equal amount of powdered seeds of *Albizia lebbeck* to treat sexual debility. Similarly, roasted fruit of *Papaver somniferum* was ground with *Terminalia chebula, Foeniculum vulgare* and black salt for the treatment of abdominal pain and sleeplessness. With regard to the method of preparation, the undertaken study was in accordance with the work carried out by Mouterde (1983). Most of the preparations were administered orally (57.58%), followed by tropical (39.62%) and inhalation (2.8%). These results were in accordance with Vijayakumar et al. (2015).

Therapeutic indications

Informant's consensus factor (F_{IC})

In order to check the agreement of informants about the knowledge of plants uses, the recorded 90 diseases were grouped into 12 different disease categories according to IDC. The Informants Consensus Factor (F_{IC}) values ranged from 0.80 to 0.91, showed much homogeneity in nearly all the described disease categories (*Table 3*). Generally, F_{IC} values range from 0 to 1, in which the high values are good indicator of high

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DOI: http://dx.doi.org/10.15666/aeer/1702_27992829

informant consensus on the species used in the treatment of a particular category of illness (Heinrich et al., 1998; Trotter and Logan, 1986). The Informants Consensus Factor (F_{IC}) analysis indicated that among the 12 disease categories skin and related symptoms had the highest F_{IC} values (0.91), treated by a large number of taxa (10 species) and 96 use reports. It was followed by circulatory problems (0.90), diabetes (0.90). The highest value of F_{IC} suggests increased data about the usefulness of plants to cure a specific disease. The most commonly used plants to treat these diseases were *Pinus roxburghii, Ranunculus arvensis Albizia lebbeck, Lepidium sativum, Chenopodium album, Otostegia limbata, Berberis lyceum, Medicago polymorpha* and *Adhatoda zeylanica*. Although the F_{IC} for gastrointestinal disorders (0.88) was less compared to some other ailment categories, however, a maximum number of plants (66 species) were recorded under this category. This category contains wide range of ailments such as abdominal pain, abdominal worms, piles, cholera, constipation, diarrhea, dysentery indigestion, flatulence, stomach disorder, intestinal problems, lack of appetite, nausea, vomiting and stomach pain (Afolayan et al., 2014; Abe and Ohtani, 2013). The plants with high F_{IC} value might contain the high amount of bioactive compounds and can be subjected to further biochemical and pharmacological analysis.

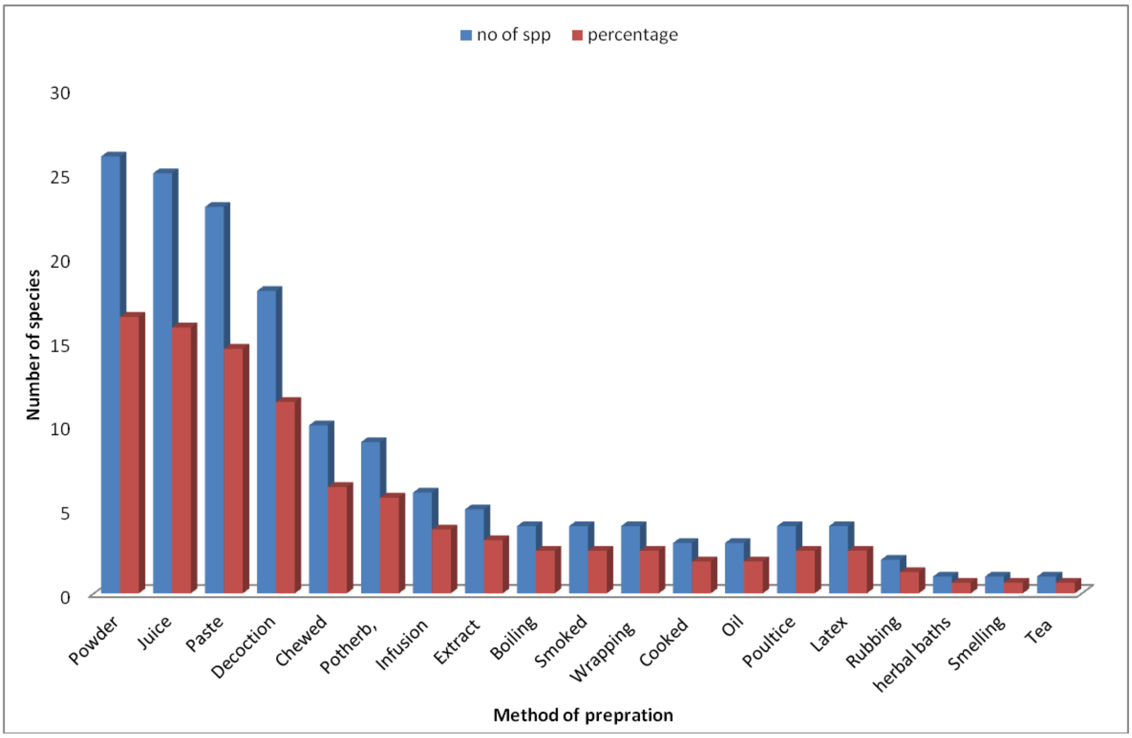

Figure 6. *Various herbal preparations/formulations from the native flora of Rajh Mehal*

Medicinal importance (MI)

The medicinal importance (MI) values can be used as a sign of the relative medicinal relevance of mentioned plant species (*Table 3*). These values can be used in finding variability among the pharmacological, environmental and cultural determinants between the investigated species and areas (Baydoun et al., 2015). The MI and F_{IC} values were higher for skin and related symptoms (9.6) because weather conditions in study area quit severe dry and prolong winter; that is why people in the study area suffer

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skin problems very commonly. After skin diseases circulatory problems (8.93) were common, then diabetes (8.8) and digestive system problems, mouth, ear and oral disease (8.45). These values were closer to the findings of Baydoun et al. (2015) and higher than the MI values reported by Carrió and Vallès (2012).

Table 3. *International classification of diseases (ICD) classification and disease categories along with various diseases versus reports, number of taxa, total reports and FIC*

Sr. no	Disease category	Symptoms	No. of taxa	Use reports	FIC	MI
1	Circulatory problems (I00-I99)	Anemia (10), blood pressure (20), blood purifier (32), heart disease (15), heart disease (29), edema (3), heart tonic (16)	14	125	0.90	8.93
2	Digestive system problems (K00-K93), (R00-R99)	Abdominal Pain (92), abdominal worms (5), piles (45), cholera (8), constipation (87) diarrhea 61 digestive problem (9) dysentery (110) indigestion (37) flatulence 18 stomach disorder (8) intestinal problems (12) lack of appetite (15) nausea and vomiting (26) stomach pain (24) scurvy (1) jaundice (94)	66	558	0.88	8.45
3	Respiratory System (J00-J99)	Asthma (45), bad breath (4), chest complaints (4), chest pain (7), flue and cold (26), cough (94) haemopty (7) pneumonia (4)	25	191	0.87	7.64
4	Diabetes (E10-E14)	Diabetes (88)	10	88	0.90	8.8
5	Musculoskeletal (M00-M99)	Back pain (43) body pain (7) joints pain (2) inflammation (69) joint pain (43) pain (76) pain and inflammation (22) swelling (33) fractured bones (8), rheumatic pain (23)	48	326	0.86	6.79
6	Infections (A00-A09)	Bacterial infection (12), ear infection (13), infection (40), skin infection (2), pus formation (6), Fever (73)	10	73	0.88	7.3
7	Skin and related symptoms (S00-T98)	Boils (65), burns (9), measles/ mumps (2), pimples (3), Small pox (7)	10	96	0.91	9.6
8	Dermatological problems (L00-L99)	Dermatitis (7), heel cracks (5), eczema (9), ringworm (7), scabies (3), skin disease (4), skin freckles (4), skin rashes (19), allergy (17) athlete foot (5)	13	60	0.80	5.8
9	Others (Tonic, cool demulcent drinks, sedative	Cool demulcent (25), hair tonic (4), hot tonic (14), nerve tonic (11), refrigerant (6), tonic (5), uterus tonic (5), tooth and gum tonic (4), sedative (7), ulcers (4), heat stock (9)	13	94	0.87	7.4
10	Antidotes T36-T50	Dog-bite (9) Insect bite (15) snake bite (27)	8	51	0.86	6.38
11	Worm insect related diseases	Anthelmintic (14) antibiotic (4) anti-lice (12) expel worms (5) intestinal worms (10) kill worms (2)	8	47	0.85	5.88
12	Oral, dental, hair and ENT	Bleeding from nose (9) bleeding gums (5) earache (41) tooth discoloration (16) eye cleaner (16) weak eye sight (22) mouth infection (21) mouth inflammation (11) mouth ulcer (32), ophthalmia (8), toothache (63), weakness (31), throat pain (38), swollen gums (2), dandruff (14) hair fall (17) headache (9), sore throat (16)	66	558	0.88	8.45

FIC = informants' consensus factor, MI = medicinal importance

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DOI: http://dx.doi.org/10.15666/aeer/1702_27992829

Relative importance (RI)

The highest RI value was obtained for *Adhatoda zeylanica* (93.75) *Zanthoxylum alatum* (91.67), *Berberis lyceum*, *Juglans regia* (87.50 each) *Punica granatum, Olea ferruginea* (83.33 each), and *Solanum surattense* (79.17) which indicated that these plants possess strong pharmacological properties and are used more frequently in the study area. The important and most commonly cited plants belonged to Asteraceae, Lamiaceae, Solanacea and, Rosaceae. Theses finding agrees with the early reports by Carmen Juárez-Vázquez et al. (2013), Yaseen et al. (2015) and Alonso-Castro et al. (2012).

Smith's salience analysis (SS)

Salience analysis (Smith, 1993) accounted for frequency of mention; on an average 60 illnesses were listed. In free-listing we interview 112 informants but for Salience analysis and ranking of illness 10 most informative informants were chosen. The *Table 4* shows salience estimates for each illness, indicating both the number of people who mentioned the illness and the order of their responses. In Smith's Salience analysis abdominal pain and worms had the highest overall salience index, mentioned by 73.21% of informants. Constipation was the second most salient illness had 70.54% of informants and Pain was the third most cited illness treated by the plants in the study had 67.8% frequency. Sleeplessness, uterus disease, weak eyesight and bleeding gonorrhea were the less salient diseases. Composite Salience was another step to reinforcement the results of Smith's Salience by narrow down the informant numbers, result was draw the on the base of ten key informants.

Table 4. *Results of ANTHROPAC analysis of Illness free-lists and Smith's salience index along with composite salience*

S. No	Illness	Frequency (%) N = 112	Inverted rank/total listed = Smith's salience index										Illness Σ	Composite salience Σ/n (n = 10)
			SS1	SS2	SS3	SS4	SS5	SS6	SS7	SS8	SS9	SS10		
1	Abdominal pain and worms	82 (73.21)	60/60= 1		58/60= 0.96			53/60= 0.883			52/60= 0.867	51/60= 0.850	4.56	0.4560
2	Constipation	79 (70.54)		60/60= 1						53/60= 0.883			1.883	0.1883
3	Pain	76(67.86)	58/60= 0.96										1.827	0.1827
4	Sexual dysfunction	72 (64.29)				56/60= 0.933							0.933	0.0933
5	Teeth problems	71 (63.39)	56/60= 0.933	58/60= 0.96									1.893	0.1893
6	Wounds	70 (62.50)					52/60= 0.867			1/60= 0.017			0.867	0.0867
7	Jaundice	69 (61.61)											0.883	0.0883
8	Skin problems	69 (61.61)	51/60= 0.850					53/60= 0.883					0.85	0.0850
9	Eczema	68 (60.71)										50/60= 0.833	0.833	0.0833
10	Cough	67 (59.82)	50/60= 0.833						1/60= 0.017				0.833	0.0833
11	Diabetes	67 (59.82)		1/60= 0.017	48/60= 0.800								0.8	0.0800
12	Diarrhea	67(59.82)	46/60= 0.767				1/60= 0.017						0.767	0.0767
13	Blood purifier	66 (58.93)				46/60= 0.768			1/60= 0.017			1/60= 0.017	0.768	0.0768
14	Cooling agent	66 (58.93)	46/60= 0.769				5/60= 0.083			46/60= 0.770			1.539	0.1539
15	Dysentery	65 (58.04)		1/60= 0.017				46/60= 0.770			1/60= 0.017		0.77	0.0770

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S. No	Illness	Frequency (%) N = 112	Inverted rank/total listed = Smith's salience index										Illness Σ	Composite salience Σ/n (n = 10)
			SS1	SS2	SS3	SS4	SS5	SS6	SS7	SS8	SS9	SS10		
16	Joints pain	65 (58.04)	44/60= 0.733										0.733	0.0733
17	Backpain	65 (58.04)			44/60= 0.734		9/60= 0.150						0.734	0.0734
18	Boils	64 (57.14)	44/60= 0.735			9/60= 0.150				1/60= 0.017			0.735	0.0735
19	Gastrointestinal disorder	64 (57.14)			9/60= 0.150			43/60= 0.717					0.717	0.0717
20	Mouth infection	63 (56.25)							3/60= 0.050			42/60= 0.700	0.7	0.0700
21	Nausea and vomiting	63 (56.25)	42/60= 0.701			42/60= 0.700							1.401	0.1401
22	Throat problems	62 (55.36)			41/60= 0.683					42/60= 0.700			1.383	0.1383
23	Weakness	61 (54.46)	39/60= 0.650						42/60= 0.700				1.35	0.1350
24	Animal or insect bites	60 (53.57)				39/60= 0.651					1/60=0.017		0.651	0.0651
25	Ear pain	60 (53.57)					42/60= 0.700					39/60= 0.652	1.352	0.1352
26	Flatulence	59 (52.68)			37/60= 0.617					42/60= 0.700			1.317	0.1317
27	Hair fall	58 (51.79)	36/60= 0.600										0.6	0.0600
28	Indigestion	57 (50.89)				34/60= 0.567			1/60= 0.017				0.567	0.0567
29	Inflammation	56 (50.0)		33/60= 0.550							1/60= 0.017		0.55	0.0550
30	Piles	55 (49.11)			31/60= 0.517			1/60= 0.017					0.517	0.0517
31	Swellings	54 (48.21)	30/60= 0.500									1/60= 0.017	0.5	0.0500
32	Urinary diseases	53 (47.32)				27/60= 0.450			5/60= 0.083				0.45	0.0450
33	Cold and flu	52 (46.43)	26/60= 0.433										0.433	0.0433
34	Eye cleaner	51 (45.54)			24/60= 0.400			5/60= 0.083					0.4	0.0400
35	Heart tonic	50 (44.64)	22/60= 0.367								5/60= 0.083		0.367	0.0367
36	Pimples	49 (43.75)				20/60= 0.333				3/60= 0.050			0.333	0.0333
37	Abortion	48 (42.86)	18/60= 0.300										0.3	0.0300
38	Amenorrhea	47 (41.96)			17/60= 0.283						3/60= 0.050		0.283	0.0283
39	Anorexia	46 (41.07)	17/60= 0.284									2/60= 0.033	0.284	0.0284
40	Antilice	46 (40.18)			3/60= 0.050				15/60= 0.250				0.25	0.0250
41	Asthma	45 (40.18)	12/60= 0.200					3/60= 0.050					0.293	0.0293
42	Athlete foot	41 (36.61)		4/60= 0.067		11/60= 0.183							0.25	0.0250
43	Blood pressure	39 (34.82)	10/60= 0.167										0.567	0.0567
44	Dandruff	36 (42.14)			9/60= 0.150			1/60= 0.017					0.167	0.0167
45	Edema	32 (28.57)	9/60= 0.151								1/60= 0.017		0.168	0.0168
46	Hot tonic	30 (26.79)					1/60= 0.017			9/60= 0.152			0.169	0.0169
47	Infection	30 (26.79)	7/60= 0.117		1/60= 0.017				1/60= 0.017				0.151	0.0151
48	Injury	25 (22.32)		6/60= 0.100				2/60= 0.033					0.133	0.0133
49	Leucorrhoea	23 (19.64)	6/60= 0.101						3/60=0.050				0.151	0.0151
50	Nerve tonic	22 (19.64)				6/60= 0.102					1/60= 0.017		0.119	0.0119
51	Ophthalmia	20 (17.86)					27/60= 0.450			5/60= 0.083			0.45	0.0450

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S. No	Illness	Frequency (%) N = 112	Inverted rank/total listed = Smith's salience index										Illness Σ	Composite salience Σ/n (n = 10)
			SS1	SS2	SS3	SS4	SS5	SS6	SS7	SS8	SS9	SS10		
52	Pneumonia	15 (13.39)			1/60= 0.017							5/60= 0.084	0.101	0.0101
53	Pus formation	14 (12.50)				4/60= 0.067							0.067	0.0067
54	Ringworm	13 (11.61)						4/60= 0.067					0.067	0.0067
55	Sedative	12 (10.71)	3/60= 0.050										0.05	0.0050
56	Sleeplessness	10 (8.93)			3/60= 0.050								0.05	0.0050
57	Uterus disease	10 (8.93)	2/60= 0.033										0.033	0.0033
58	Weak eye sight	9 (8.04)							2/60= 0.033				0.033	0.0033
59	Bleeding	5 (4.46)										1/60= 0.017	0.017	0.0017
60	Gonorrhea	3 (2.68)	1/60 = 0.017										0.017	0.0017

Use value index (UV) and relative frequency of citation (RFC)

The use value (UV) shows the range to which a species can be used and the species with high UV values were mostly exploited in the area to cure a specific aliment. *Zanthoxylum alatum* (1.19), *Olea ferruginea* (1.11), *Solanum surattense* (1.05) and *Juglans regia* (1.04) possessed the higher use values. *Table 2* showed high values of UV and RFC of different plant species. It is evident from the results that *Adhatoda zeylanica,* (0.79) *Berberis lyceum* (0.77), *Juglans regia* (0.75) and, *Punica granatum var. nana* (0.73) and *Zanthoxylum alatum* (0.72) were the most cited species in the research area. On the bases of RFC and UV we concluded, since these species are very commonly available in the area, therefore the inhabitants mostly cited these. Moreover, these species were native to that area and were present for decades, so their properties to cure different disease got popularized among the indigenous peoples. A strong significant and positive correlation ($r^2 = 0.60$) was recorded between UV and RFC showing that by increase in number of informants the knowledge about plant use in herbal medicine will also increase. These results indicate that the study can make a significant contribution to folk knowledge on the use of medicinal plants and further laboratory-based investigations could help in identifying the active ingredients of the most commonly exploited plants. The variation in data can be explained by coefficient of determination and in present study the value of R^2 was 0.36 reflecting that 33% variation in use value can be explained in terms of relative frequency of citation (*Table 5*).

Table 5. *Pearson correlation between UV and RFC*

		UV	RFC	Mean ± std. deviation
UV	Pearson correlation	1	0.606**	0.6034 ± 0.23249
	Sig. (2-tailed)		0.000	
	N	80	80	
RFC	Pearson correlation	0.606**	1	0.4250 ± 0.19266
	Sig. (2-tailed)	0.000		
	N	80	80	

$r^2 = 0.36$

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DOI: http://dx.doi.org/10.15666/aeer/1702_27992829

Discussion

This study documented the good diversity of medicinal plants coupled with their therapeutic use and medicinal importance in the indigenous communities of Rajh Mehal, AJK. The ethnobotanical inventory consists of an ample therapeutic indication of 80 medicinal plants distributed in 12 disease categories that revealed a good picture of the health disorders of the native population. The natives mostly prefer single species based preparations instead of mixtures. The high Informant Consensus (F_{IC}) suggests that current use and knowledge of medicinal plants are still strong and local inhabitants have a high dependency on medicinal plants in meeting their primary health care. Many novel uses of plants were documented after comparing with the previously published literature. Most of the documented species possessed similar uses; however, 16 species possessed various novel uses which were not previously recorded. These include: *Conyza bonariensis* (Wound), *Carthamus tinctorius* (Pneumonia), *Celtis australis* (Smallpox, anti-allergic), *Dodonaea viscosa* (Toothache, diabetes), *Ipomoea carnea* (Athlete foot), *Launaea taraxacifolia* (Diabetes, pain), *Leucaena leucocephala* (Sexual debility), *Olea ferruginea* (Mouth ulcer, throat pain), *Pinus roxburghii* (Diabetes), *Plectranthus rugosus* (Mouth infection), *Rosa brunonii* (General weakness), *Rubia cordifolia* (Amenorrhea), *Rubus ellipticus* (Cool demulcent), *Rumex nepalensis* (Edema, weakness), *Thalictrum aquilegifolium* (Sunstroke) and *Zizyphus oxyphylla* (Fever, abdominal pain). In Smith's Salience analysis indicated that abdominal pain and worms had the highest overall salience index, mentioned by 73.21% informants. Constipation was the second most salient illness had 70.54% of informants and Pain was the third most cited illness treated by the plants in the study had 67.8% frequency but Sleeplessness, uterus disease, weak eyesight and bleeding gonorrhea were the less salient diseases. Composite Salience was another step to reinforcement the results of Smith's Salience by narrow down the informant numbers, result was draw the on the base of ten key informants. On the bases of RFC and UV we can say that, these species are very commonly available in the area, therefore the inhabitants mostly cited these. Moreover, these species were native to that area and were present for decades, so their properties to cure different disease got popularized among the indigenous peoples.

Conclusion

The present study can serve as best baseline study to search active ingredients of plants which are being utilized as herbal medicine in the study area, particularly plants with novel uses. These plants might be subjected to further lab and clinical analysis for validation of results from present study. This traditional knowledge together with scientific studies can lead to powerful discovery for newer, safer and affordable medicines. Furthermore, this study could be helpful in the conservation of the traditional knowledge of medicinal plants of the remote areas like Rajh Mehal for the uplift of a good life standard and livelihood of the local communities is indispensable. High dependency of local communities on local flora is resulting in extinction of medicinal plants, so step should be taken for conservation and management of these valuable plant resources of this territory.

Acknowledgements. We are enormously thankful to the local inhabitants/informants of the study area for their assistance and knowledge sharing, without which such study is unfeasible. I want to acknowledge

COMSATS Institute of Information Technology, Islamabad, English language department for language editing.

Author's contributions. MFQ and RQ designed experiments, field data were collected by QFM, MSA and WA, analytic analysis was also done by QFM and AS, HS, MFQ wrote first draft of manuscript, RQ reviewed and made amendments in original draft of manuscript.

Conflict of interests. We declare that we have no conflict of interests.

Ethics approval. Before initiating of our survey ethical approval for the study was obtained from the COMSATS Institute of Information Technology Ethics Committee. Legal permission was taken from representatives of the municipality for conducting the interview. All respondents were asked to sign a prior informed- consent form after the objectives and possible consequences of the study had been explained. The prior informed consent (PIC) form was translated into the local Pahari language.

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NEEDLE MORPHO-ANATOMY AND POLLEN MORPHO-PHYSIOLOGY OF SELECTED CONIFERS IN URBAN CONDITIONS

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(Received 26th Oct 2018; accepted 7th Jan 2019)

Abstract. Comparison of twelve conifer species (*Abies alba, A. concolor, A. nordmanniana, A. pinsapo, Cedrus atlantica, C. deodara, Picea abies, P. omorika, P. pungens, Pseudotsuga menziesii, Taxus baccata*, and *Pinus nigra*) in the sense of needle morpho-anatomy and pollen morpho-physiology, in correlation to air pollution, was performed for the first time. Analyzed properties of species were also compared with literature sources. Listed conifers were investigated in five Belgrade parks, characterised by different degrees of air pollution, especially CO_2. Their rank, I-V, was performed from non-polluted to heavily-polluted parks. Ranking in the sense of needle morpho-anatomy and pollen morpho-physiology did not match expected ones, but park V remained the worst for many analyzed species. Trees with shorter needles had greater stomatal density, which was particularly prominent in *A. alba, A. nordmanniana, P. abies, P. omorika, P. nigra* and *T. baccata*. The pollen grains of *C. atlantica* and *T. baccata* were the most sensitive to air pollution. In some analyzed species distance of particular trees close to the heavy traffic also was in correlation with needle dimensions (*P. omorika, A. concolor, A. nordmanniana, P. nigra*), stomatal density (*A. alba, P. abies, P. omorika, P. pungens) and pollen vitality (A. pinsapo, C. atlantica, P. menziessi, P. nigra,* and *T. baccata*).
Keywords: *cedar, fir, pine, spruce, yew*

Introduction

The increasing content of air pollutants, such as CO_2, SO_2, nitrogen oxides, soot, dust, etc. is always present in urban habitats and produces an adverse impact on growth and health of the living world. Since plants absorb chemicals and deposit solid particles, green areas, such as parks, are essential for the quality of life in cities. In addition, plants suffer changes which lead to their slow development, to diminished resistance to disease and pest attacks and, ultimately, to their death. These changes could be visible

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

(macro-changes) or less visible (micro-changes). Both of them are reliable indicators of the level of vulnerability of urban habitats.

Many scienticists tried to imitate polluted habitats and trees response to them. Fumigation of *Picea omorika'*s pollen with SO_2 resulted in reduced seed production (Krug, 1990). Fumigation of the needles of seedlings of Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) with SO_2, O_3 and combination of these two gases greatly reduced soluble carbohydrates in the needles taken from *P. sylvestris*, but to a lesser content in case of needles taken from *P. abies* (Peace et al., 1995). Furthermore, when field with needles of 3-year-old seedlings of Scots pine was fumigated with relatively low concentration of SO_2, their needles emitted H_2S. This emission depended of light and SO_2 concentration (Hällgren and Fredrikson, 1982). On the other hand, some investigations showed that conifer species (Austrian pine, cypress and larch) influenced the lowest level of NO_2, O_3 and NH_3 in the air (Donovan et al., 2005). Conifers also delay ultra-fine dust particles more effectively than broadleaved trees (Freer-Smith et al., 2005). Furthermore, it should be noted that only evergreen and conifer species delay leaves and transpire throughout the whole year. Their transpiration is important especially in winter, when the level of air pollutants is extremely high, or following heavy air pollution (in the period of defoliation of deciduous trees). There are four main ways that urban trees affect air quality: (1) temperature reduction and other microclimatic effects, (2) removal of air pollutants, (3) emission of volatile organic compounds and tree maintenance emissions, and (4) energy effects on buildings (Nowak, 2002).

The purpose of the study is to evaluate, for the first time, the degree of vulnerability of twelve conifer species from five parks to air pollution by means of investigation of both morpho-anatomic leaf properties and morpho-physiological pollen traits. Furthermore, the influence of distance of trees from traffic was also taken into account. Increased content of CO_2 leads to a decrease of stomatal density (Lin et al., 2001) and number of stomata (Woodward and Bazzaz, 1988). In *Pinus crassifolia* (Qiang et al., 2003) their positive correlation (r = 0.63) were found. In one-year old needles of *P. douglasii*, Apple et al. (2000) reported that CO_2 and temperature did not influence stomatal density. They stated that effects could be more visible later (after three years) and genetic influence is very strong, so stomatal density depends on needle elongation.

Materials and methods

Plant materials, selected parks and trees

Five Belgrade parks in Serbia (Europe), exposed to different levels of air pollution, were selected in 2015 (*Fig. 1*). Listed parks were ranked from non-polluted to very heavily-polluted Popović et al. (2016).

For needle and pollen studies twelve and nine tree species were used (*Tables 1* and *2,* respectively).

Processing morpho-anatomic properties of needles

Well-developed one-year old leaves (needles) of twelve conifers were collected for laboratory testing: morpho-anatomic analyses of needles, where length, width, shape, dry mass and stomatal density were measured. For the purpose of morpho-anatomic

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DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

analyses, needles all around a tree crown were collected (ca. 20 needles). Stomata rows from abaxial needle side were obtained by the 'Kolodium method' (Wolf, 1950).

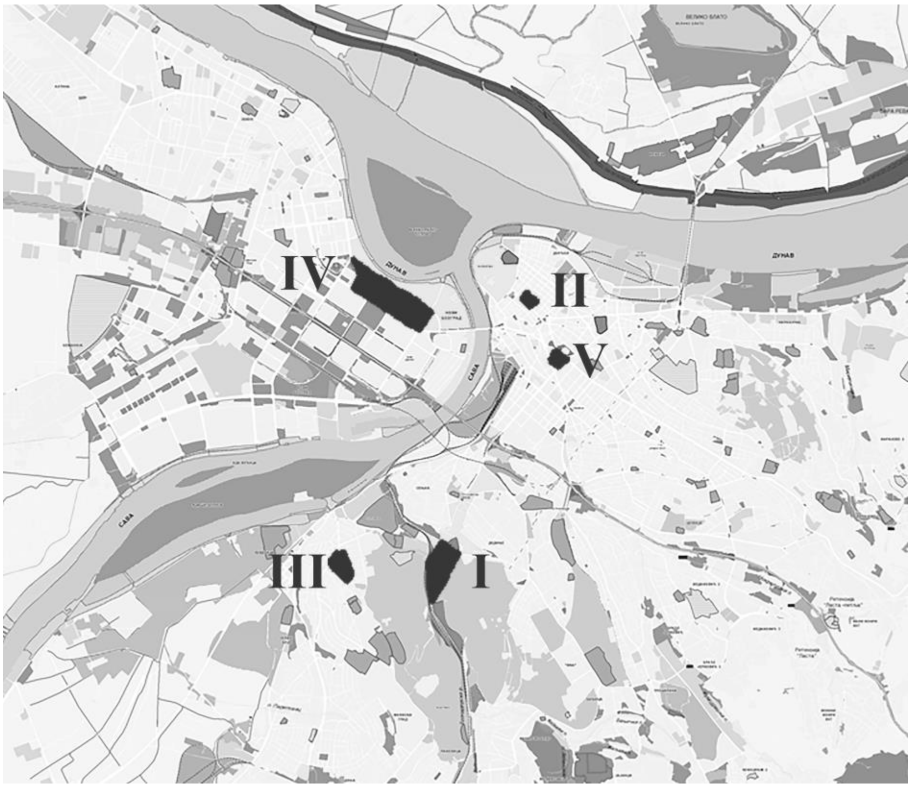

Figure 1. *Shematic view of analysed Belgrade parks performed from non-polluted to heavily-polluted parks: I – Topčider; II – Academic; III – Banovo Brdo; IV – palace 'Serbia' and V-Pioneer's park*

Table 1. *Number of trees per species sampled in five parks for needle studies*

No.	Tree species	Park No.				
		I	II	III	IV	V
1.	*Abies alba* Mill.			1		2
2.	*Abies concolor* (Gordon) Lind. ex Hildebr.			3		2
3.	*Abies nordmanniana* (Steven) Spach, 1841	3				
4.	*Abies pinsapo* Boiss.				1	
5.	*Cedrus atlantica* (Endl.) Mann. ex Carrière		3	3	4	4
6.	*Cedrus deodara* (Roxb.) G. Don)		1			
7.	*Picea abies* (L.) Karst.			1		2
8.	*Picea omorika* (Panč.) Pürkyné	1		2	2	
9.	*Picea pungens* Engelm.				2	2
10.	*Pinus nigra* J. F. Arnold	3		3	2	2
11.	*Pseudotsuga menziesii* (Mirb.) Franco			3	4	2
12.	*Taxus baccata* L.				3	2
	Sum	**7**	**4**	**16**	**18**	**18**
	Sum					**63**

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DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

Table 2. *Number of trees per species sampled in five parks for pollen studies*

No.	Tree species	Park No.				
		I	**II**	**III**	**IV**	**V**
1.	*Abies alba* Mill.			1		
2.	*Abies nordmanniana* (Steven) Spach, 1841	2				
3.	*Abies pinsapo* Boiss.				3	
4.	*Cedrus atlantica* (Endl.) Mann. ex Carrière		1	3	3	2
5.	*Picea abies* (L.) Karst.			1		
6.	*Picea omorika* (Panč.) Pürkyné			2	3	
7.	*Pinus nigra* J. F. Arnold			3	3	3
8.	*Pseudotsuga menziesii* (Mirb.) Franco			3		1
9.	*Taxus baccata* L.			2	3	2
	Sum	**2**	**1**	**15**	**15**	**8**
	Sum					**41**

Processing morpho-physiological properties of pollen grains

Pollen was extracted from ripe strobili from nine trees in parks or extracted in laboratory after immersing twigs with half-ripe strobili in water. Observations of needle stomatal density and morpho-physiological analysis of pollen were performed using a light microscope (*Leica Galen III*) and included measurements of length and width of pollen grains, as well as their germinability and length of pollen tubes at 10% of sucrose. Measurements were carried out on 50 pollen grains per tree. The needle and pollen shape coefficient were calculated by the formula: 100*width/length.

Statistical analyses

Mean values and differences between parks (at 95% level), as well as analysis of variance were calculated in *Statgraphics Centurion* XVI, Version 16.1.11. Coefficient of correlation (r) by linear regression analysis for all measured properties was performed.

Results

Morpho-anatomic properties of needles

Ranking of parks in *Tables 3* and *4* were performed from heavily-polluted (V, IV) to moderate polluted (III, II) and non-polluted areas (I). The needles of *A. alba*, *A. concolor*, *P. abies* and *P. pungens* were significantly shorter in the park V (*Table 3*). Consequently, trees with shorter needles had greater stomatal density, which is particularly prominent in *A. alba, A. nordmanniana*, *P. abies*, *P. omorika*, *P. nigra* and *T. baccata* (*Table 3; Fig. 2*). Needles of *P. omorika* and *P. nigra* were significantly larger in the park IV (*Table 3; Figs. 3* and *4*). The needles of *Taxus baccata*, predominantly collected in the proximity of traffic crossroads in the park IV were shorter than in other parks (*Fig. 4*). Interestingly, the needles of *Pseudotsuga menziesii* were larger in the park V than in the park III (*Table 3*). Larger needles very often had a larger leaf-area, perimeter and dry mass. As presented in *Table 3,* especially with respect to morphology of needles, the parks are ranked as follows (from the best to the

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DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

worst results): IV, II V, III and I. This ranking does not correlate to the ranking in terms of park pollution (I-V, from the best to the worst results). As a consequence, we could not conclude that morphological parameters of conifers are reliable indicators of air pollution. However, some species, such as *P. omorika, P. pungens, P. nigra and T. baccata* had larger needles when situated deep inside the park (far from the heavy traffic). Furthermore, *A. alba* and *P. abies* also produced better results in parks with moderately polluted air than in heavily-polluted parks. With respect to stomatal density, the greatest differences between parks were observed in case of *A. alba*, *A. concolor*, *C. atlantica* and *T. baccata*. Many species had the greatest stomatal density in the most polluted parks (V and IV), but we could not generally conclude that stomatal density of conifers is a dependable indicator of air pollution.

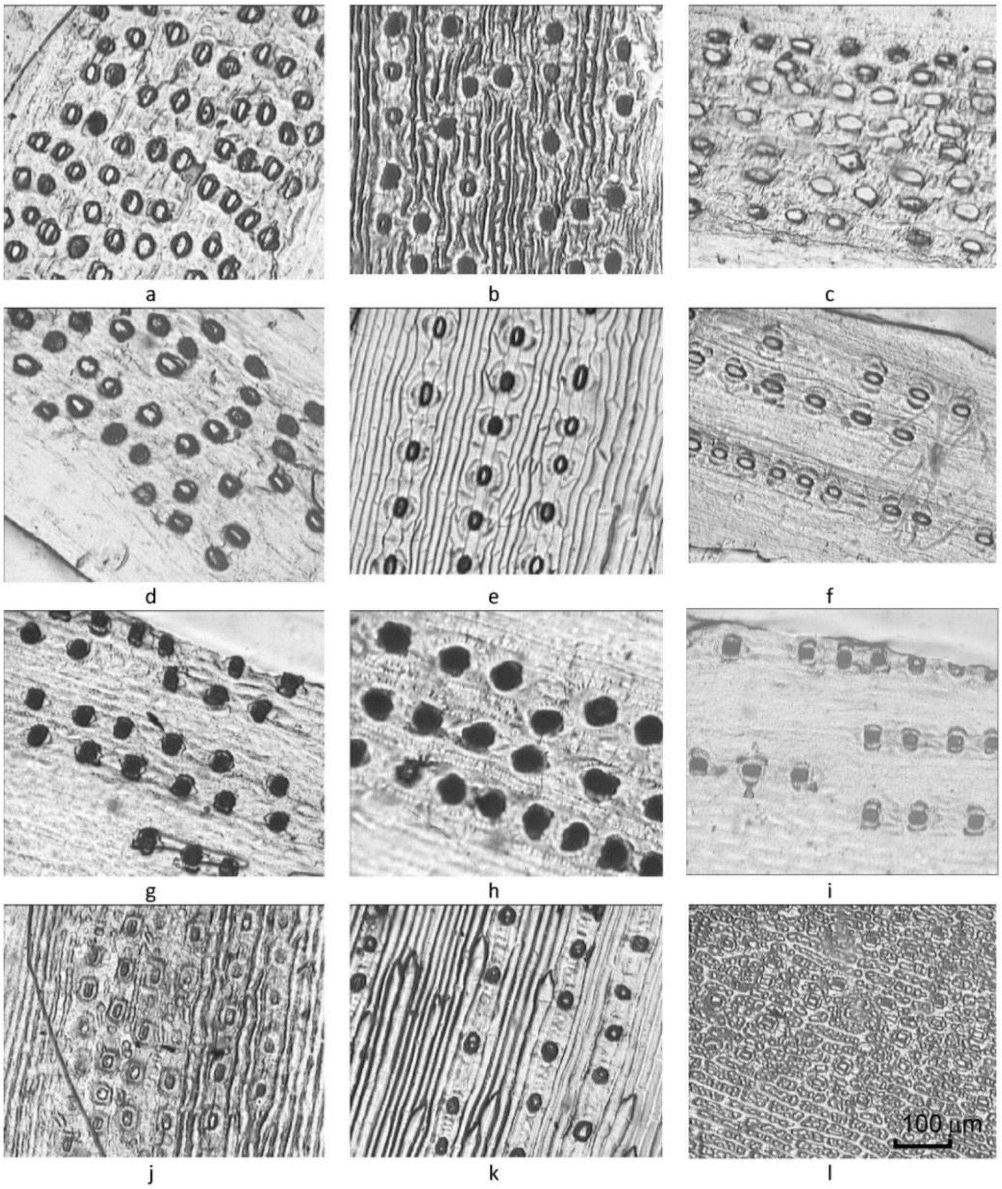

Figure 2. *Rows of stomata on the abaxial needle side (objective 10x): Abies alba - V (a), A. concolor - III (b), A. nordmanniana - I (c), A. pinsapo - IV (d), Cedrus atlantica - III (e), C. deodara - II (f), Picea abies - V (g), P. omorika – IV (h), P. pungens – IV (i), Pseudotsuga douglasii – III (j), Pinus nigra - III (k), Taxus baccata - V (l)*

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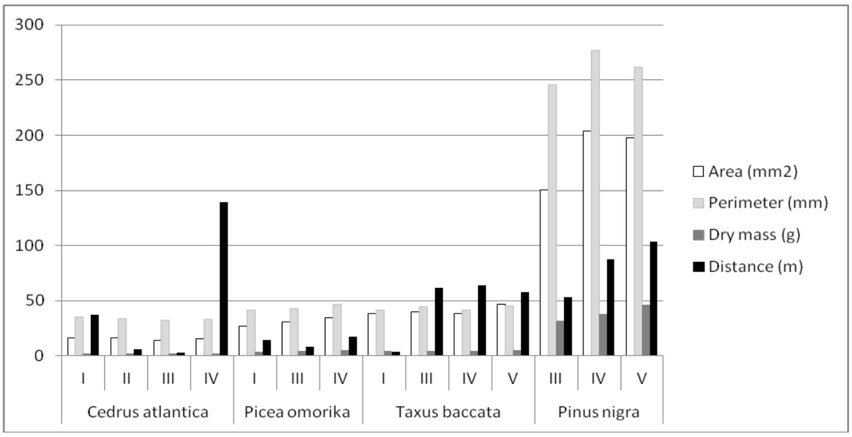

Figure 3. *Graphical illustration of average values of needle area, perimeter, dry mass and distance from traffic in Cedrus atlantica, Picea omorika, Taxus baccata, and Pinus nigra*

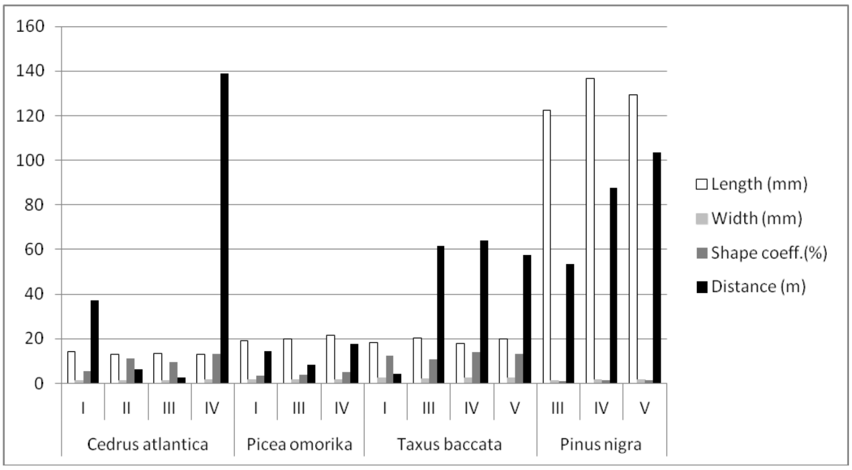

Figure 4. *Graphical illustration of needle length, width, shape and distance from traffic in Cedrus atlantica, Picea omorika, Taxus baccata, and Pinus nigra*

Table 3. *Results of the morphological analyses of conifer leaves (needles), dry mass, stomatal density and distance of trees from traffic*

Park No.	Tree No.	Needle morphology					Dry mass (g)	Stomatal density (number/ mm^2)	Distance of trees from traffic (m)
		Area (mm^2)	Perimeter (mm)	Dimensions					
				Length (mm)	Width (mm)	Shape coefficient (%)			
	Abies alba								
V	197	31.66	39.82	17.99	1.92	11.00	3.80	134.38	21.7
	199	33.34	39.35	17.56	2.11	12.52	4.20	132.82	21.6
	Mean ± SD	*32.50 ± 1.19*	*39.58 ± 0.33*	*17.78 ± 0.30*	*2.02* ± 0.13*	*11.76* ± 1.07*	*4.00 ± 0.28*	*133.60 ± 1.10*	*21.7 ± 0.07*
III	96	41.51	50.42	23.13	2.08	9.41	4.55	93.23	100.3
	Mean ± SD	*41.51 ± 0.00*	*50.42 ± 0.00*	*23.13 ± 0.00*	*2.08 ± 0.00*	*9.41 ± 0.00*	*4.55 ± 0.00*	*93.23 ± 0.00*	*100.3 ± 0.00*
Mean ± SD		**35.50* ± 5.27**	**43.20* ± 6.26**	**19.56* ± 3.10**	**2.04 ± 0.10**	**10.98 ± 1.55**	**4.18 ± 0.38**	**120.14* ± 23.32**	**65.0* ± 45.41**

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DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

Abies concolor									
V	220	77.18	84.39	39.90	2.30	6.08	14.30	68.91	24.4
	221	82.11	96.35	46.14	2.03	4.68	12.85	94.61	20.8
	Mean ± SD	*79.65 ± 3.49*	*90.37 ± 8.46*	43.02 ± 4.41	*2.17* ± 0.19*	*5.38 ± 0.99*	*13.58 ± 1.02*	*81.76 ± 18.17*	*22.6 ± 2.54*
III	248	106.70	98.07	46.20	2.83	6.19	21.33	84.58	19.6
	282	65.69	76.59	36.18	2.11	6.06	8.80	88.23	26.2
	283	89.22	95.01	45.26	2.25	5.28	13.70	93.51	22.8
	Mean ± SD	*87.40* ± 20.58*	*90.40* ± 11.62*	*42.82* ± 5.53*	*2.39* ± 0.38*	*5.78 ± 0.49*	*14.61 ± 6.31*	*88.77 ± 4.49*	*22.8 ± 3.30*
Mean ± SD		**83.96* ± 15.23**	**90.39 ± 9.24**	**42.91 ± 4.50**	**2.29* ± 0.31**	**5.61 ± 0.66**	**14.20 ± 4.53**	**85.97 ± 10.36**	**22.8 ± 2.66**
Abies nordmanniana									
I	149	31.96	40.50	18.41	1.84	10.32	3.87	116.25	74.1
	150	28.52	38.34	17.33	1.84	10.66	3.95	184.22	70.3
	153	22.98	31.31	13.83	1.82	13.23	2.40	127.97	59.7
	Mean ± SD	*28.44* ± 4.53*	*37.50* ± 4.80*	*16.91* ± 2.39*	*1.84 ± 0.01*	*11.12* ± 1.59*	*3.41 ± 0.87*	*142.81 ± 36.33*	*68.0 ± 7.46*
Mean ± SD		**28.44 ± 4.53**	**37.50 ± 4.80**	**16.91 ± 2.39**	**1.84 ± 0.01**	**11.12 ± 1.59**	**3.41 ± 0.87**	**142.81 ± 36.33**	**68.0 ± 7.46**
Abies pinsapo									
IV	8	40.07	47.14	21.34	2.23	10.86	5.60	133.99	25.6
	Mean ± SD	*40.07 ± 0.00*	*47.14 ± 0.00*	*21.34 ± 0.00*	*2.23 ± 0.00*	*10.86 ± 0.00*	*5.60 ± 0.00*	*133.99 ± 0.00*	*25.6 ± 0.00*
Mean ± SD		**40.07 ± 0.00**	**47.14 ± 0.00**	**21.34 ± 0.00**	**2.23 ± 0.00**	**10.86 ± 0.00**	**5.60 ± 0.00**	**133.99 ± 0.00**	**25.6 ± 0.00**
Cedrus atlantica									
V	26	13.96	28.70	11.07	1.29	11.88	1.80	-	77.7
	80	19.54	45.76	19.15	1.11	*5.77*	1.80	78.20	44.3
	176	16.62	37.56	15.51	1.13	*9.28*	1.95	83.13	7.7
	181	12.25	23.85	8.73	1.50	17.32	1.40	81.67	7.0
	Mean ± SD	*16.03* ± 3.19*	*35.50* ± 9.70*	*14.42* ± 4.64*	*1.20 ± 0.18*	*11.06* ± 4.86*	*1.74 ± 0.24*	*81.00 ± 2.53*	*34.2 ± 33.84*
IV	59	18.66	43.59	17.67	1.16	7.03	2.13	-	148.2
	68	13.35	25.77	9.72	1.61	19.22	2.07	-	161.9
	83	15.60	29.37	11.48	1.57	15.77	2.25	78.75	133.1
	445	15.43	33.76	13.11	1.31	10.50	1.95	56.15	113.2
	Mean ± SD	*15.73* ± 2.19*	*32.90* ± 7.70*	*12.90* ± 3.41*	*1.42* ± 0.21*	*13.13* ± 5.42*	*2.10 ± 0.12*	*67.45 ± 15.98*	*139.1 ± 20.89*
III	322	18.74	39.52	15.52	1.33	9.05	2.25	56.33	10.8
	329	10.53	27.09	10.61	1.17	11.13	1.00	77.11	32.2
	335	13.71	31.38	12.72	1.13	9.54	1.65	73.28	37.9
	Mean ± SD	*15.08* ± 4.14*	*33.78* ± 6.31*	*13.42* ± 2.46*	*1.22* ± 0.11*	*9.67 ± 1.09*	*1.63 ± 0.62*	*68.91 ± 11.06*	*29.7 ± 14.29*
II	45	18.47	40.16	13.27	1.34	8.88	2.05	51.77	113.6
	47	17.21	32.41	12.47	1.59	13.52	2.45	-	104.2
	57	12.37	28.23	11.41	1.18	10.89	1.30	65.63	5.6
	Mean ± SD	*16.02* ± 3.22*	*33.60* ± 6.05*	*13.05* ± 0.93*	*1.37* ± 0.21*	*11.10* ± 2.33*	*1.93 ± 0.58*	*58.70 ± 9.80*	*73.3 ± 59.82*
Mean ± SD		**15.71 ± 2.84**	**33.72 ± 6.89**	**13.31 ± 2.99**	**1.32* ± 0.18**	**11.30 ± 3.84**	**1.86 ± 0.40**	**70.20 ± 11.75**	**69.0 ± 56.93**

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Cedrus deodara									
II	91	19.46	39.71	15.82	1.34	8.90	2.67	128.13	18.6
	Mean ± SD	*19.46 ± 0.00*	*39.71 ± 0.00*	*15.82 ± 0.00*	*1.34 ± 0.00*	*8.90 ± 0.00*	*2.67 ± 0.00*	*128.13 ± 0.00*	*18.6 ± 0.00*
Mean ± SD		**19.46 ± 0.00**	**39.71 ± 0.00**	**15.82 ± 0.00**	**1.34 ± 0.00**	**8.90 ± 0.00**	**2.67 ± 0.00**	**128.13 ± 0.00**	**18.6 ± 0.00**
Picea abies									
V	15	34.26	44.45	20.41	1.82	9.02	2.75	74.38	36.8
	18	21.80	37.92	17.67	1.29	7.60	2.45	85.86	44.0
	Mean ± SD	*28.03* ± 8.81*	*41.18* ± 4.62*	*19.04* ± 1.94*	*1.55* ± 0.37*	*8.31* ± 1.00*	*2.60 ± 0.21*	*80.12 ± 4.52*	*40.4 ± 5.09*
III	382	28.00	45.30	17.88	1.60	8.99	3.45	-	62.1
	Mean ± SD	*28.00 ± 0.00*	*45.30 ± 0.00*	*17.88 ± 0.00*	*1.60 ± 0.00*	*8.99 ± 0.00*	*3.45 ± 0.00*	-	*62.1 ± 0.00*
Mean ± SD		**28.02* ± 6.23**	**42.56 ± 4.04**	**18.65* ± 1.52**	**1.57* ± 0.27**	**8.54 ± 0.81**	**2.88 ± 0.51**	**80.12 ± 4.52**	**51.3 ± 13.03**
Picea omorika									
IV	362	34.74	49.02	22.81	1.70	7.54	4.61	103.54	115.9
	495	34.26	44.45	20.41	1.82	9.02	5.20	105.00	51.2
	Mean ± SD	*34.50 ± 0.34*	*46.73* ± 3.23*	*21.61* ± 1.70*	*1.76* ± 0.08*	*8.28* ± 1.05*	*4.91 ± 0.42*	*104.27 ± 1.03*	*83.5 ± 45.11*
III	1	33.30	47.44	22.14	1.58	7.27	4.15	123.59	9.3
	4	27.59	38.26	17.46	1.67	9.82	3.50	108.28	19.7
	Mean ± SD	*30.45* ± 4.04*	*42.85* ± 6.49*	*19.80* ± 3.31*	*1.63 ± 0.06*	*8.55* ± 1.80*	*3.83 ± 0.46*	*115.94 ± 10.82*	*14.5 ± 7.35*
I	338	26.82	41.38	19.13	1.56	8.35	3.60	-	17.8
	Mean ± SD	*26.82 ± 0.00*	*41.38 ± 0.00*	*19.13 ± 0.00*	*1.56 ± 0.00*	*8.35 ± 0.00*	*3.60 ± 0.00*	-	*17.8 ± 0.00*
Mean ± SD		**31.34* ±3.82**	**44.11* ±4.39**	**20.39* ±2.18**	**1.67* ± 0.10**	**8.40 ± 1.05**	**4.21 ± 0.71**	**110.10 ± 9.21**	**38.6 ± 43.48**
Picea pungens									
V	23	37.44	52.75	24.60	1.77	7.47	5.80	75.47	65.9
	25	29.44	45.27	21.12	1.52	7.31	5.60	80.39	75.0
	Mean ± SD	*33.44* ± 5.66*	*49.01* ± 5.29*	*22.86* ± 2.46*	*1.65* ± 0.18*	*7.39 ± 0.11*	*5.70 ± 0.14*	*77.93 ± 3.48*	*70.5 ± 6.43*
IV	272	35.63	53.76	25.36	1.52	6.10	6.55	107.19	148.6
	273	50.09	64.51	30.42	1.83	6.08	10.70	82.03	149.5
	Mean ± SD	*42.86* ± 10.22*	*59.13* ± 7.60*	*27.89* ± 3.58*	*1.68* ± 0.22*	*6.09 ± 0.01*	*8.63 ± 2.93*	*94.61 ± 17.79*	*149.0 ± 0.64*
Mean ± SD		**38.15* ± 8.66**	**54.07* ± 7.92**	**25.37* ± 3.84**	**1.66 ± 0.16**	**6.74* ± 0.75**	**7.16 ± 2.39**	**86.27 ± 14.22**	**105.7 ± 45.53**
Pinus nigra									
V	44	211.75	276.13	136.39	1.68	1.22	49.80	77.93	96.8
	46	184.11	247.54	122.19	1.59	1.30	42.95	83.79	110.5
	Mean ± SD	*197.93* ± 19.54*	*261.84* ± 20.22*	*129.29* ± 10.04*	*1.63 ± 0.06*	*1.26 ± 0.06*	*46.38 ± 4.84*	*80.86 ± 4.14*	*102.6 ± 9.69*
IV	496	190.58	233.51	114.96	1.80	1.59	37.80	84.96	80.7
	504	192.97	267.13	131.96	1.61	1.22	36.30	-	88.5
	506	208.87	294.01	145.25	1.75	1.21	34.00	-	88.8
	507	234.46	323.62	160.18	1.63	1.02	41.60	-	92.1
	Mean ± SD	*203.97* ± 20.20*	*277.08* ±38.41*	*136.86* ± 19.25*	*1.68 ± 0.09*	*1.25* ± 0.24*	*37.43 ± 3.19*	*84.96 ± 0.00*	*87.5 ± 4.83*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

III	9	137.45	242.86	120.45	0.98	0.81	28.65	83.21	50.5
	10	127.12	211.56	105.72	1.08	1.02	28.70	80.86	53.6
	11	186.42	283.54	140.59	1.18	0.84	37.95	110.16	56.5
	Mean ± SD	*150.33* ± 31.68*	*245.98* ± 36.09*	*122.25* ± 17.50*	*1.08* ± 0.10*	*0.89* ± 0.11*	*31.77 ± 5.35*	*91.41 ± 16.28*	*53.5 ± 3.00*
Mean ± SD		**176.29* ± 34.34**	**257.28* ± 34.26**	**127.43* ± 16.81**	**1.38 ± 0.31**	**1.08* ± 0.24**	**37.53 ± 6.78**	**86.82 ± 11.70**	**81.2 ± 21.30**
				Pseudotsuga menziesii					
V	205	51.21	72.08	34.42	1.62	4.86	7.60	-	53.6
	224	41.62	60.77	28.83	1.56	5.62	4.35	147.11	26.0
	Mean ± SD	*45.73* ± 6.78*	*65.61* ± 8.00*	*31.22* ± 3.95*	*1.58 ± 0.04*	*5.24 ± 0.54*	*5.98 ± 2.30*	*147.11 ± 0.00*	*39.8 ± 19.52*
III	46	39.30	54.91	25.87	1.58	6.32	6.00	127.55	78.2
	383	48.46	64.30	30.54	1.61	5.40	5.95	142.19	71.6
	384	30.14	44.79	20.71	1.68	8.50	3.87	97.89	62.6
	Mean ± SD	*40.22* ± 9.16*	*55.63* ± 9.76*	*26.19* ± 4.92*	*1.62 ± 0.05*	*6.74* ± 1.59*	*5.27 ± 1.21*	*122.68 ± 22.57*	*70.8 ± 7.83*
Mean ± SD		**42.48* ± 8.28**	**59.74* ± 10.25**	**28.26* ± 5.15**	**1.61 ± 0.04**	**6.06* ± 1.42**	**5.55 ± 1.48**	**128.79 ± 60.66**	**55.3 ± 20.35**
				Taxus baccata					
V	58	39.29	42.80	19.00	2.40	12.85	4.45	98.44	65.8
	231	53.64	47.98	21.21	2.78	13.28	5.40	100.08	49.4
	Mean ± SD	*46.47* ± 10.15*	*45.39* ± 3.66*	*20.11* ± 1.56*	*2.59* ± 0.27*	*13.06 ± 0.30*	*4.93 ± 0.67*	*99.26 ± 1.16*	*57.6 ± 11.60*
IV	358	43.21	42.74	18.84	2.53	13.52	5.90	150.21	100.8
	791	34.36	37.63	16.56	2.26	14.17	3.45	143.83	27.1
	Mean ± SD	*38.79* ± 6.19*	*40.18* ± 3.61*	*17.70* ± 1.61*	*2.39* ± 0.19*	*13.84 ± 0.46*	*4.68 ± 1.73*	*147.02 ± 4.51*	*63.9 ± 52.11*
III	379	41.73	49.65	22.84	1.99	8.80	4.30	130.70	59.6
	380	40.85	41.26	18.35	2.28	12.80	3.65	132.71	64.2
	381	38.06	43.32	19.54	2.12	10.97	3.80	-	61.8
	Mean ± SD	*40.21 ± 1.92*	*44.74* ± 4.37*	*20.24* ± 2.33*	*2.13* ± 0.14*	*10.86* ± 2.00*	*3.92 ± 0.34*	*131.71 ± 1.42*	*61.8 ± 2.30*
I	241	38.23	41.82	18.69	2.21	12.14	3.95	137.26	26.7
	265	47.60	46.25	20.68	2.44	12.00	5.30	124.69	28.0
	340	29.64	35.55	15.76	2.02	12.98	2.75	121.95	61.5
	Mean ± SD	*38.49* ± 8.98*	*41.21* ± 5.38*	*18.38* ± 2.47*	*2.22* ± 0.21*	*12.37 ± 0.53*	*4.00 ± 1.27*	*127.97 ± 8.16*	*38.7 ± 19.73*
Mean ± SD		**40.66* ± 6.66**	**42.90* ± 4.33**	**19.15* ± 2.09**	**2.30* ± 0.24**	**12.35* ± 1.53**	**4.30 ± 0.98**	**126.65 ± 17.83**	**54.6 ± 22.96**

*Significant differences ($p < 0.05$)

Morpho-physiological properties of pollen grains

Morpho-physiological pollen properties are presented in *Table 4* and *Figures 5* and *6*. The impact of air pollution was calculated and graphically presented in four tree species: *Cedrus atlantica*, *Picea omorika*, *Taxus baccata* and *Pinus nigra* (*Fig. 6*). These four species were selected because they belong from different genera and simultaneously were present in two or more parks. The best results were obtained in the park IV (in case of *P. omorika*, *T. baccata* and *P. nigra*). The largest amount of pollen of *C. atlantica* was found in the park II, but its vitality (pollen tube length) was the

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highest in the park III. The parks were ranked as follows (from the best to the worst results with respect to morpho-physiological pollen properties): IV, II, III, I (only for *T. baccata*) and V. This ranking of parks is not similar to the rankings obtained by the analysis of morphometric values of tree needles (statistic results are not presented). However, we could generally conclude that the analysed conifers have the smallest pollen grains and the lowest pollen vitality in the park with the heaviest air pollution (V) (*Fig. 5*). The pollens of *T. baccata* and *C. atlantica* were most sensitive to air pollution (*Table 4; Fig. 5*). Furthermore, *P. omorika* and *P. nigra* showed better results when situated far from the traffic zones. Small, abnormal and non-vital pollen grains lead to lower germinability and energy of pollen germination (manifested in form of small pollen tubes) (*Fig. 5*). The most drastic examples of changes of pollen grains were observed in the park III, in case of *Cedrus atlantica* and *Picea omorika,* and in the parks IV and V, in case of *P. nigra* (*Table 4; Fig. 6*).

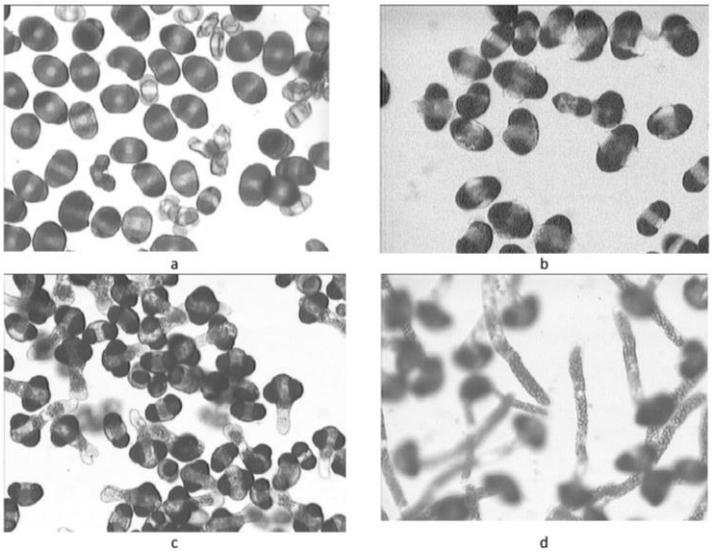

Figure 5. *Small, abnormal and non-vital pollen grains of Cedrus atlantica – V (a) and Picea omorika – V (b). Good germinability of Pinus nigra – IV (c) and C. atlantica – III (d) pollen grains*

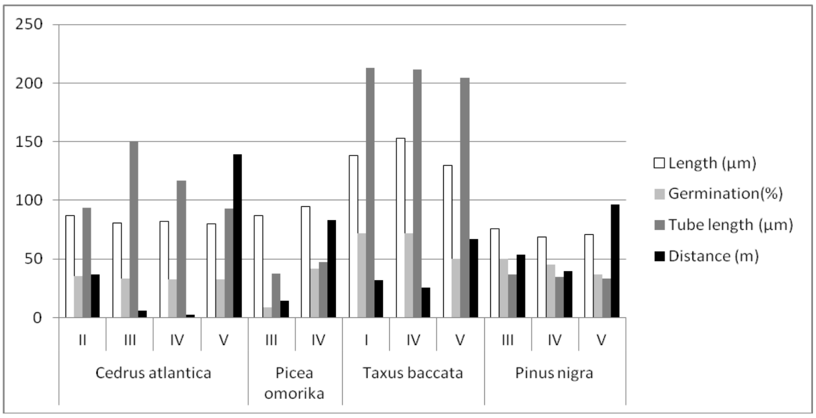

Figure 6. *Graphical illustration of average values of pollen grains length, length of pollen tubes and distance from traffic in Cedrus atlantica, Picea omorika, Taxus baccata, and Pinus nigra*

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In some analyzed species distances of particular trees from heavy traffic also were in significant correlation with needle dimensions (*A. concolor*, *A. nordmanniana and P. omorika*, r = -0.93, 0.99, and 0.56, respectively, *Fig. 7*), stomatal density (*A. alba*, *P. abies*, *P. omorika*, *P. pungens*, r = -0.99, 1.0, -0.70, and 0.71, resp., *Fig. 8*) and pollen vitality (germinability: *C. atlantica*, r = 0.73, *P. nigra*, r = 0.57 and *T. baccata*, r = 0.68, *Fig. 9;* length of pollen tubes: *A. pinsapo*, r = 0.99 and *P. menziesii*, r = 0.95, *Fig. 10*).

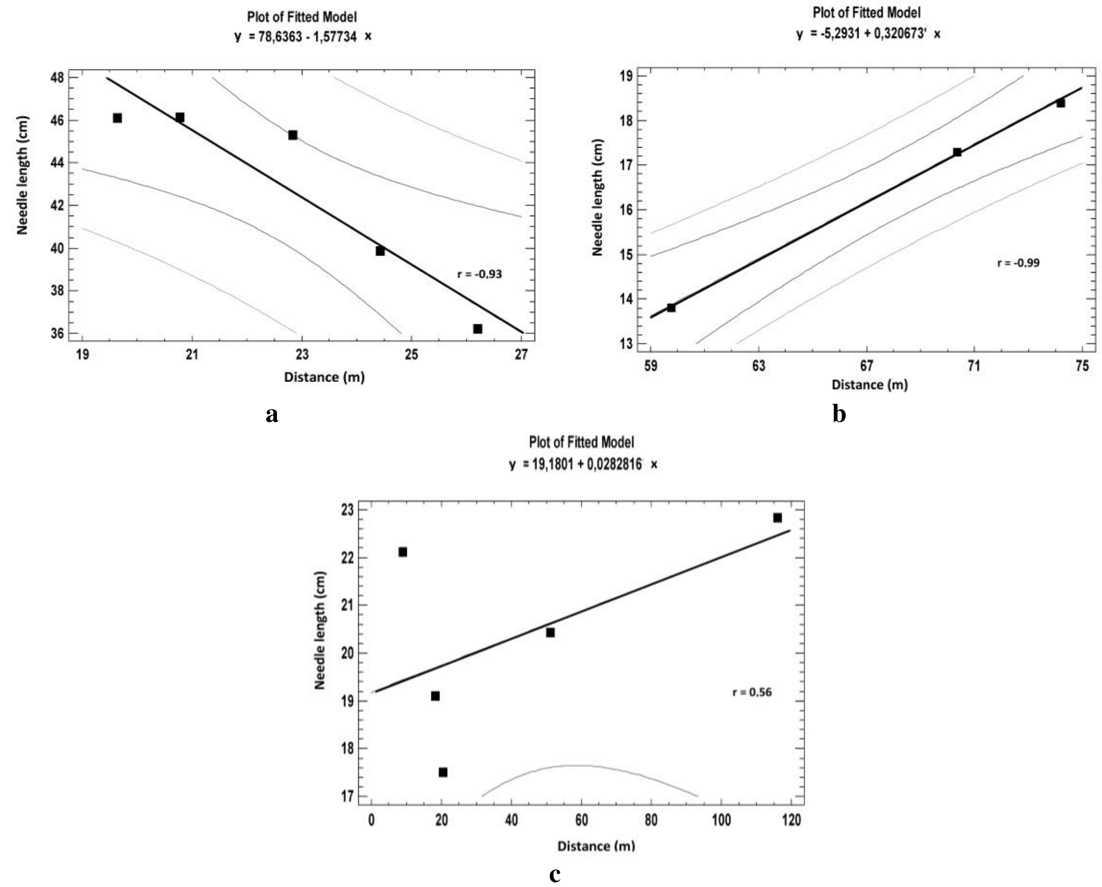
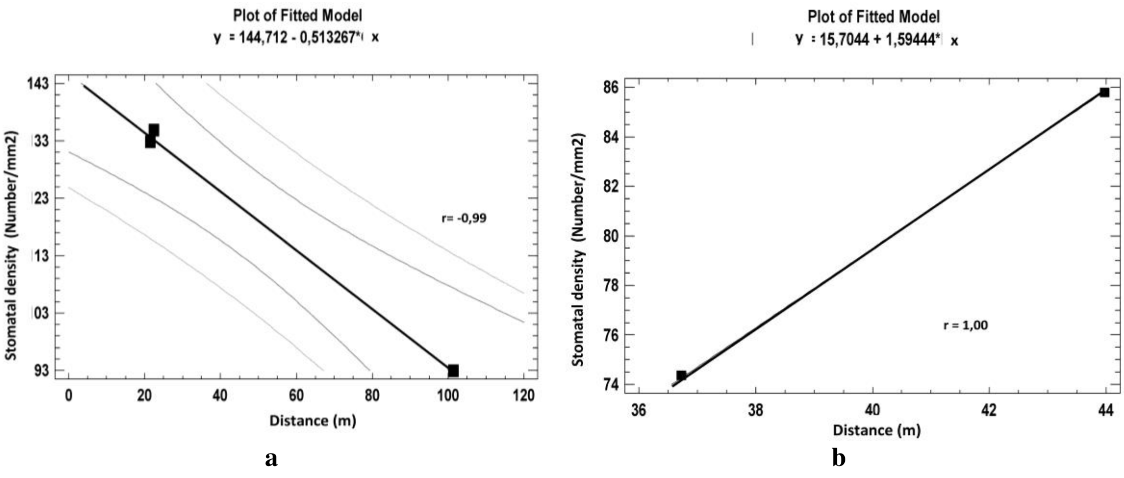


Figure 7. *Correlation between needle length and distance from traffic in Abies concolor (a), A. nordmanniana (b) and Picea omorika (c)*



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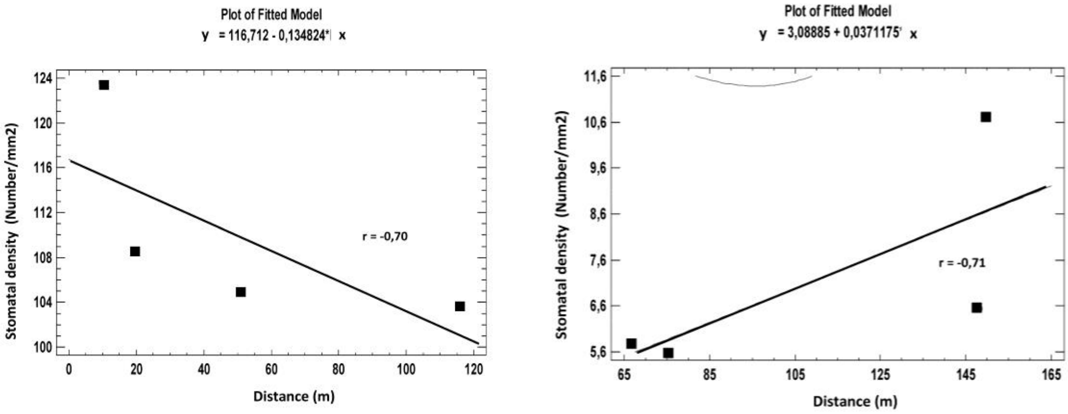
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28312848



c d

Figure 8. *Correlation between stomatal density and distance from traffic in Abies alba (a), Picea abies (b), Picea omorika (c) and P. pungens (d)*

Table 4. *Results of morpho-physiological analysis of pollen grains and distance of trees from traffic*

Park No.	Tree No.	Pollen analyses					Distance of trees from traffic (m)
		Morphological			Physiological		
		Dimensions		Shape (%)	Vitality		
		Length (µm)	Width (µm)		Germination (%)	Length of pollen tubes (µm)	
Abies alba							
III	96	149.47	103.31	69.45	48.45	39.77	100.3
Mean ± SD		**149.47 ± 0.00**	**103.31 ± 0.00**	**69.45 ± 0.00**	**48.45 ± 0.00**	**39.77 ± 0.00**	**100.3 ± 0.00**
Abies nordmanniana							
I	149	123.76	88.66	72.17	62.91	44.59	74.1
	150	139.73	96.07	69.01	51.27	52.01	70.3
Mean ± SD		**131.75* ± 11.29**	**92.36* ± 5.24**	**70.59* ± 2.23**	**57.09 ± 8.23**	**47.74* ± 5.25**	**72.2 ± 2.69**
Abies pinsapo							
IV	8	130.16	95.40	73.63	47.00	27.15	25.6
	373	142.39	95.86	68.17	46.81	51.03	96.8
	375	138.80	94.38	67.39	60.26	50.57	92.2
Mean ± SD		**137.12* ± 6.29**	**95.21 ± 0.76**	**69.73* ± 3.40**	**51.36 ± 7.71**	**42.92* ± 13.66**	**74.8 ± 39.85**
Cedrus atlantica							
V	26	79.00	54.13	68.60	18.36	71.38	86.8
	80	81.27	60.50	74.70	24.13	108.85	44.3
	Mean ± SD	*80.14 ± 1.60*	*57.32* ± 4.50*	*71.65*± 4.31*	*21.25* ± 4.08*	*92.79* ± 26.50*	*67.0 ± 30.05*
IV	59	83.03	52.73	63.66	31.40	110.39	148.2
	68	80.64	54.99	68.43	32.99	111.46	161.9
	83	82.27	50.86	61.90	52.53	128.37	133.1
	Mean ± SD	*81.98 ± 1.22*	*52.86* ± 2.07*	*64.66* ± 3.38*	*38.97* ± 12.03*	*116.94* ± 10.09*	*147.7 ± 14.41*

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III	322	79.02	51.11	64.78	3.70	101.90	10.8
	329	84.26	56.70	67.46	33.58	157.80	32.2
	335	78.04	53.17	74.20	12.86	174.34	34.9
	Mean ± SD	*80.44* ± 3.34*	*53.66* ± 2.83*	*68.81* ± 4.85*	*16.72* ± 15.31*	*150.33* ± 37.96*	*26.9 ±13.20*
II	47	86.91	55.69	64.17	3.51	93.79	4.5
	Mean ± SD	*86.91 ± 0.00*	*55.69 ± 0.00*	*64.17 ± 0.00*	*3.51 ± 0.00*	*93.79 ± 0.00*	*4.5 ± 0.00*
Mean ± SD		**81.61* ± 2.85**	**54.43* ± 3.00**	**67.54* ± 4.52**	**23.67* ± 15.85**	**119.82* ± 31.75**	**61.5 ± 61.09**
Picea abies							
III	382	114.54	74.07	64.63	24.59	75.36	62.1
Mean ± SD		**114.54 ± 0.00**	**74.07 ± 0.00**	**64.63 ± 0.00**	**24.59 ± 0.00**	**75.36 ± 0.00**	**62.1 ± 0.00**
Picea omorika							
IV	362	94.91	57.85	61.18	28.19	36.89	115.9
	367	96.99	56.43	58.31	19.54	53.26	82.4
	495	92.50	52.84	57.58	77.30	46.59	51.2
	Mean ± SD	*94.80* ± 2.25*	*55.71* ± 2.58*	*59.02* ± 1.90*	*41.68* ± 31.15*	*47.61* ± 8.23*	*83.1 ± 32.36*
III	1	86.97	53.70	61.94	2.88	27.0	9.3
	4	86.94	56.23	64.94	14.51	37.82	19.7
	Mean ± SD	*86.95 ± 0.02*	*54.96* ± 1.79*	*63.44* ± 2.12*	*8.70* ± 8.22*	*32.41* ± 7.65*	*14.5 ± 7.35*
Mean ± SD		**91.66* ± 4.58**	**55.41 ± 2.07**	**60.79* ± 2.96**	**28.48*± 28.78**	**40.31*± 10.03**	**55.7 ± 44.17**
Pinus nigra							
V	39	73.21	45.04	61.61	83.51	37.28	101.0
	45	72.19	43.96	60.96	78.27	32.81	95.1
	46	68.23	43.34	63.60	36.67	27.65	93.1
	Mean ± SD	*71.21* ± 2.63*	*44.11 ± 0.86*	*62.06* ± 1.37*	*66.15* ± 25.66*	*33.48* ± 4.82*	*96.4 ± 4.11*
IV	2	69.28	46.76	67.64	63.02	41.51	41.6
	4	64.60	40.53	62.98	22.71	27.45	34.9
	6	72.48	44.92	62.22	50.56	33.37	44.0
	Mean ± SD	*68.78* ± 3.96*	*44.07* ± 3.20*	*64.28* ± 2.93*	*45.43* ± 20.64*	*35.11* ± 7.06*	*40.1 ± 4.72*
III	9	79.42	52.60	66.42	52.49	38.46	50.5
	10	69.15	43.83	63.35	42.74	28.81	53.6
	11	78.70	51.00	64.89	54.41	40.65	56.5
	Mean ± SD	*75.76* ± 5.73*	*49.14* ± 4.67*	*64.89* ± 1.53*	*49.88 ± 6.26*	*37.18* ± 6.30*	*52.2 ± 3.00*
Mean ± SD		**71.92* ± 4.82**	**45.78* ± 3.82**	**63.74* ± 2.21**	**53.82* ± 19.24**	**34.92* ± 5.51**	**46.1 ± 25.68**
Pseudotsuga menziessi							
V	205	97.98	85.37	87.71	76.58	140.19	53.6
	Mean ± SD	*97.98 ± 0.00*	*85.37 ± 0.00*	*87.71 ± 0.00*	*76.58 ± 0.00*	*140.19 ± 0.00*	*53.6 ± 0.00*
III	46	126.56	100.32	80.02	67.42	147.70	78.2
	383	132.76	102.78	78.48	54.97	143.64	71.6

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	384	144.74	102.60	71.80	80.16	142.62	62.6
	Mean ± SD	*135.94* ± 9.24*	*102.14 ± 1.37*	*76.27* ± 4.37*	*67.51* ± 12.59*	*144.65 ± 2.69*	*70.8 ± 7.83*
Mean ± SD		**125.40* ± 19.84**	**97.49* ± 8.34**	**79.44* ± 6.53**	**69.78* ± 11.24**	**143.54 ± 3.13**	**62.2 ± 10.72**
Taxus baccata							
V	242	111.26	99.32	89.56	50.50	194.81	66.2
	243	148.42	129.00	86.30	49.88	214.13	67.4
	Mean ± SD	*129.84* ± 26.28*	*113.71* ± 20.99*	*87.93* ± 2.30*	*50.19 ± 0.44*	*204.47 ± 13.66*	*66.8m ± 0.85*
IV	791	150.07	131.50	87.70	75.40	201.62	27.1
	792	179.32	156.71	87.68	69.82	215.82	25.0
	793	129.89	116.56	90.25	71.02	216.94	24.7
	Mean ± SD	*153.10* ± 24.85*	*134.89* ± 20.29*	*88.54 ± 1.48*	*72.08 ± 2.94*	*211.46 ± 8.54*	*25.6 ± 1.31*
I	340	134.72	115.35	86.60	74.68	213.03	61.5
	669	139.98	124.83	89.73	69.76	212.78	3.2
	Mean ± SD	*138.23 ± 3.72*	*121.67 ± 6.70*	*88.69 ± 2.21*	*72.22 ± 3.48*	*212.91 ± 0.18*	*32.3 ± 41.22*
Mean ± SD		**142.72* ± 21.01**	**125.50* ± 17.73**	**88.37 ± 1.58**	**65.87* ± 10.94**	**209.88 ± 8.33**	**38.2 ± 25.41**

Significant differences ($p < 0.05$*)

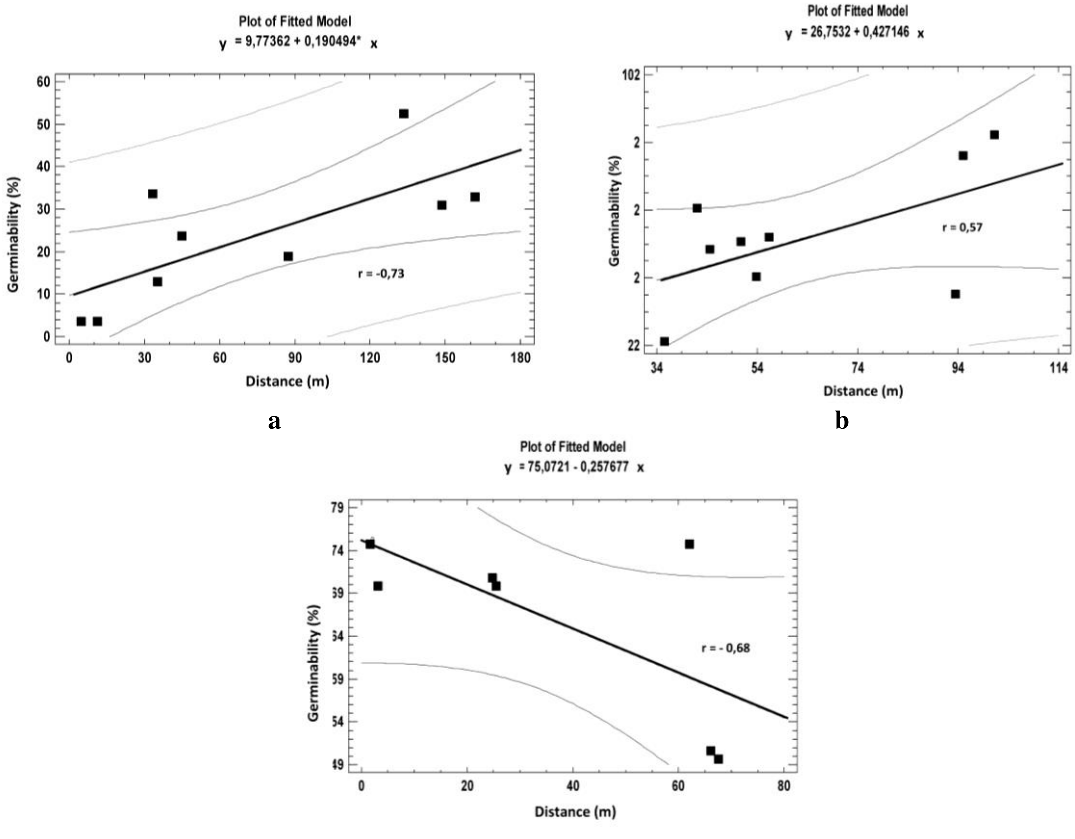

Figure 9. *Correlation between germinability and distance from traffic in Cedrus atlantica (a), Pinus nigra (b), and Taxus baccata (c)*

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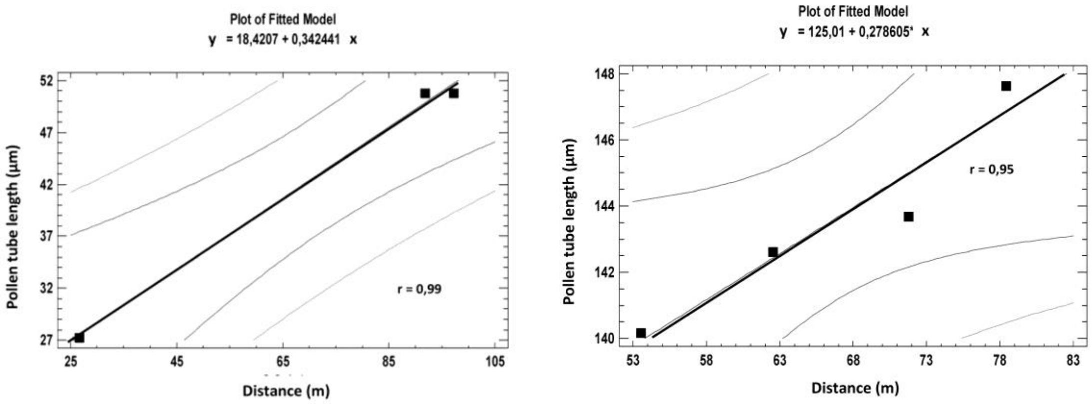

Figure 10. *Correlation between pollen tube length and distance from traffic in Abies pinsapo (a), and Pseudotsuga menziesii (b)*

Discussion

Conifer foliage is a useful indicator in bio-monitoring of air pollution (*Pinus ponderosa*). Several of investigated species Vukićević (1987) considered resistant to urban conditions (*A. concolor*, *A. nordmanianna*, *A. pinsapo*, *C. atlantica* and *P. omorika*). The average needle length and width for all twelve investigated species are in agreement with the published results of Vidaković (1982) and Vukićević (1987). The needle length and width of *A. alba* from park V also agree with previous results obtained by Pawlaczyk et al. (2005) and Pawlaczyk and Bobowicz (2008). The average needle length and width of *P. omorika* are also in agreement with the results obtained in natural habitats (Milovanović et al., 2005; Radovanović et al., 2014; Nikolić et al., 2015). Furthermore, dimensions of *Pinus nigra* from park V are in agreement with the results obtained by Matziris (1984) and Borzan et al. (2002). The values of needle length and leaf area in our *A. pinsapo* from park IV are higher than those already reported by Sękiewicz et al. (2013). The needle length of *Cedrus atlantica* also agrees with the results obtained by Jasińska et al. (2013), while needle width was about 25% higher. Needle width of *Pinus sylvestris* was higher in CO_2 polluted air, as a result of an increase of messophyl tissue (Lin et al., 2001). In some other conifers, air-polluted needles are shorter, with lower number of stomata (*Pinus pinaster*, Wahid et al., 2006). Effects of drought could be found even at the level of needle anatomy, where stomatal density increased as needle length decreased (*Pinus canariensis*, Grill et al., 2004).

Dimensions of trees and their leaves could depend on soil nutrition. Addition of nitrogen increased length, width and number of needles per shoot in *P. menziesii* (Brix and Ebell, 1969).

In *A. alba*, *A. nordmanniana* and *A. pinsapo* stomatal density was higher (*Table 1*) than in literature (Robakowski et al., 2004; Meidner and Mansfield, 1968; Sancho-Knapik et al., 2014, resp). The same situation was found in *P. abies* and *P. pungens* (Dixon et al., 1995 and Meidner and Mansfield, 1968, resp.). In *T. baccata* stomatal density is lower in park V than in other investigated parks, but similar with literature results (Stefanović, 2015).

Our results of pollen length of *P. abies* and *P. omorika* were slightly higher than those reported by Jia et al. (2014), but germination was significantly low, especially of *P. omorika* in park III (*Table 2*). In our previous investigations germination of *P.*

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omorika pollen was 54-68% (max. 94%) (Batos and Nikolić, 2013). In case of *P. menziesii,* the average values of pollen size were similar to those reported in the literature (Ho, 1968). The air pollution changed the properties of leaves and pollen grains through chemical changes. An increased content of lead in the air influenced lower pollen germination and pollen tube growth in *Pinus strobus* and *P. resinosa* (Cox, 1988). In case of *Abies alba,* contaminated pollen decreased vitality and germination up to 50%, which depended on genotype-specific response to air pollution (Kormuťák, 1996 and refs. cited therein). The same situation is found in our results of *A. alba* (*Table 2*), where decreased germinability and length of pollen tubes was also up to 60% and 50 μm, respectively. Furthermore, in some species (*P. omorika, P. nigra*, etc.) we found some abnormalities in dimensions, shape and vitality of pollen grains, which occurred slightly more frequently in more heavily contaminated parks (case of *C. atlantica*, *P. omorika*, etc., *Table 2, Figs. 5* and *6*).

We have to underline that presented results of morpho-anatomical and pollen propertiesof conifers in urban area have never been explored so detailed in the past.

Conclusions

Based on the presented results of morpho-anatomical and morpho-physiological characteristics of conifer needles and pollen, respectively, it can be concluded that the consequences of air pollution are more apparent in case of pollen. In city parks with heavy traffic (V, IV, III), pollen grains of trees were smaller and less vital. Very often results were in correlation with distance of trees from traffic. Some of examined conifer species are especially sensitive to air pollution caused by vehicles (*Picea omorika, Cedrus atlantica, Taxus baccata, Pinus nigra*), especially in pollen properties. The level of air contamination, as well as other atmospheric conditions (temperature, humidity, wind direction and strength, etc.) could vary among different years, too. All the above factors and their influence on growth, development and blooming of trees, should be taken into consideration in landscape architecture and horticulture works in future.

Acknowledgements. This work was supported by Ministry of Education, Science and Technological Development of Serbia (grant numbers 173029, 173011, 31070 and 43007), as well as by City of Belgrade, Secretariat for Environmental Protection (grant number 4011-144).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28312848

IMPACT OF CHANGES IN LAND USE AND CLIMATE ON THE RUNOFF BASED ON SWAT MODEL IN DAWEN RIVER BASIN, CHINA

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(Received 8th Oct 2018; accepted 25th Jan 2019)

Abstract. A distributed hydrological model (SWAT), which is widely used both domestically and internationally, was selected to quantitatively analyze the impact of land use and climate change on runoff in this paper in Dawen River Basin, China. The calibration and validation results obtained at Daicunba and Laiwu hydrological stations yield R^2 values of 0.83 and 0.80 and 0.73 and 0.69 and the Ens values of 0.79 and 0.76 and 0.71 and 0.72, respectively. Taking 1980-1990 as the reference period, the annual runoff increased by 288 million m^3, which was caused by changes in the land use of basin from 1991 to 2004, whereas the annual runoff decreased by 132 million m^3 due to climate change. Land use changed from 2005 to 2015, which resulted in an increase in annual runoff of 13 million m^3, and annual changes in climate caused a decrease in annual runoff of 61 million m^3. An extreme land use scenario simulation analysis shows that, compared to the current land use simulation in 2000, the runoff of cultivated land scenarios and forest land scenarios was reduced by 38.3% and 19.8%, respectively, and the runoff of grassland scenarios increased by 4.3%. Climate change simulation analysis revealed that there was a positive correlation between runoff changes and precipitation changes in the river basin. The annual total runoff in the basin decreases with rising temperatures and decreases with decreasing temperatures, which showed that the impact of precipitation variability was stronger than that of change in air temperature.
Keywords: *SWAT model; arbitrary scenario method; calibration and validation; runoff simulation*

Introduction

Climate and land use are two important factors that influence the hydrological responses of a river. Climate variability can impact flow routing time, peak-flows and volume by affecting the amount and distribution of regional precipitation and temperature (Sahoo et al., 2018; Kalogeropoulos and Chalkias, 2013; Zuo et al., 2015a), whereas land-use changes can cause changes in baseflow, annual mean discharge, and flood frequency and severity (Lin et al., 2015; Anand et al., 2018; Memarian et al., 2014). In the context of climate change, drastic human activities have a great influence on watershed hydrology. Among these activities, land-use change is the most direct manifestation of human impact (Zou et al., 2018; Zeng et al., 2015). The impact of climate change on hydrology has become more and more serious in recent years, including quantity, quality, spatial and temporal distribution of water resources, and the degree of development and utilization of water resources. The impact of climate change on water resources has been the focus of a significant amount of current research (Sunde et al., 2018). The distributed hydrological model not only considers spacial heterogeneity but also has the advantage of portraying hydrological and physical processes (Uniyal et al., 2015; Zhang et al., 2018). In recent years, distributed hydrological models have been widely used to evaluate the hydrological effects of

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climate and land use changes. In many distributed hydrological models, the SWAT (Soil and Water Assessment Tool) model has been widely used in the simulation of basin water balance, long-term surface runoff and daily average runoff (Gyamfi et al., 2016). A large number of domestic studies have adopted models to simulate the effects of climate and land-use changes on the hydrological cycle of a river basin; in particular, these models have provided a basis for large-scale complex basin applications (e.g., the Haihe River Basin (Zhang and Chen, 2009), Yellow River Basin (Wang and Zheng, 2014), and Huaihe River Basin (Yang and Chen, 2017).

International research on climate change in the hydrological field began in the 1970s (Bull and Schick, 1979; Ruddiman et al., 1977; Lettenmaier and Burges, 1978), and studies on the impact of climate change on the water cycle began in the late 1980s in China (Wu et al., 2016; Lin et al., 2015; Zeng et al., 2013; Zhang and Yang, 2014). Based on global meteorological data from 1900 to 1998 and using RS and GIS technologies, Sanjay K. Jain conducted a simulation study on the effects of land-use/cover changes on the process of runoff and sediment production in the Satluj Watershed in the Himalayas (Jain et al., 2010). Prokop found that land-use change was an important factor of hydrological changes in the Himalayas from 1930 to 2010 (Prokop and Sarkar, 2012). Wei Chao quantitatively studied the dynamic change of land use in Taian based on RS technology (Wei et al., 2016). In this paper, the SWAT model is used to simulate runoff in Dawen River Basin. Through the simulation of watershed runoff in a changing environment, the tendency and rules of runoff under the background of climate change and land use are discussed, and the impacts of climate change and land use change on water resources in the river basin can be assessed. The results of the research provide effective means for the simulation, evaluation and analysis of water resources in the basin and the optimal allocation of water resources, which is of great significance for regional economic development and sustainable development.

Materials and methods

Study area

The Dawen River is located between 35°37′30″-36°32′30″N and 116°11′15″-118°0′0″E in Shandong Province, China. The Dawen River flows from east to west and passes along Laiwu, Taian, Pingyin, and Wuyuan. The northern part of Dawen River Basin is mainly constrained by the Taishan Mountain Range and the Xiaoqing River; the eastern part is bounded by Lushan; the south is bordered by Mengshan, Yumai and Huaihe River basins; and the west is bordered by the Yellow River Basin. The east-west distance of the Dawen River Basin is approximately 176.6 km; the north-south distance is approximately 102 km; and the basin area is approximately 8726 km^2 (*Fig. 1*).

The SWAT hydrologic model

The SWAT model is a basin-scale model that was developed by the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA). It is mainly used for the simulation of water quantity and quality of surface water and groundwater (Kiniry et al., 2005). This model has been widely used in different regions around the world and mainly used for the direct evaluation of the effects of human

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activities, climate variability or other factors related to large-scale precipitation (Wang et al., 2015).

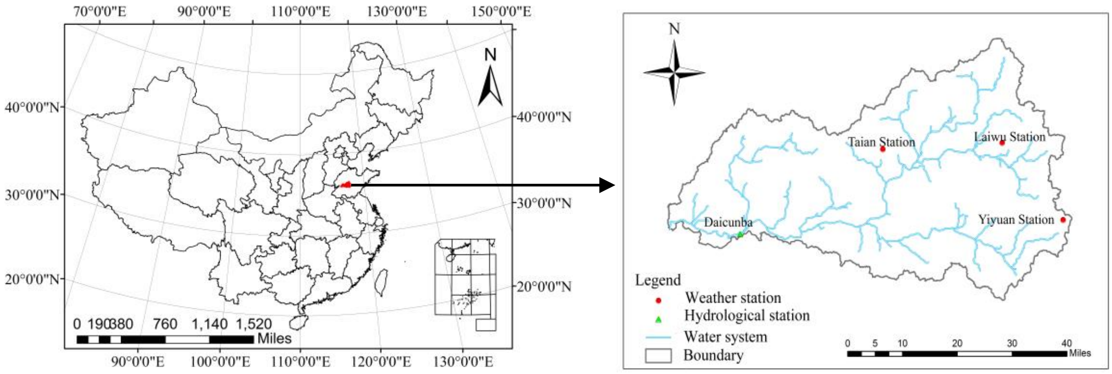

Figure 1. *Dawen River Basin location map*

The SWAT model partitions a watershed into sub-basins connected by a stream network and further delineates hydrologic response units (HRUs). By using data such as precipitation, soil properties, topography, land cover, and management, the surface runoff generated from each HRU can be calculated in each sub-watershed. The hydrologic cycle, as simulated by SWAT, is based on the water balance equation (*Eq. 1*), and the simulated processes include vadose zone processes and groundwater flow (Zhang et al., 2017).

$$SW_t = SW_o + \sum_{i=1}^{t} (R_{day} - Q_{surf} - E_a - W_{seep} - Q_{gw})_i \quad \text{(Eq.1)}$$

where SW_t is the final soil water content, SW_0 is the initial soil water content, t (days) is the time, R_{day} is the precipitation on day i, Q_{surf} is the surface runoff on day i, E_a is the evapotranspiration on day i, W_{seep} is the amount of water entering the vadose zone from the soil profile on day i (soil interflow), and Q_{gw} is the amount of return flow on day i.

The hydrologic model of the Dawen River Basin

SWAT model and database establishment

The topographic, soil, land use and hydro-meteorological data were collected or generated as follows (*Table 1*). The 90*90 DEM downloaded data is geometrically corrected by using a binomial formula. The error must be less than 0.5 pixel units to meet the requirements of the model river network division and sub-basin extraction. The soil data were sourced from the soil database of the Harmonized World Soil Database (HWSD) and the HWSD by using the USDA standard. The soil data was corrected and interpreted by ENVI 5.1. The land use map obtained by processing the classification is converted into a land use type map in a format supported by the SWAT model, get the land use map of the study area in 1985 and 2000 and 2015 (*Fig. 2*). This study used the weather observation data of three traditional meteorological stations from 1980 to 2015 (the Taian, Yiyuan and Laiwu stations).

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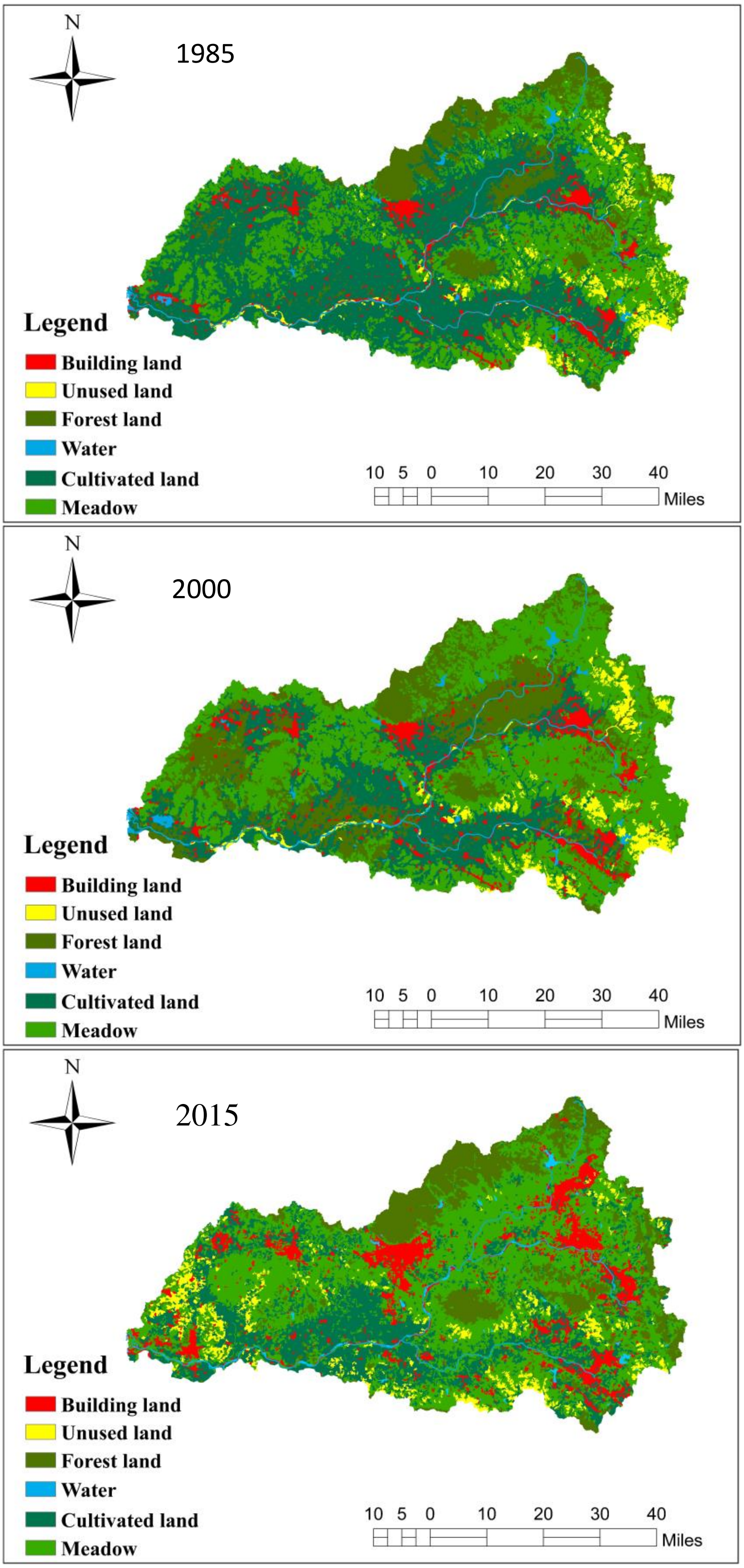

Figure 2. *Land use of the Dawen River Valley in 1985, 2000 and 2015*

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Table 1. *Data sources and basic information*

Data	Accuracy	Formatting	Source
DEM	90 m × 90 m	GRID	Geospatial Data Cloud
Land Use Map in 2000	1 km × 1 km	GRID	Chinese Research Academy of Environmental Sciences
Soil type map	1:1000000	Shapefile Feature Class	Harmonized World Soil Database
Meteorological data	Day	txt	Weather Data Sharing Network
Hydrological data	Year, Month	txt	Taian and Laiwu City Hydrographic Bureau

SWAT calibration and validation

Based on the land use data and meteorological data in 2000 and the meteorological data from 1980 to 2015, the global sensitivity analysis module in SWAT-CUP was used to analyze the sensitivity of the parameters. The value of T provides the degree of sensitivity; the greater the absolute value is, the more sensitive it is. The P value indicates that the closer the significance value of sensitivity is to 0, the more significant it is. Based on the measured data of the hydrological station, the sensitivity analysis of the parameters was performed, and parameters with strong sensitivity were selected to adjust the parameters. By using the official SWAT-CUP software, the parameters were adjusted (Khalid et al., 2018; Zhang et al., 2013). The SUFI-2 algorithm was used to determine the optimal value of the parameters by iteration (Zhang et al., 2013), and the best values of the parameters were introduced to the model through the internal tuning parameters of SWAT modelfor simulation verification. In this paper, the effective evaluation method defined by Karuse (Krause et al., 2005) is used to applicability of the simulated value of the SWAT model in Dawen River Basin was evaluated by using two indicators of correlation coefficient (R^2) and Nash-Sutcliffe efficiency coefficient (Ens). Scenarios were established to quantitatively analyze the impact of both of these indicators on river basin runoff and to separately analyze the impact of single land use type and different climate change scenarios on the runoff of the river basin. This provided an important scientific basis for water resource management and land-use planning under the influence of climate change and human activities. Relevant studies have suggested that simulations have significant effects when $R^2 > 0.6$ and Ens > 0.5 (Zuo et al., 2015b; Lotz et al., 2017).

Scenario development and model analysis

After verifying the accuracy of the model simulations, the response of the Dawen River basin to land use and climate change was analyzed by establishing five scenarios. The specific settings of these scenarios were presented in *Table 2*. In the simulation process, the parameters of the vegetation module and the soil hydraulic parameters were changed accordingly: Scenario 1 was used as the base period and compared to scenarios 4 and 5 to obtain the impact of both land use and climate change on production flows. Scenarios 2 and 3 were compared with scenario 1, and the impact of climate change on production flow was obtained. Then, scenarios 4 and 5 were compared with scenarios 2 and 3, respectively, to obtain the impact of land-use change on production flow during the corresponding period. Finally, the impact of land use and climate change at different periods on the runoff of the entire river basin were quantitatively analyzed.

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Table 2. *Model simulation scenario settings*

Scenarios	Land use	Climate data
1	1985	1980-1990
2	1985	1991-2004
3	1985	2005-2015
4	2000	1991-2004
5	2015	2005-2015

Based on the land use data in 2000, all of the land use types in the basin were set to farmland, forest land and grassland scenarios, and the corresponding soil hydrological parameters and vegetation module parameters were changed. The annual runoff under different land use scenarios was simulated from 2000 to 2015.

Based on a hypothetical climate plan (Yao et al., 2017), land use in 2000 and the possible scope of climate change, the values of the precipitation and temperature changes were obtained by using any scenario approach, and under the conditions of constant temperature and precipitation, the temperature increased by 1 °C and 2 °C, and decreased by 1 °C and 2 °C, respectively, the precipitation changes increased by 10% and 20% and decreased by 10% and 20%, respectively. 25 climate change scenarios were established. The response of runoff to climate variability was simulated through different climate variability schemes. The annual change in runoff b is solved as follows (*Eq. 2*):

$$b = (y_i - y_0) / y_0 \times 100\% \quad \text{(Eq.2)}$$

where y_i is the average annual runoff under the i climate scenario (m^3/s); and y_0 is the annual average runoff under a real scenario (m^3/s).

Results and analysis

Correction and verification results

According to the natural river network and topography of the basin, the study area was divided into 35 sub-basins, as shown in *Figure 3*. To obtain a reasonable resolution to assess soil properties, land uses, and management practices, the threshold values of land use, soil type and slope were chosen as 10%, 15% and 10%, respectively. These sub-basins were then further divided into 326 HRUs.

Through analysis, the first six sensitivity parameters were selected to adjust the parameters (*Table 3*). At the calibration period (*Table 4; Figs. 4, 5* and *6*), the simulated peak values in the flood season were lower than the measured values in 1999 and 2002, and higher than the measured values in 2000, 2005, and 2007; the R^2 and Ens values in Daicunba were 0.83 and 0.79, respectively. The simulated peak values in the flood season were lower than the measured values in 1985 and 1991, and higher than the measured values in 1982, 1987, 1988, and 1992; the R^2 and Ens values in Laiwu station were 0.73 and 0.71, respectively. The runoff simulation values of the two stations during the correction period have high precision.

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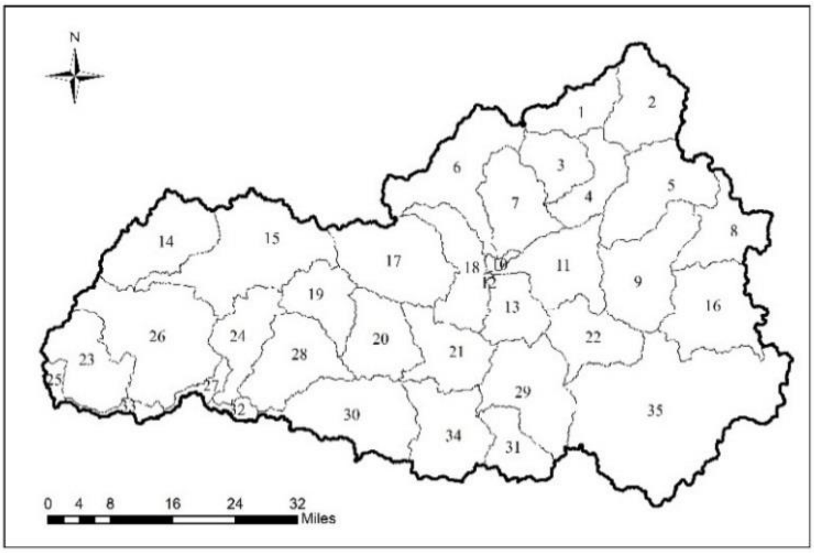

Figure 3. *Sub-basins in the Dawen River Basin delineated by the SWAT*

Table 3. *Dawen River Basin runoff sensitivity parameters*

Parameter name	Variable name	Rank	T value	P value	Ranges	The most suitable value
CN2	Curve number	1	-18.133	0	0-15	12.1687
ALPHA_BF	Base-flow recession constant	2	-2.578	0.012	0-1	0.5364
SOL_K	Saturated hydraulic conductivity	3	-2.519	0.013	0-2.0	0.6696
SOL_AWC	Available water capacity of the soil layer	4	-3.09	0.02	0.5-1.4	0.6765
ESCO	Soil evaporation compensation coefficient	5	1.8	0.08	0.3-0.9	0.5713
GWQMN	Threshold depth of water in the shallow aquifer required for return flow to occur	6	-1.463	0.147	0-5000	1763.47

Table 4. *Dawen River Basin monthly runoff simulation correction and verification*

Period	Site	R^2	Ens
Regular rate	Daicunba	0.83	0.79
	Laiwu Station	0.73	0.71
Verification period	Daicunba	0.80	0.76
	Laiwu Station	0.69	0.72

During the verification period (*Table 4; Figs. 4, 5* and *6*), the simulated peak value in the flood season was lower than the measured value in 2013, and higher than the measured values in 2009, 2012 and 2015; the R^2 and Ens values in Daicunba were 0.80 and 0.76, respectively. The simulated peak values in the flood season were lower than the measured values in 1999, and higher than the measured values in 1996 and 2001; the R^2 and Ens values in Laiwu Station were 0.69 and 0.72, respectively. The accuracy of the runoff simulation values at the two stations during the verification period is thus adequate.

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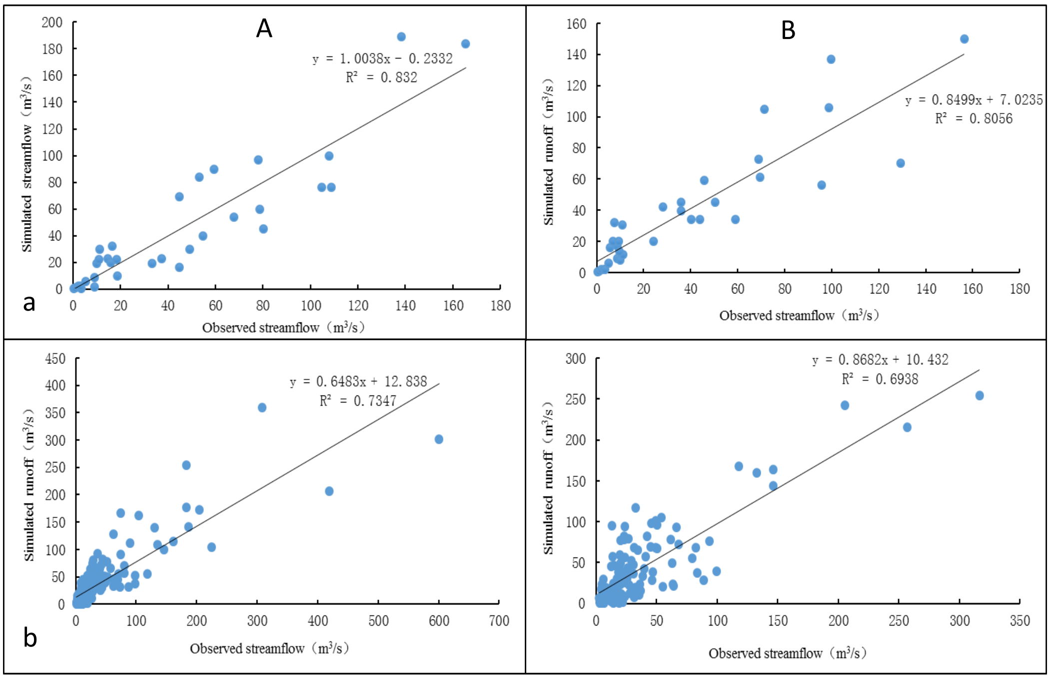

Figure 4. *Correlation between simulated and measured values of monthly runoff in the hydrological stations A: Rate period; B: Validated period; a) Daicunba; and b) Laiwu station*

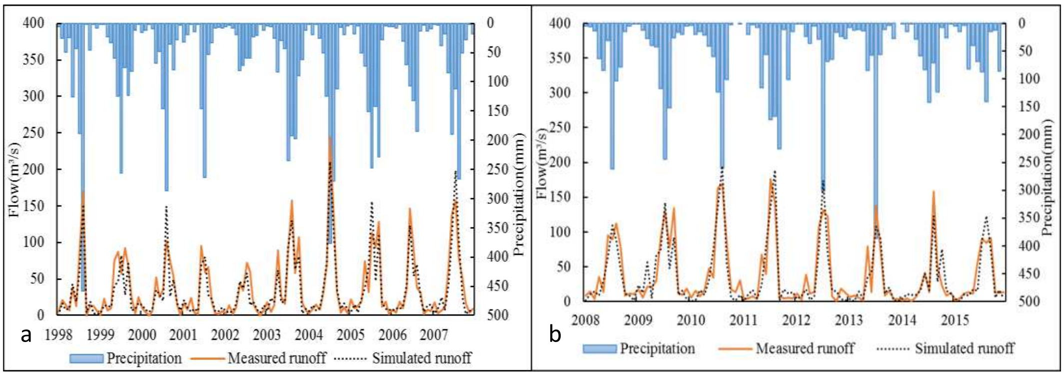

Figure 5. *Daicunba hydrological station monthly runoff simulation and actual measurement comparison chart. a: Rate period; b: Validated period*

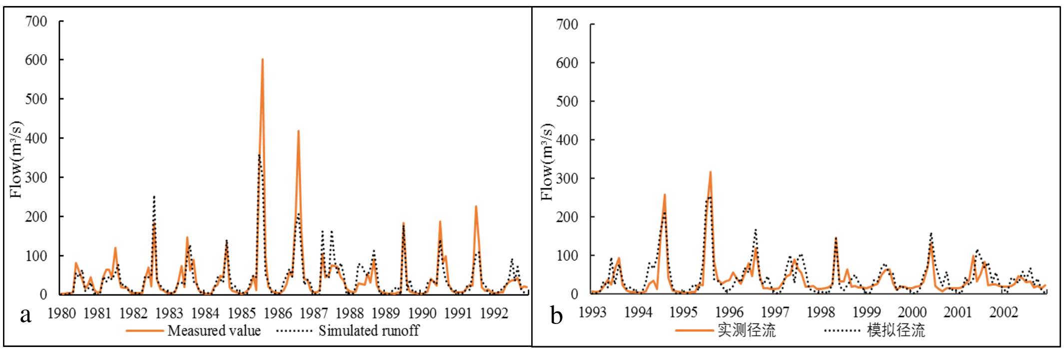

Figure 6. *Laiwu hydrological station monthly runoff simulation and actual measurement comparison chart. a: Rate period; b: Validated period*

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DOI: http://dx.doi.org/10.15666/aeer/1702_28492863

For the determination and verification of monthly runoff, the deterministics coefficient of the measured and simulated values of runoff are $R^2 > 0.6$ and Ens > 0.6. This shows that the measured values of runoff are in good agreement with the simulated values and that the SWAT model has good applicability in the study area.

Simulation scenarios

Responses of streamflow to different land use scenarios

To understand the impact of land-use changes on streamflow in this basin, the calibrated SWAT model was run under scenarios S1 S4 and S5. The percentages of land-use change rates and the changes in land use area in kilometers under these three scenarios are shown in *Figure 7* and *Table 5*. In scenario S1, the status of land use in Dawen River was mainly meadow (27.69%), forest land (19.07%), cultivated land (28.18%), and building land (12.17%). Compared to scenario S1, the area of forest land increased by 23.66%, the area of building land increased by 11.08%, and the area of cultivated land and unused land decreased by 24.38% and 6.93%, respectively, in scenario S4. Compared to scenario S4, the area of forest land decreased by 27.66% and that of unused land decreased by 3.57%, the area of the meadow increased by 10.64%, and the area of building land increased by 17.27% in scenario S5. From 1985 to 2015, the meadow area increased by 17.85% and the cultivated land area decreased by 18.3%. The water area did not change much.

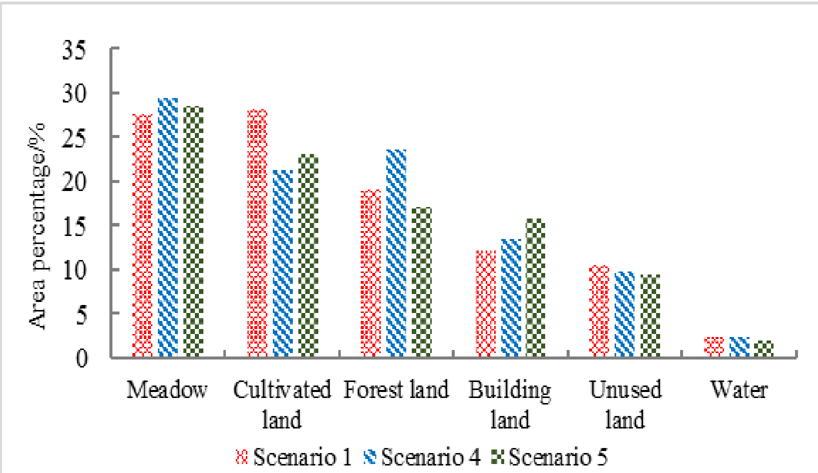

***Figure 7.** Percentages of land uses under three scenarios*

***Table 5.** Proportion of land use in the Dawen River Basin in 1985, 2000 and 2015*

Year	Meadow (%)	Cultivated land (%)	Forest land (%)	Building land (%)	Unused land (%)	Water (%)
1985	27.69	28.18	19.07	12.17	10.56	2.33
2000	29.49	21.31	23.59	13.51	9.83	2.27
2015	28.51	23.02	17.06	15.85	9.48	1.96

Based on the situational settings, a quantitative study was conducted on the runoff response to land use and climate change in Dawen River Basin (*Table 6*). The results

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show that the average annual runoff values in scenarios 1, 2, 3, 4 and 5 are 9.21, 7.89, 7.28, 8.17, and 7.41 million m^3, respectively. Scenarios 2 and 3 are compared to Scenario 1. Climate change caused an annual average runoff reduction of 1.32 and 0.61 million m^3. The comparison between Scenario 2 and Scenario 4 results in an increase of 28 million m^3 in average annual runoff due to the change in land use. The comparison between Scenario 3 and Scenario 5 results in an increase of 13 million m^3 in average annual runoff due to land use change.

Table 6. *Simulation results of land use and climate change impact on runoff in the Dawen River Basin*

Scenario	Annual precipitation (mm)	Annual average temperature (°C)	Average annual flow (10^9m^3)	Impact of land use change on runoff (10^9m^3)	Impact of climate change on runoff (10^9m^3)
1	-	-	9.21	-	-
2	755.7	13.4	7.89	-	-1.32
3			7.28	-	-0.61
4	-	-	8.17	0.28	-
5	-	-	7.41	0.13	-

Annual average runoff changes under different extreme land use scenarios

The runoff variation under three different extreme scenarios was compared to simulated runoff under the land use scenario in 2000 (*Fig. 8*). The runoff in the cultivated land scenario decreased by 38.3%, and the runoff decreased by 19.8% in the forest land scenario. However, the runoff increased by 4.3% in the grassland scenario. These results show that grassland can increase the runoff and water supply in the basin to conserve water resources. The increase in cultivated land will lead to a large reduction in runoff. Because cultivated land has the effect of inhibiting the increase of runoff, human activities have a greater impact on hydrology.

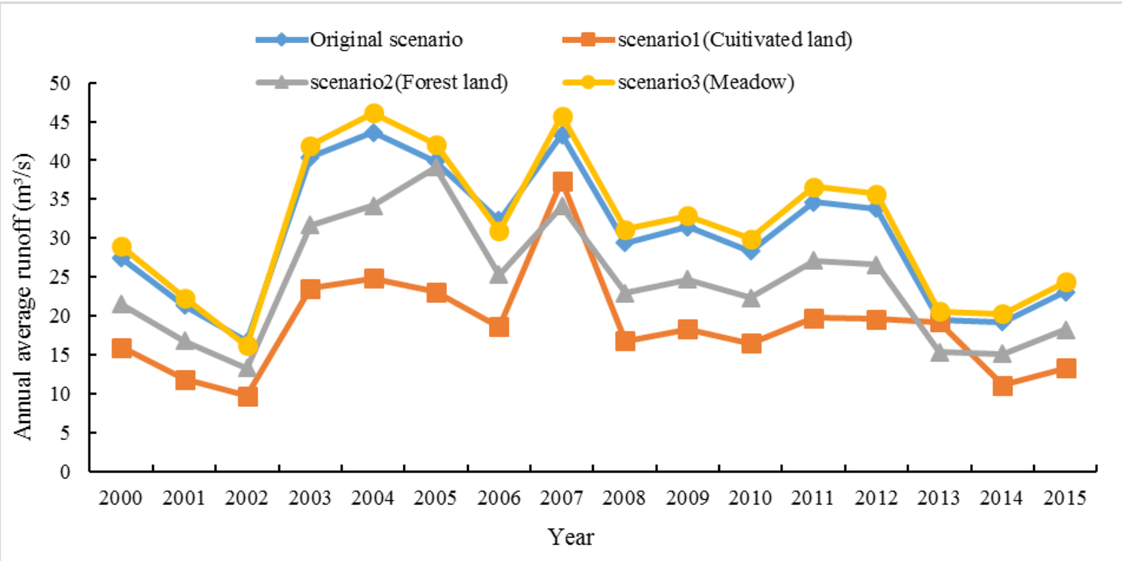

Figure 8. *Simulated runoff changes in extreme land use scenarios*

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Influence of temperature and rainfall on average runoff in the basin

It can be seen from the annual average temperature and precipitation cumulative anomaly curve in the Dawen River Basin (*Fig. 9*). From 1990 to 1995, the temperature accumulation anomaly curve showed a downward trend, which was a period of low temperature. From 1995 to 2009, the temperature accumulation anomaly curve showed an upward trend, which was a period of higher temperature. Then, the temperature accumulation anomaly curve began to rise after a short decline until 2015, and the overall situation was relatively stable. The average temperature over 26 years is approximately 13.4 °C and is growing at a speed of 0.47 °C/10a. The cumulative anomaly curve of precipitation in the Dawen River Basin from 1990 to 1998 showed a downward trend, which was a period of low rainfall. The cumulative anomaly curve of precipitation in 1998-2002 experienced a short stage of rising and falling, and the overall situation was relatively stable. From 2002 to 2015, the cumulative anomaly curve of precipitation showed an upward trend, which was a rainy period. According to this analysis, the average precipitation in Dawen River Basin during the past 26 years is 755.7 mm, and the annual average precipitation has decreased overall. The reduction rate is approximately 54.9 mm/10a.

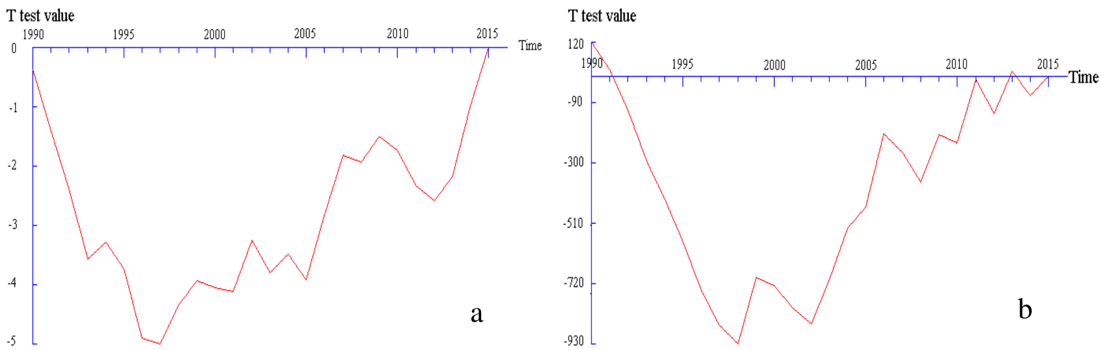

Figure 9. *Cumulative anomaly curve. a: Temperature; b: Precipitation*

It can be seen from *Figure 10* and *Table 7* that the multi-year average runoff variation in Dawen River Basin is mainly related to rainfall and temperature in the basin. First, the annual total runoff of the basin decreases with increasing temperature and decreases with decreasing temperature. The annual runoff decreased by 4.1% when the temperature increased by every 1 °C; the annual runoff decreased by approximately 3.0% when the temperature decreased every by 1 °C. This indicates that temperature is one of the main sensitive factors affecting runoff in the basin. Second, there is a positive correlation between rainfall and annual runoff in the basin. The annual runoff of the basin increases with the increase in annual rainfall, and the increase in runoff is greater than the increase in rainfall. When the annual rainfall in the basin increases by every 10%, the annual total runoff increases by approximately 18.3%. The average annual runoff of the basin decreases with the decrease of precipitation, but its reduction of rainfall is smaller than its increase of rainfall. When the annual rainfall decreases by every 10%, the annual average runoff decreases by 15.1%. Precipitation is an important factor affecting runoff in the basin. Compared to the change of temperature, the annual runoff of the basin is more affected by rainfall.

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Table 7. Simulation results of different climate change scenarios

Precipitation change / Temperature changes	-20%	-10%	0	+10%	+20%
T-2	-29.7	-18.2	-3.3	11.2	33.1
T-1	-29.1	-19.4	-3.0	10.7	34.4
0	-27.1	-15.1	0	18.3	30.7
T+1	-30.6	-20.2	-4.1	11.1	32.5
T+2	-30.3	-21.5	-4.7	12.2	31.8

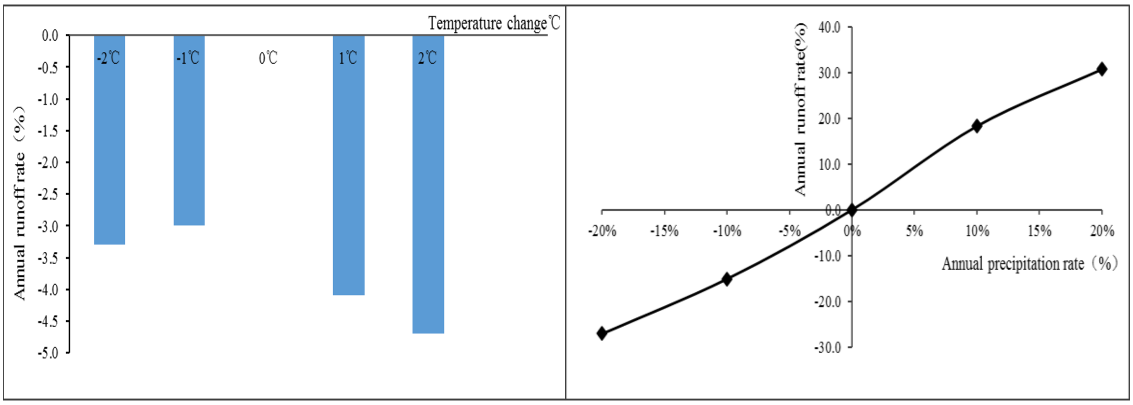

***Figure 10.** Annual runoff rate of the Dawen River Basin under changes in temperature and precipitation*

Conclusion

In this paper, the distributed hydrological model (SWAT) is applied to quantitatively simulate the impact of land use and climate change on water production in Dawen River Basin through multi-site calibration and verification. The results are shown as follows:

(1) The calibration and validation at two hydrological stations showed that the R^2 values were 0.83 and 0.80 and 0.73 and 0.69, and the Ens values were 0.79 and 0.76 and 0.71 and 0.72, respectively, which satisfied the SWAT model simulation accuracy evaluation criteria. Therefore, the SWAT model was suitable for this research basin. The parametric model can be applied directly to the actual situation in the basin.

(2) Land use and climate change in different periods have different effects on water yield in the basin. Scenarios 2 and 3 are compared to Scenario 1, indicating that climate change caused annual average runoff reductions of 1.32 and 0.61 billion m^3, respectively. The comparison between Scenario 2 and Scenario 4 results in an increase of 28 million m^3 in annual average runoff due to the change in land use. The comparison between Scenario 3 and 5 shows an increase of 13 million m^3 in average annual runoff due to land-use change.

(3) The simulation results of different land use types showed that the runoff in cultivated land decreased by 38.3%, the runoff in forest land decreased by 19.8%, and the runoff in grassland increased by 4.3%. Therefore, in the future watershed management process of Dawen River Basin, the type of land use will be adjusted to meet the water demand in the basin, increase the water output of the basin and ensure water use downstream of the basin.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28492863

(4) By analyzing the rate of runoff change in the basin under various climate scenarios, the annual total runoff in the basin decreases with the increase of temperature, and decreases with the decrease of temperature. The annual runoff increased with the increase of annual rainfall and the increase of runoff was greater than the increase of rainfall. The annual average runoff of the basin decreased with the decrease of precipitation, but its degree of decrease of rainfall was smaller than its degree of increase of rainfall.

(5) Runoff is not only affected by changes in land use but also by climate change. If the change in runoff is mainly caused by land use, the water resources planning and management policy can still be formulated based on current hydrology and meteorological data; If the main factor causing the change in runoff is climate change, then it is necessary to focus on the impact of future climate change on hydrology and water resources, and the research can also provide a reference for water resources planning management.

Acknowledgements. We express our sincere appreciation to the anonymous reviewers for their constructive suggestions. The work was supported by the special program for the National Natural Science Foundation of China (41471160). The authors would also to thank the reviewers for their very valuable comments, which greatly improved the quality of the paper.

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DOI: http://dx.doi.org/10.15666/aeer/1702_28492863

DYNAMICS OF VEGETATION COVERAGE AND RESPONSE TO CLIMATE CHANGE IN CHINA-SOUTH ASIA-SOUTHEAST ASIA DURING 1982-2013

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(Received 16th Oct 2018; accepted 16th Jan 2019)

Abstract. The NASA global inventory modeling and mapping studies released one year and a half month maximum synthetic of global dat, based on which the authors analyzed data originated from the Chinese meteorological data sharing service science and data pertaining to northwest air temperature and precipitation, climate, month and year value data sets, etc throughout the period of 1982 to 2013. The Normalized Difference Vegetation Index (NDVI) was analyzed in terms of vegetation spatial analysis, using linear regression analysis, standard deviation, the Hurst index, and the partial correlation analysis methods. The results showed that from 1982 to 2013, the overall NDVI in China, South Asia, and Southeast Asia had an increasing trend. During the 1990s, the vegetation volatility was higher in China, South Asia, and Southeast Asia. Additionally, in the three areas, the NDVI change increased dramatically after 2008, but subsequently decreased in 2010. From 1980 to 2013, the ecological environment of the eastern part of China, the northeast and the south, East Asia, Southeast Asia, India, Thailand, Bangladesh, and the eastern part of Pakistan are generally good. In Western and Northwest China and in other Southeast Asian countries, in South Pakistan, the South and back, the eastern, southern and southwestern parts, the state of the ecological environment are generally poor. From 1998 to 2005, in China, Southeast Asia, and South Asia, the NDVI is reduced. From 2006 to 2013, in China, Southeast Asia, South Asia, the NDVI increased significantly.

Keywords: *vegetation cover change, climate, China-South Asia-Southeast Asia, spatiotemporal response*

Introduction

Vegetation is connected to the atmosphere, water, and soil, comprising the core of the land surface in the earth's ecosystem. Since photosyntheses is related to the degree of vegetation coverage, changes in vegetation coverage exert a significant goal impact, as they influence the properties of the land surface, soil and water conservation, climate regulation, and overall stability of the ecosystem (Meyer et al., 1992; Sun et al., 1998).

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DOI: http://dx.doi.org/10.15666/aeer/1702_28652879

Within the interaction between the earth spheres, the hydrological processes and the soil–vegetation–atmosphere interactions and on global and regional climate and ecosystem of their feedback is the most basic frontier issue (Cao et al., 2011; Chang et al., 2011). Therefore, climate and vegetation share an inseparable relation, manifested mainly in two aspects: vegetation adaptability to climate and vegetation feedback effect on climate (Fung et al., 2000; Chen et al., 2015). Changes in vegetation influence the properties of soil surface such as moisture, which in turn will affect regional climate (Justice et al., 1986; Zhang et al., 2003). On the other hand, climate changes affect vegetation's growth; thus, the climate is the main factor influencing the distribution of the biological community, and its changes alter the structure of vegetation communities, composition, and biomass, thus shaping the forest ecological system and simultaneously decreasing biological diversity (Zhang et al., 2001; Cui et al., 2009). The Normalized Difference Vegetation Index (NDVI) characterizes surface vegetation and is sensitive to measure vegetations' growth parameters, thus comprising an effective index for quantitative characterization of vegetation growth (Yang et al., 2004; Yaşar et al., 2018). As such, the NDVI has been widely used in several areas such as agriculture, ecology, and environment. Recently, the association between NDVI and climate response has been studied by researchers of different areas (Zhang et al., 2007; Mr et al., 2017).

President Xi Jinping brought up the “One Belt And One Road” to promote close cooperation among different countries, including hazard-prone areas, such as China, South Asia, and Southeast Asia where natural disasters occur most frequently. Investigating the ecological environment of these countries may improve the prevention of regional disasters and the capacity to mitigate their effects. Additionally, it may improve the accuracy of climate change research in other areas, thus promoting the “One Belt And One Road” for an improved cooperation between countries (Wagner et al., 2014; Lu et al., 2016).

Materials and Methods

Study Area

The geographical scope of this research (*Figure 1*), followed the “One Belt And One Road” strategy, including China's south and southeast bordering countries, as follows: South Asia, including Afghanistan, Bangladesh, Bhutan, India, Nepal, and Pakistan; and Southeast Asia area including Cambodia, Laos, Myanmar, Thailand, and Vietnam (Wang et al., 2008).

Data sources

The GIMMS NDVI3g dataset was downloaded from the NOAA-Advanced Very High Resolution Radiometer (AVHRR) for the period from 1982 to 2013. GIMMS NDVI3g (http:// data.cma.cn/) has a spatial resolution of 1/12° and a temporal resolution of 15 days (Fensholt and Proud, 2012; Guay et al., 2014). The GIMMS NDVI3g dataset has been optimized to minimize the effects of the differences in sensor design between the

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AVHRR/2 and AVHRR/3 instruments as well as volcanic eruptions (Pinzon and Tucker, 2014; Liu et al., 2015a, b). To further eliminate the interference of factors such as atmosphere, clouds, and angle of the sun (Hou, 2001), the maximum synthesis MVC (maximum value composites) is used to obtain the monthly and annual NDVI values. The monthly NDVI is calculated according to the following formula:

$$MNDVI_i = Max(NDVI_1, NDVI_2) \quad \text{(Eq.1)}$$

In the formula: NDVI value of month i; i is the month number (i = 1, 2, 3 ... ... 12), - denotes the NDVI value of the first half month and the second half month, respectively. Using the same MVC method to obtain the maximum monthly NDVI as the annual NDVI value. The formula is

$$Y_{NDVI} = Max(MNDVI_1, MNDVI_2 \cdots, MNDVI_{12}) \quad \text{(Eq.2)}$$

In the formula: Y_{NDVI}--annual NDVI value; $MNDVI_1$, $MNDVI_2$…, $MNDVI_{12}$ --Monthly NDVI values for January–December.

The temperature and precipitation data in the study area are mainly from the website: (http://www.cru.uea.ac.uk/) A gridded time-series dataset. This version, released 18 November 2018, covers the period 1901-2017. Coverage: All land areas (excluding Antarctica) at 0.5° resolution.

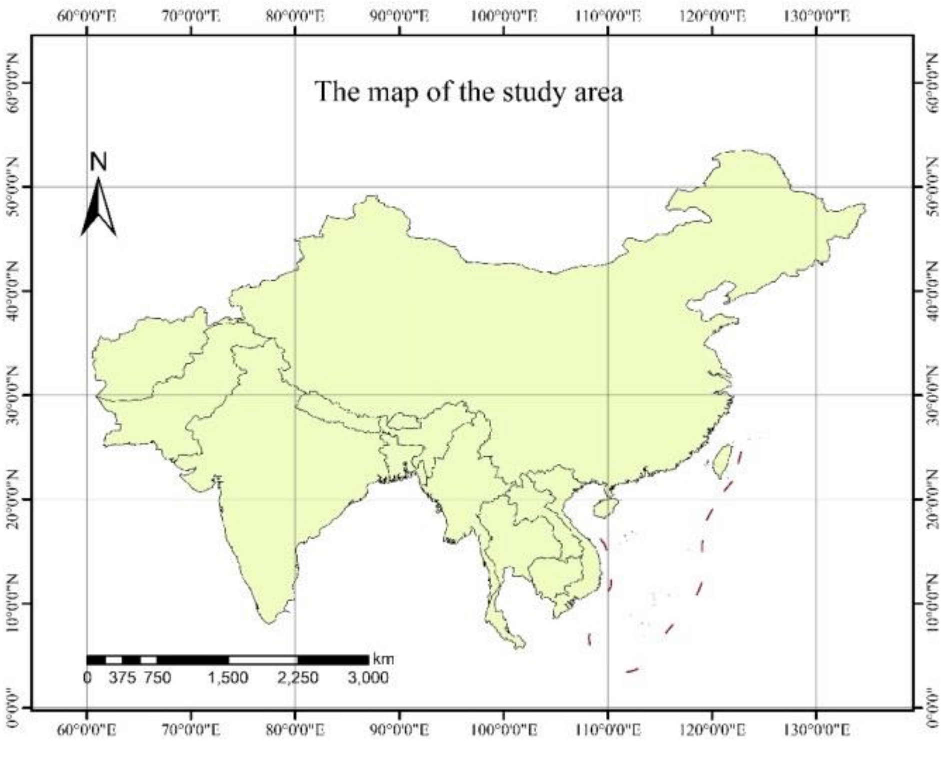

Figure 1. *Location of the study area*

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Research methods

Linear regression analysis was performed for vegetation, precipitation, and air temperature, along the factor time (Liu et al., 2015). That is, the linear regression coefficient a of vegetation NDVI and climate factor y over time t, as shown in formula (3).

$$y = at + b, \qquad t = 1, 2, \cdots, n(n \le 25) \qquad \text{(Eq.3)}$$

Sen+Mann-Kendall: The trends of changes in the spatial characteristics of vegetation were analyzed using the Sen method and tested using the Mann–Kendall method. These methods can avoid loss of data, outliers' interference, and influence of the data distribution patterns on the analysis results (Liu et al., 2013). The Sen trend was calculated using data pertaining to the NDVI from 1982 to 2013, according to the following formula:

$$\rho = median\frac{(x_j - x_i)}{j - i}, \qquad 1 < \mathrm{i} < \mathrm{j} < \mathrm{n} \qquad \text{(Eq.4)}$$

where x_j, x_i -- time series data. $\rho < 0$ shows a downward trend, whereas the reverse shows an upward trend. The Mann-Kendall method was used to test whether the trend was significant.

The Mann-Kendall test (MK test) is one of the most commonly used time series trend test methods and does not require the sample to follow a certain distribution and is suitable for non-normally distributed data. The method is based in the following formulas:

$$Q = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} sign(x_j - x_i) \qquad \text{(Eq.5)}$$

$$sign(s) = \begin{cases} 1 & (s > 0) \\ 0 & (s = 0) \\ -1 & (s < 0) \end{cases} \qquad \text{(Eq.6)}$$

$$z = \begin{cases} \dfrac{Q-1}{\sqrt{V(Q)}} & (Q > 0) \\ 0 & (Q = 0) \\ \dfrac{Q+1}{\sqrt{V(Q)}} & (Q < 0) \end{cases} \qquad \text{(Eq.7)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_28652879

where: Q - test statistic; Z - normalized test statistic; x_j, x_i - time series data; n - sample number; when $n \geq 8$, Q is approximately positive distribution, the mean and variance are calculated as follows:

$$E(Q) = 0 \qquad \text{(Eq.8)}$$

$$V(Q) = \frac{n(n-1)(2n-5)}{18} \qquad \text{(Eq.9)}$$

After standardization, Z is the standard distribution, if $|z| > z_{1-a/2}$, then it is significant trend change. $z_{1-a/2}$ is the corresponding value for the standard positive distribution table at the confidence level a. In this paper, the confidence level is 0.05, and the degree of freedom is 25- 2=23.

Standard Deviation Analysis: The standard deviation indicates the distance of the data variable from the mean, which reflects the discrete degree of a data set. The larger the value is, the farther the NDVI distance is, the greater the vegetation change (Liu et al., 2015). The formula as follow:

$$S_i = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(NDVI - \overline{NDVI}\right)^2} \qquad \text{(Eq.10)}$$

According to the standard deviation value of 5 grades of vegetation change intensity: High ($S_i \leq 2.00$), high ($2.00 \leq S_i < 4.00$), medium ($4.00 \leq S_i < 6.00$), lower ($6.00 \leq S_i < 8.00$), and low ($8.00 \leq S_i < 10.00$). The spatial characteristics of vegetation cover fluctuation in the region for many years.

Hurst index: Time series with long-term dependence in nature are ubiquitous. Hurst index is one of the effective methods to quantitatively describe the long-term dependency of time series information. The calculation principle is as follows:

For any positive integer $\tau \geq 1$, define the mean sequence $\{\xi(t)\}$

$$\langle \xi \rangle_\tau = \frac{1}{\tau}\sum_{t=1}^{\tau}\xi(t) \qquad \tau = 1, 2, \cdots, N \qquad \text{(Eq.11)}$$

Accumulation deviation:

$$X(t,\tau) = \sum_{u}^{t}(\xi(t) - \langle \xi \rangle_\tau), \qquad 1 \leq t \leq \tau \qquad \text{(Eq.12)}$$

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Very poor:

$$R(\tau) = \max_{1 \le t \le \tau} X(t, \tau) - \min_{1 \le t \le \tau} X(t, \tau), \qquad \tau = 1, 2, \cdots, \qquad \text{(Eq.13)}$$

Standard deviation:

$$S(\tau) = \left[\frac{1}{\tau} \sum_{t=1}^{\tau} (\xi(t) - \langle \xi \rangle_{\tau})^2 \right]^{\frac{1}{2}}, \qquad \tau = 1, 2, \cdots N, \qquad \text{(Eq.14)}$$

Consider the ratio R (τ)/S (τ). If there is R / S∝τH, then the time series {ξ (t)} exist Hurst phenomenon, H called Hurst index. In the double logarithmic coordinate system (lnτ, lnR / S) with the least squares fitting.

1) If 0.5 <H <1, it indicates that time series of NDVI as long-term positive correlation.
2) If H = 0.5, the time series of NDVI are independent random sequences.
3) If 0 < H < 0.5, the time series data of NDVI have anti-persistence, and the sequence has abrupt jump characteristics. H value closer to 0, the stronger the anti-persistence; closer to 1, the more positive continuity.

Results

Interannual change of NDVI in the area

Using the values of NDVI corresponding to the period between 1982 and 2013, analysis of regional mean annual variation characteristics, provided the annual NDVI variation characteristics graph below (*Figure 2*):

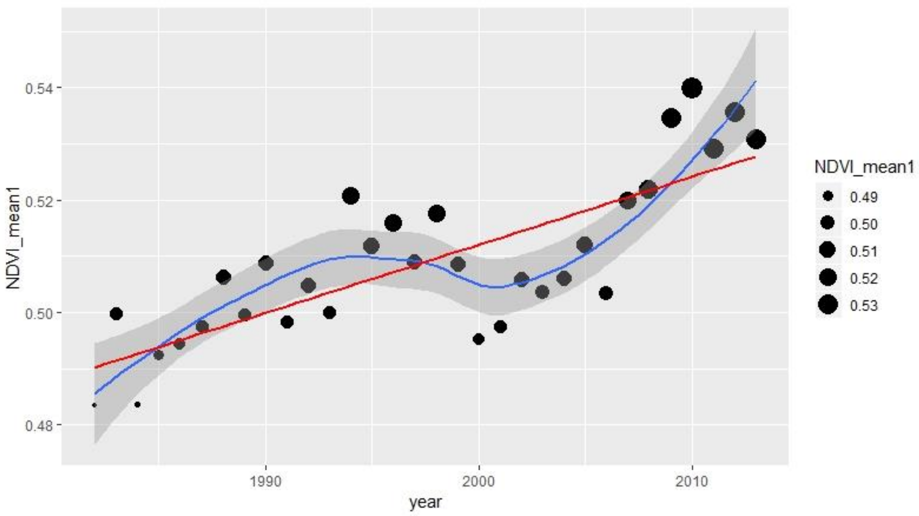

Figure 2. *Annual variation characteristics of NDVI from 1982 to 2013 in South Asia*

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As can be seen from *Figure 2*, Between 1982 and 2013, the interannual variation of the average value of NDVI in South Asia shows an increasing trend. In the late 1990s, there was a small fluctuations, that is a downward trend. High values appeared in 1994 and low values appeared in 2000. After 2000, there was a tendency to resume growth.

From *Figure 3*, the interannual variation of the NDVI regional average in Southeast Asia is the same as that in South Asia. Among them, the growth trend appeared in the 1980s. Also fluctuating in the 1990s, during which low values occurred in 1993 and high values appeared in 1994. After 2000, there is a growing trend.

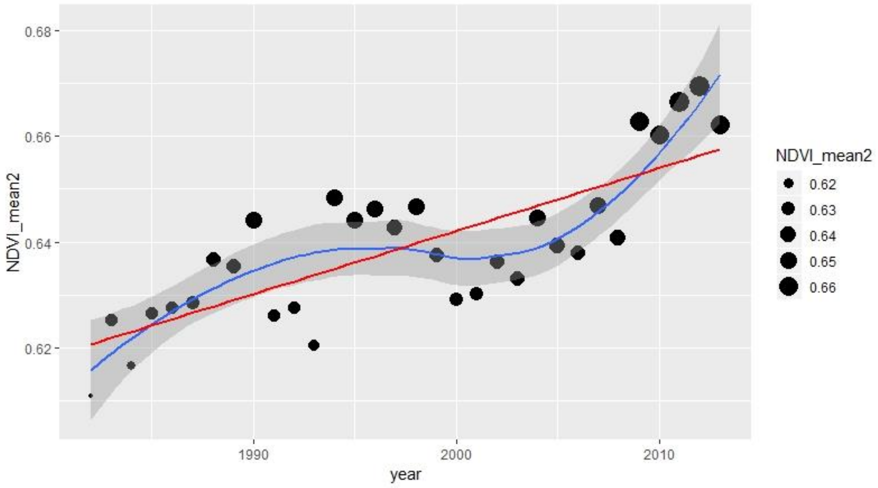

Figure 3. *Annual variation characteristics of NDVI from 1982 to 2013 in Southeast Asia*

From *Figure 4*, between 1982 and 2013, the interannual variability of China's NDVI regional average is the same as that of South Asia and Southeast Asia. It is a growth trend in the 1980s, and it has a downward trend in the 1990s, and then it has become a growth trend. The magnitude of the change is relatively large compared to other regions.

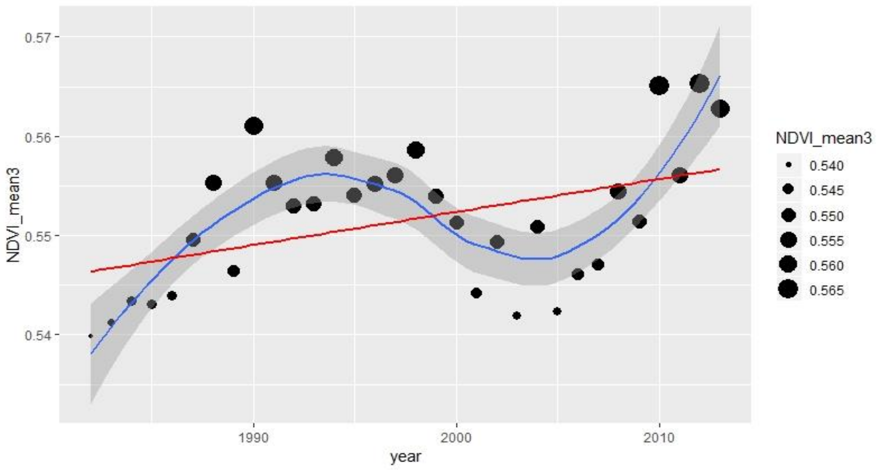

Figure 4. *Interannual variability of NDVI in China from 1982 to 2013*
(Remark: Annual average of the statistical study area)

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DOI: http://dx.doi.org/10.15666/aeer/1702_28652879

Summary, the interannual variability of NDVI in these three regions is similar, showing a growing trend in the 1980s, a downward trend in the 1990s, and a growth trend in the 2000s. In the 1990s, the interannual variation of China's NDVI regional values fluctuated significantly. These are all in sync with regional social development. In the 1990s, all regions were developing, pursuing economic development and neglecting ecological environmental protection. After the 2000s, everyone realized the importance of the ecological environment, so that they pursued economic development while also paying attention to the protection of the ecological environment. Therefore, the regional average NDVI has shown an increasing trend since the 2000s. Due to the rapid development of China, the damage to the environment was so great that there was a large fluctuation in the 1990s.

Spatial distribution of SEN trend in the study area

In the present study, the SEN method was used to analyze the spatial trends of vegetation characteristics and to test them using the Mann-Kendall method, thus determining the trend of NDVI in China, South Asia, and Southeast Asia from 1982 to 2013 (*Figure 5*).

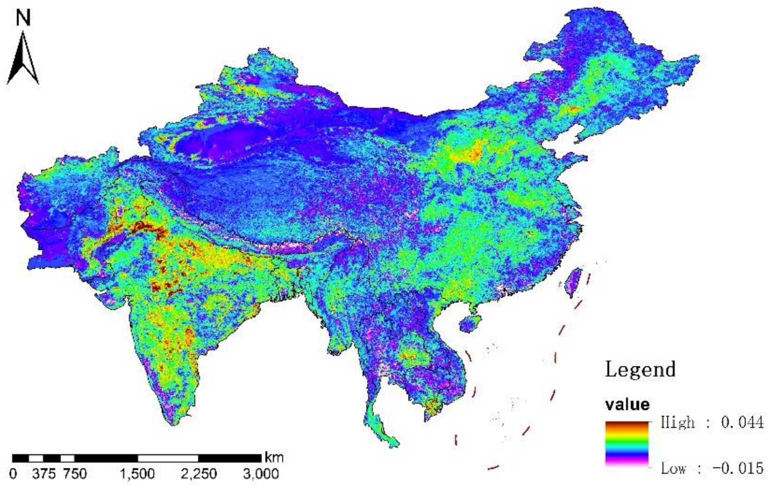

Figure 5. *Spatial distribution of SEN trend in the study area, 1982–2013*

As shown in the above picture, in China, Southeast Asian countries, and South Asian countries, most positive SEN values, indicating a good ecology. The SEN is high in the eastern, central and northeastern China and low in the west and northwest china. Most SEN values are low for Southeast Asia, Vietnam, Cambodia, and Laos and higher in eastern and southern Thailand and central Myanmar. In the South Asian countries of Bangladesh, India, eastern Pakistan, southeast and northeast, northern Afghanistan, eastern Nepal, and southern Bhutan, SEN values are high, with 0.044 being the the highest value observed.

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Spatial distribution characteristics in different periods

The spatial trend of the NDVI in the four periods of 1982-1989, 1990-1997, 1998-2005, and 2006-2013 was analyzed using Hurst exponent and partial correlation analysis (*Figure 6*).

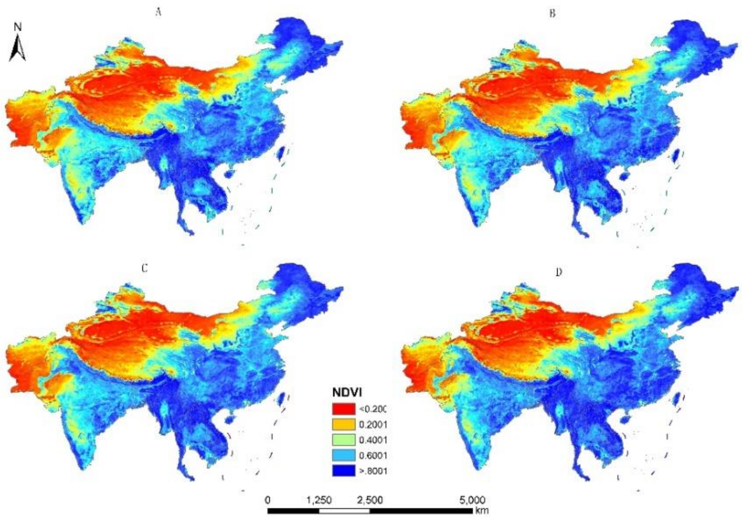

Figure 6. *Multi-year mean spatial distribution of the study area*

The NDVI of the eastern, southeastern and northeastern parts of China was above 0.6, whereas it was lower than 0.2 in the northwestern part of China, and higher than 0.6 in some parts of northern Xinjiang. In the Southeast Asian countries of Vietnam, Cambodia, Laos, Thailand, and Myanmar, as well as in South Asia; Nepal; Bangladesh; Bhutan; and central, eastern, and northern parts of India, the NDVI was higher than 0.6. With in South Afghanistan and Pakistan it was below 0.2, whereas in much of Afghanistan it was below 0.6.

In terms of spatial and temporal distribution, the NDVI decreased from 0.6001 to 0.8, especially in China and India. In Southeast Asia, Bhutan and Bhutan Bengal it remained unchanged. In the four studied time periods, the largest NDVI changes occurred from 2006 to 2013.

Overall, the NDVI values were mainly distributed over Southeast Asia, eastern and northeastern China, with the lowest values concentrated mainly in western South Asia and the Qinghai-Tibet Plateau of China. The spatial distribution of vegetation cover was related to its topographic distribution with a relatively high NDVI near the coast.

Spatial variability of vegetation cover change in the study area

The variation of vegetation coverage in the study area was calculated through the coefficient of variation, whereupon the interannual variability of vegetation coverage increased with an increasing coefficient of variation. For example, in the west and northwest of the study area, the topography is relatively high, as is the coefficient of

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variation. In the Indian Peninsula, Southeast Asia and The coefficient of variation in China's coastal areas is low. The spatial distribution of NDVI is also similar to that of the study area. The interannual variation range of NDVI is low, whereas its overall range is relatively high. Overall, the interannual variability of vegetation cover over the past 32 years was relatively low, which indicated a stable ecological change of vegetation within the study area (*Figure 7*).

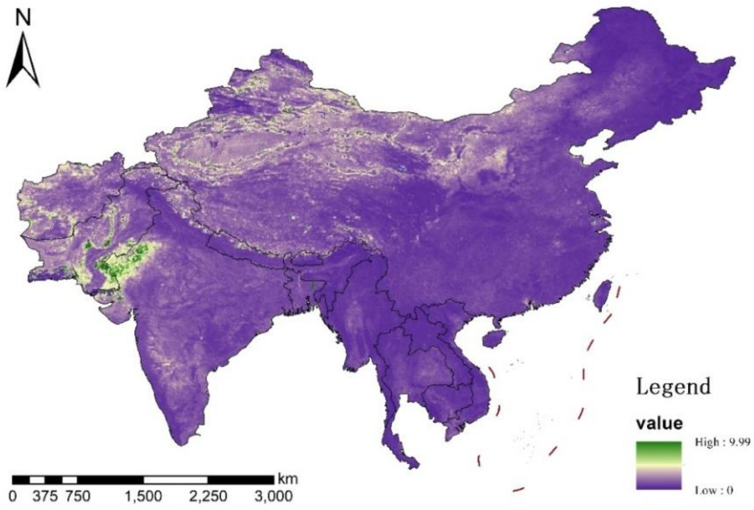

Figure 7. *1982-2013 Spatial distribution of NDVI variation coefficient in the study area*

HURST index space characteristics of sustainable change

The spatial change characteristics of vegetation coverage in the study area from 1982 to 2013 were calculated on the basis of the HURST index (*Figure 8*).

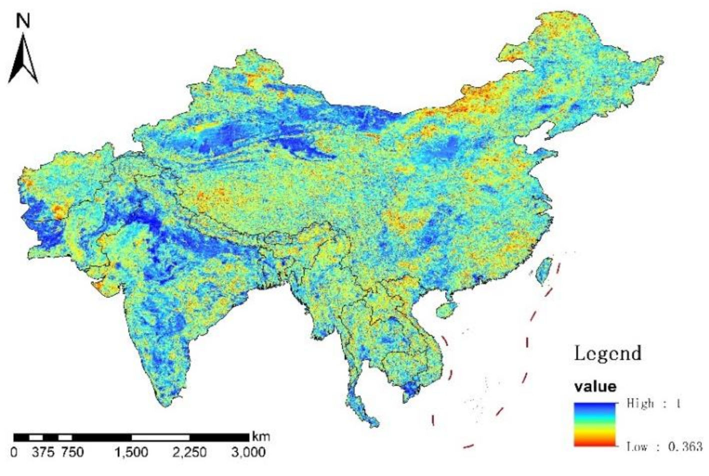

Figure 8. *1982–2013 The HURST spatial distribution in the study area*

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Regarding the spatial distribution of the HURST index, most values within the study area ranged between 0.3 and 1, with most areas exhibiting a value higher than 0.5. Most regions corresponded to northwestern China and parts of the Indian Peninsula in South Asia. Overall, the main trend of vegetation cover change in this region was a normal trend, showing that the ecological vegetation coverage in the study area improved over 32 years, further indicating an improvement of the region's ecological environment.

Spatial distribution characteristics of vegetation cover change and climate response

In the present study, the correlation between vegetation cover and precipitation within the study area was analyzed, and we concluded (*Figure 9*) that in most areas the vegetation cover was obviously correlated with precipitation, especially in South Asia and Inner Mongolia, in northern China. Only a few regions exhibited negative correlations, which were not obvious. It may therefore be concluded that the changes in vegetation cover within the research area and over the past 32 years are strongly influenced by precipitation.

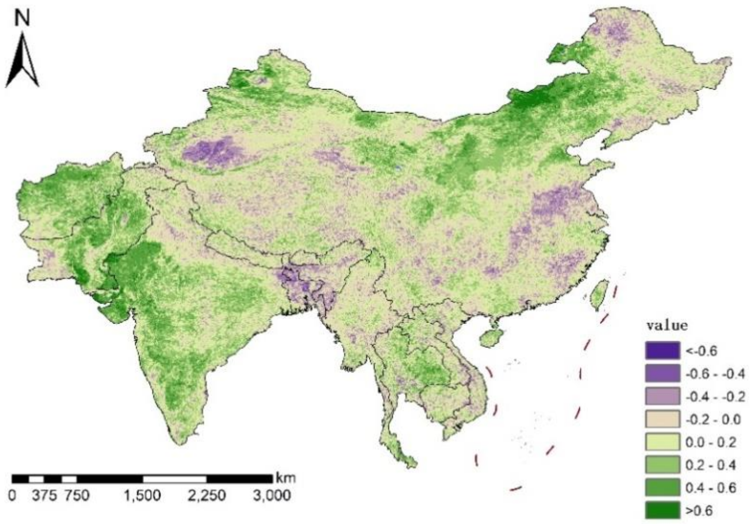

Figure 9. *1982-2013 Partial correlation NDVI and precipitation apace distribution in study area*

Moreover, as can be observed from *Figure 10*, in most areas, the response in vegetation cover changes has a positive correlation with temperature, with only a few areas showing a negative correlation, thus showing vegetation cover changes are affected by temperature.

In summary, the influence of temperature and precipitation is positively correlated with the changes of the vegetation cover, and the area of NDVI and precipitation correlation coefficient in the study area is northeast China, eastern Thailand and Central and northwest of South Asia. The correlation coefficient between NDVI and temperature is in the central, eastern, and southern parts of China; southern Vietnam; eastern Thailand;

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central Burma; and Central Asia. Therefore, it is concluded that vegetation growth is closely related to precipitation and temperature.

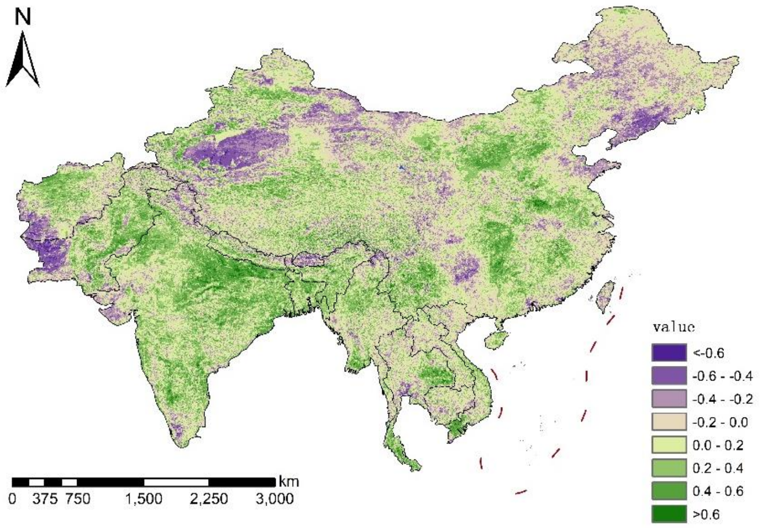

Figure 10. *1982-2013 Partial correlation NDVI and temperature apace distribution in study area*

Discussion

The present study monitored vegetation growth in three regions of China, South Asia, and Southeast Asia by analyzing data pertaining to NDVI time series (Liu et al., 2015a). Additionally, the growth status of agricultural vegetation within the study area was monitored (Zhang et al., 2013b). The results show that large-scale agricultural vegetation monitoring can be carried out by satellite remote sensing. However, due to the constraints of NDVI data resolution and the complexity of vegetation changes, several problems remain to be clarified (Zhang et al., 2013a):

1) The GIMMS-NDVI data resolution is 8 km × 8 km, and the relationship between the agricultural vegetation-climate in China-South Asia and South-East Asia cannot be fully reflected, and the study area should be more refined by using more high-resolution NDVI datasets and data from different sources.
2) Factors affecting agricultural vegetation cover include, in addition to rainfall, temperature and topography, hydrology, and soil and insect infestation, which should be taken into account in subsequent studies.
3) This paper studies changes in climate and agricultural vegetation coverage, without considering the influence of human activities. Human activities include the expansion of urbanization, agricultural modernization management, and ecological environment construction.
4) By studying the NDVI vegetation coverage, this paper studies the agricultural vegetation coverage in the study area, and does not consider the influence of other kinds of vegetation coverage on the study of agricultural vegetation coverage.

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DOI: http://dx.doi.org/10.15666/aeer/1702_28652879

Conclusions

On the basis of the analysis of the temporal and spatial NDVI trends in different climatic regions and some countries in China, South Asia, and Southeast Asia from 1982 to 2013, the following conclusions are taken:

1) Between 1982 and 2013, the average annual NDVI trends in China, South Asia, and Southeast Asia increased. Although the increase was relatively small in South Asia, it was higher in Southeast Asia and China. After the 1990s, the NDVI vegetation changes in China, South Asia, and Southeast Asia were fluctuant. After 2008, the NDVI of the three regions all increased significantly, subsequently declining by 2010.
2) The ecological environment of eastern, northeastern, and southern China; eastern Thailand; South Asia; India; Bangladesh; and eastern Pakistan was generally good in 1980 to 2013. The western and northwestern parts of the country, other countries in Southeast Asia, western Pakistan, south, back, eastern Afghanistan, south and southwest of Pakistan are generally poor in terms of ecological environment.
3) From 1998 to 2005, NDVI vegetation indices in China, Southeast Asia, and South Asia decreased, subsequently increasing over 2006 and 2013.
4) During nearly 32 years of study within the area, the interannual vegetation amplitude is small, with a relatively stable vegetation coverage, where only a few areas larger fluctuation.
5) According to the HURST index calculation, most the study area in most of the vegetation coverage were positively state change trend, a handful of areas reverse the trend.

Comprehensive above, study nearly 32 years of vegetation changes gradually in the study area, the ecological environment is improved, a further sign of the government and people pay more and more attention to ecological environment construction, so that the ecological vegetation restoration is more obvious.

Funding. This research was funded by “Management Science and Engineering” construction funding for key disciplines in Guangxi, Karst land use change ecological effect side evaluation ecological security research (GK2015010), and the perspective of rural revitalization of natural resources accounting and ecological security research in Northwest Guangxi (2018QNB13).

Conflict of Interests. The authors declare that they have no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28652879

HEAVY METAL CONTAMINATION IN THE SURFACE SEDIMENTS OF XUWEI-LIEZIKOU OFFSHORE AREA, NORTHERN JIANGSU PROVINCE, CHINA

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(Received 31st Oct 2018; accepted 28th Jan 2019)

Abstract. A total of 30 samples were collected from surface sediments of Xuwei-Liezikou offshore area, northern Jiangsu Province, China, and the concentrations of 7 heavy metals including As, Hg, Cu, Pb, Zn, Cr and Cd and their spatial distributions were determined. Geo-accumulation index (I_{geo}), pollution load index (*PLI*) and potential ecological risk index were applied to evaluate the pollution status. The mean concentrations of Cd, Pb, As, Zn, Cu and Hg were 2.62, 1.96, 1.70, 1.42, 1.32 and 1.30 times of their respective background values, which indicated there were obvious accumulations of these heavy metals in surface sediments. It was observed that the high heavy metal concentrations were in the western and northern regions of the study area except for Cu, mainly located in estuary and port regions. The sediments in the entire study area were moderately polluted, and the main pollution factors were Cd and Pb. The study area suffered from a level between low and moderate ecological risks, and Cd was at the considerable ecological risk level, Hg was a moderate ecological risk, while other metals had low ecological risk level.
Keywords: *heavy metal concentration, spatial distribution, contamination assessment, ecological risk, northern Jiangsu Province*

Introduction

The heavy metal pollution in marine environment has received much attention, becoming a global environmental issue, because of their toxicity, wide sources, persistence, ecological risk, and the non-biodegradable nature of the contamination (Irabien and Velasco, 1999; Burger and Gochfeld, 2003; Hu et al., 2011; Waheed et al., 2013; Bastami et al., 2015; Harikrishnan et al., 2017). As we all know, sediments contain and accumulate a large number of heavy metal elements (Gao et al., 2015; Fang et al., 2016; Zhang et al., 2017). The sediments are not only a 'sink' of heavy metals but also a 'source' (Matthiessen and Law, 2002; Hill et al., 2013; Machado et al., 2016; Chen et al., 2017). On the one hand, heavy metal elements enter the water through various ways, these elements enter the water where they are subsequently adsorbed by suspended substances and settle on the sea floor. On the other hand, when some physical and chemical properties of the water change, the heavy metal elements in sediments would be released and thereafter cause pollution to the water. Therefore, the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

analysis of heavy metals in sediments is necessary to our understanding and protection of the marine environment.

Xuwei-Liezikou offshore area is located in the southern part of Lianyungang city in the northern Jiangsu Province of China and is an important part of Lianyungang Port. Because of its superior geographical position, excellent port resources and rich fishery resources, the study area has become a major gathering place for mariculture, port development and coastal industry. The development of ports and a large number of land-based pollutants entering the sea are bound to exert great pressure on the marine environment (Nethaji et al., 2017; Jahan and Strezov, 2018). The evaluations of heavy metal in sediments form Lianyungang offshore area have been investigated (Zhang et al., 2013; Li and Li, 2016; Zhang et al., 2016). However, previous studies mainly focused on the study of heavy metals in columnar sediments form the northern Lianyungang offshore area (e.g. Haizhou bay), and few reports have evaluated heavy metal contents and pollution in surface sediments form the southern Lianyungang offshore area. Therefore, this study evaluates the concentration and pollution level of heavy metals (As, Hg, Cu, Pb, Zn, Cd and Cr) in surface sediments of Xuwei-Liezikou offshore area in northern Jiangsu Province, which is a supplement to the study on heavy metals of Jiangsu Province and provides reference data for controlling heavy metal pollution in the marine environment.

Materials and methods

Study area

Xuwei-Liezikou offshore area is located in the south wing of Lianyungang Port in Jiangsu Province of China, bordered by the Yellow Sea in the east, the Yangtze River Delta Economic Belt in the south, and the Bohai Economic Circle in the north. Its estuaries include the Xishu River estuary on the west side of the Dongxi Island, the Shaoxiang River estuary and Xiaowa Port on the nearshore of the Xuwei Port, and the Liezikou estuary and Guanhe River estuary in the southwest. The coastwise areas of the study sea area belong to a coastal plain. The coast stretching from the Xishu River estuary to Shaoxiang River estuary is a bedrock coast with a coastline of 40.25 km. The coast stretching from the Shaoxiang River estuary to the Liezikou estuary is an erosive muddy coast with a coastline of 32.06 km. The coast stretching from the Liezikou estuary to the Guanhe River estuary is an erosive silty muddy coast with a coastline of 39.08 km.

There are also two sea-discharging projects in the study area, including the Lingang Sewage Treatment Plant treating the sewage from the Harbor Industrial Park in the Guanyun County and the Lianyungang Zhongxin Sewage Treatment Plant treating the sewage from Lianyungang Chemical Industrial Park. The sewage source involves industries about electricity, metallurgy, chemical, shipbuilding, paper making, pesticides, and pharmaceuticals.

Sample collection and processing

In the tide period of October 2014, a survey of the marine environment was carried out in Xuwei-Liezikou offshore area, northern Jiangsu Province, China. A total of 30 surface sediment survey stations were deployed. The coordinates and details of the sampling location points are listed in *Table 1* and shown in *Fig. 1*. Sample 1, Sample 2,

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DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

and Sample 4 are located on the northeast side of Dongxi Island. Sample 3 is on the north side of the west embankment. Sample 8, Sample 11 and Sample 12 are on the northeast side of the Guanhe River estuary, and Sample 13 is on the Guanhe River estuary. Others survey samples are concentrated in the inner areas of the Xuwei Port. The survey investigated items including arsenic (As), mercury (Hg), copper (Cu), lead (Pb), zinc (Zn), chromium (Cr) and cadmium (Cd).

Table 1. *Coordinates of sampling locations*

Sample	Coordinates	Sample	Coordinates	Sample	Coordinates
1	35°04'16.70"N119°40'07.14"E	11	34°42'36.87"N120°13'39.87"E	21	34°33'19.85"N119°39'41.19"E
2	34°57'18.28"N119°33'21.48"E	12	34°37'15.93"N120°08'55.70"E	22	34°31'40.03"N119°39'44.72"E
3	34°46'50.34"N119°22'56.67"E	13	34°27'29.01"N119°59'18.82"E	23	34°32'19.82"N119°42'18.20"E
4	34°59'15.86"N119°47'26.26"E	14	34°44'46.27"N119°37'12.70"E	24	34°40'40.11"N119°42'23.17"E
5	34°42'42.91"N119°41'34.92"E	15	34°41'24.37"N119°34'09.43"E	25	34°36'43.98"N119°33'57.63"E
6	34°38'17.17"N119°38'00.08"E	16	34°39'24.12"N119°36'33.05"E	26	34°37'57.96"N119°34'30.48"E
7	34°36'13.98"N119°35'43.84"E	17	34°43'02.51"N119°38'29.32"E	27	34°39'26.66"N119°34'45.00"E
8	34°51'06.62"N120°01'06.66"E	18	34°35'34.23"N119°36'44.66"E	28	34°37'41.43"N119°35'58.95"E
9	34°37'48.48"N119°48'43.39"E	19	34°39'57.74"N119°44'50.55"E	29	34°37'15.01"N119°37'15.46"E
10	34°34'40.69"N119°44'59.25"E	20	34°37'13.56"N119°41'07.21"E	30	34°38'51.00"N119°31'32.21"E

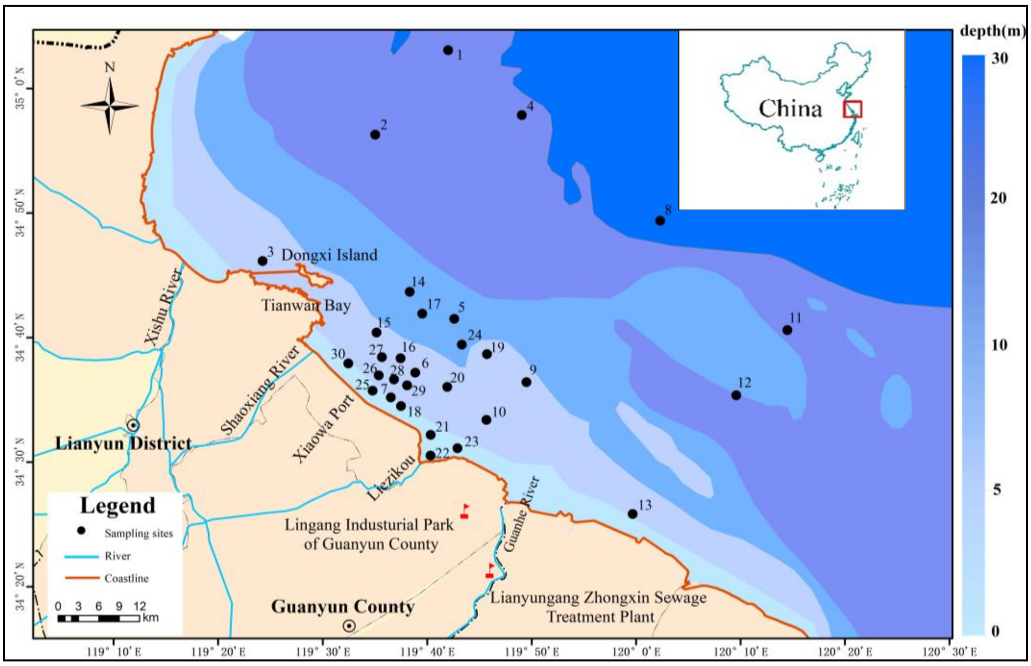

Figure 1. *Locations of surface sediment sampling sites in Xuwei-Liezikou offshore area*

The collection of sediment samples was carried out on October 10th, 2014. The methods used for the sample collection on site, the pretreatment and the preservation were in strict accordance with the *Marine Monitoring Regulations of China* (GB17378.4-2007) (AQSIQ, 2007). Samples that could not be analyzed on site were stored in a dark place after steps concerning subpackage, pre-treatments, freeze or sealing with the added fixing agent, and wrapping with plastic bags. Samples that need to be investigated were transported to the laboratory by the responsible person after the field sampling. Sediment samples were analyzed and tested at Nantong Marine

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DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

Environmental Monitoring Center. The concentrations of Cu, Zn, Pb, Cd and Cr were determined by atomic absorption spectrometer (Varian AA240FS), and for As and Hg were measured by atomic fluorescence spectrometry (Beijing Jitian Instrument Co. AFS-930).

Evaluation method

Statistical evaluation

The standard deviation (SD) and coefficient of variation (CV) were calculated for sediment samples in Xuwei-Liezikou offshore area. The inverse distance weight method was used to carry out spatial interpolation of heavy metals, and ArcGIS10.2 spatial analysis module was used to draw the spatial distribution figures of heavy metal contents.

Geo-accumulation index (I_{geo})

Geo-accumulation index (I_{geo}), originally defined by Müller (1969), has been widely used to assess heavy metal pollution in the environment (Wang et al., 2015; Malvandi, 2017; Nethaji et al., 2017; Naifar et al., 2018). The formula used for the calculation of I_{geo} is as follows:

$$I_{geo} = \log_2 \frac{C_n}{1.5B_n} \quad \text{(Eq.1)}$$

where C_n and B_n are the actual measured content of the heavy metal and the natural background value of the heavy metal, respectively. In this study, the background values of heavy metals in coastal soil in Jiangsu Province were used for B_n (Chen et al., 1985). Its degree of pollution of Müller (1981) is shown in *Table 2*.

Pollution load index (PLI)

Pollution load index (*PLI*) is an evaluation method proposed by Tomlinson and others in the study of heavy metal pollution level classification (Tomlinson et al., 1980; Badr et al., 2009), and it is convenient to apply. The index is composed of various heavy metal components in the study area. It can reflect the contribution of various heavy metals to pollution and the changing trend of heavy metals in space. The equation is shown as follows:

$$C_f^i = C_n^i / C_0^i \quad \text{(Eq.2)}$$

$$PLI = \sqrt[n]{C_f^1 \times C_f^2 \times C_f^3 \times \cdots \times C_f^n} \quad \text{(Eq.3)}$$

where C^i_f is the pollution coefficient of the heavy metal i; C_n^i is the measured concentration value of heavy metal i; C_0^i is the background value for heavy metal i. In this study, the natural background values of heavy metals in coastal soil in Jiangsu Province are adopted for C_0^i (Chen et al., 1985); n is the number of heavy metals; PLI is

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DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

the pollution load index at a certain sample site. The pollution load index is generally divided into 4 grades (Zhu et al., 2013; Maanan et al., 2015), as shown in *Table 2*.

Potential ecological risk index

In 1980, Swedish scholar Lars Hakanson proposed a potential ecological risk index method for evaluating heavy metal pollution and ecological risk based on sedimentology principles (Hakanson, 1980). This evaluation method comprehensively considers the toxicity of heavy metals, the difference of background values of heavy metals, the sensitivity of heavy metal pollution under the effect of evaluation areas, and the general rule concerning the migration and transformation of heavy metals in sediments, the calculation equations can be express as follows:

$$C_f^i = C_n^i / C_0^i \quad \text{(Eq.4)}$$

$$C_{RI} = \sum_{i}^{n} C_f^i \quad \text{(Eq.5)}$$

$$E_r^i = T_r^i \times C_f^i \quad \text{(Eq.6)}$$

$$E_{RI} = \sum_{i=1}^{m} E_r^i \quad \text{(Eq.7)}$$

where the meanings of C^i_f, C_n^i and C_0^i are the same as in *Eq.2*; n is the number of heavy metals; C_{RI} is the comprehensive contamination coefficient; E_r^i is the potential ecological risk index for heavy metal i; T_r^i is the heavy metal toxicity response coefficient that reflects the toxicity level of heavy metals and the sensitivity of organisms to heavy metal pollution, the toxicity response coefficient for As, Hg, Cu, Pb, Zn, Cd and Cr are 10, 40, 5, 5, 1, 30 and 2, respectively (Hakanson, 1980); E_{RI} is the comprehensive potential ecological risk index of the heavy metals in the study area. The relationship among the evaluation index of heavy metal pollution, its degree of pollution and the degree of potential ecological risk of Hakanson (1980) are shown in *Table 2*.

Results and discussion

Concentration of heavy metals in Xuwei-Liezikou offshore area

The contents of heavy metals in the surface sediments of Xuwei-Liezikou offshore area are showed in *Table 3*. The concentrations (mg/kg) of As, Hg, Cu, Pb, Zn, Cd and Cr were in the ranges of 4.67~17.90, 0.011~0.062, 10.00~32.30, 14.40~33.50, 40.00~84.70, 0.049~0.211 and 30.10~74.70, with the averages of 12.54, 0.03, 19.84, 22.31, 66.80, 0.11 and 42.67, respectively. The mean concentrations for these metals were ranked as follows: Zn＞Cr＞Pb＞Cu＞As＞Cd＞Hg. All the heavy metals contents were lower than Class I sediment category according to the Chinese Marine Sediment Quality standard criteria (GB 18668-2002) issued by the Administration of Quality Supervision, Inspection and Quarantine (AQSIQ, 2002), indicating that the sediment environment in the study area was generally good.

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***Table 2.** Standard for geo-accumulation index, potential ecological risk index and potential ecological risk index of heavy metals*

I_{geo}	**Pollution level**	***PLI***	**Pollution level**	C^i_f	**Single-factor pollution level**	C_{RI}	**Comprehensive pollution level**	E_r^i	**Single-factor ecological risk level**	E_{RI}	**Comprehensive potential ecological risk level**
≤0	Unpolluted	＜1	unpolluted	＜1	Low	＜8	Low	＜40	Low	＜150	Low
0～1	Unpolluted to moderate	1～2	moderate	1～3	Moderate	8～16	Moderate	40～80	Moderate	150～300	Moderate
1～2	Moderate	2～3	strong	3～6	High	16～32	Heavy	80～160	Considerable	300～600	Considerable
2～3	Moderate to strong	≥3	extreme	≥6	Severe	≥32	Severe	160～320	High	≥600	Severe
3～4	Strong							≥320	Severe		
4～5	Strong to extreme										
＞5	Extreme										

***Table 3.** Descriptive statistic of heavy metal concentrations in surface sediments in Xuwei-Liezikou offshore Area. Comparison of heavy metal concentrations of surface sediments in the study area and other representative areas in China (unit: mg/kg)*

		As	**Hg**	**Cu**	**Pb**	**Zn**	**Cd**	**Cr**	**References**
Study Area	Range	4.67~17.90	0.011~0.062	10.00~32.30	14.40~33.50	40.00~84.70	0.049~0.211	30.10~74.70	This study
	Mean	12.54	0.03	19.84	22.31	66.80	0.11	42.67	
	SD	3.523	0.013	5.200	4.994	11.342	0.042	10.357	
	CV (%)	28.10	41.89	36.21	22.39	16.98	36.54	24.27	
South Jiangsu Province		na	na	19.1	19.7	62.6	0.12	72.8	Qiu et al., 2018
Laizhou Bay		7.1	0.04	10.99	13.37	50.63	0.19	32.69	Zhang and Gao, 2015
Rizhao offshore area		17.54	0.02	15.92	29.23	42.84	0.08	43.25	Song et al., 2017
Guangdong coastal region		20.83	0.13	43.83	44.29	139.93	0.38	86.97	Zhao et al., 2016
CMSQ- I		20	0.2	35	60	150	0.5	80	AQSIQ, 2002
Background value		7.38	0.023	15.02	11.40	47.15	0.042	60.11	Chen et al., 1985

CMSQ- I is Class I sediment category of the Chinese Marine Sediment Quality standard criteria (GB 18668-2002) issued by the Administration of Quality Supervision, Inspection and Quarantine (AQSIQ, 2002)

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Compared to the background values of heavy metals in coastal soil in Jiangsu Province (Chen et al., 1985), the mean concentrations of Cd, Pb, As, Zn, Cu and Hg were higher than the background values in the Jiangsu province except for Cr, which were 2.62, 1.96, 1.70, 1.42, 1.32 and 1.30 times of their respective background values, indicating there were obvious accumulations of these heavy metals in surface sediments. The standard deviation (SD) and coefficient of variation (CV) indicated moderate variability for As, Pb, Zn, Cr (15%＜CV＜36%) and high variability for Hg, Cu, Cd (CV＞36%) according to Phil-Eze (2010). These coefficients of variation of the heavy metals were higher, and the heavy metal concentrations varied over a wide range, which suggested an imbalance in the spatial distribution.

We compared the heavy metal concentrations in the study area with those other representative areas in China are also listed in *Table 3*. The mean concentrations of all heavy metals in study area were lower than those found in sediments of Guangdong coast. The mean Cu and Zn concentrations in study area were higher than those in South Jiangsu Province, Laizhou Bay and Rizhao offshore area. The mean concentrations of Cd and Cr in study area were lower than those in South Jiangsu Province. The mean As, Pb and Cr concentration was higher in study area than in Laizhou Bay, but lower than in Rizhao offshore area. The mean Cd and Hg concentration was higher in study area than in Rizhao offshore area, but lower than in Laizhou Bay.

Spatial distribution of the heavy metal concentrations

The spatial distributions of heavy metals in surface sediments of Xuwei-Liezikou offshore area are presented in *Fig. 2*.

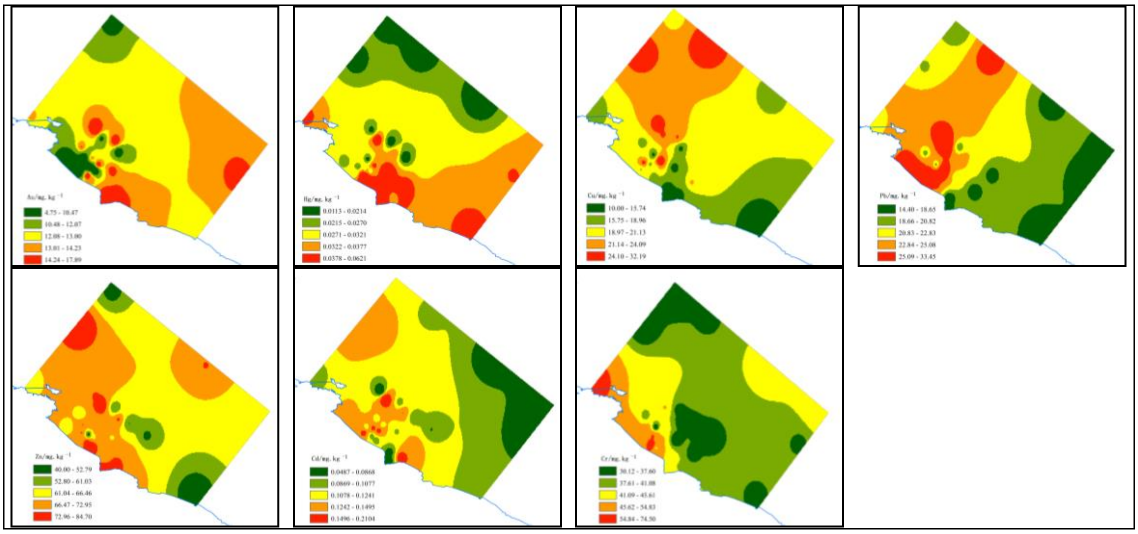

Figure 2. *Spatial distribution of heavy metals in Xuwei-Liezikou offshore Area*

The spatial distributions of Zn, Hg, Cd and As were similar, these elements had high concentration values in the western region of the study area, which was consistent with the major rivers inlets. The high concentration values of Pb were in the northern of the study area mainly located in the Xuwei Port. Cu tended to be higher in the northwest and lower in the southeast. In general, the heavy metal high-values areas, the western and northern region of the study area, were mainly located at the river estuaries and

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Xuwei Port. The result showed that land-based pollutants and ship pollutants had an important influence on heavy metal pollution in the study area.

Assessment of heavy metal contamination

Geo-accumulation indices of the heavy metals

The geo-accumulation indices (I_{geo}) of the heavy metals in Xuwei-Liezikou offshore area are shown in *Table 4*. The I_{geo} values of As, Hg, Cu, Pb, Zn, Cd and Cr were in the ranges of -1.25~0.69, -1.61~0.85, -1.17~0.52, -0.25~0.97, -0.82~0.26, -0.37~1.74 and -1.58~-0.27, with the averages of 0.11, -0.26, -0.23, 0.35, -0.11, 0.76 and -1.12, respectively. The mean I_{geo} values were ranked as follows: Cd>Pb>As>Zn>Cu>Hg>Cr. According to Müller (1981), the mean I_{geo} values in the study area ranged form -2 to 1, showing unpolluted and unpolluted to moderate polluted. The result indicated that the study area has not been obviously polluted as a whole. However, the I_{geo} values of Cd ranged from -0.37 to 1.74, and 10% of the sampling sites were unpolluted, 33% were unpolluted to moderately polluted, 57% were moderately polluted. The sampling sites that were moderately polluted for Cd were mainly distributed in the western region of the study area (*Fig. 3a*), indicating that the land-based pollutants had an important impact on the sediment environment in the study area. Meanwhile, it should be noted that the I_{geo} values form 87% of the sampling sites for Pb ranged form 0 to 1, indicating unpolluted to moderately polluted, the high I_{geo} values were located in the Xuwei port (*Fig. 3b*). Overall, the surface sediments were a low degree of pollution throughout the study area, and Cd and Pb were the main pollution factors.

Table 4. *Geo-accumulation indices (I_{geo}) and contamination degree of surface sediments*

I_{geo}	As	Hg	Cu	Pb	Zn	Cd	Cr
Min	-1.25	-1.61	-1.17	-0.25	-0.82	-0.37	-1.58
Max	0.69	0.85	0.52	0.97	0.26	1.74	-0.27
Mean	0.11	-0.26	-0.23	0.35	-0.11	0.76	-1.12
Compared with I_{geo} (% of sample in each class)							
$I_{geo}\leq 0$	30%	57%	77%	13%	63%	10%	100%
$0<I_{geo}\leq 1$	70%	43%	23%	87%	37%	33%	0%
$1<I_{geo}\leq 2$	0%	0%	0%	0%	0%	57%	0%

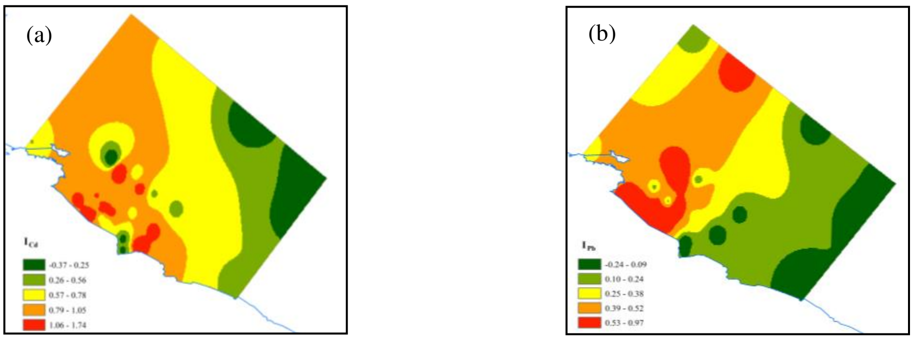

Figure 3. *Spatial distribution of the geo-accumulation indices for Cd and Pb*

Pollution load indices of the heavy metals

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

The pollution load indices (*PLI*) of the heavy metals for surface sediments of Xuwei-Liezikou offshore area are presented in *Table 5*. The *PLI* values in the study area ranged from 1.15 to 1.91, with the averages of 1.44, the Xuwei-Liezikou offshore area was considered to be moderately polluted. 100% of all sample sites showed moderately polluted, indicating that the study area was affected by human activities. The high *PLI* values were in the western and northern region of the study area (*Fig. 4a*). The result was similar to the distribution characteristics of main pollution elements.

Table 5. *The pollution load indices of the heavy metals in the study area*

Sample	*PLI*	Sample	*PLI*	Sample	*PLI*
1	1.18	12	1.35	23	1.61
2	1.51	13	1.15	24	1.41
3	1.55	14	1.40	25	1.52
4	1.36	15	1.43	26	1.49
5	1.28	16	1.27	27	1.48
6	1.84	17	1.91	28	1.44
7	1.89	18	1.63	29	1.40
8	1.15	19	1.18	30	1.41
9	1.28	20	1.45	Min	1.15
10	1.54	21	1.30	Max	1.91
11	1.31	22	1.51	Mean	1.44

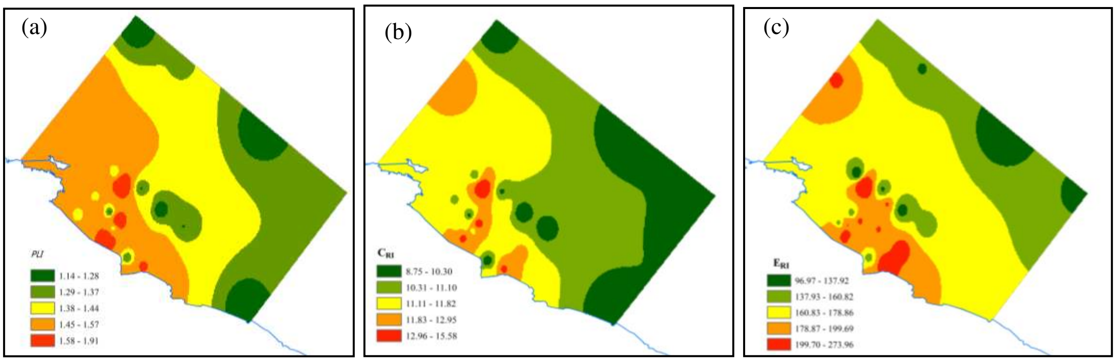

Figure 4. *Spatial distribution of PLI,* C_{RI} *and* E_{RI} *of heavy metals*

Potential ecological risk index in Xuwei-Liezikou offshore area

The contamination factor (C^i_f) values for the heavy metals in Xuwei-Liezikou offshore area are showed in *Table 6*. The mean C^i_f values of As, Hg, Cu, Pb, Zn, Cd and Cr were 1.70, 1.30, 1.32, 1.96, 1.42, 2.62 and 0.71, ranked as follows: Cd>Pb>As>Zn>Cu>Hg>Cr. 67% of the sampling sites for Cd were moderate pollution, while 33% (mainly located in the nearshore region) showed high pollution. 100% of the sampling sites for Pb were moderate pollution, and the high C^i_f values for Pb located in Xuwei Port. The C^i_f values of others elements showed low to moderate pollution. The C_{RI} values of the study area ranged from 8.75 to 15.6, with the mean of 11.21, indicating a moderate pollution. The high C_{RI} value distributed in the western and northern regions of the study area, mainly located in the estuary and port regions (*Fig. 4b*).

The potential ecological risk indices for the heavy metals in Xuwei-Liezikou offshore area are showed in *Table 7* and *Fig. 4c*. The mean E_r^i values for As, Hg, Cu, Pb, Zn, Cd and Cr were 17.03, 55.42, 6.60, 9.78, 1.42, 81.78 and 1.42, ranked as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

follows: Cd > Hg > As > Pb > Cu > Cr=Zn. The mean E_r^i values for Cd showed a considerable ecological risk, Hg was a moderate ecological risk, the others elements were a low ecological risk. The E_{RI} values ranged from 96.97 to 274.62, with an average value of 173.46, which were a low to middle ecological risk (*Table 2*). The high E_{RI} values distributed in the western region of the study area (*Fig. 4c*), mainly located in the estuary regions, which was similar to the Cd distribution characteristics (*Fig. 2*). These results showed that the risks were mainly from Cd.

Table 6. *Contamination factor of the heavy metals in surface sediments*

	C^i_f							C_{RI}
	As	**Hg**	**Cu**	**Pb**	**Zn**	**Cd**	**Cr**	
Min	0.63	0.49	0.67	1.26	0.85	1.16	0.50	8.75
Max	2.43	2.71	2.15	2.94	1.80	5.02	1.24	15.60
Mean	1.70	1.30	1.32	1.96	1.42	2.62	0.71	11.21
Compared with C^i_f / C_{RI} (% of sample in each class)								
$C^i_f \leq 1$ ($C_{RI} \leq 8$)	10%	33%	23%	0%	3%	0%	93%	0%
$1 < C^i_f \leq 3$ ($8 < C_{RI} \leq 16$)	90%	67%	77%	100%	97%	67%	7%	100%
$3 < C^i_f \leq 6$	0%	0%	0%	0%	0%	33%	0%	0%

Table 7. *Potential ecological risk indices of the heavy metals in surface sediments*

	E_r^i							E_{RI}
	As	**Hg**	**Cu**	**Pb**	**Zn**	**Cd**	**Cr**	
Min	6.33	19.65	3.33	6.32	0.85	34.71	1.00	96.97
Max	24.26	108.35	10.75	14.69	1.80	150.71	2.49	274.62
Mean	17.03	55.42	6.60	9.78	1.42	81.78	1.42	173.46

Conclusion

The mean concentrations (mg/kg) of As, Hg, Cu, Pb, Zn, Cd and Cr were 12.54, 0.03, 19.84, 22.31, 66.80, 0.11and 42.67 in surface sediments of Xuwei-Liezikou offshore area, respectively. The averages of Cd, Pb, As, Zn, Cu and Hg were higher than the background values in the Jiangsu province except for Cr, which were 2.62, 1.96, 1.70, 1.42, 1.32 and 1.30 times of their respective background values, indicating there were obvious accumulations of these heavy metals of the study area. The spatial distributions of heavy metals showed that Zn, Hg, Cd and As had high concentration values in the western region of the study area, which was consistent with the rivers inlets, and the high concentration values of Pb were in the northern of the study area mainly located in the Xuwei Port. The results of contamination assessment showed the study area was moderately polluted, and the main pollution areas were in the western and northern region. The pollution mainly came form Cd and Pb. For the main pollution factors, further studies on their sources and impacts can be conducted to provide data for environmental protection. The study area suffered from a level between low and moderate risks, and Cd was an important factor causing ecological risks. The estuary areas where were prone to ecological risk should be paid attention to.

Acknowledgements. This project was funded by the National Natural Science Foundation of China (41373112).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28812892

EFFECTS OF DIETARY *VACHELIA KARROO* LEAF MEAL INCLUSION ON MEAT QUALITY AND HISTOLOGICAL PARAMETERS IN PEDI BUCKS FED A *SETARIA VERTICILLATA* HAY-BASED DIET

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(Received 1st Nov 2018; accepted 20th Dec 2018)

Abstract. The objective of this study was to investigate the effect of dietary *Vachellia karroo* leaf meal inclusion on growth performance, meat quality and histological parameters in bucks**.** A total of thirty yearling Pedi bucks with a mean initial body weight of 16.7 ± 3.3 kg were randomly assigned to five treatments in a completely randomized design. *Vachellia karroo* leaf meal inclusion levels of 20, 25, 30, 40 and 50% were fed to the animals for 60 days. The results showed that dietary treatments had no effect on the final live weights and average daily gains of bucks. Similarly, yields of different carcass components and meat sensory attributes were not affected by the *V. karroo*. Bucks fed 25% *V. karroo* leaf meal had higher meat L* values than those on diets 30 or 40% inclusion levels. The livers of bucks fed 20, 25 or 30% *V. karroo* leaf meal were not adversely affected. However, severe hepatocyte degeneration was noticed in the liver tissues of bucks fed 40 or 50% leaf meal. Dietary *V. karroo* did not cause any serious damage to the kidneys of the bucks. Thus, *V. karroo* leaf meal inclusion levels of 20 to 30% are recommended.
Keywords: *goats, tannin, carcass characteristics, sensory evaluation, acacia*

Introduction

Pedi goats in communal areas of Limpopo province, South Africa depend on *Vachellia karroo* (*Acacia karroo*) leaves, particularly during the dry season (Jamala et al., 2013). *Vachellia karroo* leaves contain high levels of extracted condensed tannins ranging from 55 – 110 g/kg DM and have potent antioxidant properties (Mokoboki et al., 2005; Moon et al., 2014). Antioxidant activity of these plants is attributed to their phenolic compound content (Velasco and Salinas, 2011). Antioxidant activity of plant extracts such as condensed tannins have positive effect on meat quality and oxidative stability (Moyo et al., 2012; Qwele et al., 2013). On the other hand, high condensed tannin contents in the diet negatively affect nutrient availability (Gxasheka et al., 2015). Reports on the effects of tanniferous diets on small ruminant productivity and meat quality are not conclusive. Some authors found that tannin-rich diets positively influence chevon quality (Priolo and Vasta, 2007; Mapiye et al., 2009; Marume, 2010; Bakare and Chimonyo, 2011; Ngambu et al., 2012, 2013), whereas, others found no clear evidence (Mapiye et al., 2010). Additionally, *Vachellia karroo* leaf meal inclusion levels for optimum Pedi bucks meat quality were not found in the literature.

Kidney and liver are vital organs in the animal's body and play vital role in the detoxification process. Dysfunction in liver and kidney may lead to diseases such as ascites and uremia (Karimi et al., 2014). Ruminants browsing on tanniferous forage have adapted rumen micro-organisms that detoxify many, but not all plant secondary

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_28932909

metabolites (Cheeke, 1988). A large intake of tannins in the diet may cause kidney irritation and liver damage due to the detoxification process as a consequence of increased enzymatic demand (Van Soest, 1994; Mahgoub et al., 2008). Previous studies have reported a negative effect of condensed tannins on the histopathology of Boer goats (Mbatha et al., 2002). According to the authors, increased dietary tannin levels induced thickening and/or keratinization of epithelial tissue in the reticulum, rumen, omasum and abomasum. Increased tannin levels also resulted in a loss of epithelial cells, erosion of microvilli and shortened villi height in the duodenum (Mbatha et al., 2002). Karimi et al. (2014) reported histological changes in the kidney and liver parenchyma of GhezelxArkhar Merino crossbred lambs fed low tannin sorghum grain. Goats are predominantly browsers and they are able to consume large amounts of tannin-rich forage, which may increase the risk of exposure to sub-clinical systemic toxicity (Gilboa, 1995). Hence, monitoring of these organs in nutritional studies is imperative. There is paucity of information on the effect of tanniferous diets on the liver and kidney of goats. It is hypothesized that increased dietary tannin may result in variable damage of these organs. This study investigated the effects of *Vachellia karroo* leaf meal inclusion on meat quality and histology of livers and kidneys of Pedi bucks.

Materials and methods

Study site

The study was conducted at the University of Limpopo Experimental farm, South Africa (latitude 27.55° S and longitude 24.77° E). The ambient temperatures at the study site range between 20 and 36 °C during summer and between 5 and 25 °C during winter. Mean annual rainfall is 446.8 mm with the dry season occurring between April and October and the rainy season occurring between November and March.

Collection, drying and storage of plant material

Vachellia karroo leaves were harvested during the summer months (November-January). Branches of each shrub were removed manually and placed in a shed. Leaves were allowed to air dry on the branches and then removed by carefully beating the branches with sticks. The leaves were stored in air-tight bags until feeding time. Detailed reviews on the botanical description of *V. karroo* have been described by Barnes et al. (1996). *Setaria verticillata* (L.) P. Beauv. is a perennial grass belonging to the tribe Panacea and is widely grown by commercial farmers in Limpopo Province of South Africa. The grass is well grazed during summer and is suitable for hay making. Botanical authentication of the plant materials was done at the Larry Leach Herbarium, Department of Biodiversity, University of Limpopo.

Animal, management, diet and experimental design

All animal procedures were approved by the Animal Research Ethics Committee of the University of Limpopo, South Africa. A total of thirty yearling Pedi bucks (*Capra hircus*), (a local indigenous breed in Limpopo province of South Africa) with a mean initial body weight of 16.7 ± 3.3 kg were randomly assigned to five treatments in a completely randomized design. Each treatment had three replicates with two goats per replicate. The animals were housed in individual holding pens (1 × 3 m^2) that were installed in a well ventilated shed with one side open to natural light and roofed to

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protect animals against the sun and rain. Yearling Pedi bucks were selected because they are the ones fattened for meat in the province. All animals were drenched with an anthelmintic (Valbazen® broad spectrum dewormer, manufactured by Pfizer Animal NY, USA) and sprayed with Diazintol® (Alfasan International, Holland) before the start of the experiment.. The goats were individually fed ad libitum once a day at 8:00 am, allowing a 15% refusal of each diet (Kaitho et al., 1996) and they had free access to clean water and a salt block. Prior to the trial, the animals were given the dietary treatment for 14 d ad libitum for adaptation, and the feeding trial lasted for 60 d. The grass and *V. karroo* leaves were chopped and thoroughly mixed to avoid diet selection by the animals when fed (*Table 1*). *Vachellia karroo* leaf meal inclusion levels of 20, 25, 30, 40 and 50 were used in the present study. These inclusions include low and high tannin levels as indicated in the literature (Brown et al., 2017). Feed intake was recorded on a daily basis. Intake was calculated by subtracting leftovers from the feed given. Dry matter values of the feeds and feed refusals were determined. The goats were weighed three times, at the start of the experiment, on day 54 and on the 60th day when data collection ended. Goats were weighed before morning feeding to avoid feed effect. Average daily gains were calculated as differences between final and initial body weights divided by number of feeding days.

Table 1. *Feed composition of the experimental diets*

Diets		
Diet code	***Vachellia karroo*** **(%)**	***Setaria verticillata*** **hay (%)**
$V_{20}S_{80}$	20	80
$V_{25}S_{75}$	25	75
$V_{30}S_{70}$	30	70
$V_{40}S_{60}$	40	60
$V_{50}S_{50}$	50	50

Nutrient composition of *V. Karroo* and *S. verticillata* grass was previously reported by Brown and Ng’ambi (2017). The same applies to the nutritive values of dietary mixtures of the browse and grass hay used in the present study (*Tables 2* and *3*).

Table 2. *Nutrient composition of Vachellia karroo leaves and Setaria verticilllata hay*

Nutrient	*Vachellia karroo*	*Setaria verticillata hay*
Dry matter (g/kg)	971	962
Organic matter (g/kg DM)	921	914
Crude protein (g/kg DM)	127	79
Acid detergent fibre (g/kg DM)	325	507
Neutral detergent fibre (g/kg DM)	380	779
Condensed tannins (%DM)[1]	2.0	0
Total phenols (%DM)[2]	1.9	0

[1]Condensed tannins as percentage DM leucocyanidin equivalent
[2]Expressed as tannic acid equivalent (%)

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Table 3. *Chemical composition of the dietary mixtures of Vachellia karroo leaves and Setaria verticillata grass hay*

	Diet				
Nutrient	**$V_{20}S_{80}$**	**$V_{25}S_{75}$**	**$V_{30}S_{70}$**	**$V_{40}S_{60}$**	**$V_{50}S_{50}$**
Dry matter (g/kg)	952	958	940	952	970
Organic matter (g/kg DM)	915	915	916	916	917
Crude protein (g/kg DM)	89	91	93	98	103
Acid detergent fibre (g/kg DM)	470	461	452	433	415
Neutral detergent fibre (g/kg DM)	699	679	659	613	579
Condensed tannins (%DM)[1]	0.4	0.5	0.6	0.8	1.0
Total phenols (%DM)[2]	0.3	0.4	0.5	0.7	0.9

[1]Condensed tannins as percentage DM leucocyanidin equivalent
[2]Expressed as tannic acid equivalent (%)

Slaughter and carcass evaluation

Prior to slaughter and muscle sample collection, the goats were fasted for 16 h with free access to drinking water and their body weight was recorded immediately after fasting. The animals were slaughtered humanely in the abattoir facility at the University Experimental Farm. The weights of the hot carcass and internal organs were recorded. The gastro-intestinal (GI) tract, including alimentary canal, reticulo-rumen and intestines were weighed after the removal of ruminal contents. Carcasses were chilled at 4 °C for 24 h. After this period, cold carcass weight (CCW) was determined.

Meat quality determination

The pH value at 1 h in *Longissimus thoracic et lumborum* muscle was measured after evisceration by using a pH meter equipped with a penetrating electrode (Crison pH25, CRISON instruments S. A., Alella, Spain). Before measurement, pH meter was calibrated with standard pH buffer (pH 4.0, 7.0 and 10.0). At 24 h postmortem, the entire *Longissimus thoracic et lumborum* was removed for pH, meat color and cooking loss. Meat color (L* = lightness, a* = redness and b* = yellowness) was measured using a spectrophotometer with a D-65 illuminant and an aperture size of 50 mm (45/0 BYK-Gardener instrument GmbH, Germany). Before measurement, the spectrophotometer was calibrated with a white tile (model CR-A43). Three readings were taken by rotating the instrument 90° between measurements, in order to obtain a representative average value of the color, and avoiding connective tissues and intramuscular fat. The readings were taken 1 and 24 h post-mortem. The meats were allowed to bloom for 1 h prior to color analysis.

For cooking loss analysis, blocks of *longissimus thoracic et lumborum* muscle, measuring approximately 7 × 4 × 4 cm, were used to determine cooking loss (Babikerm et al., 1990) and shear force values (Chrystall et al., 1994). The muscle was weighed, placed in a water tight PVC plastic bag and cooked in a water bath at 85 °C for 45 min, until an internal temperature of 70 °C was attained. The samples were cooled and re-weighed. Cooking loss (CL) was calculated using the following

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formula: Cooking loss % = [(weight before cooking – weight after cooking) / weight before cooking] × 100 as described by Ding et al. (2010). After measurement of cooking loss, cooked samples were used to determine meat Warner Bratzler shear force. Three sub-samples (cut parallel to the muscle fibers with a cross-section of 1 × 1 cm and at least 3 cm long) were removed from each cooked muscle. The sub-samples were sheared perpendicular to the fiber direction with an Instron Universal Testing Machine (Model 3344, Instron Industrial Products, GC, USA) equipped with a Warner-Bratzler (WB) shear force apparatus (crosshead speed at 400 mm/min, one shear in the center of each core). The measurements were read in Newton. Dressing percentage was calculated as the ratio of hot carcass weight divided by slaughter body weight and the result multiplied by 100. Water holding capacity (WHC) of the meat was measured as the amount of water expressed from a fresh meat sample (1 g) held under pressure (60 kg) using the filter-paper press method (Trout, 1988).

Meat sensory evaluation

Meat samples used for consumer sensory evaluation were obtained from each carcass and were cut 24 h post-slaughter. The meat samples were cut into cubes (2 × 2 cm), which were placed in watertight PVC plastic bags and cooked in a boiling water bath at a temperature of 85 °C for 45 min (Babikerm et al., 1990). Salt was added to taste. Twenty trained consumer panelists from the University of Limpopo were used for the consumer sensory assessment of meat. Panelists were screened and selected following guidelines from Cross et al. (1978). The panelists were taught how to infer and record scores for each variable. The waiting period between meat sample tastings was 10 min. Distilled water was served to panelists to freshen their mouths between sub-sample assessments to avoid crossover effects. Five-point descriptive scales were used to evaluate the sensory attributes (*Table 4*).

Table 4. *Evaluation scores used by the sensory panel*

	Sensory attribute					
Score	**Tenderness**	**Juiciness**	**Flavor**	**Taste**	**Aroma**	**Overall acceptability**
1	Too tough	Too dry	Very bad flavor	Dislike extremely	Dislike extremely	Dislike extremely
2	Tough	Dry	Poor flavor	Dislike	Dislike	Dislike
3	Neither tough nor tender	Neither dry nor juicy	Neither bad nor good	Neither like nor dislike	Neither like nor dislike	Neither like nor dislike
4	Tender	Juicy	Good flavor	Like	Like	Like
5	Extremely tender	Extremely juicy	Very good flavor	Like extremely	Like extremely	Like extremely

Histological analysis

Liver and kidney samples from each goat were collected and preserved in 10% neutral buffered formalin for 24 h. Subsequently, liver and kidney tissues were dehydrated using standard histological techniques in graded ethanol series and embedded in paraffin wax for histopathology examination (Sanchez-Chardi et al., 2008). From each sample, 60-65 µm sections were cut and mounted on glass slides

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before staining with haematoxylin and eosin. Slides were examined under light trinocular microscopy at 400X (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA). Each slide was photographed with a DVC digital camera (Digital Video Camera Company, Austin, TX) mounted on a BH-2. The process was as described by Sanchez-Chardi et al. (2009).

Chemical analysis

Dry matter, organic matter and crude protein were determined using the methods described by AOAC (2005). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the method of Van Soest (1994). Total phenolics were determined using Folin-Ciocalteau methods and expressed as tannic acid equivalent (% DM) (Water and Mole, 1994). Condensed tannins were determined using the Butanol-HCl method and expressed as leucocyanidin equivalent (%DM) (Porter et al., 1986). For each measurement, duplicate analyses were done.

Statistical analysis

Statistical analysis was performed using the GLM procedure of SAS (SAS, 2010). Initial live weight was fitted as covariate for carcass traits. The data are expressed as the mean ± SEM and analyzed using one-way analysis of variance (ANOVA). Fisher's Protected Least Significant Difference (LSD) test was used for the post hoc analyses. A $P < 0.05$ was considered statistically significant for all data.

Results

Nutrient composition of Vachellia karroo and Seteria verticillata grass hay

Vachellia karroo contained 127 g CP/kg DM and 921 g OM/kg DM. It also contained phenolic compounds. *Seteria verticillata* grass hay contained 79, 779 and 507 g of CP, NDF and ADF/kg DM, respectively. *Setaria verticillata* hay has no traces of phenolic compounds (*Table 2*). The nutritive values of dietary mixtures of *V. karroo* and *S. verticillata* hay are presented in *Table 3*. Diet containing 50% *V. karroo* leaf meal contained 103 g CP/kg DM. The condensed tannin and total phenolic contents were 1.0 and 0.9%, respectively. A diet containing 20% *V. karroo* leaf meal inclusion had 89 g CP/kg DM, 0.4% condensed tannin and 0.3% total phenolic content.

Feed intake and growth performance

Daily dry matter intakes were similar ($P > 0.05$) across the dietary treatments, ranging from 638 to 786 g per goat. Similarly, goats consumed the same ($P > 0.05$) daily amounts of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents. *Vachellia karroo* inclusion did not ($P > 0.05$) affect the final body weights and average daily gains of goats (*Table 5*).

Slaughter performance and internal organ weight of bucks

As shown in *Table 6*, dietary *V. karroo* leaf meal inclusion had no effect ($P > 0.05$) on hot and cold carcass weights of bucks. Similarly, the internal organ weights of bucks were not affected ($P > 0.05$) by the dietary treatments.

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***Table 5.** Effect of Vachellia karroo leaf meal inclusion level on diet intake and growth performance of yearling Pedi bucks fed a Setaria verticillata grass hay-based diet*

Variable	Treatment					SEM	*P* value
	$V_{20}S_{80}$	$V_{25}S_{75}$	$V_{30}S_{70}$	$V_{40}S_{60}$	$V_{50}S_{50}$		
Intake (g/goat/day)							
Dry matter	752	786	638	661	686	70.3	0.526
Organic matter	688	720	585	606	630	64.5	0.531
Crude protein	66	72	59	64	70	6.83	0.647
NDF	525	533	421	409	397	45.9	0.135
ADF	353	362	288	286	285	31.5	0.232
Initial body weight (kg)	16.0	17.3	13.7	16.1	15.2	2.55	0.815
Final body weight (kg)	22.8	24.1	21.4	23.8	24.6	2.61	0.800
Average daily gain (g)	113	114	129	129	157	23.8	0.204

SEM: Standard error of the means; NDF: Neutral detergent fiber; ADF: Acid detergent fiber

***Table 6.** Effects of Vachellia karroo leaf meal inclusion level on slaughter performance and internal organ weight of bucks fed a Setaria verticillata grass hay-based diet*

Variable	Treatment					SEM	*P* value
	$V_{20}S_{80}$	$V_{25}S_{75}$	$V_{30}S_{70}$	$V_{40}S_{60}$	$V_{50}S_{50}$		
Pre-slaughter weight (kg)	22.8	24.1	21.4	23.8	24.6	1.61	0.686
Hot carcass weight (kg)	7.8	8.3	6.9	8.2	8.9	0.64	0.361
Cold carcass weight (kg)	7.4	7.8	6.1	7.1	8.0	0.57	0.196
Head (g)	1882.3	1888.9	1772.8	1706.8	1987.3	141.8	0.674
Skin (g)	2092.2	2459.9	2328.3	2399.1	2486.3	210.8	0.695
Feet (g)	635.1	640.7	636.9	636.2	667.2	34.1	0.341
Kidney (g)	71.9	76.4	60.9	65.8	66.6	5.36	0.350
Liver (g)	310.0	334.8	275.8	286.8	284.8	24.87	0.487
Lung (g)	177.5	226.9	230.3	227.7	219.9	17.78	0.261
Heart (g)	106.6	96.8	96.0	96.4	119.3	8.01	0.218
Genital scrotum (g)	177.8	215.2	165.1	179.6	143.9	27.2	0.493
Empty alimentary canal (g)	1722.8	1449.7	1432.5	1792.7	1403.3	139.7	0.223
Empty reticulo-rumen (g)	585.0	543.8	458.8	555.8	446.6	52.6	0.310
Empty intestine (g)	790.8	561.2	636.6	862.9	544.0	92.5	0.125

SEM: Standard error of the means

Meat quality and consumer acceptability

As shown in *Table 7*, there was no observed effect of dietary *V. karroo* leaf meal inclusion on the $pH_{1\ h}$ and $pH_{24\ h}$. Dietary *V. karroo* did not affect (P > 0.05) the meat color 1 h post-mortem, water holding capacity, shear force, cooking loss and dressing percentage. However, there was treatment effect on meat color at 24 h post-mortem. Goats fed 25% *V. karroo* leaf meal had higher (P < 0.05) L* intensity than those on diets 30 or 40% inclusion levels. On average, no differences (P > 0.05) existed among treatments with respect to tenderness, juiciness, flavor, taste, aroma and overall acceptability (*Table 8*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 7. *Effect of Vachellia karroo leaf meal inclusion level on meat quality of bucks fed a Setaria verticillata grass hay-based diet*

Variable	Treatment					SEM	*P* value
	$V_{20}S_{80}$	$V_{25}S_{75}$	$V_{30}S_{70}$	$V_{40}S_{60}$	$V_{50}V_{50}$		
Muscle pH							
pH_1	6.9	6.9	6.9	6.7	6.6	0.202	0.673
pH_{24}	5.9	5.9	5.7	5.8	5.9	0.156	0.744
Meat color 1 h post-mortem							
L*[1]	34.4	27.6	29.7	28.0	25.0	2.244	0.120
a*[2]	14.8	17.4	15.3	15.9	18.1	1.555	0.547
b*[3]	6.9	6.9	6.5	5.7	5.7	0.613	0.503
Meat color 24 h post-mortem							
L*[1]	34.2^{ab}	37.3^{a}	27.2^{b}	29.2^{b}	33.6^{ab}	2.295	0.041
a*[2]	16.4	15.6	20.1	17.6	15.1	2.071	0.493
b*[3]	8.3	6.7	10.9	6.5	7.9	1.730	0.421
Water-holding capacity (g)							
Initial	13.6	13.2	12.9	12.1	12.1	1.993	0.294
Final	13.1	12.8	12.3	11.7	11.5	1.963	0.308
Shear force (N)	36.6	35.1	31.4	31.8	33.3	4.729	0.920
Cooking loss (%)	38.2	38.0	35.7	37.7	40.3	1.799	0.449
Dressing percentage	35.5	36.8	35.9	34.6	39.7	1.675	0.318

^{a,b}Means with different superscripts in the same row are significantly different ($P < 0.05$)
SEM: Standard error of the means
[1]Lightness
[2]Red intensity
[3]Yellow intensity

Table 8. *Consumer acceptability of meat from bucks fed dietary mixture of Vachellia karroo and Setaria verticillata grass hay*

Sensory characteristic	Treatment					SEM	*P* value
	$V_{20}S_{80}$	$V_{25}S_{75}$	$V_{30}S_{30}$	$V_{40}S_{60}$	$V_{50}S_{50}$		
Tenderness	3.18	3.22	3.83	3.09	3.41	0.345	0.553
Juiciness	3.01	3.25	3.46	2.77	3.07	0.358	0.662
Flavor	3.15	3.29	3.59	3.46	3.51	0.305	0.802
Taste	3.21	3.34	3.55	3.64	3.51	0.305	0.852
Aroma	3.16	3.20	3.55	3.49	3.35	0.286	0.792
Overall acceptability	3.26	3.49	3.84	3.63	3.40	0.322	0.676

SEM: Standard error of the means

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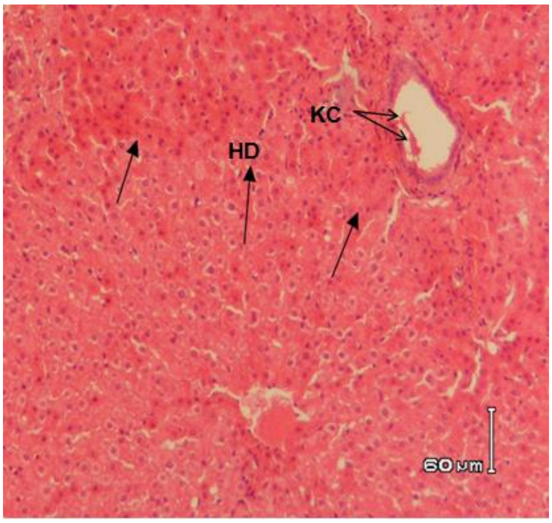
Effect of dietary Vachellia karroo on histological changes in the liver and kidney tissues of bucks

Histological analysis of the liver tissues of bucks fed 20, 25 or 30% *Vachellia karroo* leaf meal showed moderate hepatocellular hydropic degeneration, dilation of central veins and proliferation of sinusoidal kupfer cells (*Fig. 1a, b* and *c,* respectively). The hepatocytes of bucks fed diets containing 40 or 50% *V. karroo* leaf meal had severe hepatocellular hydropic degeneration with multifocal single cell and clustered cell necrosis (*Fig. 1d* and *e,* respectively). The kidney tissues of bucks fed 20, 25 or 30% *V. karroo* leaf meal had early dilatation of glomerular uriniferous spaces and mild renal tubular nephrosis (*Fig. 2a, b* and *c,* respectively), while those fed 40 or 50% *V. karroo* leaf meal had dilatation of uriniferous spaces and ectasia of some proximal convoluted tubules (*Fig. 2d* and *e*, respectively). Intra-tubular proteinaceous fluids with protein cast formation were also noticed in the kidney of bucks on diets 40 or 50% *V. karroo* leaf meal.



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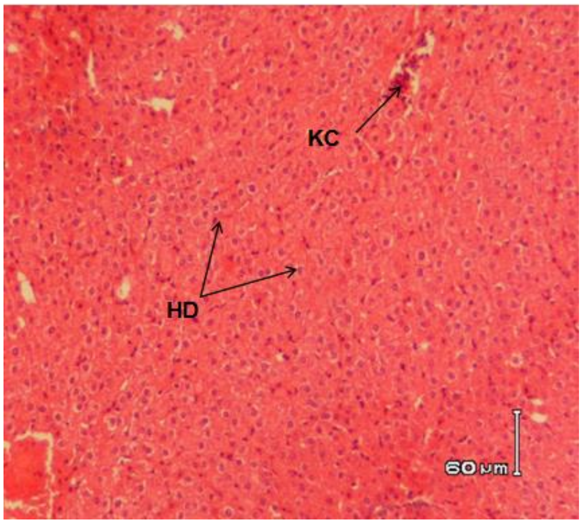

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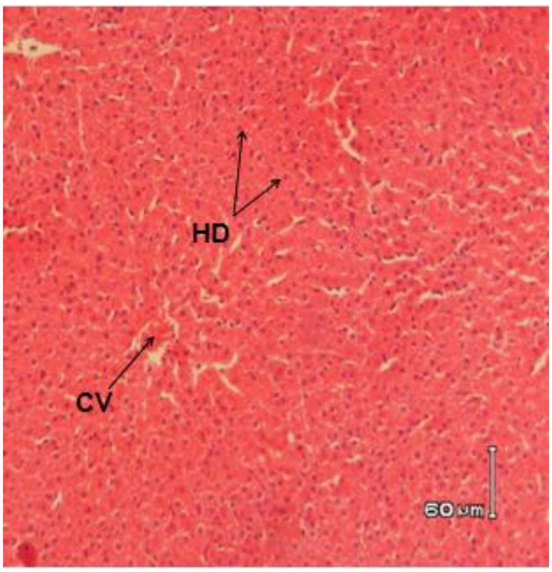

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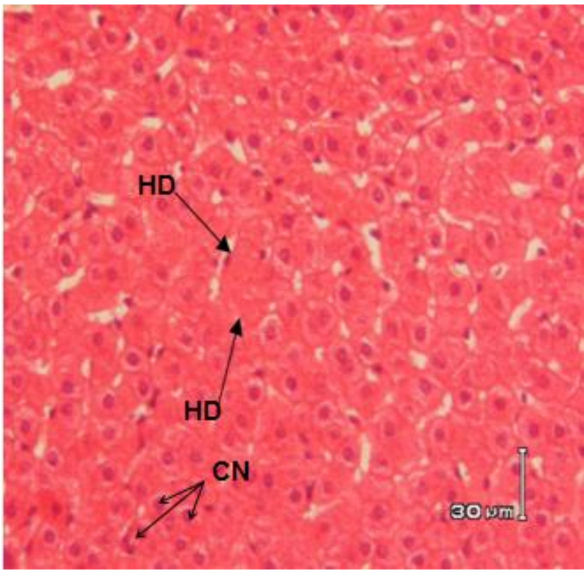

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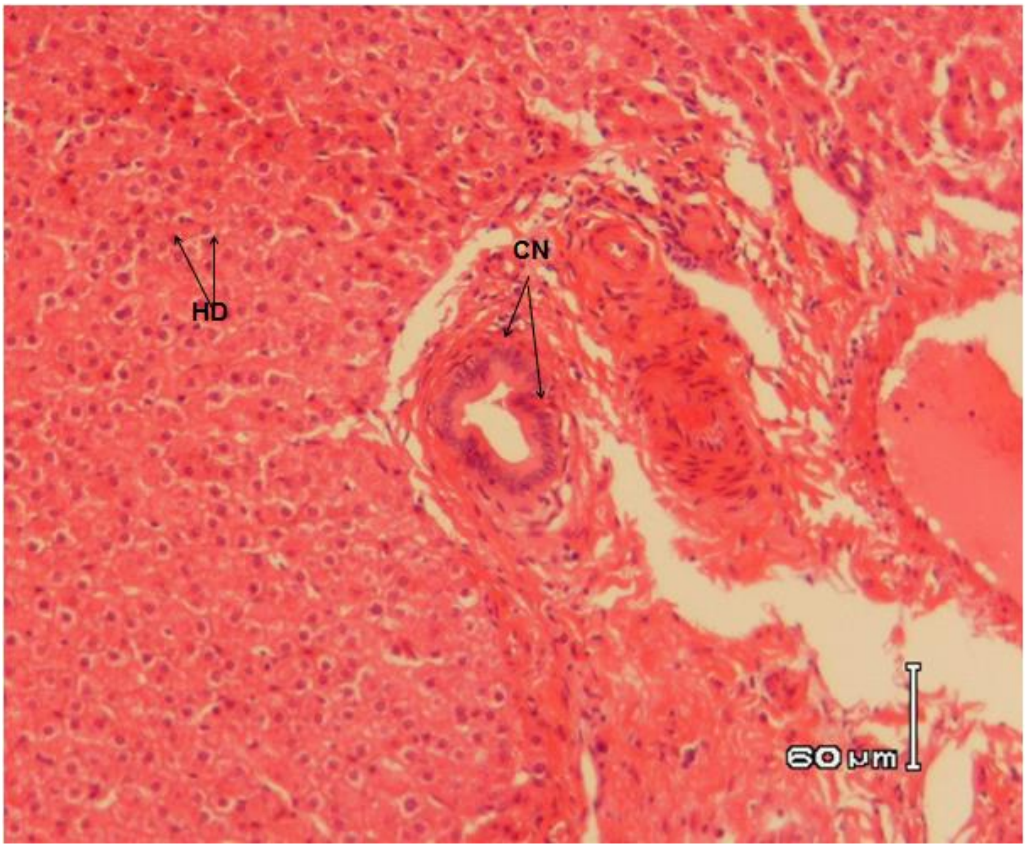
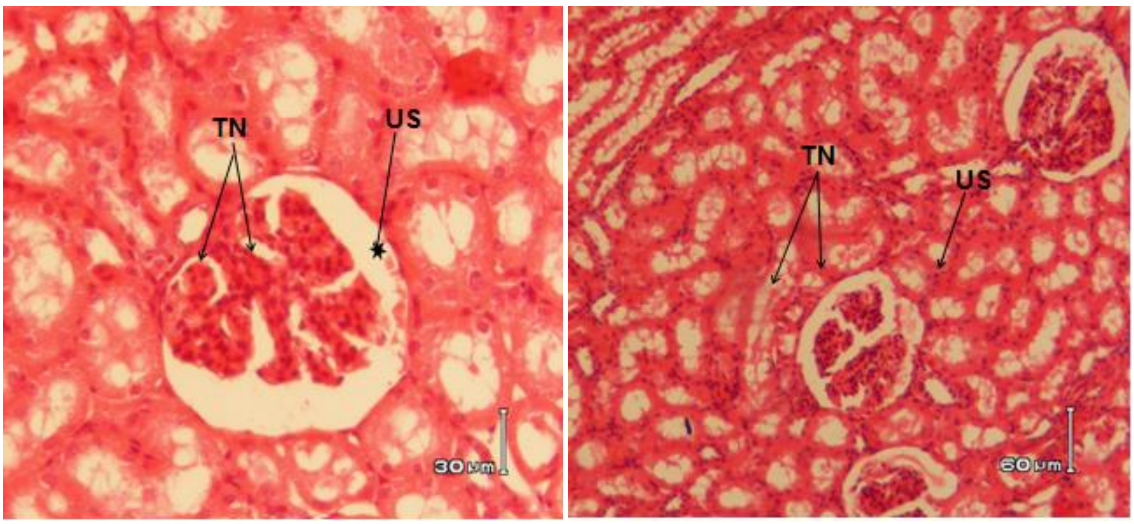


e

Figure 1. *Effect of V. karroo leaf meal inclusion on liver histology of bucks.* ***a*** *Moderate hepatocellular hydropic degeneration and proliferation of sinusoidal kupfer cells (20% V. karroo).* ***b*** *Moderate hepatocellular hydropic degeneration and proliferation of sinusoidal kupfer cells (25% V. karroo).* ***c*** *Moderate hepatocellular hydropic dilatation of central veins (30% V. karroo).* ***d*** *Severe hepatocyte degeneration and multifocal single cells (40% V. karroo).* ***e*** *Severe hepatocyte degeneration, multifocal single cell and clustered cell necrosis (50% V. karroo). Arrows point to hepatocellular hydropic degeneration (HD), kupfer cells (KC), central veins (CV) and cell necrosis (CN)*



a **b**

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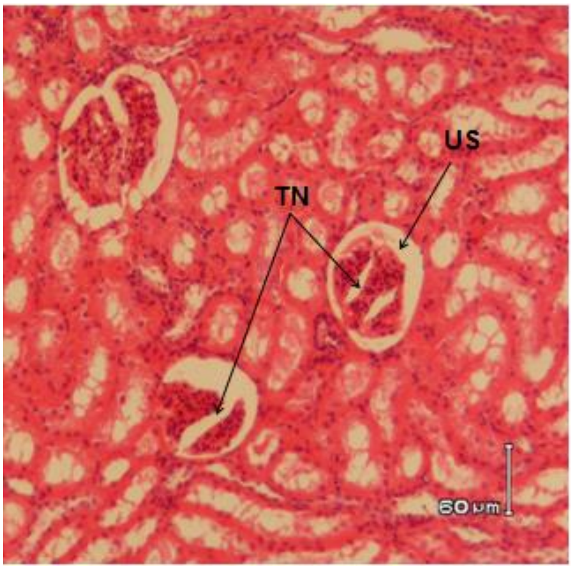

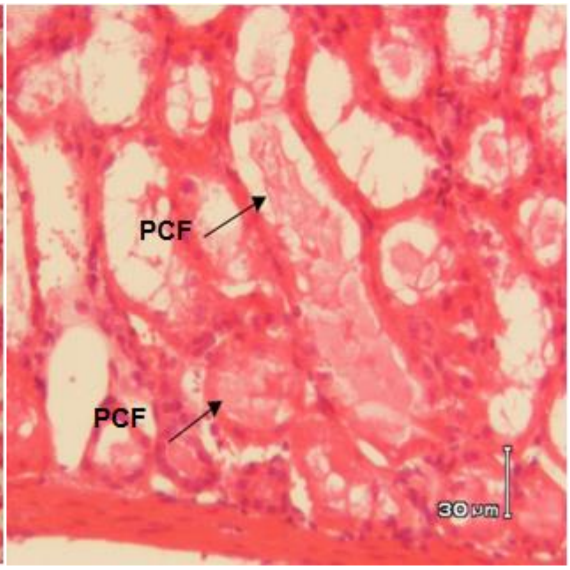

c **d**

e

Figure 2. *Effect of V. karroo leaf meal inclusion on kidney histology of Pedi bucks.* ***a*** *Early dilatation of glomerular uriniferous space and mild renal tubular nephrosis (20% V. karroo).* ***b*** *Dilatation of glomerular uriniferious spaces and mild to moderate renal tubular nephrosis (25% V. karroo).* ***c*** *Dilatation of uriniferous spaces, moderate renal tubular nephrosis (30% V. karroo).* ***d*** *Dilatation of uriniferous spaces, intra-tubular proteinaceous fluid with protein cast formation (40% V. karroo).* ***e*** *Dilatation of uriniferous space, intra-tubular proteinaceous fluid with protein cast formation. Arrows points to tubular nephrosis (TN), uriniferous space (US) and protein cast formation (PCF)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_28932909

Discussion

In the present study, *V. karroo* inclusion had no effect on diet intake of bucks. Similar results were reported for kids fed tanninferous *Ageritum conyzoides*, *Eupotarium odoratum* and *Crystellina* parasitica (Das et al., 2011). It has also been reported elsewhere that diets high in CT reduce intake of goats (Holechek et al., 1990). On the contrary, Solaiman et al. (2010) reported increased feed intake in Kiko crossbred male kids fed CT forage (*Sericea lespedeza*). Condensed tannins in forage legumes adversely affect feed intake through astringency in the oral cavity (Lamy et al., 2011). However, in the present study, diets with higher levels of CT were well-tolerated by goats. This may be due to the fact that the goats developed mitigatory systems against tannin toxicity such as secretion of proline-rich proteins in their saliva or proliferation of microorganisms resistant to condensed tannins in their digestive tracts (Lamy et al., 2011; Muir, 2011).

Dietary treatment did not improve growth performance of Pedi bucks. This is similar to the findings of Dludla (2010) who reported no differences in body weight gain of goats fed forages with different tannin concentrations. Wright (2015) observed similar results in Kiko-cross goats fed tanninferous *Sericea lespedeza*, pine bark powder and combination of *Sericea lespedeza* and pine bark powder. However, Ngambu et al. (2013) reported improved growth performance when Xhosa lop-eared goats were supplemented with 200 g of fresh *V. karroo* leaves. The lack of significant differences in body weight gain in the present study could be due to the fact that the dietary treatments met the nutrient requirements for a growing goat and hence nutrient supply was adequate for growth (McDonald et al., 2011).

Vachelia karroo leaf meal inclusion had no effect on the carcass weight and yields of Pedi bucks. Wright (2015) reported similar finding in Kiko-cross goats fed *Sericea lespedeza*, pine bark powder and combination of *Sericea lespedeza* and pine bark powder. The result obtained in the present study was expected since the dietary treatments did not influence average daily weight gains of goats.

According to Priolo et al. (2001), meat pH values can be influenced by dietary treatments. In the present study, *V. karroo* leaf meal inclusion had no effect on meat pH values. Previous studies demonstrated that the consumption of tanninferous feeds had no effect on chevon pH (Mapiye et al., 2010). However, Ngambu et al. (2013) observed significant differences in meat pH when goats were supplemented with *V. karroo* leaf meal. The discrepancies in the results could be due to the amount of dietary tannins ingested by the animals in each study. The ultimate pH (pH_u) of a carcass usually varies from 5.3 to 6.8 (Mostert, 2007). Under-nutrition is a primary cause of high pH_u in meats since animals do not have the possibility to accumulate sufficient glycogen reserve in their muscles (Bray et al., 1989). However, the experimental diets in the present study met the nutrient requirements for a growing goat and hence differences in meat pH values were not expected. Additionally, animals stressed prior to death are more likely to have a high pH_u. This is due to depletion of muscle glycogen resulting in lower lactic acid production. The animals in this study were not stressed prior to slaughter.

The present study indicated that goats fed 25% *V. karroo* leaf meal produced meat which was lighter than those on diets 30 or 40% leaf meal. Normally, consumers prefer lighter meat than dark meat. Verna et al. (1989) found similar result when lambs were fed two sorghum varieties with different tannin contents. Effect of tanninferous feed on meat lightness have also been reported by other authors (Priolo et al., 2001). It has been hypothesized that tannins present in forages are responsible for the differences found in

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meat color (Priolo et al., 2005). The effect of tannins on meat color is attributed to the reduced microbial biosynthesis of vitamin B_{12} (Priolo and Vasta, 2007). An increase in intramuscular and marbling fat is another factor that contributes to meat lightness (Baublits et al., 2004). However, the mechanism of action of forage tannins on meat color is not clear and merits further studies.

Dietary treatments had no effect on water holding capacity, shear force, cooking loss and dressing percentage of buck meat. These findings are similar to the report of Mapiye et al. (2010) who reported that beef from steers supplemented with *V. karroo* leaves had no effect on shear force, WHC, and cooking loss. Further research is needed to deepen the knowledge in this area. Meat sensory attribute values were also similar across the dietary treatments. Ngambu et al. (2012) however reported significant differences in consumer sensory attributes of meat from indigenous Xhosa lop-eared goat breed supplemented with different amounts of *V. karroo* leaves. It is not clear how dietary tannins affect the sensory attributes of goat meat and this may require further studies.

The liver is a primary site of detoxification and is generally the major site of intensive metabolism, hence, it is prone to various disorders as a consequence of exposure to toxins (Ganong, 2005). In the present study, goats fed 20, 25 or 30% *V. karroo* leaf meal inclusion had moderate hepatocyte degeneration, dilation of central veins and proliferation of sinusoidal kupfer cells. This is similar to the findings of Karimi et al. (2014), who reported mild congestion in central veins and sinusoidal abnormality when Merino lambs were fed tanninferous sorghum-based diets. Hervas et al. (2003) reported moderate hydropic degeneration in the hepatocytes of sheep dosed intra-ruminally with 3.0 g quebracho tannin extracts. The results indicate that the livers of the goats fed *V. karroo* leaf meal up to 30% were not adversely affected. However, severe hepatocyte degeneration was noticed in the liver of goats fed diets with 40 or 50% *V. karroo* leaf meal. Lesions associated with natural oak toxicosis characterized by hepatic and renal damage have been reported in cattle (Spier et al., 1987). Histological changes such as shrinkage of hepatocyte, degeneration and necrosis of hepatocytes around the central veins and degeneration of some hepatic sinusoids have been reported in lambs fed high tanninferous sorghum grain diets (Karimi et al., 2014). The present results showed that high *V. karroo* leaf meal inclusion levels of 40 or 50% in the ration may cause damages to the liver of bucks and hence such inclusion levels are not recommended.

The kidney is also an important organ for elimination of waste or toxic materials in the body. Exposure of the kidney to toxins may cause serious damages to this organ (Junqueira and Carneiro, 2003). However, the present study showed that the experimental diets did not cause any serious damage to the kidneys of the goats. This agrees with the findings of Silanikove et al. (1996) who reported no damage to the kidneys of goats fed tannin-containing leaves. On the contrary, Karimi et al. (2014) observed that high levels of sorghum grains in the diets induced histological changes in the kidneys of sheep. Some ruminants on tanninferous forage tend to develop adapted rumen microorganisms that detoxify plant secondary metabolites (Alonso-Diaz et al., 2010). The detoxification process may, however, cause adverse effects in ruminants as a consequence of increased enzymatic demand in the liver, kidney, gut mucosa and other tissues (Van Soest, 1994).

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Conclusions

Dietary *V. karroo* leaf meal levels in the present study did not improve feed intake, growth performance, carcass weight and carcass components of bucks. This may be an indication that the diets supplied adequate nutrients for growth of the goats. *Vachelia karroo* leaf meal did not adversely affect meat tenderness, juiciness, flavor, taste, aroma and overall acceptability. This is good for goat meat consumers, and requires further studies to confirm the responses. Meat pH, color, water-holding capacity, shear force, cooking loss and dressing percentage were not influenced by high and low *V. karroo* leaf meal levels. This is an indication that the diets had no adverse effects on these parameters. However, the effect of *V. karroo* on meat lightness was variable, depending on the inclusion level but without any recognizable pattern.

Vachelia karroo leaf meal inclusion levels of 20, 25 or 30% did not have adverse effects on livers and kidneys of bucks. However, severe hepatocyte degeneration was noticed in the liver of bucks fed diets with *V. karroo* leaf meal inclusion levels of 40 or 50%. Thus, *V. karroo* leaf meal inclusion levels of 20 to 30% are suggested. However, more detailed studies are recommended to confirm these results.

Acknowledgements. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF.

Conflict of interests. The authors declare no conflict of interests.

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MORPHOLOGICAL AND GRAIN YIELD RESPONSES OF SOYBEAN (*GLYCINE MAX* L.) TO TIME AND INTENSITY OF DEFOLIATION UNDER APPLICATION OF AMINO ACID

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(Received 1st Nov 2018; accepted 25th Jan 2019)

Abstract. The use of amino acids (AA) to increase the uptake of nutrients has been increasing dramatically in recent years. This investigation was conducted in the two successive seasons of 2016 and 2017 on soybean in Golestan Province, Iran, to study the effect of time and intensity of defoliation on quantitative and qualitative characteristics of soybean (*Glycine max* L.) under the application of AA. A two-year factorial experiment was conducted based on randomized complete block design with three replications. The first factor included the defoliation intensity at three levels 0, 50 and 100%, the second factor included the defoliation time in five levels V1, V3, V5, V7, and R1 (growth stages), and the third factor was the application and non-application of AA. Results indicated that the effects of the defoliation time and intensity, as well as the foliar application of AA, were significant on morphological traits, yield and yield components, and oil and protein contents. In the quadruple interaction, the highest of grain yield (4040 $kg.ha^{-1}$) was observed in the application of AA under defoliation in V5 with 50% intensity during the first year. The lowest value of this trait was achieved in non-application of AA under 100% defoliation in R1 growth stage during both years of the experiment (1585 and 1525 $kg.ha^{-1}$, respectively). The maximum grain oil and protein yield were observed in 100% defoliation in V7 growth stage under AA application during the first-year experiment (978.5 and 1499 $kg.ha^{-1}$, respectively). Also, the lowest value of the traits was observed in 100% defoliation in the V5 growth stage under non-application of AA during the second year (225.6 and 471.9 $kg.ha^{-1}$, respectively). According to the present study, soybean is regarded generally as a defoliation-tolerant crop. Also, foliar application of AA could be used as an effective amendment for preventing defoliation injury in yield and yield components of soybean.
Keywords: *foliar application, harvest index, oil content, pod, grain yield*

Introduction

Soybean (*Glycine max* L.) is an important industrial legume as human and animal feed in the world with an average of 18-22% oil, 38-56% vegetable protein in its seeds. Soybean is the most widely grown oilseed in the world as a main or second crop (Arslan et al., 2018). Soybean is originated from China. Domestication and cultivation of soybean by humans in China date from about the 11th century BC or a little earlier according to oldest records (Hymowitz and Shurtleff, 2005). The plant is introduced from the ancient world to the new world in the middle of the 17th century and gained its worldwide importance at the beginning of the 20th century (Arslan et al., 2018).

Plants are continuously exposed to biotic and abiotic stresses (Sadak et al., 2015). Plant response to defoliation depends on more than just the total amount of leaf area that

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DOI: http://dx.doi.org/10.15666/aeer/1702_29112929

is lost (Ahmadi et al., 2009). It is also known that defoliation intensity may vary along nutrient availability gradients and that defoliation may alter competition relationship between species (Alhamd and Alrababah, 2008). The ability of soybean to prevent substantial reduction in yield after the loss of leaves caused by defoliators and disease depends on several factors among including intensity of, the phenological stage of development at the time of defoliation, duration of defoliation, the ability of cultivar to tolerate or compensate for defoliation, and environmental conditions, especially rainfall, temperature and solar radiation (Nardino et al., 2016). Regarding the percentage of defoliation, research has established levels for the control of insect pests, when the defoliation is greater than 30% in the vegetative phase, or 15% in the reproductive phase. However, these recommendations are based on work done in the 70-80's decades. In this sense, another key issue for reduction of income is the degree to which defoliation reduces light interception by the canopy (Nardino et al., 2016). Earlier studies addressed the effect of leaf removal on growth and yield. In soybean, during the reproductive period, defoliation levels reduced the rate of the natural trend of losing leaf area. The yield was affected only by 67 and 100% defoliation applied at R6, while main agronomic traits such as date of harvesting maturity, plant loading, and height were not affected by defoliation (An et al., 2003). The defoliation of maize leaf up to 50% at the time of feed shortage did not have adverse in grain and stover yield components of maize (Khaliliaqdam et al., 2012). During the reproductive period, levels of defoliation reduced the rate of the soybean natural trend of losing leaf area (Pickle and Caviness, 2006).

The amino acid (AA) is a well-known biostimulant which has positive effects on plant growth, yield and significantly mitigates the injuries caused by biotic or abiotic stresses (Kowalczyk and Zielony, 2008). Saeed et al. (2005) on soybean found that treatments of AA significantly improved growth parameters of shoots and fresh weight as well as pod yield. Foliar application of nutrients has been recognized by many researchers, as a very efficient method of plant nutrition (Stiegler et al., 2013). Amino acid foliar applications are biostimulants in plants because they enhance the nutrient uptake efficiency because of increases in the leaf cuticle permeability (Moreira and Moraes, 2017), better plant growth, and higher plant biomass and grain yield, and they reduce abiotic stresses (Mendes et al., 2016). Researches are still necessary to verify the effects from amino acid foliar fertilizers under intensity and time of defoliation to improved quality and quantity of soybean plant. The objective of this study was to evaluate the influence of intensity and time of defoliation under amino acid application on yield and yield components and grain oil and protein contents of the soybean plant.

Materials and methods

Experimental design, site, and soil chemical analysis

In order to evaluate the effect of intensity and time of defoliation on quantitative and qualitative traits of soybean (Katol cultivar) under application of amino acid condition, a two-year factorial experiment was used based on randomized complete block design with three replications in, Golestan Province, Iran (Aliabad Katoul city; lat. 34° 54', long. 54° 56' E, altitude 142 m) during two consecutive years 2015-16 and 2016-17. The average annual minimum and maximum temperature and rainfall were 12.5 and 23.6 °C, and 182 mm (*Table 1*). The first factor included the intensity of defoliation soybean leaf in three levels 0, 50 and 100%, the second factor included time of

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defoliation in five levels (V1, V3, V5, V7, and R1; soybean growth stages), and the third factor was the use and non-use of amino acids.

Table 1. *Monthly weather characteristics of the test area in the year of implementation*

Parameters	Apr	May	Jun	Mar	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
Maximum air temperature (°C)	20.1	27.6	31.4	34.7	35.0	32.7	25.0	18.8	13.6	13.0	9.9	16.8
Minimum air temperature (°C)	9.6	15.2	18.9	22.2	22.2	20.3	13.8	9.1	2.6	3.4	2.8	5.2
Air temperature mean (°C)	14.9	21.4	25.2	28.5	28.6	26.5	19.4	13.9	8.1	8.2	6.4	11.0
Relative humidity (%)	77	74	68	64	62	64	71	77	73	72	78	74
Rainfall (mm)	147.4	46.6	30.5	22.2	31.3	68.9	36.2	73.0	53.6	6.7	100.3	44.1
Total number of rainy days	16	14	7	8	6	8	7	9	13	5	18	9
Total amount of evaporation (mm)	72.4	145.3	191.4	198.8	231.0	183.7	109.8	64.6	53.4	41.3	29.9	64.0
The number of frosty days	0	0	0	0	0	0	0	0	9	0	3	2

Agronomical operations were carried out in accordance with the tradition of the area. The previous planting on the farm was wheat. In order to determine some physical and chemical properties of the soil, a sample from 0-30 cm depth was prepared and sent to the laboratory and the amount of fertilizer was taken according to the laboratory's recommendations (*Table 2*). After fertilization, cultivation was carried out with a soybean row known as Fatahi's row planter (is a famous brand). The size of each plot was 2 × 3 m (with five rows). The distance between blocks and the distance plot in each block were 2 and 1 m, respectively. The pesticide used in the first stage consisted of Thiodicarb (1 $kg.ha^{-1}$) and secondly, 2 L/ha of Chlorpyrifos and thus, 250 cc Imidacloprid. Hand weeding weeds and 5 irrigations steps were performed.

Table 2. *Physicochemical properties of soil in the experimental farm at depths of 0 to 30 cm*

Soil texture	Sand (%)	Silt (%)	Clay (%)	K (mg/kg)	P (mg/kg)	N total (%)	Organic carbon (%)	Percentage of neutralized matter	pH	EC (dS/m^2)
Clay loam	22	50	28	261	8.8	0.15	1.46	12.5	7.4	2.4

Underuse of AA conditions, spraying with the recommended dose of 1 kg per 1000 liters of water (250 $g.ha^{-1}$) was performed at V3, V5, V7, and R1 growth stages (*Fig. 1*). The AA used by the Nutramin-WSP brand is Biomega, which contains 14-15% nitrogen, 0.3-0.7% calcium, 0.4-1.2% phosphate, 1.1-1.5% potassium, 0.3-0.6% ammonium, and 90% amino acids. The intensity and time of defoliation (by hand) was done also determined by dividing the soybean morphology stages. For this purpose, at 0% level (no defoliation of leaves), 50% (defoliation of 50% the leaves) and 100% (defoliation of 100% the leaves) was performed (*Fig. 2*).

Yield and yield components

To measure morphological and yield characteristics, at the R7 stage, five samplings from each experimental plot were examined for yield components (with marginal effect) and plant height: the height of the first pod from the surface, number of branches, number of pods, number of seeds per pod, and 100-seed weight were measured. In order to determine the grain yield after removing the marginal effects, two rows of middle

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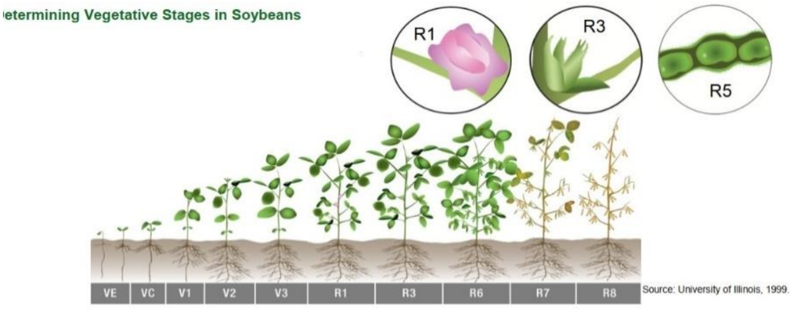
were harvested for grain yield. In other words, yield components were measured according to plant levels, but grain yield was measured according to surface levels. After calculating the biological yield, the harvest index (from grain yield to biological yield) was also determined (Divsalar et al., 2015).



VE	Cotyledons appear above the soil surface and provide nutrients for 7 to 10 days.
VC	Cotyledons have fully expanded and unifoliate leaves have unfolded. Four dormant growing points are present at the base of the petiole (axil) of unifoliate leaves and cotyledons.
V1	First trifoliate: Second true node, but first node at which a trifoliate leaf is produced. Nodules visible. New V stages develop every 5 days with normal temperatures.
V2	Two fully developed trifoliates unfold. The plant is roughly 8 inches tall. Nodules are actively fixing nitrogen. Cotyledons have fallen off plant. Lateral roots proliferating rapidly in the top 6 inches of soil.
V3-V4	A dramatic increase in the number of nodules visible on roots take place by these stages. This is typically the time that iron chlorosis deficiency syndrome is highly visible in impacted fields.
V5-Vn	Lateral roots extend 15 inches away from the main stem and grow to the centre of 30 inch row. Branches begin developing on the lowest nodes. The total number of nodes that the plant may produce is set at V5.
R1	Beginning bloom: R1 stage is reached when one flower is open at any node on the main stem.
R2	Full bloom: An open flower at one of the two uppermost nodes of the main stem with a fully developed leaf.
R3	Beginning pod: Pods are 3/16 of an inch long at one of the four uppermost nodes on the main stem.
R4	Full pod: Pod is ¾ of an inch long at one of the four uppermost nodes on the main stem. This is the most critical period for seed yield. Any stress from R4-R6 can cause more yield reduction than at any other time in plant development.
R5	Beginning seed: Seed in one of the four uppermost nodes with fully developed leaves is 1/8 of an inch long.
R6	Full seed: Pod containing a green seed filling the pod cavity is present at one of the top four nodes.
R7	Beginning maturity: One normal pod on the main stem has reached its mature pod colour. At this stage, the crop is safe from a killing frost.
R8	Full maturity: Ninety-five percent of the pods on the plant have reached their mature colour. Approximately 5 to 10 days of good drying weather is needed to bring crop to less than 15 percent moisture.

Figure 1. *Determining vegetative stage in soybeans*

Figure 2. *Experimental site and planting farm*

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Grain oil and protein content

To measure the oil content and seed protein, about 50 seeds were randomly selected from each treatment. After powdered, the percentage of oil by Soxhlet method and protein percentage were also measured by Kjeldahl method (Divsalar et al., 2015). To calculate oil yield and protein yield, oil percentage and protein percentage were multiplied in grain yield, respectively.

Statistical analysis

Analysis of variance was performed to test for statistical differences among the treatments, and means were statistically analyzed using least significant difference test (LSD) at P < 0.05 level of significance by using SAS software (Statistical Analysis Software, 9.2).

Results

Plant height

Results indicated that the effect of year (Y), amino acid (AA), defoliation time (DT), and defoliation intensity (DI) were significant on plant height (*Table 3*). The maximum plant height was achieved in first Y (93.02 cm), foliar application of AA (92.16 cm), DT in V1 (94.03 cm), and DI in 0 and 50% (96.0 and 92.8 cm, respectively). Mean comparison results showed the interaction effects of Y × AA and DT × DI were significant on plant height. The highest and lowest plant height were observed DT in V1 and DT in control treatment (98.16 cm) and DT in V5, V7, and R1 under DI in 100% (74.14, 72.33, and 71.66 cm, respectively) (*Fig. 3A*). In interaction Y × AA, the maximum this trait was observed in the first Y under application of AA (99.8 cm), and the lowest value of this trait was achieved in the second Y under non-application of AA (84.4 cm) (*Fig. 3B*).

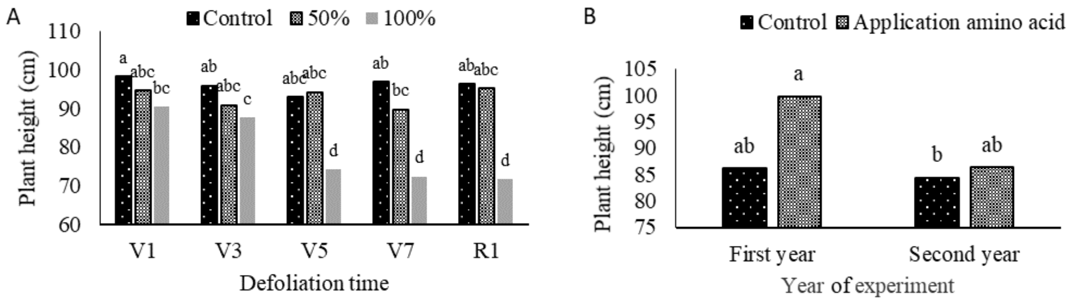

Figure 3. *Effect of intensity (0, 50, and 100%) and time (V1, V3, V5, V7, and R1) of defoliation (A); and defoliation time (V1, V3, V5, V7, and R1) and amino acid (control and application) (B) on plant height of soybean (columns with different letters are significantly different at P = 0.05, using LSD test)*

First pod height from the surface

In the current study, the effects of Y, DT, and interaction DT × DI were significant on the first pod height from the surface. The highest means of this trait was observed in the first Y (21.18 cm) and DT in R1 (22.0 cm) (*Table 3*). Mean comparison of

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interaction DT × DI showed the highest value of the trait was achieved in DT in R1 and DI in 100% (24.75 cm) (*Fig. 4*).

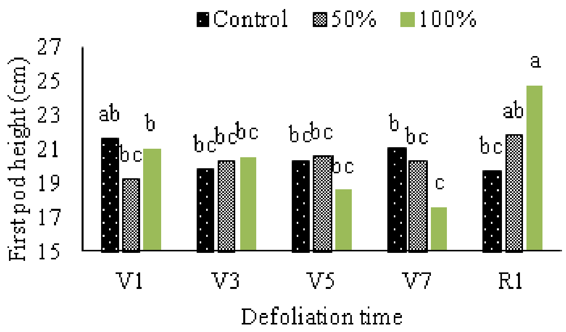

***Figure 4.** Effect of intensity (0, 50, and 100%) and time (V1, V3, V5, V7, and R1) of defoliation on first pods height from the surface (columns with different letters are significantly different at P = 0.05, using LSD test)*

Number of branches

The effects of Y, AA, DT, Y×A, and DT×DI was significant on number of lateral branches. Also, the effects of Y, DT, DI, Y×DI, and AA×DI were significant on number of main branches (*Table 3*). The highest number of lateral branch was observed DT in V3 under DI in 100% (6.93 no.). The lowest value of this trait was achieved in DT in R1 under DI in 100% (3.32 no.) (*Fig. 5A*). In the interaction DT × AA, the highest number of lateral branch was observed first Y under non-application of AA (6.98 no.) and the lowest value of this trait was observed in the second Y under non-application AA (4.15 no.) (*Fig. 5B*).

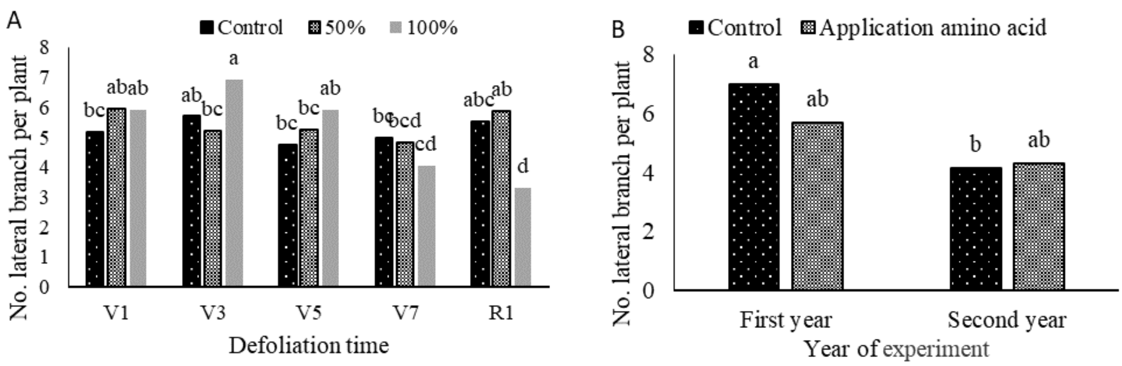

***Figure 5.** Effect of intensity (0, 50, and 100%) and time (V1, V3, V5, V7, and R1) of defoliation (A); and defoliation time (V1, V3, V5, V7, and R1) and amino acid (control and application) (B) on number of lateral branch per plant (columns with different letters are significantly different at P = 0.05, using LSD test)*

Number of the total pods

Results showed that the effect of Y, DT, and DI was significant on number of total pods (*Table 3*). The highest of the number of total pods was observed in the first Y

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(69.05 no.), DT in V1, V3, and V5 (64.8, 67.0, and 62.3 no., respectively), and DI in 50% (63.4 no.) (*Table 3*).

Number of pod free grain

The effects of Y and Y × AA was significant on number of pod free grain (*Table 4*). The highest number of pod free grain was observed in first Y (5.83 no.). In interaction Y × AA, the highest number of pods free grain was observed in the first Y under non-application and application of amino acid (4.97 and 6.69 no., respectively). The lowest value of the trait was achieved in the second Y under both amino acid treatments (*Fig. 6*).

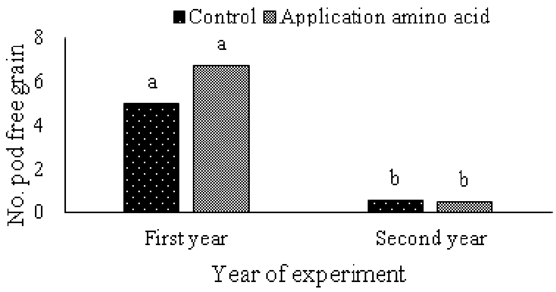

Figure 6. *Effect of the year (first and second) and amino acid (control and application) on number of pod free grain of soybean (columns with different letters are significantly different at P = 0.05, using LSD test)*

Grain number per plant

Results indicated that the effects of Y, DT, DI, and DT × DI was significant on grain number of soybean (*Table 4*). The first Y had superiority in term of grain number in compared to the second Y. Among the DT treatments, defoliation in the V1 growth stage had the highest grain number (152.2 no. per plant). By increasing defoliation intensity observed reduction the grain number per plant, so that the zero and 100% intensity of defoliation has the highest and lowest means of the trait (147.7 and 122.5 no per plant, respectively). In the interaction DT×DI, the highest grain number was achieved in V1 and V5 growth stages under 50% defoliation (164.7 and 163.5 no., respectively). The lowest value of the trait was observed in R1 growth stages under 100% defoliation (74.7 no.) (*Fig. 7*).

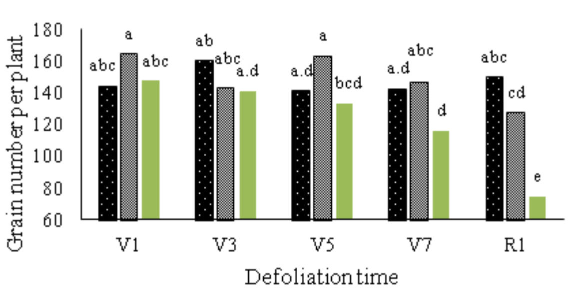

Figure 7. *Effect of intensity (0, 50, and 100%) and time (V1, V3, V5, V7, and R1) of defoliation on grain number of soybean (columns with different letters are significantly different at P = 0.05, using LSD test)*

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Table 3. *Analysis of variance indicating the effects of intensity and time of defoliation on plant height, first pod height, number of lateral branches, number of the main branch, and number total pods of soybean under application of amino acid*

Treatments	Plant height (cm)	First pod height (cm)	No. lateral branch per plant	No. main branch per plant	No. total pod per plant
Year (Y)					
First	93.02 a	21.18 a	6.33 a	33.85 a	69.05 a
Second	85.75 b	19.73 b	4.25 b	30.40 b	52.31 b
LSD (*P = 0.05*)	2.75	1.18	0.57	1.69	5.75
Amino acid (AA)					
Control	85.50 b	20.98 a	5.85 a	31.30 a	57.90 a
Application	92.16 a	20.08a	4.89 b	32.71 a	62.67 a
LSD (*P = 0.05*)	2.79	1.20	0.58	1.72	5.83
Defoliation time (DT)					
V1	94.3 a	20.6 ab	5.68 ab	34.7 a	64.8 a
V3	91.4 ab	20.1 b	5.95 a	32.1 abc	67.0 a
V5	87.1 bc	19.4 b	5.31 abc	32.8 ab	62.3 a
V7	86.0 c	19.9 b	4.62 c	30.8 bc	58.0 ab
R1	87.9 bc	22.0 a	4.90 bc	30.0 c	51.0 b
LSD (*P = 0.05*)	4.36	1.87	0.90	2.68	9.09
Defoliation intensity (DI) (%)					
0	96.0 a	20.4 a	5.23 a	34.6 a	62.5 ab
50	92.8 a	20.4 a	5.42 a	34.8 a	63.4 a
100	79.2 b	20.4 a	5.23 a	26.9 b	56.0 b
LSD (*P = 0.05*)	3.37	1.45	0.70	2.08	7.04
Interaction effect					
Y × A	**	NS	**	NS	NS
Y × DT	*	NS	NS	NS	NS
Y × DI	NS	NS	NS	*	NS
AA × DT	NS	NS	NS	NS	NS
AA × DI	NS	NS	NS	*	NS
DT × DI	**	*	**	NS	NS
Y × AA × DT	NS	NS	NS	NS	NS
Y × AA × DI	NS	NS	NS	NS	NS
Y × DT × DI	NS	NS	NS	NS	NS
AA × DT × DI	NS	NS	NS	NS	NS
Y × AA × DT × DI	NS	NS	NS	NS	NS

LSD: least significant difference; NS: non-significant. Columns with different letters are significantly different at P = 0.05, using LSD test

100-grain weight

The effects of Y, AA, DT, DI, Y×AA, and Y×AA×DT×DI was significant on 100-grain weight (*Table 4*). In the first year (19.98 g), non-application of AA (20.01 g), defoliation in V1 growth stage (20 g), and control treatment of defoliation intensity (20.11 g) showed the highest mean of 100-grain weight (*Table 4*). In the quadruple

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interaction, the highest 100-grain weight was observed in control DI in V3 growth stage under non-application of AA during the first year (20.8 g) and lowest was achieved in 100% defoliation of leaf in V5 growth stage under application of AA during the second year (18.58 g) (*Table 5*).

Table 4. *Analysis of variance indicating the effects of intensity and time of defoliation on no. pods free seed per plant, no. grain per plant, 100-grain weight, biological yield, grain yield, and harvest index of soybean under application of amino acid*

Treatments	No. pods free grain per plant	No. grain per plant	100-grain weight (g)	Biological yield (kg.ha^{-1})	Grain yield (kg.ha^{-1})	Harvest index (%)
Year (Y)						
First	5.83 a	150.2 a	19.98 a	9698.3 a	3233.8 a	33.40 b
Second	0.49 b	129.4 b	19.61 b	8059.7 b	2795.6 b	34.56 a
LSD (*P = 0.05*)	0.54	9.87	0.15	356.4	106.6	0.99
Amino acid (AA)						
Control	3.19 a	134.9 a	20.01 a	8611.0 b	2832.1 b	32.93 b
Application	3.14 a	143.3 a	19.64 b	9070.5 a	3145.1 a	34.73 a
LSD (*P = 0.05*)	0.54	10.01	0.15	361.5	108.1	1.00
Defoliation time (DT)						
V1	3.50 a	152.2 a	20.0 a	9378.5 a	3290.4 a	35.4 a
V3	3.41 a	148.1 ab	19.8 ab	9204.9 a	3141.3 ab	34.2 ab
V5	3.02 a	146.0 ab	19.8 ab	8997.9 a	2978.0 bc	33.0 b
V7	2.88 a	135.2 b	19.7 b	8418.1 b	2850.2 c	33.8 b
R1	3.00 a	117.4 c	19.6 b	8395.8 b	2813.6 c	33.2 b
LSD (*P = 0.05*)	0.85	15.6	0.24	563.5	168.6	1.57
Defoliation intensity (DI) (%)						
0	3.00 a	147.7 a	20.11 a	9548.8 a	3299.8 a	34.85 a
50	3.03 a	149.1 a	19.84 b	9300.0 a	3213.8 a	34.80 a
100	3.46 a	122.5 b	19.44 c	7788.3 b	2530.5 b	32.30 b
LSD (*P = 0.05*)	0.66	12.09	0.18	436.5	130.6	1.21
Interaction effect						
Y × AA	**	NS	**	NS	NS	NS
Y × DT	NS	NS	NS	**	**	NS
Y × DI	NS	NS	NS	NS	NS	*
AA × DT	NS	NS	NS	NS	NS	NS
AA × DI	NS	NS	NS	NS	NS	NS
DT × DI	NS	**	NS	**	**	*
Y × AA × DT	NS	NS	NS	NS	NS	NS
Y × AA × DI	NS	NS	NS	NS	NS	NS
Y × DT × DI	NS	NS	NS	*	*	NS
A × DT × DI	NS	NS	NS	NS	NS	NS
Y × AA × DT × DI	NS	NS	**	*	**	NS

LSD: least significant difference; NS: non-significant. Columns with different letters are significantly different at P = 0.05, using LSD test

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Biological yield

Results indicated that the effects of Y, AA, DT, DI, Y×DT, DT×DI, Y×DT×DI, and Y×AA×DT×DI were significant on biological yield (*Table 4*). The highest mean of biological yield was observed in the first Y (9698.3 $kg.ha^{-1}$), the application of AA (9070.5 $kg.ha^{-1}$). Also, defoliation in V1, V3, and V5 growth stages under intensity 0 and 50% showed the maximum biological yield (*Table 4*). In the quadruple interaction, the highest of biological yield was observed in the application of AA under defoliation in V1 with 50% intensity during the first year (11750 $kg.ha^{-1}$) and also, in the application of AA under defoliation in V5 with 50% intensity during the first year (11733.3 $kg.ha^{-1}$). Results indicated that the lowest mean of the trait was achieved in no-application of AA under 100% defoliation in the R1 growth stage during second Y (5225 $kg.ha^{-1}$) (*Table 5*).

Table 5. *Interaction effects of year, amino acid, defoliation time, and defoliation intensity on 100-grain weight, biological yield, and grain yield of soybean*

Year	Amino acid	Defoliation time	Defoliation intensity	100-grain weight (g)	Biological yield ($kg.ha^{-1}$)	Grain yield ($kg.ha^{-1}$)
First year	Control	V1	Control	20.5±1.2 a.d	9616.7±1200.3 b.l	3025±190.8 f.o
			50%	19.93±0.21 c.m	10816.7±1350.3 a.d	3615±258.2 a.f
			100%	20.07±0.46 b.k	9900±1525.6 b.k	3058.3±191.1 f.o
		V3	Control	**20.8±0.56 a**	10033.3±775.1 a.j	3176.7±648.5 e.m
			50%	20.4±0.75 a.e	8833.3±568.6 f.q	3085±118.2 e.n
			100%	19.4±1.2 j.r	8033.3±1164.4 l.t	2736.7±520.7 j.p
		V5	Control	20.27±0.23 a.f	10316.7±938.5 a.h	3380±138.1 b.i
			50%	20.47±0.55 a.d	9133.3±1985.8 c.o	3245±406.1 e.l
			100%	20.73±0.25 ab	10200±888.8 a.i	2825±135.9 j.o
		V7	Control	20.63±0.49 abc	10733.3±1106 a.d	3286.7±110.9 c.k
			50%	20.23±0.21 a.g	9350±390.5 c.n	3060±236.4 f.o
			100%	20.07±0.59 b.k	7083.3±775.1 q.w	2501.7±115.4 n.r
		R1	Control	20.4±0.6 a.e	9316.7±1101.5 c.n	3325±464.9 b.j
			50%	20.4±0.1 a.e	10400±2201.7 a.g	3508.3±431.4 a.g
			100%	19.63±0.4 f.p	5666.7±378.6 vwx	**1585±199.8 u**
	Application	V1	Control	20.47±0.4 a.d	10900±624.5 abc	3875±360.9 abc
			50%	20.13±0.81 a.i	**11750±300 a**	3895±82.3 ab
			100%	19.67±0.71 f.p	8716.7±361.7 g.p	3481.7±311.3 a.g
		V3	Control	19.37±0.91 k.r	10533.3±1183.6 a.f	3468.3±363 a.g
			50%	19.53±0.91 g.q	10300±1175.8 a.h	3440±122.9 b.h
			100%	19.47±0.21 i.r	9566.7±513.2 b.m	3330±312.8 b.j
		V5	Control	20.47±0.7 a.d	10666.7±1056.3 a.e	3658.3±444.6 a.e
			50%	19.63±0.67 f.p	**11733.3±2670.4 a**	**4040±912 a**
			100%	19.63±0.12 f.p	10366.7±1661.6 a.h	3495±373.6 a.g
		V7	Control	19.67±0.29 f.p	11300±1361.1 ab	3906.7±162.6 ab
			50%	19.9±0.44 d.m	9700±526.8 b.l	3353.3±265.4 b.i
			100%	19.03±0.74 p.s	7800±492.4 m.u	2518.3±516.2 n.r

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		R1	Control	19.47±0.32 i.r	10233.3±1600.3 a.h	3456.7±308.5 a.h
			50%	19.87±0.15 d.n	10500±650 a.g	3456.7±137.1 a.h
			100%	19.23±0.68 m.s	7450±1068.9 o.v	2226.7±460.6 p.t
Second year	Control	V1	Control	20.4±0.42 a.e	8362.5±1538 j.s	3030±169.7 f.o
			50%	19.5±0.42 h.q	7700±70.7 n.u	2500±70.7 n.r
			100%	19.7±0.71 e.p	8350±883.9 j.s	2925±388.9 g.o
		V3	Control	20.2±0.14 a.h	8325±2333.5 j.s	2915±445.5 g.o
			50%	20±0.28 c.l	9025±424.3 d.o	3442.5±449 b.h
			100%	19.50±0.1 h.q	7937.5±265.2 l.t	2585±219.2 m.q
		V5	Control	20.1±0.42 a.j	7137.5±654.1 p.w	2475±530.3 o.s
			50%	20.10±0.1 a.j	9587.5±2740 b.m	2935±827.3 g.o
			100%	18.9±0.14 qrs	5537.5±583.4 wx	**1470±11.1 u**
		V7	Control	19.9±0.14 d.m	8187.5±2103.6 k.t	2700±495 k.p
			50%	19.7±0.14 e.p	6037.5±1043 u.x	1882.5±371.2 stu
			100%	19.10±0.1 o.s	6412.5±300.5 t.x	1955±268.7 r.u
		R1	Control	19.4±0.28 j.r	8425±777.8 i.s	3085±120.2 e.n
			50%	19.25±0.64 m.s	7512.5±441.9 p.u	2660±14.1 l.p
			100%	18.75±0.07 rs	**5225±1378.9 x**	**1525±247.5 u**
	Application	V1	Control	19.88±0.45 d.m	8018.8±1049.9 l.t	3196.3±512.6 e.l
			50%	19.8±0.32 d.o	8750±1589.4 f.q	3263.8±210.8 d.k
			100%	20.03±0.22 b.l	9156.3±1826.2 c.o	3213.8±613.1 e.l
		V3	Control	20.33±0.63 a.f	9962.5±1379.2 a.k	3388.8±395.8 b.i
			50%	19.65±0.4 f.p	8900±1479.6 e.p	3160±338.7 e.m
			100%	19.15±0.57 n.s	8362.5±948.1 j.s	2825±445.5 i.o
		V5	Control	20.08±0.25 b.k	7962.5±592.5 l.t	2866.3±558.7 h.o
			50%	19.48±0.97 i.q	8406.3±1128.7 j.s	3021.3±439.1 g.o
			100%	**18.58±0.32 s**	6668.8±2115.6 s.x	1992.5±540.5 q.u
		V7	Control	19.63±0.34 f.p	8593.8909.1 h.r	3180±579.6 e.l
			50%	19.7±0.64 e.p	8043.8495.6 l.t	3042.5±94.3 f.o
			100%	19.13±0.29 o.s	6831.31015.8 r.x	2191.3±430.2 p.t
		R1	Control	20.23±0.56 a.g	10737.51667.6 a.d	3845±485.6 a.d
			50%	19.33±0.26 l.r	8581.31498.1 h.r	2986.3±358.4 g.o
			100%	19.1±0.42 o.s	5487.51080.4 wx	1687.5±218.5 tu

Columns with different letters are significantly different at P = 0.05, using LSD test. The bold number showed the highest and lowest means of trait

Grain yield

The effects of Y, AA, DT, DI, Y×DT, DT×DI, Y×DT×DI, and Y×AA×DT×DI was significant on grain yield (*Table 4*). In the first Y (3233.8 kg.ha^{-1}), AA application (3145.1 kg.ha^{-1}), defoliation in V1 growth stage (3290.4 kg.ha^{-1}), and DI 0 and 50% (3299.9 and 3213.8 kg.ha^{-1}, respectively). In the quadruple interaction, the highest grain yield was observed in the application of AA under defoliation in V5 with 50% intensity during the first year (4040 kg.ha^{-1}). The lowest value of this trait was achieved in non-

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application of AA under 100% defoliation in R1 growth stage during both years of experiments (1585 and 1525 kg.ha^{-1}, respectively) (*Table 5*).

Harvest index

The effects of Y, AA, DT. DI, Y×DI, DT×DI were significant on harvest index (*Table 4*). The highest of harvest index was observed in the second Y (34.56%), AA application (34.73%), defoliation in V1 growth stage (35.4%), 0 and 50% defoliation intensity (34.85 and 34.80%, respectively) (*Table 4*). Interaction effect of DT×DI showed the highest mean of harvest index in defoliation in V1 and R1 growth stages under control intensity treatments (36.2 and 35.7%, respectively). The lowest value of the trait was observed in 100% defoliation during the R1 growth stage (29.7%) (*Fig. 8*).

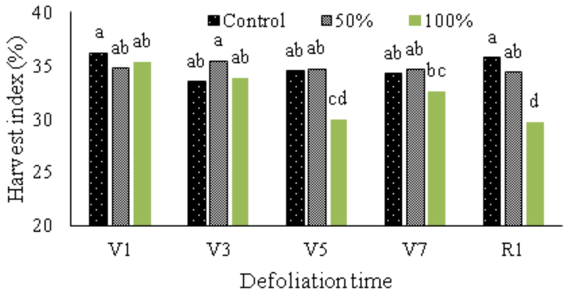

Figure 8. *Effect of intensity (0, 50, and 100%) and time (V1, V3, V5, V7, and R1) of defoliation on harvest index of soybean (columns with different letters are significantly different at P = 0.05, using LSD test)*

Grain oil content

Analysis of variance results showed that the effects of Y and Y×DT×DI were significant on oil percentage. Also, the effects of Y, AA, DT, DI, Y×AA, DT×DI, Y×AA×DT, Y×DT×DI, and Y×AA×DT×DI was significant on oil yield (*Table 6*). The highest grain oil yield was achieved in first Y (726.4 kg.ha^{-1}), AA application (689.7 kg.ha^{-1}), defoliation in the V3 growth stage (699.7 kg.ha^{-1}), and zero percentage of intensity defoliation (700.5 kg.ha^{-1}) (*Table 6*). In the quadruple interaction, the maximum grain oil yield was observed in 100% defoliation in V7 growth stage under AA application during the first-year experiment (978.5 kg.ha^{-1}). Also, the lowest value of the trait was observed in 100% defoliation in the V5 growth stage under non-application of AA during the second year (225.6 kg.ha^{-1}) (*Table 7*).

Table 6. *Analysis of variance indicating the effects of intensity and time of defoliation on oil yield and percentage and protein yield and percentage under application of amino acid*

Treatments	Oil percentage	Oil yield (kg.ha^{-1})	Protein percentage	Protein yield (kg.ha^{-1})
Year (Y)				
First	22.3 a	726.4 a	33.66 a	1087.55 a
Second	19.9 b	564.8 b	31.23 a	874.10 b
LSD (*P =0.05*)	2.01	71.2	2.08	109.5

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Amino acid (AA)				
Control	20.68 a	583.8 b	32.94 a	921.9 b
Application	21.48 a	689.7 a	32.09 a	1022.9 a
LSD (*P = 0.05*)	1.53	81.1	1.49	49.9
Defoliation time (DT)				
V1	20.26 a	649.1 b	31.55 a	996.1 b
V3	21.81 a	699.7 a	32.87 a	1053.8 a
V5	20.96 a	606.5 c	31.78 a	904.0 c
V7	21.62 a	658.2 b	33.30 a	1004.3 b
R1	21.09 a	614.6 c	32.73 a	945.8 c
LSD (*P = 0.05*)	1.11	12.49	2.07	49.8
Defoliation intensity (DI) (%)				
0	21.38 a	700.5 a	32.2 a	1052.0 a
50	21.26 a	650.1 b	32.4 a	984.5 a
100	20.81 a	586.3 c	32.6 a	905.8 b
LSD (*P = 0.05*)	1.13	52.3	0.86	77.2
Interaction effect				
Y × AA	NS	*	NS	NS
Y × DT	NS	NS	NS	NS
Y × DI	NS	NS	NS	NS
AA × DT	NS	NS	NS	NS
AA × DI	NS	NS	NS	NS
DT × DI	NS	*	NS	*
Y × AA × DT	NS	*	NS	NS
Y × AA × DI	NS	NS	NS	*
Y × DT × DI	*	**	NS	*
AA × DT × DI	NS	**	NS	**
Y × AA × DT × DI	NS	*	NS	*

LSD: least significant difference; NS: non-significant. Columns with different letters are significantly different at P = 0.05, using LSD test

Grain protein content

Results indicated that the effects of Y, AA, DT, DI, DT×DI, Y × AA × DI, Y × DT × DI, AA × DT × DI, and Y × AA × DT × DI were significant on grain protein yield (*Table 6*). In the quadruple interaction, the highest grain protein yield was observed in 100% defoliation in V7 growth stage under AA application during the first-year experiment (1499 $kg.ha^{-1}$). Also, the lowest value of the trait was observed in 100% defoliation in the V5 growth stage under non-application of AA during the second year (471.9 $kg.ha^{-1}$) (*Table 7*).

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Table 7. *Interaction effects of year, amino acid, defoliation time, and defoliation intensity on grain oil and protein yield of soybean*

Year	Amino acid	Defoliation time	Defoliation intensity	Grain oil yield ($kg.ha^{-1}$)	Grain protein yield ($kg.ha^{-1}$)
First year	Control	V1	Control	606.7±173.7 g.n	1068.6±110.6 b.n
			50%	607.9±131.1 g.n	954±202.5 f.q
			100%	515.6±168.4 k.q	896.5±310.1 h.q
		V3	Control	685.7±111.1 c.k	1004±150.5 d.p
			50%	719.7±92.3 b.i	1121±206.8 b.k
			100%	768.5±94 b.h	1077.9±110.7 b.m
		V5	Control	822.7±87.8 a.e	1188.2±223 b.f
			50%	617.9±31.9 a.e	940.9±44 f.q
			100%	495.9±162.8 k.q	869.5±338.8 k.q
		V7	Control	802.4±67.2 a.f	1239.7±160.9 b.e
			50%	670.4±178.3 c.l	1024.2±240.9 d.o
			100%	731.1±333.6 b.i	1073.6±436.3 b.n
		R1	Control	595.8174 h.n	880.4±254.6 i.q
			50%	570.7±107 i.n	936.8±223.8 f.q
			100%	667.1±225.6 c.m	1057.5±345.6 b.o
	Application	V1	Control	825.2±121.4 a.d	1138.7±164.2 b.h
			50%	849.8±95.5 abc	1116.5±186.9 b.k
			100%	768±72.7 b.h	1120.4±150.4 b.k
		V3	Control	882.2±91.8 ab	1297.2±186.4 ab
			50%	725.4±110.8 b.i	1147.6v124.1 b.h
			100%	776.8±95.4 b.h	1125.9±234.3 b.j
		V5	Control	804.9±118.6 a.f	1109.4±97.2 b.k
			50%	828±73.5 a.d	1133.1±69.6 b.i
			100%	654.8±116.1 d.m	987±69.1 e.p
		V7	Control	769.3±145.9 b.h	1140.2±265.9 b.h
			50%	732.7±130 b.i	1075.4±173.1 b.m
			100%	**978.5±170.5 a**	**1499±275.2 a**
		R1	Control	791.3±61.7 a.g	1162.1±120.2 b.g
			50%	636.3±39.8 d.n	951.6±59.5 f.q
			100%	892.7±208.4 ab	1289.6±301 abc
Second year	Control	V1	Control	522.9±17.8 j.p	962.1±163 f.q
			50%	474±143.9 m.r	727±101.4 q.u
			100%	458.2±175.7 n.r	819.7±76.2 n.r
		V3	Control	517.3±77 k.q	812.7±97.7 o.r
			50%	683.2±139.8 c.k	1075.3±133 b.m
			100%	560.7±79.9 i.n	828.1±30.2 m.r
		V5	Control	540±74.7 i.o	769±214.4 p.t
			50%	543.5±207 i.o	876.4±318.8 j.q
			100%	**225.6±57.2 s**	**471.9±14.6 v**

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DOI: http://dx.doi.org/10.15666/aeer/1702_29112929

	V7	Control 50% 100%	565.9±135.7 i.n 355.7±97.6 o.s 325.2±110.4 qrs	915.7±85.3 g.q 604±134.8 r.v 554.2±117.2 s.v
	R1	Control 50% 100%	521.4±136.6 j.p 489.8±130.5 l.q 294.7±107.4 rs	804.1±25.3 o.s 848.7±68.5 l.r 503.3±211.5 uv
Application	V1	Control 50% 100%	630.9±211.1 e.n 713±216.4 b.j 640.7±208.4 d.n	960.1±138.3 f.q 1011.9±113.3 d.p 1017.5±196.7 d.p
	V3	Control 50% 100%	731.1±137.4 b.i 629.9±114.9 e.n 637.5±205.5 d.n	1087.4±181 b.l 1041.8±95.9 c.o 917±196.4 g.q
	V5	Control 50% 100%	624.1±122.5 f.n 674.4±173.2 c.l 337.9±126.7 p.s	894.2±91.8 h.q 958.7±162.3 f.q 553.6±166.3 s.v
	V7	Control 50% 100%	673.7±162.1 c.l 657.365.2 c.m 456.594 n.r	1032.5±213.4 d.o 963.9±60.2 f.q 716.4±133.4 q.v
	R1	Control 50% 100%	824.9157.2 a.d 584.7193.2 h.n 353.772.7 o.s	1253.3±184.4 a.d 925.4±170.3 g.q 547.4±75.3 tuv

Columns with different letters are significantly different at P = 0.05, using LSD test. The bold number showed the highest and lowest means of trait

Discussion

Effect of defoliation time and intensity

Defoliation caused by insects is common biotic stress in soybean [*Glycine max* (L.) Merr.] production (Xiangjun et al., 2009). The effects of defoliation on yield and yield components of soybean have been well studied, but time and intensity of defoliation have not been demonstrated. Defoliation affected soybean yield through a combination of reduced light interception resulting in decreased canopy photosynthesis, loss of leaf storage material, and/or shortening of the effective grain-filling period (Xiangjun et al., 2006). In the experiments of Xianjun et al. (2006, 2009), the effect of defoliation of soybean under salinity stress or application of nitrogen fertilizer were investigated. The effect of defoliation on growth and yield characteristics in plants varied with time and intensity of defoliation (Xiangjun et al., 2009). In the present study, effects of time and intensity of defoliation were significant on morphological traits, and yield and yield components such as plant height, first pod height, numbers of lateral and main branch, number of total pod, number of grain, 100-grain weight, biological yield, grain yield, and harvest index (*Tables 3* and *4*). Results illustrated by increases defoliation intensity was observed decreasing on yield and yield components. Also, defoliation in the reproductive growth stage (R1) had significantly reduced on studied traits. Several studies have shown strong relationships between the leaf-area index, defoliation intensity, light interception rate, and yield (Hammond et al., 2000; Xiangjun et al.,

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DOI: http://dx.doi.org/10.15666/aeer/1702_29112929

2006). Defoliation decreased soybean dry-matter production by reducing the effective leaf area for light interception and carbon fixation (Klubertanz et al., 1996). On the other, it may enhance growth through two mechanisms: compensatory regrowth and delayed leaf senescence, including delayed leaf abscission and increased leaf photosynthetic rates (Xiangjun et al., 2006). These mechanisms may aid soybean in tolerating defoliation during vegetative and early reproductive stages; however, the ability of these mechanisms to function in the presence of additional stresses is not known. In this regard, Ugese et al. (2011) reported that the defoliation time and intensity significantly affected on seedlings of *Vitellaria paradoxa,* which confirms the results of this study. Non-defoliation and mild defoliation intensity (50%) would not have a significant effect on yield reduction, but the severe defoliation intensity (100%) was caused decreasing in the studied traits. In this regards, the yield and growth reduction under severe stress conditions (100% defoliation) can be attributed to reduced photosynthetic parameters (net photosynthesis, transpiration rate, stomatal conductance, and chlorophyll content) (Zobiole et al., 2009, 2010). Defoliations in the early stages of growth apparently does not affect yield and yield components positively or negatively, an observation that is consistent with other types of crops, such as corn and garlic (Olfati et al., 2010). Xiangjun et al. (2009) reported that although defoliation temporarily reduced soybean dry weight and N accumulation during 15 days after defoliation.

Our finding that increasing levels of defoliation leads to progressive declines in grain oil and protein yield, but not significantly affected protein percentage (*Table 6*). This result agreed with the report by Turnipseed (1972) and Xiangjun et al. (2009) and that defoliation caused no decrease in seed protein content. Nitrogen (one of the important elements in protein synthesis) absorption following defoliation may be promoted. Although root growth usually is reduced following defoliation, the increase in nutrient uptake rate per unit root mass was found in a sedge (*Kyllinga nervosa* Steud.) plants (McNaughton and Chapin, 1985).

Effect of amino acid application

Amino acids are molecules with the following structure: a central carbon atom (C), usually asymmetric, attached to a carboxylic acid group (COOH), an amino group amidogen (NH_2) and hydrogen (H) atom (Moreira and Moraes, 2017). The AAs have several functions, and the most important are: i) protein synthesis, ii) intermediate compounds in the synthesis of endogenous plant hormones, iii) chelating effect on nutrients and other compounds, iv) greater resistance to drought stress and high temperatures, and v) greater disease resistance (Castro, 2009; Moreira and Moraes, 2017). In the current study, results indicated that the effect of the amino acid application was significant on yield and yield components as well as oil and protein yield (*Tables 4* and *6*). Application of amino acid significantly increased plant height, biological yield, grain yield, harvest index, and oil and protein yield. It is well documented that plants are capable of utilizing amino acids as nitrogen (N) and carbon (C) sources (Thornton and Robinson, 2005). Amino acid foliar fertilization has generally been sprayed onto plants to increase the crop yield (Souza et al., 2018). Amino acid foliar applications are biostimulants in plants because they enhance the nutrient uptake efficiency because of increases in the leaf cuticle permeability (Moreira and Moraes, 2017), better plant growth, and higher plant biomass and grain yield, and they reduce abiotic stresses (Azimi et al., 2013; Gazola et al., 2014; Mendes et al., 2016). Moreira and Moraes

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DOI: http://dx.doi.org/10.15666/aeer/1702_29112929

(2017) found increases in common bean (*Phaseolus vulgaris* L.) yield after applying amino acid. Despite demonstrating these positive results regarding the efficient use of amino acids, some studies indicated that plant responses may vary under the influence of many factors such as the molecular weight of amino acids, anatomical features, growth stage, climate conditions and time of application (Fernández et al., 2013).

In all studied traits, the means of the traits from each growing season was different (*Tables 3, 4,* and *6*). We found 3233.8 kg.ha^{-1} (first year) and 2795.6 kg.ha^{-1} (second year) which means a reduction of 13% in the grain yield (*Table 4*). Similar to wheat and soybean, this reduction was caused by hydric stress at the beginning of the soybean development because this stage is the crucial moment for successful crop development, and the effects reflect on crop yield (Souza et al., 2018).

Conclusion

Results indicated that the defoliation time and intensity significantly decreased morphological traits and yield components as well as oil and protein contents. Server defoliation intensity (100%) during the R1 growth stage (reproductive stage) showed an intensive reduction effect. While defoliation in early growth stages had a negligible effect on the traits studied. On the other hand, the use of low molecular weight amino acid is aimed to increase nutrient uptake by leaves, with the consequent increase in productivity. The findings of this study showed that, on the average of two years of assessment, the foliar application of amino acid increased morphological traits, yield, and yield components. According to the present study, soybean is regarded generally as a defoliation-tolerant crop. Also, foliar application of amino acid could be used as an effective amendment for preventing defoliation injury in yield and yield components of soybean.

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DOI: http://dx.doi.org/10.15666/aeer/1702_29112929

DETERMINATION OF HEAVY METALS IN WOODEN TREES AND ASH RESIDUES IN KOSOVO

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(Received 2nd Nov 2018; accepted 16th Jan 2019)

Abstract. In this study, we examined the content of Fe, Mn, Cd, Pb, Cu, Zn and Ni in fresh wooden trees and ash wood residues. The material of analysis was sampled in four different zones in Kosovo. Initially, the samples of wood were grinded and dried in room temperature, and then they were treated with HNO_3 and H_2O_2. The content of heavy metals was determined with atomic absorption spectrophotometer technique. The following results show that we have higher concentrations of some elements such as Cu, Zn and Ni, in the ash residues more than the Environmental Protection Agency standard (EPA Ecological Soil Screening Level) predicts for these elements to be as wastes in the soil. In order to preserve our health and environment, we must be cautious towards the amount of waste we dispose in the ground, for the reason that they straightforwardly end up in our food chain.
Keywords: *environmental, pollution, atomic absorption spectroscopy, EPA*

Introduction

The environmental problem in Kosovo in recent years has been a big and very serious problem. According to various measurements and numerous reports, our country was ranked among the world's first countries in terms of pollution. The Hydrometeorological Institute dealing with environmental monitoring in Kosovo has cited that the main causes of pollution are industries, transport and fossil fuels. According to this Institute, the air pollution has been increasing so drastically in the five last years that in some cases the permissible values have been exceeded (Kosovo Environmental Protection Agency, 2017)

Today it is a known fact through different researches that accumulation of heavy metals is related to different industrial activities, such as mining, smelting, energy and fuel production, traffic activities, agriculture etc. (Balabanova et al., 2014; Mooreet al., 2013; Yan et al., 2012; Kouamé et al., 2013; Jusufi et al., 2017). In our day, what is also known is that plants uptake these metals from various reactions: redox and ionic exchange reactions, precipitation-dissolution etc. (Mirecki et al., 2015). It is impossible to imagine life without trees because of their role in economic, environmental and industrial aspects, as well as spiritual, historical and aesthetic (Seth, 2004). However, in terms of environmental pollution, trees can also serve as long term accumulators of heavy metals and act as bio indicators of contamination.

In this paper, we evaluate the chemical distribution of Cd, Mn, Pb, Cu, Ni, Zn and Fe in fresh wood and their ashes. The importance of determining the scale of contamination in ashes is enormous, since the possibility of wood ending up in environment as ash residue is huge. Also, knowing the forms of toxic metals present in wood ash could indicate how much the emitted particulates pose risks to the human health and the environment (Odlare et al., 2007; Pettersson, et al., 2008).

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DOI: http://dx.doi.org/10.15666/aeer/1702_29312941

Materials and methods

Area of study

Kosovo forests cover around 41 % (U.S. Department of State, 2008; Knaus and Warrander, 2010) of the entire territory of the country. Most of the forests are located in the southwest. There are various types of forestry in Kosovo, but for the most part are conifers. The regions with the highest diversity are the regions of the Sharr Mountains and the Albanian Alps. Kosovo's forests are threatened by fires and illegal logging. For this study, we chose four different regions in Kosovo where wood samples were collected.

Treatment procedure

In order to measure the heavy metal content, samples were inspected in fresh wood and calcinated form. The types of wood were beech and oak. The wood samples were mangled in fine parts before being grinded.

Initially, wood samples were left to dry at 60 °C for several hours before further study. For the analysis, 1 g of dried wood up to constant weight were taken and were placed in Teflon containers, where 12 ml of concentrated nitric acid (65%) was added. The mixture was heated at 105 °C and in small portions 4 ml of hydrogen peroxide (30%) were introduced to the mixture. The samples were left to evaporate until drying, then 1 ml of HCl were added. The mixture was filtered and leveled up to 50 ml with distilled water.

To treat the wood ash, fresh wood samples were initially calcinated at 400 °C for about two hours. 0.5 grams of calcinated sample were taken and placed in Teflon containers. 6 ml of HNO_3 were added and placed in heater at 105 ° C. Additionally, 2 ml of H_2O_2were added and after the sample was dried, it was removed and 0.5 ml HCl were added. The mixture was filtered and leveled up to 25 ml with distilled water.

Concentrations of heavy metals (Fe, Mn, Cd, Pb, Cu, Zn and Ni) were measured using the atomic absorption spectroscopy (AAS) technique.

Results and discussion

The purpose of this study was to determine the heavy metal content in wood which is burnt in conditions similar to domestic. A study of this form is very important since there are different studies of heavy metal uptake in vegetation (Peng et al., 2006; McLaughlin et al., 2011; Mingorance etal., 2007), but there are few data which examine the concentration of heavy metals in wood (Algreen et al., 2012; Świetlik et al., 2013).

The bioaccumulation of heavy metals in trees happens owing to climate, atmospheric depositions, the concentrations of heavy metals in soil, the nature of soil on which the trees are grown and the degree of maturity of the plants at time of harvest (Lake et al., 1984; Voutsa et al., 1996). Wood ash is one of the inorganic residues of wood burning, whereas CO_2 and water are the organic remains (Hannam et al., 2002; Reimann et al., 2008). The ash contents vary among types of trees, soil and climate. Campbell (1990) and Naik (2003) pointed out that it can also vary according to the method and manner of combustion, efficiency of the boiler, and other supplementary fuels used with wood.

Table 1 presents a summary of the wood samples: the ash content in percentage, moisture and the wood type.

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Table 1. *List of analyzed samples and their properties*

Sample	Regions	Ashes %	Moisture	Type
1	Drenica	<0.9	7.4	Beech/oak
2	Ferronickel	<0.7	8.58	Beech/oak
3	Sharrcem	<0.8	6.96	beech
4	Sharr	<0.6	11.88	beech

The moisture was determined by weight loss and was used to correlate the results with dry weight, while the ash content was calculated before and after the burn, and was left at 105 °C to constant weight. Studies show that the percentage of ash content in wood can vary from 0.1 to 1.4% (Fengel and Wegener, 1989; Prosiński, 1984; Rowellet al., 2005; Szczepkowski and Nicewicz, 2008).

Table 2 presents the following basic statistical data of dry wood samples in mg/kg: average (AVG), range (min-max), standard deviation (SD), variance, first and third quartiles (Q1 and Q3) and median.

Table 2. *List of analyzed elements in fresh wood*

Elements	AVG (mg/kg)	Min-max	SD	Variance	Q1	Q3	Median
Mn	400.475	(20.4-1440)	694	481034	29	1102	71
Fe	105.81	(0.149-172)	72.1	5191.2	40.6	171	81.1
Zn	19.6	(10.05-39)	11.52	132.67	11.4	27.8	16.6
Pb	5.26	(0.05-18.9)	9.98	99.54	0.25	18.9	1.65
Cu	4.96	(0.6-10.85)	4.96	24.58	1	10.85	7.02
Ni	0.812	(0.05-2.55)	1.284	1.649	0.074	2.55	0.55
Cd	0.748	(0.4-0.943)	0.246	0.061	0.488	0.932	0.825

The concentrations of heavy metals are ranked in descending order of average concentration as follows: Mn>Fe>Zn>Pb>Cu>Ni>Cd. The average concentration of Zn is 19.6 mg/kg and ranges from 10.05-39 mg/kg. The results for lead and copper in wood content were similar, ranging from 0.049 to 18.9 mg/kg for lead with the average of 5.26 mg/kg, and the average for copper was 4.96 mg/kg, whereas it ranged between 0.6 to 10.85 mg/kg. The lowest concentration of heavy metals was found for Ni and Cd, with the average of Ni being 0.812 mg/kg and the average of Cd 0.75 mg/kg.

Table 3 presents the following basic statistical data of ash residue samples in mg/kg: average (AVG), range (min-max), standard deviation (SD), variance, first and third quartiles (Q1 and Q3) and median.

The mobility of an element, the form it occurs in the environment and other conditions determine whether that element is likely to enter the food chain. Highly mobile elements such as Mn or Zn are mainly taken up by the root system and are then transported from there to the leaves. Concentration of manganese was shown to be very high, and it can be explained as a result of the high content of this easily assimilative element in soil (Szczepkowski and Nicewicz, 2008). In our study it ranges from 5.99 to 2596 mg/kg. Kuokkanen et al. (2009) also found higher concentrations of this element.

If we compare the average values of the concentrations of heavy metals, we find that their order in ash samples is Fe> Mn> Zn> Cu> Ni>Pb> Cd. The global average of

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DOI: http://dx.doi.org/10.15666/aeer/1702_29312941

cadmium content in soil is 0.41 mg/kg (Kabata-Pendias and Mukherjee, 2007), while in the surface layer of soil in Europe the average concentration of cadmium concentration is 0.15 mg/kg (Salminen et al., 2005). The standard of soils (Dutch list) defines the target concentration of Cd in soil to be 0.8 mg/kg. According to ecological soil screening levels established by USEPA (2003) (Eco-SSL) that serves for soil contamination, the maximum concentration of Cd is 32 mg/kg. In our samples, Cd ranges from 0.95 mg/kg to 7.85 mg/kg with an average of 4.51 mg/kg. By this definition of EPA, cadmium in our samples results to be in tolerated concentration.

Table 3. *List of analyzed elements in ashes residue*

Elements	AVG mg/kg	Min-max	SD	Variance	Q1	Q3	Median
Mn	978.25	(52.99-2596)	1192	1420019	67	2236	632
Cd	4.51	(0.95-7.85)	2.84	8.05	1.77	7.14	4.63
Pb	42.25	(32.35-48.05)	7.32	53.51	34.54	47.91	44.3
Cu	87.5	(38.25-195)	72.9	5308.6	40.5	163.6	58.4
Ni	58.8	(36.3-82.4)	20.2	407.4	39.6	78.6	58.3
Zn	229.39	(0.05-670)	300	89907	26	538	124
Fe	2095.013	(0.05-4705)	1956	3825547	393	4055	1838

The concentration of Cu in our samples ranges between 38.25 to 195 mg/kg, with an average of 87.5 mg/kg. The Dutch list defines the intervention concentration of Cu in soil to be 190 mg/kg. Eco-SSL classifies Cu as an element that should not exceed the concentration of 70 mg/kg when discharged on the soils. If we compare the average concentration of this element in ashes 87.5 mg/kg, we see that the maximum amount of copper is exceeded by almost 13%.

Zinc is an element with relatively low toxicity. In general, zinc toxicity is restricted to taking high doses. The global average of zinc content in soil is 90 mg/kg (Xie et al., 2000), while in the soils in Europe is around 48 mg/kg (Salminen et al., 2005). In our research for ash content of heavy metals, the average concentration of Zn is 229.39 mg/kg, ranging between 0.05 and 670 mg/kg. Eco-SSL maximum value for Zn is 160 mg/kg, thus it is over 40% higher than what EPA recommends.

The significance of nickel to the living organisms has been revealed in the last decades of the last century, bearing in mind by that time that nickel was considered to be toxic metal to the organism. Since then, this element is considered as "potential essential" for humans, although its role in the organisms is still relatively unknown. According to Kabata-Pendias and Mukherjee (2007), the global average content of Ni in soil is 29 mg/kg, while in the surface layer of soil in Europe is 18 mg/kg (Salminen et al., 2005). According to EPA's program issued for *ecological* soil screening levels, nickel should not exceed the amount of 38 mg/kg. In our ash samples it ranges from 36.3 to 82.4 mg/kg with the average of this metal being 58.8 mg/kg. As a conclusion, we see that the concentration of nickel is higher by 20 mg/kg.

For the concentrations of Pb we can say that we have lower concentration than the standard Dutch list. This list sets the maximum allowed concentration of Pb to 530 mg/kg. However, the comparison of these elements is different, because Pb is recognized as a much more toxic and dangerous element even in lower quantities. According to Kabata-Pendias and Mukherjee (2007) the average concentration of Pb in

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soils of the world is 27 mg/kg, while in surface layer of soils in Europe is 23 mg/kg (Salminen et al., 2005). Eco-SSL defines the maximum concentration of lead as a discharged element to be 120 mg/kg. In our samples it varies from 32.35 mg/kg to 48.05 mg/kg.

It should be noted that these metals in the soil often come from various anthropogenic and natural pollutants, so their concentration is not dictated exclusively by ash residues. However, to avoid increasing the concentration of these metals, the most appropriate and ecofriendly methods should be identified.

To compare the results of heavy metals in fresh wood samples and their ash forms, a diagram is presented in *Figure 1*.

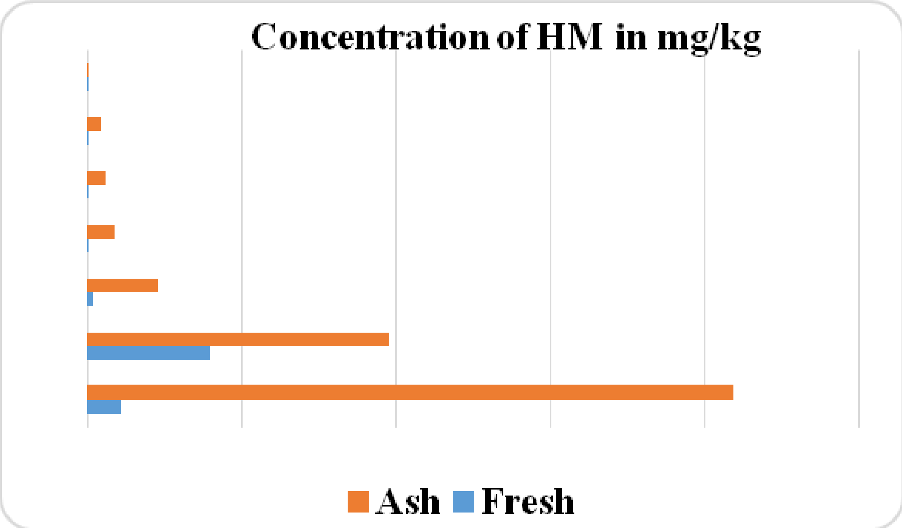

Figure 1. *Comparison of heavy metals in fresh and ash residues in woods*

In all cases we have a higher concentration for Zn, Fe, Mn, Ni, Cu, Pb and Cd. This is justified by the fact that ashes contain precisely these elements. Algreen et al. (2014) found similar results as our average results for Cd, Cu, Zn and Ni.

Correlation analysis

Table 4 shows the analysis of the similarity correlation of elements: Cd, Mn, Pb, Cu, Ni and Zn in measured areas. The statistical calculations were done by using the software Minitab 18.

Kosovo, though a rather small place in territory, is known as a rich country for many minerals. These elements in the environment can come from natural reserves, but anyway the anthropogenic influence has an impact on increasing their concentration.

From the correlation analysis it can be inferred that we have similarity sources of Mn and Pb in with more than 99% and Cd with Ni of about 99%. This shows that these elements occur as natural resources in Kosovo, but these elements can come also due to anthropogenic atmospheric pollution. On the other hand, we have a fairly large similarity of Fe with Cu, which stands at about 75 %.

Figures 2–7 show distribution of heavy metals in fresh wood and in their ash samples. The histograms show the frequency of different concentration brackets expressed in mg/kg.

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***Table 4.** Correlation analysis elements*

	Cd	Mn	Pb	Cu	Ni	Zn
Mn	-0.944					
	0.056					
Pb	-0.928	0.994				
	0.072	0.006				
Cu	-0.675	0.822	0.774			
	0.325	0.178	0.226			
Ni	-0.988	0.981	0.974	0.73		
	0.012	0.019	0.026	0.27		
Zn	0.344	-0.187	-0.079	-0.401	-0.244	
	0.656	0.813	0.921	0.599	0.756	
Fe	0.085	-0.234	-0.148	-0.741	-0.117	0.576
	0.915	0.766	0.852	0.259	0.883	0.424

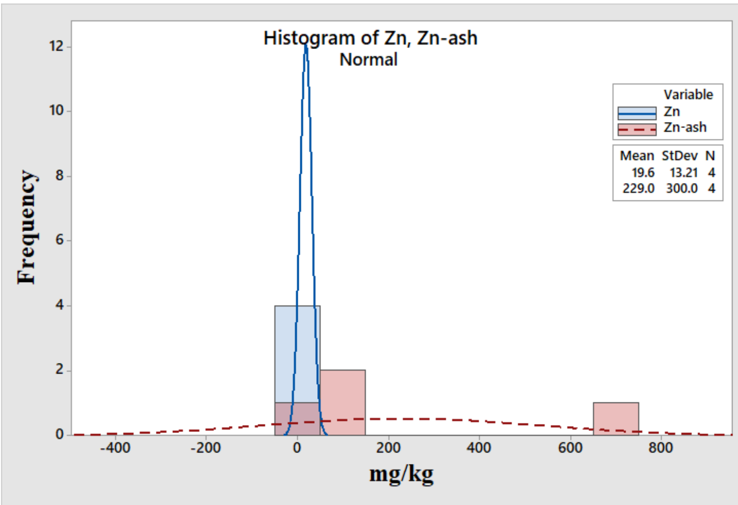

***Figure 2.** Distribution of Zn in wood and ashes in mg/kg*

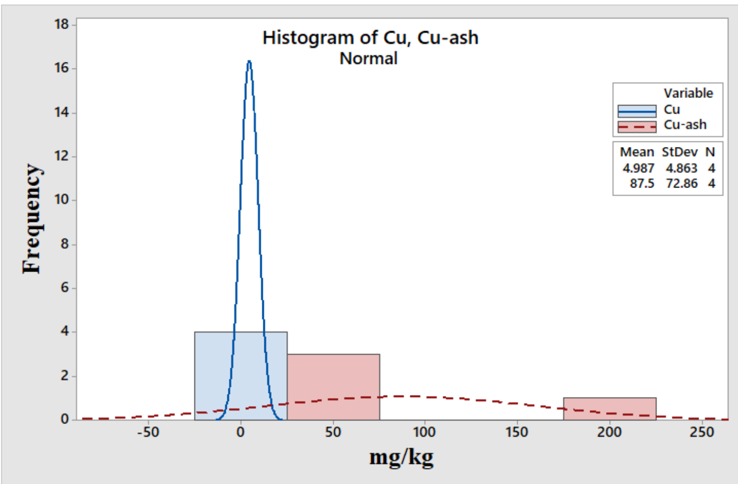

***Figure 3.** Distribution of Cu in wood and ashes in mg/kg*

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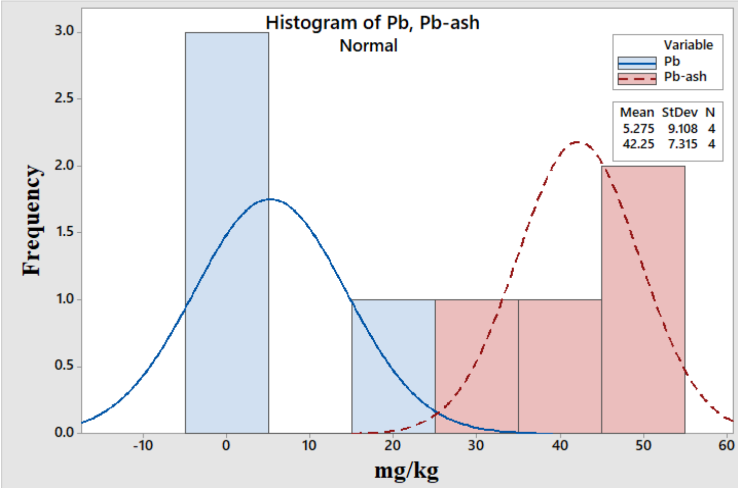

Figure 4. *Distribution of Pb in wood and ashes in mg/kg*

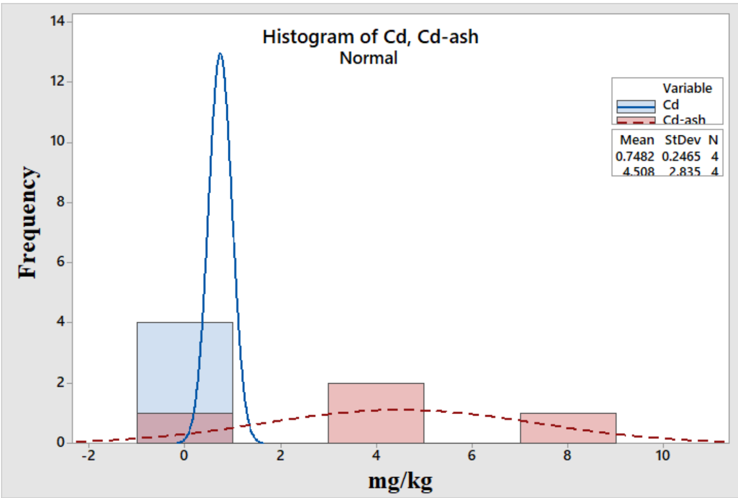

Figure 5. *Distribution of Cd in wood and ashes in mg/kg*

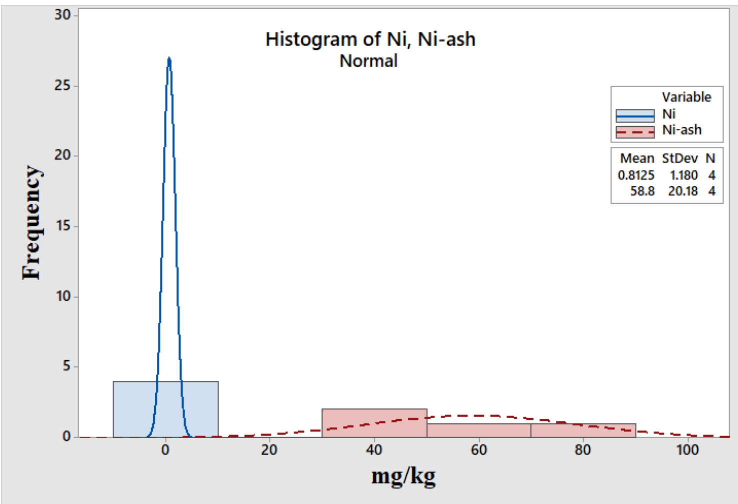

Figure 6. *Distribution of Ni in wood and ashes in mg/kg*

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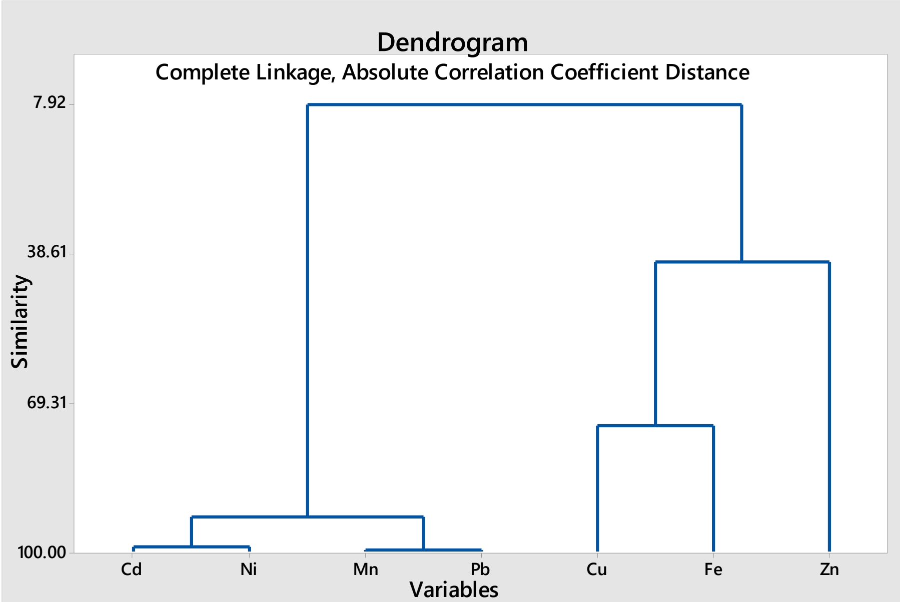

Figure 7. *Dendrogram showing the similarities of elements*

If we compare the results between fresh wood samples and ash residues, we see a significant difference in the concentration of heavy metals. In the case of manganese, its concentration is more than two times higher in ashes than in fresh wood; whereas in Fe, the concentration in ash residue is almost 20 times higher. In zinc, copper, lead, nickel and cadmium we also have significant increases in the concentration of heavy metals in ash samples. A part of these large increases in concentration can be explained by the fact that ashes themselves contain precisely these heavy metals.

Conclusion

The aim of this paper is the examination of the concentration of heavy metals in wood samples and in their ash form. In Kosovo, such a research is very important due to the many environmental problems and mismanagement of waste. In this country, the ash waste is typically disposed of in the ground, water or atmosphere without any of the necessary precautions. In this study, we have analyzed heavy metals and the consequences that can come from improper disposal of ash in the environment. The concentration of heavy metals is slightly higher than foreseen by the relevant standards due to pollution and lack of institutional control in these areas.

The presence of heavy metals in the ash samples from this study can lead to possible water contamination and environment in general.

In order to avoid environment pollution from ash, it is suggested to choose wood from unpolluted areas, free of contaminants, and the wood to undergo complete and careful burning.

If these conditions are met, then this ash can be used in various fields: regulation of pH levels in soil, heavy metal adsorption, remediation of pollutants in various forms, use in construction etc. But under the current conditions with the high concentrations of heavy metals, it is necessary to identify methods of prior treatment before considering the appropriate way of reusing the ash residue.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29312941

CONTAMINATION OF THE ENVIRONMENT BY PATHOGENIC BACTERIA IN A LIVESTOCK FARM IN LIMPOPO PROVINCE, SOUTH AFRICA

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(Received 2nd Nov 2018; accepted 11th Jan 2019)

Abstract. The aim of this study was to investigate the occurrence and diversity of pathogenic bacteria in the environment at a livestock farm in Limpopo Province. Environmental samples were collected from three sampling locations: cattle camp (CC), sheep camp (SHC), and goat camp (GC). Samples were processed and analysed for total bacterial counts in the Biotechnology Laboratory, University of Limpopo, South Africa. Identifications were done with Matrix Assisted Laser Disorption Ionisation Time of Flight Mass Spectometry (MALDI-TOF MS) using the simplified on plate technique. The colony forming unit formula per gram of environmental sample (CFU/g) was used to determine total bacterial counts. High counts were observed in CC and low counts in SHC for both soil and faecal samples. *Bacillus species* were the most dominant across the sampling locations for soil samples. *Bacillus cereus* occurred in 36 samples (33%), however, the frequency of isolation dominated in the CC with (44.4%), GC (22.2%) and SHC (13%). *Staphylococcus aureus* had the least frequency (2.2%) followed by *Listeria monocytogene*s (4.4%). In faecal samples, the occurrence and level of contamination with bacterial species varied across sampling locations with *Escherichia coli* dominating in the CC with 20% frequency, and abscent from SHC and GC.
Keywords: *MALDI-TOF MS, livestock diseases, soil, Escherichia coli, Bacillus cereus*

Introduction

The rural communities of South Africa depend on livestock farming in various ways such as the provision of meat, milk, manure, improved livelihoods and socioeconomic relief (Meissner et al., 2013; Becker, 2015; Madziga et al., 2013). However, livestock diseases present a major threat to animal health. The increase in the circulation of infectious agents over the past decade as well as the emergence of antibiotic resistance and environmental pollution has placed human and animal health at risk. This highlights the importance of human-animal ecosystem interfaces in the evolution of emergence of pathogens (Destoumieux-Garzon et al., 2018). A better knowledge of causes and consequences of certain human activities, lifestyles, and behaviour in ecosystems is crucial for a rigorous interpretation of disease dynamics.

Threats from old and new pathogens continue to emerge as a result of changes in the climatic conditions. Young growing animals on pasture are especially prone to spore forming bacteria of the *Bacillus* and *Clostridium spp*. Livestock acquire the spores

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

during grazing on contaminated pastures (Lange et al., 2010; Cooper and Valentine, 2016). Once the bacteria are in abundance on pasture, cases of the disease usually occur frequently in susceptible herds. Affected farms are usually those with irregular vaccination programs as farmers seek to minimise production costs (Maas, 2012). Limited studies focused on the ecology of transmission of livestock diseases (Morand and Figuié, 2016; Morand and Lajaurie, 2017; Cantor et al., 2017). In endemic areas, *Bacillus* and *Clostridium spp* may be present in soil and faeces (Dharmasena and Jiang, 2018). Christianssonn et al. (1999) reported bacterial contaminants of soil particularly *Bacillus cereus* to contaminate teats and udders of pasture fed cows leading to milk contamination and food borne diseases. Over the last few decades, several pathogens have either emerged or re-emerged. Livestock from small scale production systems in Limpopo Province suffer endemic diseases as they are kept under scavenging conditions which expose them to virulent factors.

In most cases, there is little attention to disease control and prevention in these livestock production systems (Gibbs, 2005). The extent of the diversity of microorganisms in soils determines the quality of pastures, since a wide range of pathogenic bacteria are involved in pasture contamination from livestock manure (Bagge et al., 2010). Bacterial species have always been of considerable medical and economic importance and the world is faced with changing landscape of infectious diseases due to pathogenic bacteria that affect man and animals posing significant threats to health and welfare of livestock (King et al., 2006). According to the 'one health', 60% of all human infections are directly or indirectly linked to livestock disease outbreaks (FAO et al., 2007). The documents and publications on the "One Health" approach, and the strategic framework developed around it, have largely focused on the battle against emerging zoonoses originating in domestic (Day, 2011) or wildlife (Dantas-Torres et al., 2012) and/or their interactions (Mencke, 2013). Bagge et al. (2010) reported manure to be the common pasture contaminant resulting in livestock infections. Based on the farm records, diseases and conditions such as blackleg, pulpy kidney, pink eye and mastitis are endemic at the farm. Therefore, identification of the causative agents in the farm is crucial for implementation of prompt therapeutic and control measures (Neumann et al., 2002). Therefore, the objectives of the study were to determine the total bacterial counts from environmental samples collected from the cattle and small stock camps in the farm using the colony forming unit (CFU) formula per gram of environmental sample and to identify the pathogenic bacteria isolated from soil and faecal samples.

Literature review

Arable land and pastures contaminated with the faeces of sick animals contribute considerably to pathogen transfer resulting in farm environment pollution (Trawińska et al., 2006; Nageswaran et al., 2012; Czekalski et al., 2012). Furthermore, the soil and manure in high producing farms provide diverse biological and physicochemical environments to microorganisms which pose a significant threat to humans and animals (Godwin and Moore, 1997; Whitman, 1998; and Douglas et al., 2003). Stecher and Hardt (2008) reported that bacteria in the gastro-intestinal tracts of animals can be excreted at high levels in faeces thereby contaminating soils and pastures. These bacteria may persist due to differences in local soil conditions (Dapilly and Neyrat, 1999; Gale, 2004). Extensive livestock farming poses a threat to the environment, as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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livestock shed millions of bacteria through their faeces and contaminate the soil and pastures thereby elevating the risk of disease breakouts. Nonetheless, disease outbreaks can be prevented through vaccination when local strains are recognised and safeguarding of human and animal health can be ensured (Radostitis et al., 2000; Amin et al., 2013; Manyi-Loh et al., 2016).

Livestock practices influence the microbial composition of faeces shed by animals (Wang et al., 2004; Manyi-Loh et al., 2016). The ability of these pathogens in manure to pollute, contaminate and infect the environment and livestock depends on the pathogen's ability to survive in manure following excretion or the hygiene practices to prevent its entry into the food chain (Wang, 2004). Studies on diversity of spore forming bacteria in cattle manure reported pathogenic bacteria of particular concern for animal health as, *Clostridium spp,* particularly (*C. chauvoei*, *C. botulinum*, *C. tetani*) and *Bacillus spp* (*B. anthracis* and *B. cereus*) (Bagge et al., 2010; Adak et al., 2002*;* Pachepsky et al., 2006; Pell, 1997; Plaut, 2000; Chauret et al., 1999). *Campylobacter spp* (*C. jejuni* and *C. coli*)*, Salmonella enterica* (Adak et al., 2002*;* Pachepsky et al., 2006; Pell, 1997; Plaut, 2000), *Yersinia spp* (Y. enterocolitica and Y. pestis), *Leptospira spp* (*L. interrogans*) and *Coxiella burnetii* (Aitken et al., 2005; Nightingale et al., 2004), *Mycobacterium avium* sub species *paratuberculosis, Listeria monocytogenes, Escherichia coli O157* (Robert et al., 2017) are also reported as major threats to animal and human health. These bacterial species can cause serious clinical diseases in farm animals, when they are ingested in feed and have also been implicated in cases of food borne diseases in humans such as *L. mocytogenes* in human listeriosis.

Salihu et al. (2009) reported that *Ca. jejuni* and *Ca. fetus* cause abortion, stillbirths and birth of weak lambs in sheep during late pregnancy. The campylobacter species has highly been associated with feed contamination mostly in poultry as a result of faecal contamination. This alarms the need to identify these species at farm level so as to put hygiene strategies like proper manure management in place to prevent cross contaminations. Moreover, the depletion of medicinal plants due to increased population alarms the need to recognise local pathogen strains so that reliable drugs and vaccines can be produced for proper management of livestock diseases (Nishteswar, 2014). Thus, it is necessary that local pathogen strains be recognised so that vaccines can be developed for better disease management at farm level.

There is ample evidence showing that soil amendments that harbor enteric pathogens, such as raw animal manure or incompletely composted manure, are a means of introducing these pathogens in crop production systems (Kim et al., 2009; Nicholson et al., 2005; Ziemer et al., 2010). Products from mixed farming, including meat, eggs, and fresh produce, are at greater risk of cross-contamination as they are grown in the same facility and are currently considered to be high-risk foods (Adl et al., 2011). The source of contamination of fresh produce with enteric pathogens can frequently be traced back to environmental reservoirs associated with farm animals such as poultry, cattle, swine, goat, and sheep (Brinton et al., 2009; Park et al., 2014).

Materials and methods

Description of the study area

The study was carried out in a livestock farm in Limpopo Province, South Africa, coordinates: 23°49' S; 29°41' E. During the study period, no animal diseases or mortalities were reported. The total number of livestock kept at the farm was 225

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

comprising 82 cattle, 77 sheep and 66 goats. The farm practices mixed livestock farming. Extensive livestock production system increases the chances of disease outbreak as livestock will have access to pastures and water supplies that may be contaminated with disease causing agents such as bacteria (McGuirk, 2015). The farm relies on chemoprophylaxis as a disease management tool, however, the practice is irregular. Environmental samples were collected from different resting points within camps in the farm. *Figures 1-4* depict the environment from where samples were collected.

Figure 1. *Collection of soil samples from resting points in the cattle camp in a livestock farm in Limpopo Province*

Figure 2. *Collection of faecal samples from resting points in the cattle camp in a livestock farm in Limpopo Province*

Figure 3. *Livestock water source in the vicinity of resting points from where environmental samples were collected*

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

Figure 4. *Collection of faecal pellets from small stock camps*

Collection of environmental samples

Environmental samples (soil and faeces) analysed in this study were collected from the vicinity of sheep and goat housing facilities as well as cattle grazing pastures. Samples are collected over a period of three months, from January to March 2018. The samples were labelled according to the site of collection as cattle camp samples (CCS), sheep camp samples (SHCS) and goat camp samples (GCS). Hundred soil samples comprising of sixty CCS, twenty SHCS and twenty GCS were collected from locations that were perceived as high risk areas for bacterial contamination in a random zigzag pattern. Unpolluted and polluted top soil samples in the vicinity of faecal droppings were collected at different depths. Samples taken from below the soil surface were collected using a soil auger according to the procedure described by Brooks (2016). Seventy faecal samples comprising of thirty CCS, twenty SHCS and twenty GCS were collected. Cattle faecal samples were collected as cowpats on grazing land while sheep and goat faecal samples were collected as pellets. Approximately 100-200 g environmental samples were collected in sterile sealable plastic bags. The samples were then transported on ice, in a Styrofoam cooler box and transported to the University of Limpopo, Biotechnology Laboratory for microbial analysis

Microbial analysis of environmental samples

All the required consumables for the study were procured from Prestige Laboratories (Pty) Ltd, Durban, South Africa. Microbial analyses were performed under sterile conditions in a laminar flow with a Bunsen burner to avoid contamination from the environment. The collected samples were cultured on selective and general media for isolation of pathogenic bacteria.

Reinforced clostridia agar, Reinforced clostridia broth, sheep blood agar, anaerobic basal broth and nutrient agar were prepared and used for detailed investigation of pathogenic bacteria according to the manufacturer’s instructions. Environmental samples were serially diluted to reduce the bacterial concentration of the original soil sample to levels low enough for single colonies to be grown on agar plates, allowing for calculation of the initial counts of bacteria in the environmental samples (Koch, 1883).

Purification of bacteria and determination of total counts

Bacterial culture and purification were carried out according to the methodology of Bagge et al. (2009) while colony counts were determined according to Brugger (2012) (*Eq. 1*). Samples cultured on sheep blood agar grew and triggered haemolyses within

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

24 h of incubation at 37 °C. The isolates appeared flat and raised with the shape being filamentous to irregular, round and punctiform. They were smooth or wrinkled and the colour ranged from grey, cream to white and with few colonies being yellow (*Figs. 5-7*). Different colonies subcultured on nutrient agar for purification were able to grow under anaerobic conditions within 24 to 36 h of incubation at 37 °C.

Figure 5. *Plates of colonies cultured on sheep blood agar*

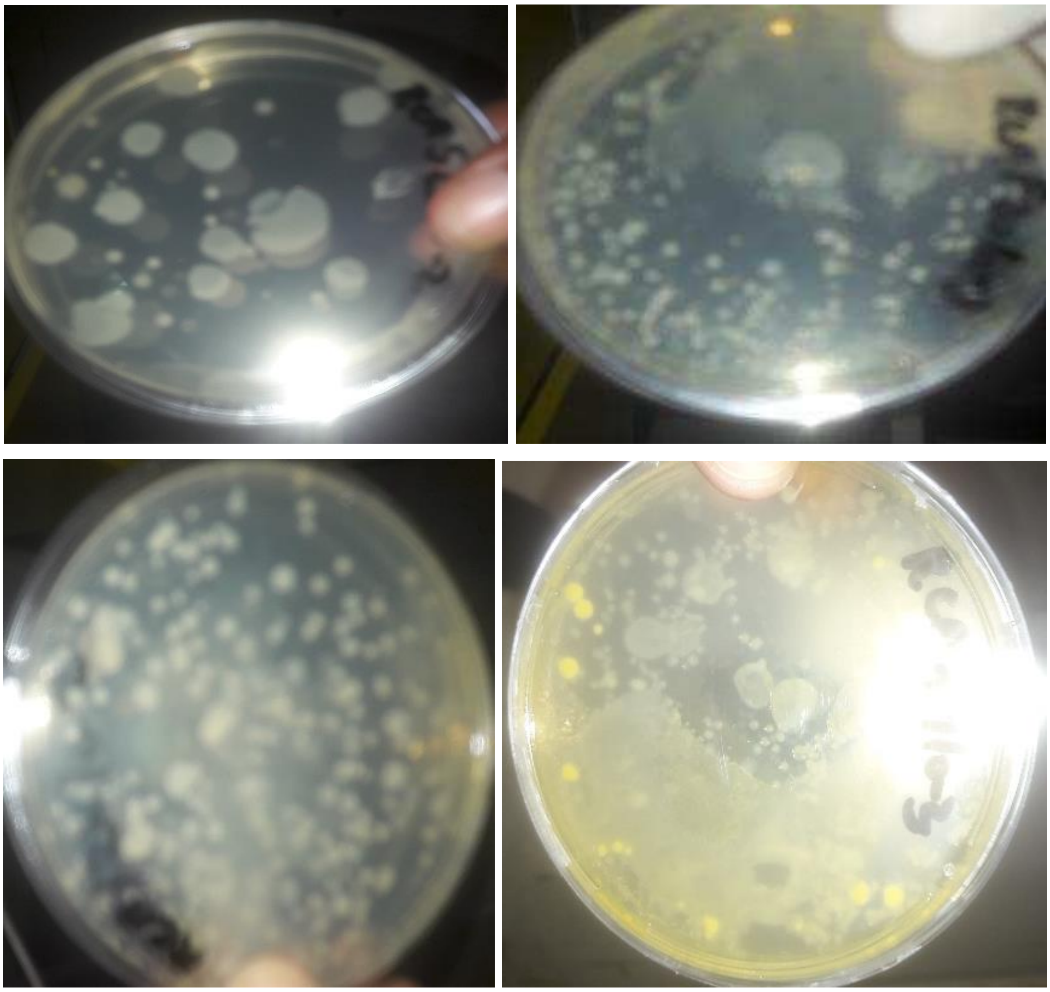

Figure 6. *Plates of colonies cultured on Reinforced Clostridia and nutrient agar*

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

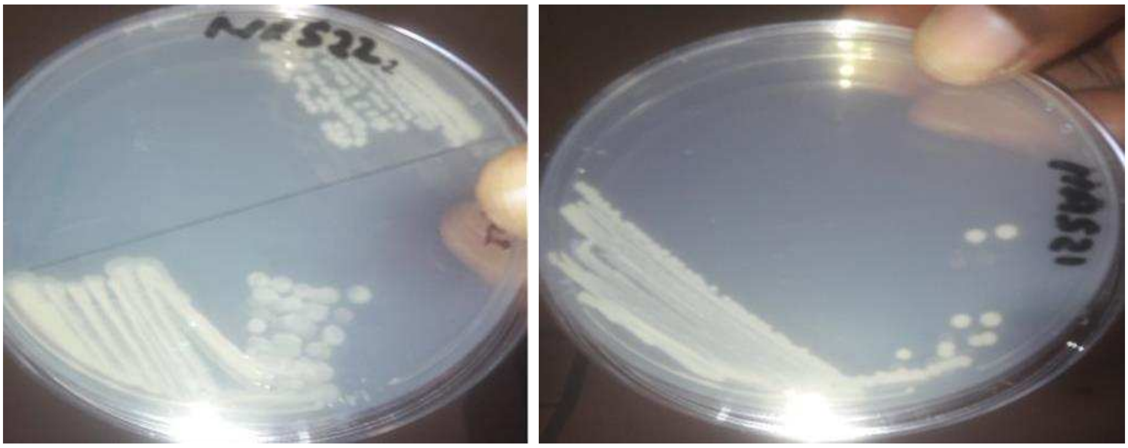

***Figure 7**. Plates with a four-way streaking of pure cultures on nutrient agar*

Proteomic identification of colonies

All bacterial isolates reported in this study were analysed by MALDI-TOF MS, using a Microflex LT bench top mass spectrometer (Bruker Daltonics, Maldi Biotyper, Bremen, Germany). The software for the control of the instrument was FlexControl 3.3 and Maldi Biotyper 3.1 (Bruker Daltonics) for the analysis of the spectra and comparison with the database. A bacterial test standard provided by the manufacturer was included in every run for calibration purposes. Default settings (acquisition of mass spectra in the linear positive mode within the 2e20 kDa range, ion source 1 (IS1) 20 kV, IS2 18.05 kV, lens 6.0 kV, linear detector 2,560 V) were applied for all the detections. A rapid, on-plate method that requires less time and reagents for its performance was followed in this study (Matsuda et al., 2012).

Data presentation

The numbers of viable bacteria per gram were obtained using a mathematical formula for colony forming units (CFU) per gram of environmental sample. Diversity, occurrence and frequency of isolation of pathogenic bacteria identified in environmental samples using MALDI-TOF MS are presented with descriptive statistics. Microsoft Excel, 2013 was used to compare the level of contamination in faecal and soil samples.

$$\mathrm{CFU}/g = \frac{\text{Number of colonies per agar plate} \times \text{Dilution factor}}{\text{Volume of culture plated}} \quad \text{(Eq.1)}$$

Results

Colony forming units per gram of environmental sample

Colony forming units were calculated using the formula of Harley (2005) (*Eq. 1*) based on average colony counts per plate. This was done to estimate the number of viable bacteria per gram of environmental sample. All samples were of similar weight (1 g) and exposed to same dilution factors of 10^{-5}, 0.1 ml of bacterial culture were plated per agar plate. Based on these calculations, there were differences in the average colony forming units of different sampling locations. The results indicated that the cattle

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camp had a higher level of contamination as compared to the goat and sheep camps for both soil and faecal samples. The highest counts were observed in samples from the cattle camp and the lowest counts were observed in samples from the sheep camp for both soil and faecal samples (*Figures 5* and *6*).

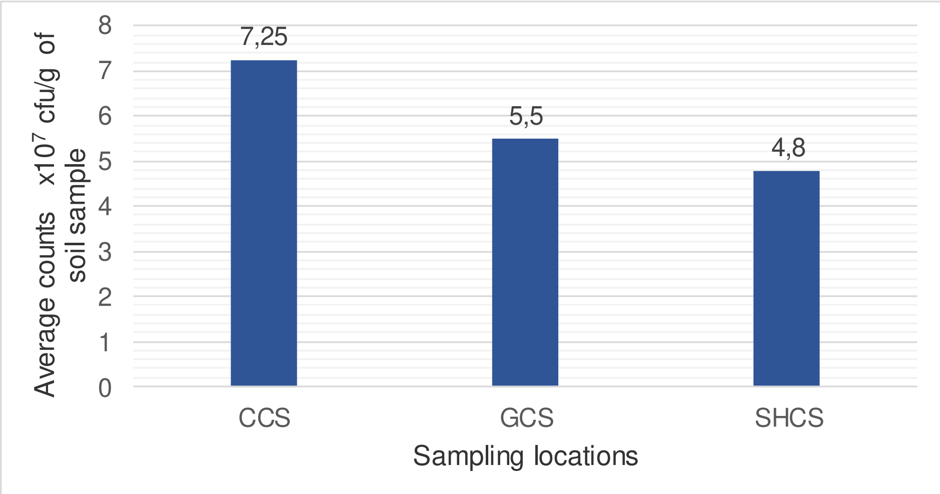

Figure 5. *Average colony forming units (CFU) per gram of soil sample from different camps within the farm*

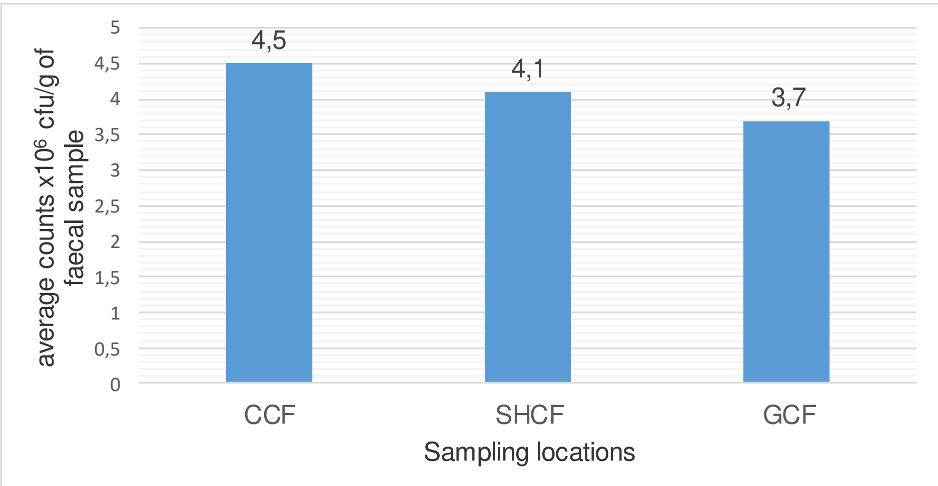

Figure 6. *Average colony forming unit per gram of faecal sample from different camps in the farm*

Diversity of bacterial species identified in soil and faecal samples

During the study period, a total of hundred and seventy environmental samples collected for microbial analysis of pathogenic bacteria were analysed by MALDI-TOF MS using the simplified on-plate method. Hundred and thirty-four (79%) of the samples comprising of 108 soil and 26 faecal samples were successfully analysed and tested positive for different bacterial species. The remaining thirty-six samples (21%) could

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

not be identified by the technique used. *Tables 1* and *2* indicate the bacterial isolates identified upto genus/species level for faecal and soil samples respectively. Based on these results, it was observed that different farm environments harbour different bacterial species. However, minor similarities were observed with few bacterial species where *B. cereus* and *E. coli* were isolated in both manure and faecal samples. Using (*Eq. 2*) *Bacillus cereus* were identified as the most dominant species isolated from soil samples in 36 positive samples (33%), followed by *B. mycoides* in 24 positive samples (22%) (*Table 3*). Regarding faecal samples, 26 samples tested positive with *E. coli* being the most prevalent species, identified in 9 positive samples (34.6%), followed by *Acinetobacter baumannii* identified in 8 samples (30.8%), *Acinetobacter baumanii* in 5 samples (19.2%), *B. cereus* and *B. psedomycoides* were the least dominant with (7.7%) each (*Table 4*).

Table 1. *Bacterial species identified from faecal samples by MALDI-TOF MS using the simplified on-plate method (n = 26)*

Bacteria species	No of positive samples	Cut off values proposed for this study			
		≥3	2.00-2.999	1.699-1.999	0.00-1.599
*Escherichia coli**	9	-	8	1	-
*Bacillus cereus**	2	-	-	2	-
Bacillus pseudomycoides	2	-	-	-	2
Acinetobacter genomospecies	8	-	2	6	-
Acinetobacter baumanii	5	-	4	1	-
Total	**26**	**0**	**14**	**10**	**2**

*Pathogenic bacteria

Table 2. *Bacterial species identified from soil samples by MALDI-TOF MS using the simplified on-plate method (n = 108)*

Cut off values proposed for this study					
Bacteria species	**No of positive Samples**	**≥3**	**2.00-2.99**	**1.6-1.99**	**0.00-1.599**
*Bacillus cereus**	36	-	6	30	-
*Listeria monocytogens**	2	-	-	-	2
Lysinibacillus fusiformis	4	-	-	2	2
Bacillus megaterium	1	-	-	1	-
*Staphylococcus aureus**	1	-	-	1	-
Enterobacter cloacae	4	-	-	3	1
Enterobacter cancerogenus	1	-	-	-	1
Bacillus simplex	1	-	-	1	-
Enterobacter absburiae	2	-	-	2	-
Bacillus mycoids	24	-	-	20	4
Bacillus weihenstephanesis	12	-	-	8	4
*Escherichia coli**	4	-	3	1	-
*Seratia marcescens**	5	-	2	2	1
*Bacillus licheniformis**	4	-	-	3	1
Bacillus endophyticus	1	-	-	1	-
Pseudomonas resinovarans	1	-	-	-	1
*Pseudomonas aeruginosa**	2	-	-	2	-
Pseudomonas monteilii	2	-	-	1	1
Pseudomonas corrugate	1	-	-	1	-
Total	**108**	**0**	**11**	**79**	**18**

*Pathogenic bacteria

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

***Table 3.** Occurrence and percentage frequency of bacteria from soil of different camps in the farm (n = 108)*

Bacterial species	No. of positive samples in the farm	% frequency in the farm	Occurrence			No of positive samples in the camp and % frequency					
			CCS	SHCS	GCS	CCS		SHCS		GCS	
						+NSC	%FC	+NSC	%FC	+NSC	%FC
*Bacillus cereus**	36	33.3	+	+	+	20	28	6	50	10	41.6
*Listeria monocytogenes**	2	1.9	+	-	-	2	2.8	0	0	0	0
Lysinibacillus fusiformis	4	3.7	-	+	+	0	0	1	8.3	3	12.5
Bacillus megaterium	1	0.9	+	-	-	1	1.4	0	0	0	0
*Staphylococcus aureus**	1	0.9	+	-	-	1	1.4	0	0	0	0
Enterobacter cloacae	4	3.7	+	-	-	4	5.6	0	0	0	0
Enterobacter cancerogenus	1	0.9	+	-	-	1	1.4	0	0	0	0
Bacillus simplex	1	0.9	+	-	-	1	1.4	0	0	0	0
Enterobacter absburiae	2	1.9	+	-	-	2	2.8	0	0	0	0
Bacillus mycoides	24	22.2	+	-	-	24	33	0	0	0	0
Bacillus weihenstephanesis	12	11.1	+	-	-	12	17	0	0	0	0
*Escherichia coli**	4	3.7	+	-	-	4	5.6	0	0	0	0
*Serratia marcescens**	5	4.6	-	-	+	0	0	0	0	5	20.8
*Bacillus licheniformis**	4	3.7	-	+	-	0	0	4	33.3	0	0
Bacillus endophyticus	1	0.9	-	-	+	0	0	0	0	1	4.2
Pseudomonas resinovarans	1	0.9	-	+	-	0	0	1	8.3	0	0
*Pseudomonas aeruginosa**	2	1.9	-	-	+	0	0	0	0	2	8.3
Pseudomonas monteilii	2	1.9	-	-	+	0	0	0	0	2	8.3
Pseudomonas corrugate	1	0.9	-	-	+	0	0	0	0	1	4.2
Total	**108**	**100**				**72**	**100**	**12**	**100**	**24**	**100**

CCS = Cattle camp soils; SHCS = Sheep camp soils; GCS = Goat camp soils; +NSC = Number of positive samples in camp; % FC = Percentage frequency in the camp; *Pathogenic bacteria; + = present; - = absent

***Table 4.** Occurrence and percentage frequency of bacteria from faecal samples of different camps in the farm (n = 26)*

Bacteria	No. of positive samples in the farm	% frequency in the farm	Occurrence			Number of positive samples in the camp and % frequency					
			CCF	SHCF	GCF	CCF		SHCF		GCF	
						+NSC	% FC	+NSC	% FC	+NSC	% FC
*Escherichia coli**	9	34.6	+	-	-	9	64.2	0	0	0	0
*Bacillus cereus**	2	7.7	+	-	-	2	14.3	0	0	0	0
Bacillus psedomycoides	2	7.7	+	-	-	2	14.3	0	0	0	0
Acinetobacter baumannii	8	30.8	+	+	+	1	7.1	3	100	4	44.4
Acinetobacter genomospecies	5	19.2	-	-	+	0	0	0	0	5	55.6
Total	**26**	**100**				**14**	**100**	**3**	**100**	**9**	**100**

CCF = Cattle camp faeces; SHCF = Sheep camp faeces; GCF = Goat camp faeces; + = Present; - = Absent; *Pathogenic bacteria; +NSC = Number of positive samples in the camp; % FC = Percentage frequency in the camp

In the present study, the levels of environment contamination by pathogenic bacteria varied with respect to sampling location. However, there were some similarities with respect to the bacterial isolates prevailing across sampling locations (*Tables 3* and *4*).

Percentage frequencies in *Tables 3* and *4* were calculated using *Equation 2:*

$$\%Frequency = \frac{\text{Number of samples from a camp positive for a bacterial species}}{\text{Total number of positive samples from the farm/camp}} \times 100 \quad \text{(Eq.2)}$$

Discussion

This study utilised MALDI-TOF MS to identify bacterial isolates from environmental samples. The results indicated that 134 (79%) environmental samples out of the 170 collected tested positive for various bacterial species with MALDI-TOF MS identification. Urwlyer and Glaubitz (2015) reported MALDI-TOF MS to have a revolutionized speed and precision of microbial for clinical isolates to outperform conventional methods. This is evidenced in their study were MALDI-TOF MS showed the lowest number of false identification (4%) and 60% accuracy at genus level. In contrast, the biochemical-based system assigned 25% of genera incorrectly. In this study, MALDI-TOF MS provided identification at the genus level of 67% of the bacterial isolates. This agrees with Dupont et al. (2010), Justeen et al. (2011), Nagy et al. (2012), Wieser et al. (2012), Lee et al. (2015) and Florio et al. (2018) who reported the capacity of MALDI-TOF MS to identify bacterial microorganisms at the genus and species level according to the cut of values in this study (score value 2.000 - 2.299) ranging from 65.2 to 83.9%. Average colony forming units in both soil and faecal samples fell within the range reported by previous researchers for environmental samples (Okoh et al., 1999; Ogunmwonyi et al., 2008). Total bacterial counts were relatively higher in soil samples suggesting high contaminations as compared to faecal samples.

These results agree with the reports of Trawińska et al. (2006) who indicated that soil in the vicinity of high-production farms is commonly microbial-contaminated as arable land and pastures contaminated with the faeces of sick animals, especially, contribute considerably to pathogen transfer into the soil. Boes et al. (2005) and Ngole et al. (2006) reported that micro-organisms survival in the soil environment is favoured by high temperature and moisture. Oliver et al. (2006) stated that rainfall events result in faecal microbes being washed from the cowpats into the surrounding soils where their survival could be enhanced. This finding is further supported by the results of Muirhead (2009) who reported an increase in soil *E. coli* concentrations on the grazed camps which they believed coincided with an increase in rainfall during their study period. Furthermore, Kress and Gifford (1984) and Stoddard et al. (1998) reported that heavy rain or irrigation on a fresh cow pat is likely to result in far greater microbial mobilization and leaching. Mobilization and leaching of bacteria during rainfall could be the reason for high percentage prevalence of bacteria species in soil compared to faecal samples recorded in the current study. Bacteria leach with water into the soil, where they incubate and multiply resulting in contamination of pastures were animals graze (Kress and Gifford, 1984).

In the present study, *Bacillus species* was identified to be the most prevalent species in soil samples. This is in agreement with Gutiérrez- Mañero et al. (2003) and Amin (2015), who reported bacillus as naturally occurring soil bacteria and most abundant

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

genus in the rhizosphere of soil. From this genus, the most dominant species appeared to be *B. cereus,* which when allowed an opportunity to invade mammalian tissues is an opportunistic pathogen that may cause severe or local systematic infections such as endophthalmitis and septicaemia (Kotiranta et al., 2000). *B. cereus* has been reported to cause serious clinical diseases in farm animals, thereby causing economic loses to farmers. Songer and Post (2005), Nieminen et al. (2007) and Salih (2015) reported *B. spp* to occasionally cause mastitis in cattle. Furthermore, *B. cereus* has been reported as an important food borne pathogen. Tewari and Abdullah (2015) reported highly toxic strains of *B. cereus* responsible for food-related fatalities. Ranieri et al. (2009) and Gundogan and Avci (2014) reported *Bacillus spp* to be ubiquitous in nature. The ability of its spores to tolerate different environmental conditions including elevated temperatures result in these species being the most common isolated bacteria from food, air, soil, and faecal samples. However, the reports of Bavykin et al. (2004) indicating that bacillus is the most common genus in faecal samples are in contrast with the findings of this study as these bacteria tested positive only in faecal samples of cattle with a frequency occurrence of 6.6%.

In faecal samples, *E. coli* was the most frequently isolated pathogen. The dominance of *E. coli* in faecal samples reported in our study agrees with reports by previous researchers (Tahamtan et al., 2006; Raji et al., 2006; Hiko et al., 2008; Hashemi et al., 2010). Hogan et al. (1999) and Abakpa et al. (2015) reported *E. coli* to be naturally present in faeces of warm blooded animals. *E. coli* occurrence has been previously used as an indicator of faecal contamination, signaling the possible presence of faecal pathogens such as *Salmonella* and *Shigella* species (Odonkor and Addo, 2018). In a study by Rodrigues et al. (2015), MALDI TOF-MS identified 83% *E. coli* prevalence in faecal samples, however, the pathogenicity of the strains were not reported. Schmidt et al. (2015) reported *E. coli* to be free-living commensals in animal intestines. The prevalence of this bacterial species was found to be high in the cattle camp in comparison with the small stock camps. Sima et al. (2009), Kiranmayi et al. (2010) and Rahimi et al. (2012) reported domestic animals as the sources of *E. coli*. However, the major animal carriers seem to be healthy domesticated ruminants, primarily cattle and, to a lesser extent sheep.

These findings agree with the results of our study as *E. coli* were only identified in cattle faeces with zero prevalence in sheep and goat faecal samples. However, large variations have been described in the shedding patterns of individual animals, the proportion of shedding animals on farms that harbor them and, over time, in the amount of shedding on the same farm (Smith et al., 2010). Among cattle, shedding occurs intermittently (Hancock et al., 1997; Kulow et al., 2012; Sharma et al., 2012), and it was reported that, at any time, up to 50% of the healthy animals excrete *E. coli* in their stool (Lim et al., 2010). Freshly deposited faeces contain the nutrients required by bacteria, and replication presumably depends on the faeces retaining water and attaining suitable temperatures for growth. Cow pats are able to retain moisture when exposed to sunlight. The pats quickly form a skin, which thickens to a well-defined crust within 48 h favouring bacterial growth (Van Kessel et al., 2007). However, exposure of sheep and goat pellets to the sun results in drying of the pellets due to size and less moisture contained making the environment unfavourable for bacterial growth. Topp et al. (2003) studied the relationship between soil moisture content and prevalence of *E. coli* bacteria and concluded that elevated soil moisture levels were associated with increased number of bacteria in the soil. This could explain the low counts of *E. coli* in soil samples of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

current study as soil samples were collected in early summer where moisture content in soil is believed to be relatively low. Of the twenty-two bacterial isolates isolated from the environmental samples in this study, some species have been reported previously to carry virulent strains which are responsible for livestock infections particularly in cattle, sheep and goats. These pathogenic bacteria include: *E. coli* (Olson, 2001; Chekabab et al., 2013), *B. cereus* (Nieminen, 2007; Manyi-Loh, 2016; Robert et al., 2017), *L. monocytogens* (Nightingale et al., 2004; Nicholson et al., 2005), *Staphylococcus aereus* (Toroitich, 2013), *Pseudomonas aeruginosa* (Radostits et al., 2000), *Seratia marcescens* and *B. licheniformis* (Olson, 2001).

Pathogenic bacteria in farm environments pose a major epidemiological threat (Amin et al., 2013). Although *E. coli* have been reported as harmless commensals of the intestines of warm blooded animals, the strain *E. coli* O157:H7 has been reported to be harmful (Titilawo et al., 2015). Shearer et al. (2003) reported *E. coli* as one of the major bacterial pathogens associated with livestock infections in farms. Healthy colonized cattle and other ruminants are the most significant animal reservoir harboring *E. coli* (Ferens and Hovde, 2011). Previous studies linked approximately 75% of the human *E. coli* outbreaks to food products of bovine origin (Callaway et al., 2009; Munns et al., 2016). Other reservoirs that may impact transmission include sheep (La Ragione et al., 2012; Soderlund et al., 2012; Gencay, 2014), goats Pao et al.(2005), La Ragione et al.(2009), Mersha et al.(2010), Alvarez-Suarez et al.(2016) and Swift et al.(2017).

According to Tomita and Hart (2001) *E coli* bacteria possess several pathogenic factors responsible for their pathogenicity, among which exotoxin A (*tox*A) and exoenzyme S (*exo*S) are the two major fatal toxins which are associated with subclinical mastitis infection in bovines (Toroitich, 2013) in a cross-sectional study to determine the prevalence of mastitis and identify the associated risk factors reported the most predominantly isolated bacterium to be *Staphylococcus aureus* with a prevalence of 36% followed by *E. coli* with a prevalence of 27.2% and *Pseudomonas* were least isolated with less than 1% prevalence.

In the present study, *L. monocytogenes* tested positive in cattle camp soils (4.4%). This falls in the same range as the findings of Mohammed et al. (2009) (5.4%) and less compared to the findings from previous studies by Moshtaghi et al. (2003) (17.7%) and Nightingale et al. (2004) (22.2%) and Locatelli et al. (2013) (38.1%). Several animal derived *L. monocytogenes*-contaminated food products, including raw milk, pasteurized milk, chocolate milk, butter, soft cheeses, and processed meat and poultry products, have been implicated as sources of human listeriosis cases and outbreaks (Buchanan et al., 2017). It has been reported that manure from infected or shedding animals represent direct links between human infections and *L. monocytogenes* in farm animals and farm environments as a result of consuming animal-derived food products that are not processed before consumption such as raw milk and raw foods of plant origin (Nightingale et al., 2004).

Previous studies reported *Campylobacter* species and *Clostridium* species to be pathogenic bacteria of livestock commonly isolated from environmental samples (Baserisalehi et al., 2007; Bagge, 2009). However, in the present study, all samples tested negative for *Campylobacter* species. *Clostridium species* were detected in 2 samples (5%). Bandelj et al. (2016) reported a 10% prevalence of *clostridium difficile* in cattle faecal samples. The use of selective media may have disadvantaged growth of other bacteria species in the samples. Olson (2001), reported the *Campylobacte*r genus to have fastidious growth requirements making conventional detection and

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DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

identification difficult. This could be the reason why all samples in the present study tested negative for *Campylobacter species*. The differences in bacterial contamination in the sampling locations in this study may be due to different management practices in the livestock camps were cattle are kept in their camp continuously resulting in piling of faecal samples increasing microbial habitat. In comparison with sheep and goats which are housed at night resulting in few fresh pellets in their camps. Spiehs and Goyal (2007), Hutchison et al. (2005) and Manyi-Loh et al. (2016) indicated that the levels and types of pathogens occurring in livestock faeces vary with animal species, dietary sources, health status and age of the animal.

Conclusions and recommendations

This study showed that diverse bacterial species contaminate the livestock grazing environment at the study farm. Although samples were collected from different locations within the farm, the bacteria isolated from soil samples were generally similar. However, with regard to faecal samples, bacterial isolates differed from one camp to the other entailing that different livestock harbour different bacteria in their faeces. Of the bacterial species isolated in the study, some genus have been reported to be highly pathogenic. This study demostrated contamination by opportunistic, food-borne bacteria like *B. cereus* and *L. monocytogenes* in the farm environment and the need for good hygiene practices to prevent its entry into the food chain. Rapid microbial identification is necessary for quick implementation of relevant disease management strategies at farms. The findings of this study show that MALDI-TO MS can identify bacteria rapidly in environmental samples. This method can be used to identify potential disease risk in an environment and allow for appropriate control measures.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29432963

THE DIFFERENCES AMONG MELON GENOTYPES AND VARIETIES UNDER SALT STRESS BASED ON CERTAIN MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES – MIXTURE MODELING AND PRINCIPAL COMPONENT ANALYSIS (PCA)

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(Received 7th Nov 2018; accepted 8th Feb 2019)

Abstract. Present study aimed to determine the effects of salt stress in 13 melon genotypes and 4 commercial melon varieties collected from the Van Lake Basin. Two doses of salt applications were conducted at 0 mM and 50 mM NaCl concentrations and the plants were cultivated in 3 repeats under 25 ± 2 °C temperature and 16/8 light/dark periods based on the randomized block experimental design in climate chamber conditions. In order to determine the effect of stress, traits such as 0-5 scale, shoot and root lengths, shoot diameters, leaf number, fresh and dry weights of shoots and roots, leaf relative water content, membrane injury index, stoma widths and lengths, stomatal areas and stoma densities were evaluated. The reaction of the genotypes against stress was determined via mixture modeling and PCA analysis. In PCA analysis, three PCA components explained 71.48% of the total variation at 0 mM, while four components explained 69.53% of the total variation at 50 mM. As a result of the mixture modeling analysis, it was observed that 4 sub-populations for 0 mM and 3 sub-populations for 50 mM were formed and it was revealed that the salt-tolerant genotypes were in the sub-population 3 and salt-susceptible genotypes were in the sub-population 1.
Keywords: *Cucumis melo L.,·soil salinity,·susceptibility,·tolerance, Van Lake Basin*

Introduction

Especially in arid and semi-arid regions with low precipitation, soil salinity, which occurs with the high surface evaporation due to the effect of incorrect applications such as excessive irrigation and fertilization, is among the increasingly significant abiotic stress factors (Maksimovic and Ilin, 2012; Yıldız and Balkaya, 2016). While salinity problem exists in 65% of global agricultural lands, this rate is around 20%in Turkey (Yetisir et al., 2016). Especially, 20% of the 230 million hectares of irrigated global farmlands face salinity problem (Peleg et al., 2011). Salinity causes stress in plants through different mechanisms. The plant initially undergoes water stress due to low osmotic pressure that occurs in the soil solution (Ashraf and Harris, 2004). Furthermore, ion imbalance and toxicity occur due to the accumulation of high amounts of Na^+ and Cl^-ions (Lauchli and Grattan, 2007; Aktas et al., 2009). Generally, in the salt stress induced by the Na^+ and Cl^-ions, the function and structure of the proteins deteriorate (Tuteja et al., 2012) and harmful effects occur in the function of metabolites at high concentrations of Na^+ (Agarwal et al., 2013).

Since the tolerance mechanisms, including different genes under environmental conditions, have a complex structure (Ashraf and Harris, 2004), plants develop extremely diverse responses to salt stress due to their genotypic differences (Çulha and Çakırlar, 2011). While Levitt (1972) indicated that plants often cope with the negative effects of salt stress through escape, avoidance or tolerance strategies, Munns and Tester (2008) claimed that plant tolerance against soil salinity was ensured through

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

three components, namely, Na^+ exclusion, tissue tolerance against Na^+, and osmotic tolerance. It was reported that the most effective solution to salt stress is the development of varieties with tolerance (Dasgan and Koc, 2009).

Harlan (1951) indicated that Turkey, which is the second highest melon producer globally (FAO, 2013), was a micro-center for cucurbits, including melon (Sari et al., 2008). Furthermore, it was reported that Turkey is one of the gene centers for melon (Erdinc et al., 2013). Van province, which is located in the Eastern Anatolia region in Turkey, is known as one of the gene centers for cantaloupe melon (Turkmen et al., 2008). Thus, melon could be considered among the basic economic products of Turkey (Dasgan et al., 2012). It was noted that the threshold of Electrical Conductivity (EC) for the melon with moderate sensitivity against salt was 1 dS m^{-1} (Maksimovic and Ilin, 2012). It was reported that although most cultivated plants such as beans, carrots and onions were known to be susceptible to salt (Petropoulos et al., 2017), melon exhibited a medium tolerance to salt stress, yet the condition was different among varieties due to the presence of tolerant and susceptible varieties (Damianos and Savvas, 2016).

Selection and breeding studies, conducted to develop high-yield commercial varieties, resulted in a reduced genetic diversity, and inevitably led to a reduction in genetic variations and a lower tolerance of cultivars to abiotic stress conditions. It was considered that genotypes with qualified breeding properties could provide gene resources for future breeding programs through determination of high tolerance genotypes via screening studies.

Mixture modeling intends to recognize previously unobserved homogenous sub-populations involving an apparently heterogeneous data set (Wang et al., 1996; Dalrymple et al., 2003; Martinez et al., 2009) utilizing Akaike's information criteria (AIC) and Bayesian information criteria (BIC) (Yeşilova et al., 2010) to characterize and distinct sub-populations. Mixture modeling is a novel methodology and has two significant benefits than the cluster and factor analysis (Muthén and Muthén, 2014). The first, for every observation, mixture modeling is settled by the likelihood of consideration inside the subgroup classes. The second, mixture model gives the parameter estimates for every subgroup (Mao et al., 2013). Present study aimed to determine the effects of salt stress in melon genotypes via mixture modeling and PCA analysis.

Materials and methods

Materials

Fourteen genotypes collected from different regions in Lake Van Basin (*Fig. 1*), 3 hybrid cultivars and 1 foreign standard cultivar were used in the present study where the reactions of the melon genotypes to salt stress were examined (*Table 1*).

NaCl treatment

In the study designed with three replications with 5 plants per repetition in randomized block experimental design, the seeds were sown into non-drainage 3-L pots having 2:1 ratio of sterile peat: perlite mixture. The seedlings were irrigated with Hoagland solution (Aktas et al., 2009). For salt application, based on previous studies (Yasar et al., 2006; Kusvuran et al., 2007b; Yarsi et al., 2017) the most suitable dose was chosen and when the seedlings reached into two-true-leaf stage, 50 mM NaCl

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

concentration was applied gradually for 2 days until a final concentration of 100 mM was obtained. The study was terminated after 18 days of salt application.

Figure 1. *The locations where the genotypes used in the study were collected*

Table 1. *Passport information of melon accessions and cultivars used in the study*

Accessions	Location	Accessions	Location
YYU-1	Van-Sihke-Kiratlı	YYU-21	Van-Unseli
YYU-4	Van-Sihke-Kiratlı	YYU-22	Van-Ercis
YYU-6	Van-Sihke-Kiratlı	YYU-23	Van-Ercek-Irgatli
YYU-10	Van-Sihke	YYU-29	Van-Ercek-Irgatli
YYU-11	Van-Sihke-Kiratlı	**Cultivar**	**Location**
YYU-13	Van-Sihke-Kiratlı	Kırkağaç F_1 (Tolerant)	YükselTohum
YYU-14	Van-Sihke-Kiratlı	Lokum F_1 (Tolerant)	YükselTohum
YYU-15	Van-Sihke-Kiratlı	Napolyon F_1 (Susceptible)	YükselTohum
YYU-18	Van-Cakirbey	Galia (Susceptible)	Standard
YYU-20	Van-Unseli		

Seedling parameters

The morphological parameters such as shoot and root length, shoot diameter, leaf number, shoot-root fresh and dry weights were determined at the end of the experiment. The root: shoot ratio (Dry weight-DW%) (R: S) was determined after the dry weights were obtained.

Leaf relative water content (LRWC)

In order to determine the leaf relative water content in melon plants, primarily the fresh weights (FW) of the 3^{rd} and 4^{th} leaves of the three randomly selected plants in each repetition were determined and were stored in sterile distilled water for 4 h for the leaves to reach the maximum turgor weight (TW) and at the end of this duration their

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turgor weights were measured. Leaf specimens, which were tested for turgor weight, were placed in an oven at 80 °C temperature and dry weights (DW) were determined. After measurements were completed, LRWC was calculated using *Equation 1* (Yamasaki and Dillenburg, 1999), consequent to the completion of measurements:

$$LRWC = [(FW - DW)/(TW - DW)] \times 100 \quad \text{(Eq.1)}$$

Membrane injury index (MII)

The membrane injury index refers to the amount of electrolyte released from the cell. The amount of electrolyte released from the cell under stress conditions was determined with the methods developed by Dlugokecka and Kacperska-Palacz (1978) and Fan and Blake (1994). The discs retrieved from the third plant leaves were measured for their EC values after being kept in deionized water for 6 h at room temperature, then disc leaves were left in water at 100 °C for 10 min to retrieve the EC values again, and *Equation 2* was used for the calculations:

$$MII = \left[\frac{Lt - Lc}{1} - Lc\right] \times 100 \quad \text{(Eq.2)}$$

Lt: EC value before autoclaving of stressed leaf/EC value after autoclaving of stressed leaf.
Lc: EC value before the control leaf is autoclaved / EC value after the control leaf is autoclaved.

Visual evaluation of salt stress (0-5 scale)

In the scale, used for visual evaluation of the salt injury in the seedlings, the scale values were as follows (Dasgan, 2002; Kuşvuran et al., 2007a):

0- No effect; 1-Local yellowing and curling of leaves, slow growth; 2- Necrosis and chlorosis in 25% of the leaf; 3- Necrotic spots on the leaves and defoliation by 25-50%; 4- Necrosis by 50-75% and death of several plants; 5- Formation of severe necrosis in leaves by 75-100% and predominant deaths in plants.

Stomatal traits

In order to determine the stoma density (StD) (unit mm^2), stomatal area (StA) (µm^2), stoma width (StW) and length (StL) (µm), the lower epidermis of the 4th plant leaf was stripped and spread on a slide with two drops of water (Kurtar et al., 2016). The stoma count was calculated with LAS EZ 3.0 software under three-field light microscope (LEICA DM500) with 40× magnification in three randomly selected 0.08 mm^2 tissue specimen sections spread on the slide.

Stomatal area was calculated with *Equation 3* (Orsini et al., 2013):

$$StA = \pi \times (SW \times 0.5) \times (SL \times 0.5) \quad \text{(Eq.3)}$$

Statistical analysis

In the evaluation of the data obtained with the measurements and observations conducted in the study, the extent of the effects of salt stress on genotypes and varieties

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were based on the variation rates when compared to the control group and the data were compared using *Equation 4:*

$$\text{Percent change} = \left[\frac{\text{Control} - \text{Salt treatment}}{\text{Control}}\right] x100 \quad \text{(Eq.4)}$$

The mixture modeling statistical analyzes were performed with Mclust (mixture cluster) extension in R 5.2.3 statistical software (R Development Core Team, 2017). 0-5 scale, growth parameters, leaf relative water content, membrane injury index and stomatal features were classified with the Mclust Software. The principal component analysis (PCA) was conducted to identify the patterns of variation within the sets of melon accessions based on 14 properties using the PRINCOM procedure described by SAS (SAS Institute, 2015). Furthermore, Pearson correlation analysis was conducted with SPSS Software in order to determine the correlations among the variables.

Results and discussion

Seedling parameters

The leaf number, shoot diameter, shoot and root length values are presented in *Table 2.* It was observed that leaf number decreased in all genotypes and varieties due to salt stress, and the highest proportional decrease occurred in the genotype YYU29 (-54.24%) followed by cv. Lokum F_1 with a -52.61% decrease. YYU6 was the genotype that exhibited the lowest proportional decrease in leaf number due to salt stress (-7.46%). While the highest decrease in shoot diameter, -20.33%, was determined in genotype YYU10 when compared to the control group, the shoot diameter of 66.67% of the genotypes increased in saline conditions. It was established that salt stress affected the shoot length negatively in all melon genotypes and varieties. On the contrary, root length increased in half of the genotypes under salt stress. While YYU29 genotype and cv. Lokum F_1 were among the most adversely affected melons by salt stress in terms of shoot length (-62.93% and -62.59%, respectively), YYU15 genotype exhibited the lowest decrease in shoot length with a -21.42% decrease, when compared to the control group. Similar to the shoot diameter, YYU10 genotype was the most affected genotype by salt stress, similar to the decrease in root length (-25.31%). When compared to the control group, the highest increase in root length was observed in YYU6 genotype with 41.50%.

Although all genotypes and cultivars were adversely affected by salt stress, it was found that the shoot fresh weights of YYU18 and YYU6 genotypes were the least affected parameters (-13.94% and -18.55%, respectively). It was determined that the genotype with the highest decrease was YYU4, with -56.78%. In the presence of salt stress, increases in shoot dry weight were observed in only 3 genotypes, YYU6, YYU18 and YYU1 (20.00%, 6.76% and 1.11%, respectively). It was determined that about 67% of the genotypes exhibited an increase in root fresh weight in saline conditions and for root dry weight, the increase was 72%. The highest rate of increase was observed in YYU6 genotype in both cases (*Table 3*). The same trend was also reflected in the root to shoot ratio and 89% of the genotypes demonstrated an increase in root: shoot ratio due to salt stress. While, the highest increase was observed in the YYU6 genotype

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

(403.24%), only YYU11 and YYU10 genotypes exhibited a decrease of -7.65% and -1.19%, respectively (*Table 4*).

It was determined that salt application commonly affected plant growth negatively. Hence, the first response of the plants to salt was the reduction of growth rate and toxic effect symptoms on the leaves and shoot ends (Dasgan et al., 2002). While all varieties and genotypes were adversely affected in leave number, shoot length and shoot fresh weight, it was established that there existed a variation between genotypes in shoot diameter, root length, shoot dry weight, root fresh and dry weight, and root:shoot rate. The reduction in leaf number ranged between 7.46 to 54.24%. Yetişir et al. (2016) also reported a reduction between 20 and 90% in Turkish gourd genotypes due to salt treatment. The soil salinity and low osmotic potential in the root zone reduce the water intake of plants (Lauchli and Grattan, 2007). These conditions create a negative effect especially on the fresh weight of plants that are salt intolerant. It was determined that once the amount of salt applied to the soil increased, the growth of plants, and thus the amount of dry matter decreased, hence in turn, the plant growth and the dry matter amount were affected more negatively (Romero et al., 2001). Although the root system is directly exposed to salinity, leaf growth is more susceptible to salt stress than root growth; therefore, root: shoot ratio increases under salt stress (Çulha and Çakırlar, 2011). Orsini et al. (2013) found that the root: shoot rate increased in plants under salt stress when compare d to control conditions. Thus, it was determined that there was a significant correlation between R: S ratio and RFW ($r = 0.501$, $p < 0.01$) and RDW ($r = 0.620$, $p < 0.01$) (*Table 5*).

Table 2. *Several growth parameters of melon accessions and cultivars with/without salt application*

Accession	Leaf number per plant			Shoot diameter (mm)			Shoot length (cm)			Root length (cm)		
	0 mM	**50 mM**	**Change (%)**	**0 mM**	**50 mM**	**Change (%)**	**0 mM**	**50 mM**	**Change (%)**	**0 mM**	**50 mM**	**Change (%)**
YYU1	6.11	4.38	-28.31	4.09	4.52	10.51	49.56	30.50	-38.46	16.41	22.38	36.38
YYU4	7.56	4.00	-47.09	4.31	3.90	-9.51	66.00	36.00	-45.45	19.80	16.27	-17.83
YYU6	4.56	4.22	-7.46	4.13	3.98	-3.63	43.11	31.67	-26.54	13.88	19.64	41.50
YYU10	5.11	3.11	-39.14	4.18	3.33	-20.33	45.44	27.22	-40.10	16.79	12.54	-25.31
YYU11	5.33	3.11	-41.65	5.38	4.84	-10.04	40.56	26.78	-33.97	22.50	19.90	-11.56
YYU13	4.86	3.22	-33.74	4.65	4.19	-9.89	39.80	30.00	-24.62	23.70	19.33	-18.44
YYU14	4.89	2.89	-40.90	4.53	4.62	1.99	42.56	26.56	-37.59	16.33	18.82	15.25
YYU15	5.00	4.50	-10.00	3.42	3.63	6.14	47.89	37.63	-21.42	13.44	16.65	23.88
YYU18	6.67	5.11	-23.39	3.45	3.98	15.36	63.56	43.33	-31.83	13.89	18.78	35.21
YYU20	8.22	4.44	-45.99	4.06	4.61	13.55	94.44	39.00	-58.70	18.83	19.22	2.07
YYU21	8.22	4.88	-40.63	4.27	5.19	21.55	84.86	44.25	-47.86	17.33	19.56	12.87
YYU22	6.89	4.78	-30.62	3.52	4.19	19.03	77.14	42.56	-44.83	18.63	15.22	-18.30
YYU23	8.00	5.11	-36.13	3.48	4.32	24.14	88.00	44.11	-49.88	14.66	16.03	9.35
YYU29	7.78	3.56	-54.24	4.05	4.48	10.54	81.22	30.11	-62.93	23.75	17.82	-24.97
Galia	5.56	4.75	-14.57	3.29	3.92	19.15	51.38	30.75	-40.15	14.19	13.90	-2.04
Kırkağaç F_1	7.78	4.78	-38.56	3.69	4.02	8.94	104.71	43.22	-58.72	15.04	17.56	16.76
Lokum F_1	8.44	4.00	-52.61	3.37	3.97	17.80	87.89	32.88	-62.59	15.63	15.38	-1.60
Napolyon F_1	7.22	4.67	-35.32	3.44	3.44	0.00	68.38	32.78	-52.06	15.88	17.36	9.32

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

***Table 3.** Several growth parameters of melon accessions and cultivars with/without salt application*

Accession	Shoot fresh weight (g)			Shoot dry weight (g)			Root fresh weight (g)			Root dryweight (g)		
	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)
YYU1	15.15	11.94	-21.19	0.90	0.91	1.11	0.56	1.10	96.43	0.04	0.07	75.00
YYU4	19.02	8.22	-56.78	1.34	0.77	-42.54	2.07	0.82	-60.39	0.08	0.06	-25.00
YYU6	9.65	7.86	-18.55	0.55	0.66	20.00	0.40	1.33	232.50	0.01	0.08	700.00
YYU10	12.50	5.45	-56.40	0.79	0.66	-16.46	0.61	0.39	-36.07	0.04	0.03	-25.00
YYU11	16.60	7.62	-54.10	1.29	0.87	-32.56	1.30	0.68	-47.69	0.10	0.06	-40.00
YYU13	18.26	9.73	-46.71	1.09	0.89	-18.35	1.54	1.58	2.60	0.10	0.08	-20.00
YYU14	16.94	8.78	-48.17	1.13	0.81	-28.32	0.98	1.42	44.90	0.05	0.09	80.00
YYU15	13.00	9.94	-23.54	0.61	0.52	-14.75	0.43	0.79	83.72	0.02	0.05	150.00
YYU18	11.41	9.82	-13.94	0.74	0.79	6.76	0.58	0.83	43.10	0.03	0.06	100.00
YYU20	15.74	11.29	-28.27	1.25	1.09	-12.80	0.85	1.71	101.18	0.05	0.12	140.00
YYU21	19.31	14.15	-26.72	1.61	1.27	-21.12	1.06	1.24	16.98	0.06	0.09	50.00
YYU22	14.87	9.29	-37.53	0.92	0.86	-6.52	0.77	1.20	55.84	0.03	0.06	100.00
YYU23	13.67	6.19	-54.72	0.88	0.70	-20.45	0.71	0.86	21.13	0.03	0.05	66.67
YYU29	19.74	9.41	-52.33	1.55	0.84	-45.81	1.71	1.21	-29.24	0.07	0.07	0.00
Galia	10.11	7.76	-23.24	0.53	0.50	-5.66	0.49	0.85	73.47	0.03	0.05	66.67
Kırkağaç F_1	16.57	10.27	-38.02	1.23	1.00	-18.70	0.90	1.59	76.67	0.05	0.09	80.00
Lokum F_1	22.25	11.24	-49.48	1.52	0.91	-40.13	1.27	1.26	-0.79	0.07	0.08	14.29
Napolyon F_1	16.91	9.70	-42.64	1.15	0.68	-40.87	1.27	1.23	-3.15	0.06	0.07	16.67

***Table 4.** Several growth and physiological parameters of melon accessions and cultivars with/without salt application*

Accession	Root:shoot ratio (DW %)			Leaf relative water content (%)			Membrane injury index (%)	0-5 scale
	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)	50 mM	50 mM
YYU1	0.0433	0.0797	84.06	82.22	70.14	-14.69	21.49	1.78
YYU4	0.0570	0.0823	44.39	78.45	68.95	-12.11	33.96	2.22
YYU6	0.0247	0.1243	403.24	100.74	76.27	-24.29	15.59	2.00
YYU10	0.0503	0.0497	-1.19	63.80	72.44	13.54	23.35	2.22
YYU11	0.0693	0.0640	-7.65	76.39	75.34	-1.37	41.35	2.22
YYU13	0.0507	0.0820	61.74	82.78	71.72	-13.36	10.20	2.11
YYU14	0.0440	0.1010	129.55	76.36	72.15	-5.51	28.12	1.56
YYU15	0.0320	0.0850	165.63	79.92	78.52	-1.75	19.04	1.78
YYU18	0.0470	0.0763	62.34	71.39	74.59	4.48	18.01	1.33
YYU20	0.0410	0.1077	162.68	80.25	68.68	-14.42	26.51	1.78
YYU21	0.0400	0.0720	80.00	77.66	71.66	-7.73	11.60	1.67
YYU22	0.0367	0.0740	101.63	73.14	66.70	-8.81	24.30	1.89
YYU23	0.0340	0.0723	112.65	75.42	75.16	-0.34	40.04	1.67
YYU29	0.0400	0.0787	96.75	82.00	70.08	-14.54	-6.18	1.89
Galia	0.0500	0.0743	48.60	78.87	77.69	-1.50	6.00	1.89
Kırkağaç F_1	0.0420	0.0863	105.48	82.15	71.28	-13.23	17.42	1.45
Lokum F_1	0.0430	0.0917	113.26	79.10	72.06	-8.90	19.29	2.33
Napolyon F_1	0.0503	0.0980	94.83	82.38	74.86	-9.13	-6.54	1.33

Table 5. *Correlations among parameters evaluating salt stress*

	SFW	SDW	RFW	RDW	RS	LRWC	LN	SD	SL	RL	StL	StW	StA	StD	MII	S
SFW	1	0.849**	0.433**	0.367**	-0.253**	0.231*	0.736**	0.164**	0.644*	0.127**	0.074	-0.084	0.017**	-0.149**	-0.402**	-0.553**
SDW		1	0.542**	0.529**	-0.134	0.064	0.648**	0.312	0.568	0.278	0.040	-0.142**	-0.048	0.048**	-0.183	-0.290**
RFW			1	0.750**	0.501**	-0.049	0.199**	0.342**	0.111	0.476	-0.026	0.070**	0.030**	0.152	-0.046	0.081
RDW				1	0.620**	-0.118	0.061	0.473**	-0.105	0.420	-0.047	0.079**	0.023**	0.275**	0.108	0.207*
RS					1	-0.256**	-0.379**	0.255	-0.476**	0.294**	-0.138	0.081**	-0.055	0.382**	0.322**	0.588**
LRWC						1	0.208	-0.155**	0.186	-0.106*	0.232*	0.002*	0.131	-0.174	-0.205	-0.340**
LN							1	-0.183**	0.898*	0.034**	-0.047	-0.286**	-0.186**	-0.109*	-0.369**	-0.585**
SD								1	-0.228	0.363	0.175	0.163	0.203**	0.116**	0.139	0.094
SL									1	0.022**	0.001	-0.254**	-0.143**	-0.083	-0.367**	-0.583**
RL										1	0.145	0.143	0.191**	0.055**	0.095	0.084
StL											1	0.363	0.826	-0.181	-0.210*	-0.158
StW												1	0.807	-0.188	0.034	-0.059
StA													1	-0.241	-0.130	-0.151
StD														1	0.252**	0.380**
MII															1	0.647**
S																1

$^{*}p < 0.05$, $^{**}p < 0.01$
SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, RS: R:S ratio (DW %), LRWC: Leaf relative water content, LN: Leaf number, SD: Shoot diameter, SL: Shoot length, RL: Root length, StL: Stoma length, StW: Stoma width, StA: Stomatal area, StD: Stoma density, MII: Membrane injury index, S: 0-5 Scale

Leaf relative water content (LRWC) and Membrane Injury Index (MII)

Among the genotypes and varieties examined in the present study, there was an increase in LRWC only in YYU10 and YYU18 (13.54 and 4.48%, respectively) in saline conditions, and it was determined that the LRWC decreased in all remaining genotypes and varieties due to salinity. While the injury rate varied between 6 and 41% in MII, which was evaluated 18 days after the salt treatment, it was determined that the YYU29 genotype and cv. Napolyon F_1 cultivar yielded negative values of -6.18% and -6.54%, respectively (*Table 4*). Salt stress decreased water uptake due to osmotic effect and furthermore led to membrane damage due to ion toxicity (Munns, 2002). The decrease in LRWC refers to low turgor pressure at limited water conditions (Katerji et al., 1997) and is, therefore, an important indicator of salt tolerance in cultivated plants (Sarabi et al., 2017). Membrane injury is caused by ion imbalance, which is caused by osmotic inconsistency inside and outside the cell under stress conditions such as salinity and drought (Ghoulam et al., 2002). It was found that membrane injury was lower in tolerant genotypes when compared to susceptible genotypes (Asha, 2007). Previous studies indicated that membrane injury increased due to salt stress and thus, MII could be used to determine the stress-effect rate in plants (Jamil et al., 2012).

Visual evaluation of salt stress (0-5 Scale)

In the 0-5 scale, which commonly assists the visual determination of leave damage, it was observed that cv. Lokum F_1 had the highest scale score (2.33) and YYU4, YYU10 and YYU11 genotypes were among the most affected genotypes with a scale score of 2.22. It was observed that YYU18 genotype had the lowest score with 1.33. Furthermore, it was found that 33.3% of the genotypes were more affected by salinity and their scores were above 2.0 (*Table 4*). Several studies reported that the scale findings could be used in estimating the reactions under salt stress by melon (Kusvuran

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

et al., 2007a), tomato (Daşgan et al., 2002), bean (Kıpçak and Erdinç, 2016) species. It was determined that there were negative correlation between the findings of the 0-5 scale utilized in present study on SFW ($r = -0.553$, $p < 0.01$), LN ($r = - 0.585$, $p < 0.01$) and SL ($r = - 0.583$, $p < 0.01$) and there were positive correlations between R:S ($r = 0.588$, $p < 0.01$), and MII ($r = 0.647$, $p < 0.01$) findings (*Table 5*).

Stomatal traits

It was observed that 78% of the melon cultivars and genotypes exhibited a decrease in stoma length, 56% exhibited a decrease in stomatal width and 61% exhibited a decrease in stomatal area under salt stress, and stoma density increased in 94%. The only negative ratio was observed in stoma density with 11.11% in YYU21 genotype and it was found that the highest increase in LRWC was observed in YYU18 genotype (216.67%), which had prominent scale scores. Thus, it could be observed that the stoma density (StD) increased as StL, StW and StA values decreased under salt stress (*Table 6*). It was reported that this mechanism is a functional plant response to suppress salt stress, regulate the respiration and preserve plant performance (Orsini et al., 2011) and it was also indicated that increased StDdue to increase in salt stress led to an increase in photosynthesis (Chaves et al., 2009). Although several studies reported similar findings, indicating that salt stress led to a decrease in stoma dimensions and areas and an increase in stoma density (Orsini et al., 2013; Kurtar et al., 2016), Sarabi et al. (2017) reported that stoma density decreased due to the increase in salinity and interpreted this as a counter adaptation against salt stress.

Table 6. *Stomatal characteristics of melon accessions and cultivars with/without salt application*

Accession	Stoma length (µm)			Stoma width (µm)			Stomatal area (µm²)			Stoma density (unit/mm²)		
	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)	0 mM	50 mM	Change (%)
YYU1	18.10	15.73	-13.09	14.33	10.53	-26.52	202.25	129.95	-35.75	104.17	108.33	3.99
YYU4	14.20	21.47	51.20	13.23	15.57	17.69	153.84	261.87	70.22	54.17	100.00	84.60
YYU6	21.77	17.73	-18.56	14.77	14.10	-4.54	250.70	196.56	-21.60	75.00	104.17	38.89
YYU10	17.93	13.47	-24.87	13.60	11.03	-18.90	193.76	117.48	-39.37	62.50	91.67	46.67
YYU11	20.43	20.77	1.66	14.77	14.83	0.41	236.58	242.78	2.62	145.83	166.67	14.29
YYU13	21.33	18.53	-13.13	19.13	14.20	-25.77	323.66	206.91	-36.07	62.50	95.83	53.33
YYU14	21.47	12.47	-41.92	14.13	22.43	58.74	239.40	219.67	-8.24	100.00	158.33	58.33
YYU15	19.30	18.63	-3.47	13.80	14.77	7.03	209.13	216.80	3.67	95.83	145.83	52.18
YYU18	15.47	15.13	-2.20	12.43	10.27	-17.38	156.30	122.26	-21.78	75.00	237.50	216.67
YYU20	15.77	11.67	-26.00	10.97	10.80	-1.55	135.07	99.28	-26.50	225.00	241.67	7.41
YYU21	15.73	23.70	50.67	12.47	12.87	3.21	153.37	246.64	60.81	187.50	166.67	-11.11
YYU22	15.63	15.50	-0.83	10.53	11.77	11.78	129.46	142.85	10.34	70.83	183.33	158.83
YYU23	16.40	16.27	-0.79	12.70	12.07	-4.96	163.54	154.94	-5.26	95.83	100.00	4.35
YYU29	23.30	19.53	-16.18	17.20	14.80	-13.95	314.50	227.72	-27.59	58.33	129.17	121.45
Galia	19.93	16.57	-16.86	17.30	13.17	-23.87	270.78	170.55	-37.02	83.33	100.00	20.00
Kırkağaç F_1	22.07	14.53	-34.16	12.57	12.10	-3.74	218.41	139.27	-36.23	158.33	287.50	81.58
Lokum F_1	14.80	19.20	29.73	10.77	12.83	19.13	132.51	195.74	47.72	95.83	229.17	139.14
Napolyon F_1	20.23	19.10	-5.59	12.43	13.20	6.19	195.05	197.00	1.00	87.50	229.17	161.90

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

Principle component analysis (PCA)

Principal component analysis (PCA) method was used to determine the traits that led to the variation. Eigen value and variances obtained in the analysis and the properties that led to the differences among the genotypes were determined (Sönmez et al., 2015). 71.48% of the total variance in fourteen different traits was grouped in 3 PCA groups for the control application and the 69.53% in the 50 mM salt application were grouped in 4 PC groups, and it was found that the two applications were similar in total variance. In the control group, the variation ratios for three principal components were 35.42%, 25.26%, and 10.80%, respectively, and these ratios were observed as 28.16%, 18.56%, 11.62%, and 11.19% in the 4 principal components of in 50 mM salt application group (*Table 7*). In a study conducted by Sarabi et al. (2017) on salt stress using physiological and biochemical parameters, it was reported that the total variance was 97.17% in the PCA analysis and the first principal component explained 66.96 of the variance. Furthermore, Shelke et al. (2017) determined that 82.65% of the total variance of 95.07% was explained by the first principal component. It is considered that in both studies, the researchers were able to increase the variance by working with an excessive number of salt concentrations and the variances could stem from the reactions that plants exhibited indifferent concentrations.

Table 7. *Principal component analysis (PCA) of characters associated with melon accessions based on salt stress*

	PC axis						
	0 mM			**50 mM**			
	PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC4**
Eigen values	4.96	3.54	1.51	3.94	2.60	1.63	1.57
Explained proportion of variation (%)	35.42	25.26	10.80	28.16	18.56	11.62	11.19
Cumulative proportion of variation (%)	35.42	60.68	71.48	28.16	46.72	58.34	69.53
Characters	**Eigen vectors**						
	0 mM			**50 mM**			
	PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC4**
Shoot fresh weight	0.39	0.02	0.17	0.40	-0.02	-0.22	0.24
Shoot dry weight	0.42	0.01	0.11	0.34	0.06	0.13	0.49
Root fresh weight	0.37	0.17	0.02	0.40	0.08	0.24	-0.31
Root dry weight	0.40	0.18	-0.12	0.46	0.08	0.19	-0.08
R:S ratio (DW %)	0.18	0.27	-0.41	0.33	0.07	0.08	-0.51
Leaf relative water content	-0.02	0.06	0.61	0.04	-0.17	-0.55	0.03
Leaf number	0.36	-0.22	0.20	-0.03	0.59	-0.17	-0.02
Shoot diameter	0.15	0.30	-0.28	0.25	-0.08	0.14	-0.03
Shoot length	0.30	-0.26	0.26	0.21	-0.31	-0.18	-0.03
Root length	0.23	0.27	-0.12	0.24	0.21	0.09	0.45
Stoma length	-0.07	0.40	0.37	-0.15	-0.03	0.39	0.27
Stoma width	-0.12	0.42	0.09	-0.22	0.07	0.50	0.01
Stomatal area	-0.10	0.47	0.24	0.00	0.47	-0.23	0.14
Stoma density	0.13	-0.17	-0.06	-0.07	0.48	-0.04	-0.22

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

In the control group, it was found that 42% of the variance in PC1 was explained by SDW, StA explained 47% of the variance in PC2 and LRCW explained 61% of the variance in PC3, which were the highest ratios. The highest ratio in PC1, which is one of the principal components that occurred under salt stress, was observed with RDW (46%), in PC2, the highest ratio was observed with LN (59%), in PC3, the highest ratio was observed with LRWC (55%) and in PC4, the highest ratio was observed with SDW (49%). SDW and LRWC exhibited the highest variance in 0 and 50 mM, furthermore, SI (17%), which exhibited low variance in 0 mM, reached a higher value due to salt stress (48%) (*Table 7*). Genotypes and varieties were classified in two groups of 0- and 50-mM salt treatment (*Fig. 2*); YYU4, YYU20, YYU21 genotypes and cv. Lokum F1 in the control group and the YYU18, YYU20 genotypes and cv. Kırkağaç in the 50 mM salt treatment group were in the same group and the remaining genotypes and varieties were in the second group.

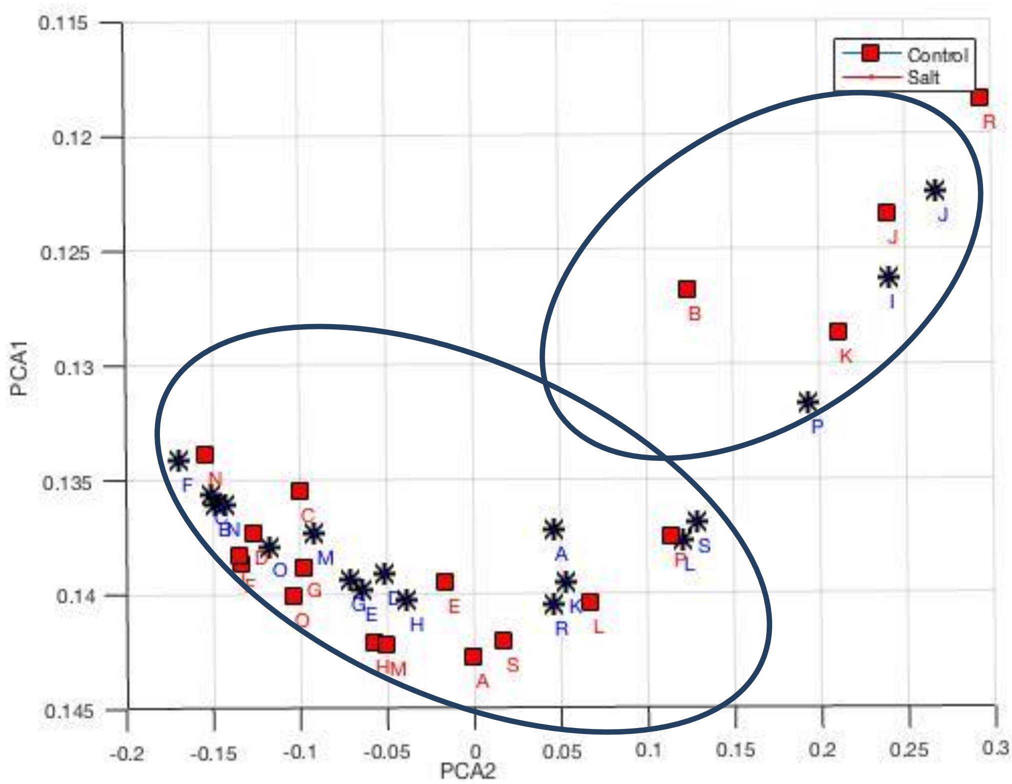

Figure 2. *The first three principle components principle component analysis (PCA) plot conducted using 14 parameters (A: YYU1, B: YYU4, C: YYU6, D: YYU10, E: YYU11, F: YYU13, G: YYU14, H: YYU15, I: YYU18, J: YYU20, K: YYU21, L: YYU22, M: YYU23, N: YYU29, O: Galia, P: Kırkağaç F$_1$, R: Lokum F$_1$, S: Napolyon F$_1$)*

Mixture modeling

The number of homogeneous sub-groups for the genotypes was determined with the Gaussian mixture model through the evaluation of inspected properties in order to determine the reactions of melon genotypes to salt stress. Based on all properties scrutinized in the present study and using the smallest AIC and BIC criteria (Yeşilova et al., 2016), it was determined that genotypes could have 4 homogenous sub-groups under

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

stress-free conditions and 3 sub-groups under conditions with salt stress (*Table 8*). Thus, it was found that the correct classification rate of entropy was 98% for 0 mM and 93% for 50 mM. In the control application, based on the sub-group distribution with respect to their traits, it was found that the highest mean values for all other traits except StD were observed in the second and fourth sub-groups, where no significant dispersion was observed in the lowest values and the majority of the lowest means were in the first group (*Table 8*). It was also determined that the high mean values were significantly observed in the first group based on the distribution of the variable means, and the low mean values were in the third group (*Table 8*). In other words, it is possible to state that the genotypes and varieties in the first group tend to increase their tolerance against salt stress. Thus, genotypes in the third group could be considered as more susceptible to salt.

Table 8. *Estimated means of variables for model with four sub-population at 0 and 50 mM in melon genotypes*

Characters	**0 mM**				**50 mM**		
	Sub-group 1	**Sub-group 2**	**Sub-group 3**	**Sub-group 4**	**Sub-group 1**	**Sub-group 2**	**Sub-group 3**
Shoot fresh weight	10.73	30.84	17.52	18.36	11.06	10.31	6.70
Shoot dry weight	0.63	2.11	1.26	1.35	0.93	0.76	0.72
Root fresh weight	0.53	1.99	0.99	1.67	1.55	0.98	0.70
Root dry weight	0.025	0.095	0.051	0.102	0.094	0.058	0.048
R:S ratio (DW %)	0.040	0.045	0.014	0.063	0.102	0.078	0.066
Leaf relative water content	77.95	86.33	79.35	78.55	71.16	76.75	71.52
Leaf number	5.18	10.58	7.79	5.72	4.14	5.28	3.50
Shoot diameter	3.68	3.83	3.84	4.96	4.41	4.07	3.96
Shoot length	50.60	106.25	83.50	44.58	35.22	41.69	30.12
Root length	15.01	17.34	17.49	21.33	19.12	16.26	16.88
Stoma length	19.16	19.00	16.69	20.26	17.96	16.14	17.15
Stoma width	14.34	12.08	11.70	16.71	14.20	11.84	13.61
Stomatal area	218.36	187.33	115.22	268.36	201.96	150.11	183.66
Stoma density	89.69	100.01	131.09	80.57	178.63	167.72	131.58
Membrane injury index	-	-	-	-	15.75	19.40	22.80
0-5 scale	-	-	-	-	1.81	1.57	2.20

It was found that the resulting homogeneous sub-groups exhibited variations in distribution of genotypes for 0 mM, and majority of genotypes were in the 1st and 3rd sub-groups, where the means were low. Furthermore, based on the mean values obtained with the variables, it was determined that the genotypes and varieties in the first group were tolerant to salt, those in the second group had moderate tolerance, and those in the third group were susceptible to salt under 50 mM salt treatment. Accordingly, it was determined that YYU4, YYU10 and YYU11 genotypes could be susceptible to salt, YYU15, YYU18, YYU23 genotypes and cv. Galia could be medium-tolerant, YYU6, YYU13, YYU14, YYU20, YYU21 and YYU29 genotypes and cv. Kırkağaç F_1 and cv. Lokum F_1 varieties could be tolerant to salt stress (*Table 9*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

Table 9. *Mixture model results with correct classification values for four accession sub-populations*

Accession	0 mM				50 mM		
	Sub-population1	Sub-population2	Sub-population3	Sub-population4	Sub-population1	Sub-population2	Sub-population3
YYU-1	0.987	0	0.013	0	0	0.996	0.004
YYU-1	1	0	0	0	1	0	0
YYU-1	0.001	0	0.999	0	0	0	1
YYU-4	0	0	0	1	0	0	1
YYU-4	0	0	0	1	0.99	0.001	0.009
YYU-4	0	0	1	0	0.004	0.023	0.973
YYU-6	1	0	0	0	0	0.018	0.982
YYU-6	1	0	0	0	1	0	0
YYU-6	1	0	0	0	1	0	0
YYU-10	1	0	0	0	0	0.004	0.996
YYU-10	1	0	0	0	0	0	1
YYU-10	0.932	0	0.065	0.003	0	0	1
YYU-11	0	0	0	1	0	0	1
YYU-11	0	0	0	1	0	0	1
YYU-11	1	0	0	0	0.954	0	0.046
YYU-13	0	0	0	1	0.992	0.01	0.002
YYU-13	0	0	0	1	1	0	0
YYU-13	0	0	0	1	0.04	0	0.96
YYU-14	0.019	0	0.972	0.009	1	0	0
YYU-14	0	0	0	1	1	0	0
YYU-14	1	0	0	0	0	0	1
YYU-15	1	0	0	0	0	0.999	0.001
YYU-15	1	0	0	0	0	1	0
YYU-15	1	0	0	0	0	0	1
YYU-18	0.988	0	0.012	0	0	0.537	0.463
YYU-18	0	0	1	0	0.006	0.994	0
YYU-18	1	0	0	0	0	1	0
YYU-20	0	0	1	0	1	0	0
YYU-20	0	0	1	0	1	0	0
YYU-20	0	0	1	0	0.997	0.003	0
YYU-21	0	0	1	0	1	0	0
YYU-21	0	0.001	0.999	0	1	0	0
YYU-21	0	0	1	0	0.013	0.990	0
YYU-22	0	0	1	0	0.913	0.090	0
YYU-22	0	0	1	0	0	1	0.004
YYU-22	0.998	0	0.002	0	0	0.14	0.865
YYU-23	0	0	1	0	0	1	0
YYU-23	0	0	1	0	0	0.936	0.064
YYU-23	1	0	0	0	0	0.035	0.964
YYU-29	0	0	0	1	0	0	1
YYU-29	0.926	0	0.073	0.001	0.982	0.001	0.017
YYU-29	0	1	0	0	1	0	0
Galia	1	0	0	0	0	0.763	0.237
Galia	1	0	0	0	0.02	0.98	0
Galia	1	0	0	0	0	0	1
Kırkağaç F_1	0	0	1	0	0.999	0.001	0
Kırkağaç F_1	1	0	0	0	0.001	0.994	0.004
Kırkağaç F_1	0	1	0	0	1	0	0
LokumF_1	0	0	0.999	0	0.001	0.027	0.972
LokumF_1	0.981	0	0.019	0	1	0	0
LokumF_1	0	1	0	0	1	0	0
NapolyonF_1	0	0	1	0	0	1	0
NapolyonF_1	1	0	0	0	0.001	0.001	0.998
NapolyonF_1	0	1	0	0	0.998	0.002	0
Total Acc.	24	4	17	9	22	14	18

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DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

Conclusion

Salt stress is one of the most important stress factors for the majority of cultivated plants. Several studies were conducted on the subject. Tolerance to abiotic stress factors such as salinity displays a complex structure, and therefore, makes it difficult to develop tolerant varieties. Tolerance varies between plant varieties and even between variety genotypes. In the present study, it was determined that variations existed between the tolerances of studied genotypes against salt stress based on the examined traits. It was concluded that the examined traits could be used to determine the effect of salt stress, and the conducted correlation analysis demonstrated that there were correlations among various traits. It was found that traits such as leaf number, shoot length, shoot fresh weight and leaf relative water content of all genotypes were adversely affected under salt stress. While stoma length, stoma width and stomatal area decreased under salt stress, it was determined that the stoma intensity increased. It was observed that shoot dry weight, leaf relative water content and were among the traits that best explained the variance both salt stress and the control group in the PCA analysis. Mixture modeling analysis indicated that there was no significant difference between the 0 mM application sub-groups, however, especially in the 50 mM application, the distribution of the lowest and highest mean values of the variables was clearer; hence, the variance between the genotypes and varieties could be determined with mixture modeling analysis based on the reactions they exhibited against salt stress.

Acknowledgements. The authors would like to express their gratitude for the financial support provided by Van Y. Y. U. Scientific Research Projects Project no: 2015-ZF-B067.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29652981

CO_2 EMISSION TRENDS AND RISK ZONE MAPPING OF FOREST FIRES IN SUBTROPICAL AND MOIST TEMPERATE FORESTS OF PAKISTAN

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(Received 25th Nov 2018; accepted 16th Jan 2019)

Abstract. Due to high temperatures and dry summer in most subtropical regions, forest fires are of a regular occurrence. Fires emit CO_2 and other greenhouse gases which retroact on the ecological systems. This study provides CO_2 emission estimates from forest fires and risk zones in subtropical and temperate forests of Pakistan. CO_2 emission was calculated using average dry matter g/m^2, burned area, combustion factor (CF) and burning efficiency (E_f) following the guidelines of Inter-governmental Panel on climate change (IPCC). Fire risk zones were created by using GIS tools considering anthropogenic and natural geological factors. Results showed that average dry organic matter is 13837 ± 5774.64 gm^{-2}, while average annual CO_2 emission is 7280 ± 5369 Gg with 56.6% average annual increase of CO_2 emission. Meanwhile, highest emission of about 22799 Gg was recorded in 2009 corresponding to about 145.6 ha of burnt forests. Additionally, over 56% of the area or 9.33% (extreme risk zone) and 45.20% (high risk zone) is threatened by fire. Forest fire trends are mainly due to an increase anthropogenic activities and changes in weather conditions. Understanding forest fire threats and trends, will aid the government authorities in making appropriate conservation programs to curtail these forest fire threats and carbon dioxide emission trends.
Keywords: *burnt biomass, burning efficiency, Murree forest division, remote sensing, risk assessment*

Introduction

Globally, human activities and forest fires continue to reduce forest cover. This forest cover change distorts the forest and surrounding ecosystem functions, biodiversity conservation and hence forest regeneration (Tanvir et al., 2006; Beckline

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_29833002

and Yujun, 2014). Forest fires occur at regular intervals and reduce plant growth and regeneration hence exacerbating soil erosion (Jaiswal et al., 2002; Mukete, 2018a).

Forest fires are also deleterious to trees where trees become more susceptible to fungal and insect attacks thus affecting wood quality and volume (Abd El-Kawya et al., 2011). Globally, tree cover loss was observed to have attained a record 2.97 million km^2 in 2016 (Weisse and Goldman, 2017), the loss is 51% higher than the previous year with forest fires being the primary cause of this spike. In the Brazilian Amazonian region tree cover loss was estimated at over 370,000 km^2 nearly three times more than in 2015, this increase mainly occurred in the states of Pará and Maranhão, which were heavily affected by fire in late 2015 and early 2016 (Chanthalath, 2017)

Forest fires are the greatest potential risk to stored terrestrial carbon and each year an estimated 2-3 PgC are released into the atmosphere corresponding to about 3-4 million km^2 of burnt forests (Giglio, 2006; Hansen et al., 2013; Weisse and Goldman, 2017). Forest fires cause changes in the earth's biological and physical characteristics which over the years, affect carbon exchange (Harden et al., 2000; Vilen and Fernandes, 2011). According to Wekesa et al. (2016), about 82-97% of released carbon dioxide (CO_2) constitutes total carbon stock. Therefore, emission of greenhouse gases such as CO_2 and methane (CH_4) from forest fires directly affects climate (Simpson et al., 2006; Hansen et al., 2013)

Forest fire risk zones are areas from where a fire is likely to have started and spreads to other areas (Leblon, 2005). The availability of forest fire risk zone mapping aids in the precise evaluation of the fire (Jaiswal et al., 2002). This risk-mapping involves the multi-criteria evaluation method that includes factors such as slope, elevation, aspect, land-use, climatic conditions, proximity to roads and settlements (Bacani, 2016; Mukete et al., 2018b). One of the most common practices of risk zoning is the assigning of the subjective layer to all classes according to the sensitivity of fire and ignition capabilities (Dong et al., 2005). For instance, Ito and Penner (2004) used GIS-grid based system and multi-criteria to map peat swamp forests in Pekan District, south of Pahang, Malaysia to develop weighted fire index model for fire hazards. However different studies use different methods such as meteorological data and occurrence of forest fires for the establishment of forest fire models and risk zone assessment (Alonso-Betanzos et al., 2003; Zhang et al., 2011). The other studies have developed forest fire models by maintaining environmental and physiographic factors that affect the wildfire (Mitchener and Parker, 2005; Thompson et al., 2017). Similarly, the combination of satellite remote sensing and modeling have opened up new opportunities for the quantitative assessment of pre and post- forest fires.

In Pakistan, sub-tropical and temperate forests are located in the northern part of the country along the Himalayan foothills (Mannan et al., 2018a; Amir et al., 2018). This dry sub-tropical climate makes sub-tropical and temperate forests vulnerable to forest fires. This is particularly from altitudinal variations which influence the spreading of forest fires at different elevations (Ali, 2013; Saeed et al., 2019). In Pakistan, each year a considerable area of subtropical and moist temperate forests come under fire mainly due to natural causes, human and accidents. According to FAO (2015) and Hansen et al. (2013) more than 80% fires in these forests are due to anthropogenic activities.

Although biotic and abiotic factors facilitate the assessment of heterogeneous distribution and occurrence of fire density, Pakistan lacks active fire management plans both at regional and national levels. These limits and diffuses the control and monitoring of the increasing trends in national carbon emissions (Krawchuk et al.,

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2009; Saeed et al., 2016, 2018; Mannan et al., 2018b; Adnan et al., 2018) which has therefore necessitated an urgent need for national fire management plans. This study strives to fill this knowledge gap by estimating carbon emissions in the subtropical and temperate forests of Pakistan. It focuses on carbon emission trends within the last two decades as well as the anthropogenic, topographic and climatic factors. Understanding forest fire threats and trends, will aid the competent government authorities in developing appropriate conservation policies such as to curtail these forest fire threats and carbon dioxide emission trends.

Materials and methods

Description of study area

The subtropical and moist temperate forests are primarily located at the foot of the Himalayan mountain ranges (550-2600 altitude) between 33° 46' 50.60" N to 33° 56' 01.48" N latitude and 73° 09' 36.76" E to 73° 33' 29.66" E longitude (Ashraf et al., 2014), as shown in *Figure 1*. The area spreads across over 46898 ha and it is administratively controlled by Murree Forest Division (MFD) and includes three forest types. These are subtropical broad-leaved evergreen forests, subtropical Chir pine forests and moist temperate forests. Common plant species include *Pinus roxburghii* (Chir-pine), *Pinus wallichiana* (Blue pine), *Abies spp, Cedrus deodara* (Diar), *Quercus incana* (Oak), *Aesculus indica* (Chentnut), *Dodonea spp* (snatha), *Olia spp* (Indian olive) (Shahzad et al., 2015)

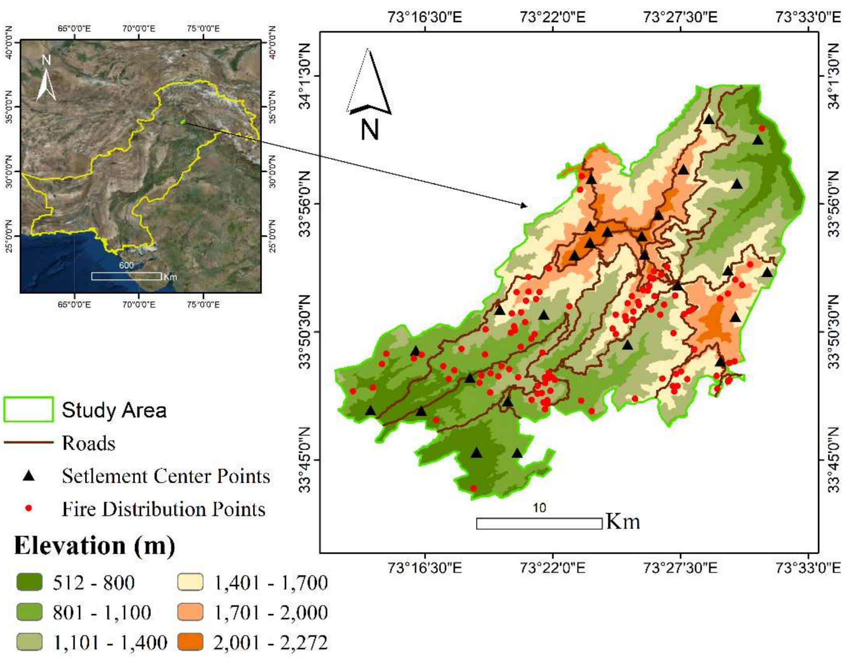

Figure 1. *Map of the study area, showing fire distribution, settlements centre points and roads*

The mean annual precipitation is 1800 mm, the mean annual max and min temperature are 24.3 °C and 11.4 °C, while the mean annual relative humidity is 53.06%, mean annual wind velocity is 3.22 km/h and mean annual solar energy is 18.4 J

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(PMD, 2015). Geologically the study area is composed of Cretaceous and Tertiary sedimentary strata having deep relief due to tectonic uplift during the tertiary period (Ashraf et al., 2014).

Data acquisition

The image of the study area was obtained from satellite (Sentinel-2), the cloud free image of 22nd October, 2017 was selected that is freely available on European space agency (ESA) website https://sentinel.esa.int/web/sentinel/home, topographic maps at scale of 1:25000 of the study area was obtained from survey of Pakistan (http://www.surveyofpakistan.gov.pk/). Digital elevation model (DEM) with the 30 m resolution was obtained from United States Geological Survey (USGS) https://earthexplorer.usgs.gov/. Forest type map, forest fire data (1998-2017) and Fire watch tower information were obtained from the office of the Murree forest division (MFD). Roadmap of Murree was obtained from National Highway Authority Islamabad (NHA) and Settlements map from the office of Pakistan Statistical Bureau (PSB) as shown in *Table 1*. The field data were collected by taking sample plots randomly distributed in the study area for biomass and CO_2 emission calculations.

Table 1. *Dataset and sources*

Data	Source	Abbreviation	Unit/resolution/Scale
Sentinel-2	European Space Agency (ESA)	Sent-2	10 m
Digital elevation model	Earth explorer (USGS)	DEM	30 m
Topographic map	MFD (Punjab forest department)	MFD	1:25000
Elevation	Derived from DEM	Elev	Meters (m)
Aspect	Derived from DEM	Asp	Classes 1-4
Slope	Derived from DEM	Slope	Classes 1-4
Distance from roads	Rod map from (NHA)	DisRd	Meters (m)
Distance from settlements	Settlements map from SBP	DisSet	Meters (m)
Watch towers	MFD	MFD	
Mean Annual Precipitation	PMD &NOAA	mm	Millimeters (mm)
Mean Annual Temperature	PMD &NOAA	Temp	Degree centigrade (°C)
Land-uses	From Satellite Image	Land-use	30 m

Biomass and CO_2 emission estimation

A total of 150 plots of size 20 m × 30 m were randomly sampled as shown in *Figure 2* and the stem volume (m^3/ha) measured for all the trees found within. In each sample plot, the diameter of all trees greater than 4 cm was measured at breast height (DBH). Using the Abney level, the height of 20 trees per species was recorded which were used to construct DBH/height function of a specific plot.

We first arranged the trees present in the sample plot into different classes based on their diameter (Dia classes), density of each diameter class i.e. no of trees per hectare were calculated and the basal area (B.A) of each diameter class was by using *Equation 1*.

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$$B.A = Density \times DBH \times \pi r^2 \, (^{m^2}/_{ha}) \qquad \text{(Eq.1)}$$

Stem volume was measured using *Equation 2* as described by Newbery (2009).

$$Stem\ Volume \left(^{m^3}/_{ha}\right) = B.A(m^2/ha) \times h(m) \times f.f \qquad \text{(Eq.2)}$$

where B.A is the basal area, h is height and form factor f.f is the volume of cylinder to actual tree form (Tenzin et al., 2017). In the Indian sub-continent, f.f is usually 0.37 for conifers and 0.6 for broad leaves is commonly used (Haripriya, 2002). Biomass t/ha was calculated by multiplying stem volume with the wood density (W.D) t/m^3 of particular species using IPCC proposed guidelines (IPCC, 2006b). The wood density values were also confirmed by previous research and reports (Sheikh, 1993; *Eq. 3*).

$$Stem\ Biomass \left(^{t}/_{ha}\right) = StemVolume \left(^{m^3}/_{ha}\right) \times W.D(^{t}/_{m^3}) \qquad \text{(Eq.3)}$$

Total Biomass t/ha (above ground biomass, understory vegetation biomass, litters and dead wood) was measured by using Biomass expansion factor (BEF) for subtropical and temperate forests of Pakistan as described by Haripriya (2002) and IPCC (2006a) (*Eq. 4*).

$$Total\ Biomass(^{t}/_{ha}) = Stem\ Biomass(^{t}/_{ha}) \times BEF \qquad \text{(Eq.4)}$$

Biomass carbon values of understory vegetation (USV) were measured by establishing sub-plot of 4 m × 4 m in each sample plot; the USV vegetation includes herbs, weeds, and grasses etc. The vegetation in each sub-plot was harvested by using sickle. The average fresh weight of the harvested material in kg/m^2 was recorded and all harvested vegetation was put into brown paper labeled bags. Then the paper bags were dried in gravity convection oven, model no (SG03) at 72 °C for 48 h and their dried weights in kg/m^2 were measured for Biomass Carbon Calculation. Similarly, we collected litter; dead wood and cones present in the sub-plot and measure the average weight kg/m^2. Tree biomass, USV biomass, litters and deadwood were added and the total biomass t/ha was calculated (Brown et al., 1982; Saugier et al., 2001; Malhi et al., 2004; Vilen and Fernandes, 2011), as shown in *Equation 5.*

$$Average\ dry\ organic\ matter\ B\ (\mathrm{g\ dm/m^2}) = Average\ Biomass\ (^{t}/_{ha}) \times 100 \qquad \text{(Eq.5)}$$

B is the average organic matter in particular ecosystem per unit area (g dm/m^2), after the dry organic matter was calculated, we used (Seiler and Crutzen, 1980; Vilen and Fernandes, 2011) formula for the calculation of total burned biomass (BBM) as shown in *Equation 6*.

$$BBM = A \times B \times CF \qquad \text{(Eq.6)}$$

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BBM is the burned dry biomass (g dry matter per unit time) in particular ecosystem burned. Where A is the annually burnt area m^2/year, B is Average organic matter per unit area g/m^2 and CF is the Combustion factor or biomass burning efficiency. Combustion factor is the exposed biomass that is actually consumed during combustion (Ito and Penner, 2004). Only a portion of the biomass is burnt but most biomass remains in the form of standing trees, charcoal or dead organic matter. This handicap was resolved by using 0.74 combustion factor for subtropical/tropical grasslands and 0.45 for temperate forests according to guidelines of IPCC (2006a; *Eq. 7*).

$$E_c = BBM \times E_{fc} \quad \text{(Eq.7)}$$

The total burnt biomass BBM was then converted into emitted CO_2 by using the equation, where the CO_2 emission factor (E_{fc}) that described the weight of CO_2 emitted per weight of dry burnt biomass. The amount of released compound by the amount of dry fuel consumed g/kg of dry fuel consumed is the emission factor (Andreae and Merlet, 2001; Saranya et al., 2016). According to IPCC (2006a) the emission factor E_{fc} 1531 g/kg for sub-tropical and temperate region is used to calculate the average annual carbon emission for the last two decades (1998-2017).

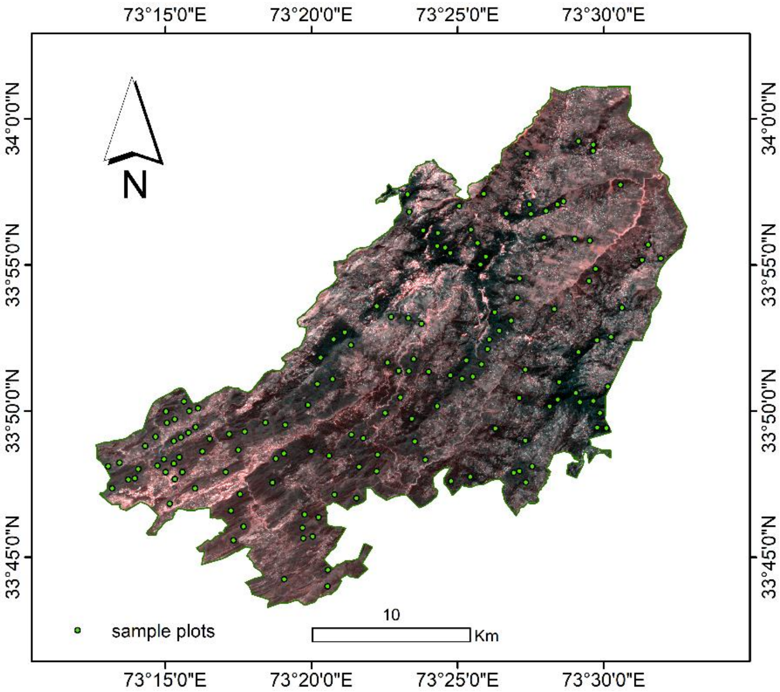

Figure 2. *Sample plots distribution*

Forest fire risk zoning and fire risk index

Forest fire risk zone map was generated by using vegetation type (broad-leaved forest, chirpine forest, and moist temperate forest), land-use (dense forest (DF), open forest (OF), bare land (BL), settlements (SM), agricultural land (AL), topography (slope, aspect, elevation), human factors (distance from roads, distance from settlements) and watch tower visibility in MFD. The topographic sheet of the study area

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and forest distribution map at the scale of 1:25000 of the study area was obtained from the office of the MFD**.** The topographic map and forest distribution map were scanned and exported into the processing software ArcGIS 10.2 and ENVI 5.1. The Shapefile was clipped by using the topographic sheet as a reference. Sentinel-2 image having 10 m resolution was downloaded for European space agency (ESA). First, the image pre-processing was performed in ENVI 5.1 and the geometric distortion of the image was removed from the topographic sheet as well as from the GPS by selecting 20 Ground Control Points (GCPs) around the study area. Image pre-processing is necessary before the classification or distribution for establishing a relationship between acquired data and biophysical processes (Abd El-Kawy et al., 2011; Iwan et al., 2004; Mannan et al., 2018). The geometrically corrected image was then used to clip the region of interest (ROI). After that, the image was classified into five different classes such as dense forest (DF), open forest (OF), agricultural land (AL), settlements (SM), and barren land (BL), Road (RD) with corresponding classes by assigning per-pixel signatures. On the bases of different digital number (DN) value of landscapes different colours assigned to differentiate them. After that Radiometric, the atmospheric and topographic correction was performed by using ArcGIS 10.2 and ENVI 5.1.

Training samples for each predetermined class were selected by delimiting polygons and spectral signatures for each land cover type derived from satellite imagery were recorded using pixels inside polygons. Supervised classification was performed by using a maximum likelihood algorithm. Accuracy of the land-use change was assessed by using confusion matrix in ENVI 5.1. We took 50 ground truth points in each land-use, for accuracy assessment. The kappa statistics was calculated by using *Equation 8*.

$$K\ (kappa) = \frac{Observed - Expected}{1 - Expected} \quad \text{(Eq.8)}$$

To improve the quality of classification and to classify the un-classed pixels, post classification smoothing was performed. Post classification smoothing is used to improve the classification quality, un-classified pixel (Harris and Ventura, 1995). The Land cover classification as shown in *Figure 6a,* gives meaningful information about the class value and also links the spectral characteristics of the image, that can be displayed as a map for researchers and scientists to evaluate the land-use classes (Weber and Dunno, 2001).

Spatial distribution and statistics of forest type were calculated from forest distribution map (*Fig. 7a*). The forest distribution map was first scanned imported to processing software ArcGIS 10.2. Geometric correction of the map was performed from the topographic sheet by selecting 50 Ground Control Points (GCP's) around the study area, DEM (30 m) was used to calculate the slope, elevation and aspect, as shown in *Figure 8a, b, c.* Reclassification tool was used for making their risk zone classes. Roadmap and settlements map of MFD was obtained from NHA and PBS, which was used to construct fire risk distance corridors. Euclidian distance tool in ArcGIS 10.2 was used for making 100 m, 200 m, 300 m and 400 m corridors of roads and settlement center points of the study area (see *Fig. 7b*).

For Fire rating index (FRI) determining first of all the risk ratings were assigned to the variable factors responsible for forest fire according to their fire causing potential, which was determined by referring previous research and historical record, as shown in *Table 2*. The higher rating shows that variable has a high influence on fire risk. Secondly, each variable is classified into classes i.e. (extreme, high, moderate, low and

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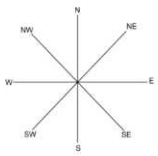
no risk) and finally all the layers were integrated through GIS and by using *Equation 9* FRI is calculated.

$$FRI = 10(lu_i + ft_j) + 5sl_k + 3asp_l + 2ele_m + 2set_n + 2rd_o \qquad \text{(Eq.9)}$$

In FRI, *lu* is the land-use having (6 classes), *sl* is slope factor (4 classes), *asp* is aspect (9 classes), *ele* is elevation (4 classes), *set* is distance from settlements (4 classes) and *rd* is the distance from roads (4 classes). The superscripts (*I,j,k,l,m,n,o*) are the sub-classes. Finally, weighted overlay tool was used for overlay analysis and all the layers were combined to form final fire risk zone map of the study area shown in *Figures 3* and *10.*

Table 2. *Fire hazard factors*

Factors	Classes	Assigned value	Risk factor
Land-use (10)	Dense forest	5	Extreme
	Open forest	4	High
	Barren land	1	No risk
	Agricultural land	3	Moderate
	Roads	1	No risk
	Water	1	No risk
	Settlements	1	No risk
Forest type (10)	Sub-tropical broad leave evergreen forest	4	High
	Subtropical Chir pine (*Pinus roxburghii*) forest	5	Extreme
	Moist temperate forest	3	Moderate
Elevation (2)	500-1000 m	5	Extreme
	1000-1500 m	4	High
	1500-2000 m	3	Moderate
	2000-2500 m	2	Low
Slope (5)	0-10°	2	Low
	11-20°	3	Moderate
	21-40°	4	High
	<41°	5	Extreme
Aspect (3) 	N (0-22.5)	2	Low
	NE (22.5-67.5)	3	Moderate
	E (67.5-112.5)	3	Moderate
	SE (112.5-157.5)	4	High
	S (157.5-202.5)	5	Extreme
	SW (202.5-247.5)	5	Extreme
	W (247.5-292.5)	3	Moderate
	NW (292.5-337.5)	3	Moderate
	N (337.5-360)	2	Low
Distance from settlements (2)	0-100 m	5	Extreme
	100-200 m	4	High
	300-400 m	3	Moderate
	400-500 m or >	2	Low
Distance from roads (2)	0-100 m	5	Extreme
	100-200 m	4	High
	200-300 m	3	Moderate
	300-400 m or >	2	Low

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Visibility analysis

Fire watch towers are very important for early detection of forest fires. There are two fire watch towers in the study area which include the 17 m high and 12 km maximum range of visualization Ban fire tower and the 20 m high and 15 km of maximum visualization range Charihan fire tower. The visibility analysis of the study area was carried out with the view-shed tool in ArcGIS 10.2 by using DEM and fire towers geographical location (*Fig. 9* and *Table 3*)

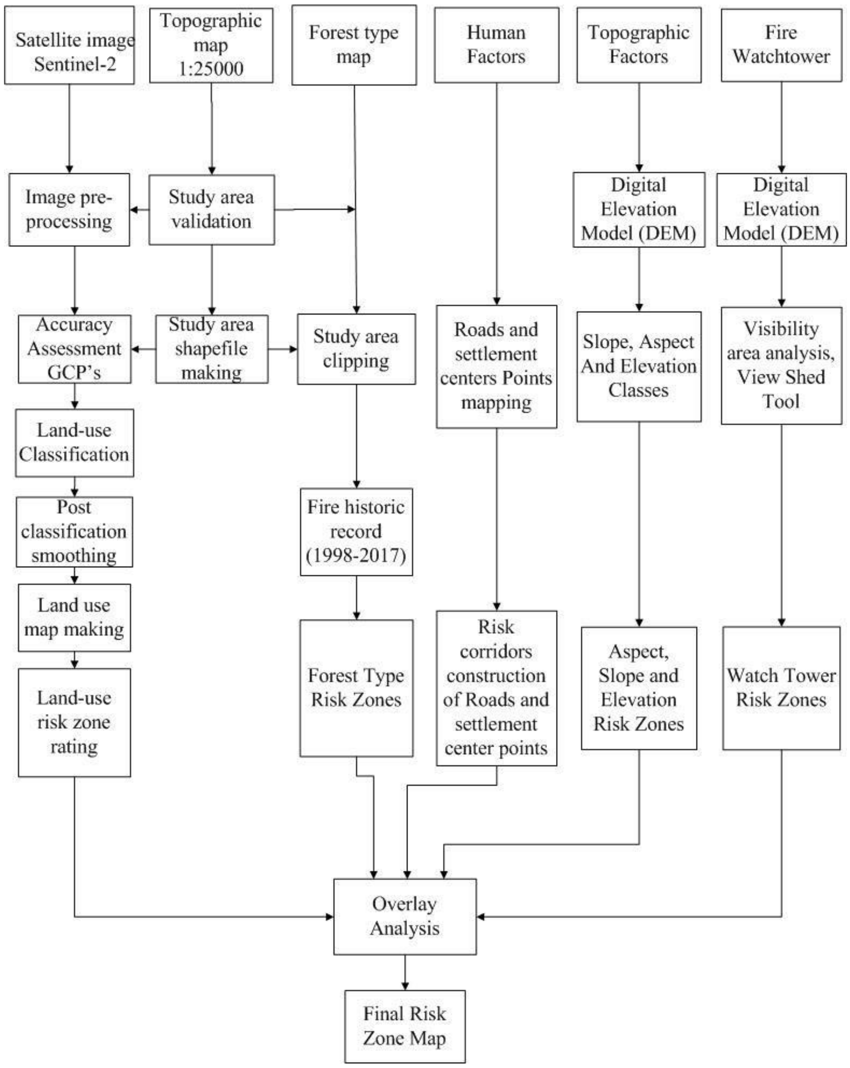

Figure 3. *Flow Chart*

Table 3. *Visibility analysis of the study area*

Towers	Visible area		Invisible area	
	Area (ha)	**Percent (%)**	**Area (ha)**	**Percent (%)**
Ban tower	2192.203	4.67	44706.59	95.32
Charihan tower	6637.86	14.15	40260.92	85.84
Total visible area	8302.636	17.70	38596.15	82.29
Common visible area	999.3	2.13		

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Results and discussions

We performed field survey by random forest sampling in the study area. The study area includes Conifers and Broad-leaved trees. Conifers like *Pinus roxburghii, Pinus wallichiana, Abies pindrow, Picea smithiana* have more tree volume due to their tall height as compared to broad leaved trees like *Olea spp, Quercus incana, Aesculus indica* (Mannan et al., 2018a). The results showed that the average density in the study area is 556.68 (±128.19 std. dev.) trees per ha, while average BA is 40.27 (±10.124 std. dev.) m^2/ha, the average volume is 349.26 (±111.06 std. dev.) m^3/ha and mean DBH is 35.42 (±15.54 std. dev.) cm.

Forest fires and CO_2 emission trend

We have obtained forest fire data for the last two decades that include fire origin location, size, date of occurrence, forest type and ignition cause form MFD. The historical record shows that accidental fires are 72.2% of the total fires, while 25% are deliberate and 2.6% are lightning-caused fires. Accidental fires mostly occur due to cigarette disposal in or around forests, careless fire handling during forest picnics, vehicle transportation, and the burning of farm residues along forests.

The increasing trend of forest fire has been observed in the study area, the average annual fire in the first decade (1998-2007) was 276 ha while it reached to 651 ha in the second decade (2008-2017), with the total 928.79 ha burned as shown in *Figure 5*.

The average dry organic matter in the forests was found to be 13837 (±5774.64 std.dev.) g/m^2. The total estimated CO_2 emission in last 2 decades as obtained from the study is 145.60 (±5369.68 std. dev.) Tg CO_2, with estimated average annual CO_2 being 7280 (±5369 std. dev.) Gg CO_2. However, the largest fire events were observed in 2009 with 22799 Gg CO_2 emission. This was due to the fact that 2009 was recorded as driest summer year after 1980 (PMD, 2015). During the last two decades 788 (±175 std. dev.) Gg CO_2 was emitted in the month of June followed by 25.40 (±1.5 std. dev.) Gg CO_2 in May. The statistical analysis showed that average area burned in the first decade (1998-2007) was 27.69 ± 7.37^a (ha) which is significantly less than the second decade (2008-2017) which was 66.927 ± 7.37^b (ha), whereas the month of June found most critical for forest fire occurrence, with average forest fire of 23.13 ± 3.12^a (ha) significantly higher than 12.40 ± 3.12^b (ha) in May, while the occurrence of forest fire in the other months of the year was very less and showed non-significant relation with each other.

The historic fire record also showed that average net annual increase of CO_2 emission is 56%. According to Yi and Bao (2016) increasing temperatures and CO_2 concentration cause an increase in fuel availability. This increases the photosynthetic process which results into more plant growth and water use efficiency thus inducing more fires and CO_2 emission. *Figures 4* and *5* show that the rate of CO_2 emission increases steadily with increasing linear trend. Therefore, increases in temperatures exacerbate evapotranspiration which speeds-up the hydrological cycle (Finney et al., 2005). This ultimately causes more rainfall, vegetation growth, large fires and CO_2 emission. For instance, new settlements in Murree have been reported in the last decade, encroachment of forest area by land grabbers is a big issue, 1158 ha of forest land have encroached (Ashraf et al., 2014).

About 80% of fires occurred in areas accessible to humans, having road density and proximity to settlements, thus the patterns and occurrences of forest fires are related to human disturbances. Most of the fire events occurred in May and June burning over

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653.2 ha, as shown in *Figure 5*. This may be attributed to the fact that, pine trees particularly *Pinus roxburghii* shed dry needles in the summer season and also because May and June constitute the hottest months. Additionally, this may be related to the fact that a ban on green felling was imposed in 1992. This put a halt to management activities such as thinning, pruning, line clearing and prescribed burning which in combination with burning are the most effective methods of fire prevention (Finney et al., 2005; Kuenzi et al., 2008)

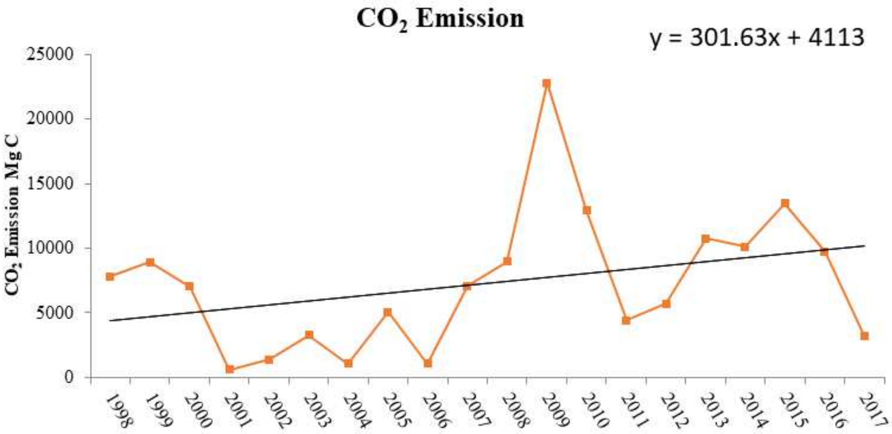

Figure 4. *CO_2 emission trend*

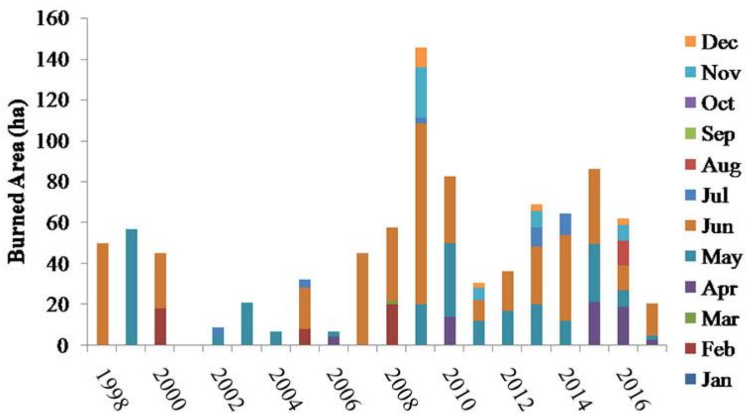

Figure 5. *Forest fire trend*

Fire risk zones and fire risk index

The Land-use map showed that 21.9% of the area includes DF which is in extreme risk zone, while 31.4% OF is in high-risk zone. Also, areas under low risks of fires AL are 6.6% and 39.9% which include water, roads, and barren lands (*Table 4* and *Fig. 6b*).

In the DF for instance, due to higher vegetation density, fuel is more available compared to the OF or BL. Thus, fire occurrence is more plausible and this is corroborated by another study by Martin et al. (2016) their study observed that forest fires are 70% more like to occur in dense vegetation as compared to sparse vegetation. The accuracy of land use map was assessed by formula as shown in *Equation 8,* and kappa statistics was 0.85.

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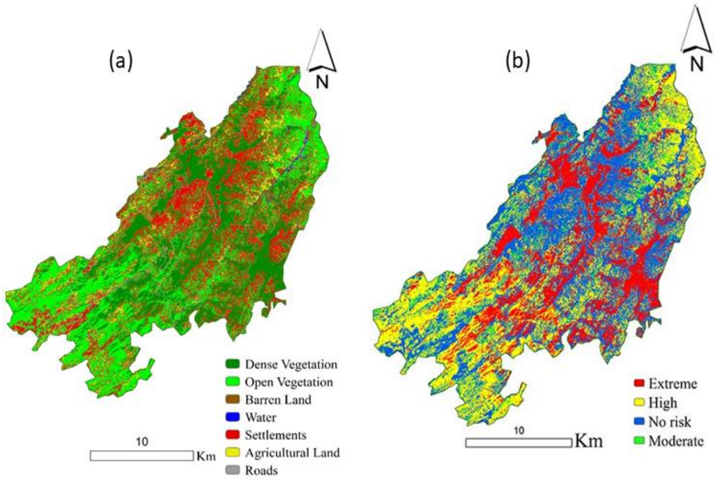

Figure 6. *Land-use map (a) and land-use risk zones (b)*

Similarly, from the forest distribution map, three forest types including broad-leaved evergreen forests, chirpine forests, and moist temperate forests were observed. Here, each forest type has its susceptibility level towards forest fires, and based on historical records obtained from the study area, Chirpine forests are most susceptible to forest fires mainly due to falling of dry needles in summer. Hence, their categorizations as extreme high-risk zones while the broad-leaved forests are categorized as high-risk zones and moist temperate forests as moderate risk zones. In a related study, Kumar et al., 2015 categorized Chirpine forests *(Pinus roxburghii)* as the major contributor to forest fires in Kangra region of Indian western Himalaya. Furthermore, the forest type map showed that more than half 51.14% of the area in the extreme risk zone, while 36.7% in a high-risk category and 12% in the moderate risk category (*Fig. 7a*).

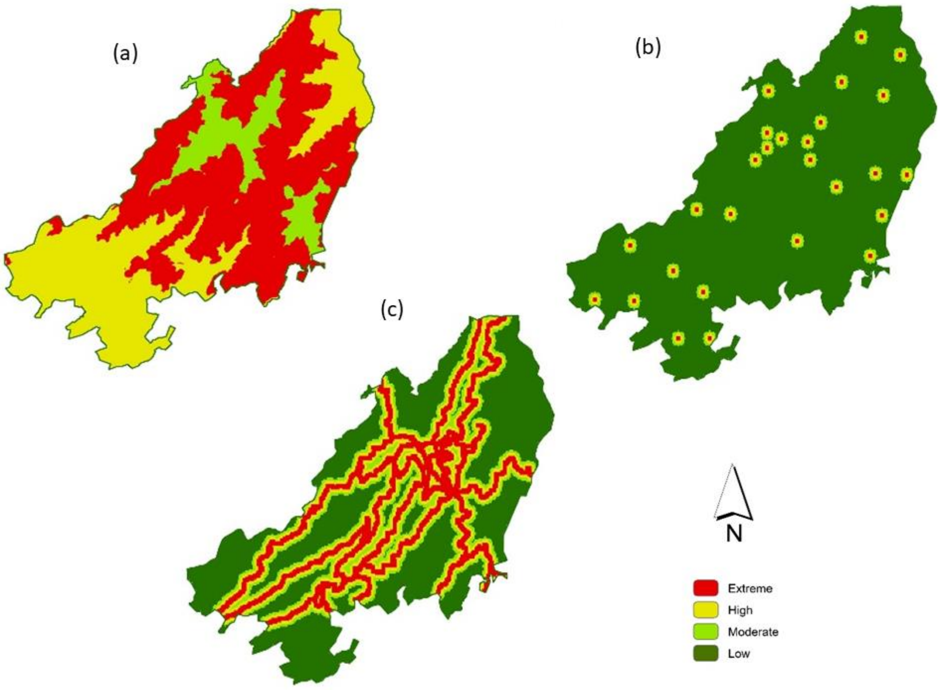

Figure 7. *Risk zones of forest type (a), settlement center (b), roads (c)*

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However, in sub-tropical broad-leaved evergreen forest which is present at lower altitudes of Himalayan Mountains, fire ignition chances are slightly lower due to the association of many plant species. According to Ye et al. (2017), various broadleaf species are fire resistant especially in the eastern parts of China. At higher elevations of Himalayan mountains, the chances of fire occurrence are lower, due to climatic conditions, studies also showed that 85% of fire occurred at the elevation lower than1000 m, hardly any fire was recorded above 4000 m (Martin et al., 2016),

Topography which includes slope and elevation is also important role in the ignition and expansion of forest fires. Topography is an important factor in forest fire ignition and it was given much importance in studying forest fire causes in western United States (Liu et al., 2015). In slope aspect the southern (S) and south-west (SW) slopes are dry in nature due to the sun's direct impact thus leading to higher weights (Brosofske et al., 2007). Due to less moisture, high temperatures, robust winds and low fuel moisture, chances of fire ignitions are higher in southern aspects (Finney et al., 2005). Similarly, there are less chances of fire occurrence on north (N), north east (NE), west (W), and North West (NW) slope as sunrays not fall directly upon them. From the aspect risk map, 22.04% of the area faces the south and is thus located in extreme fire risk zones (S) and south-west (SW) slope. Meanwhile, 17.02% is located in the high-risk zone facing south-east (SE) slope and 50.66% is located in the moderate risk zone facing north-west, west, north east (NE) and east (E) as shown in *Figure 8c* and *Table 4.* Fire spreads quickly along steep slopes than gentle slopes because the flame angle is closer to the surface and where wind effects supply thermal energy through convection (Zhong et al., 2003). Similarly, steep slopes are more venerable for fire spreading slope map showed 0.26%, 12.51%, 59.43% and 27.78% in extreme, high, moderate and low risk zones respectively (*Fig. 8a* and *Table 4*).

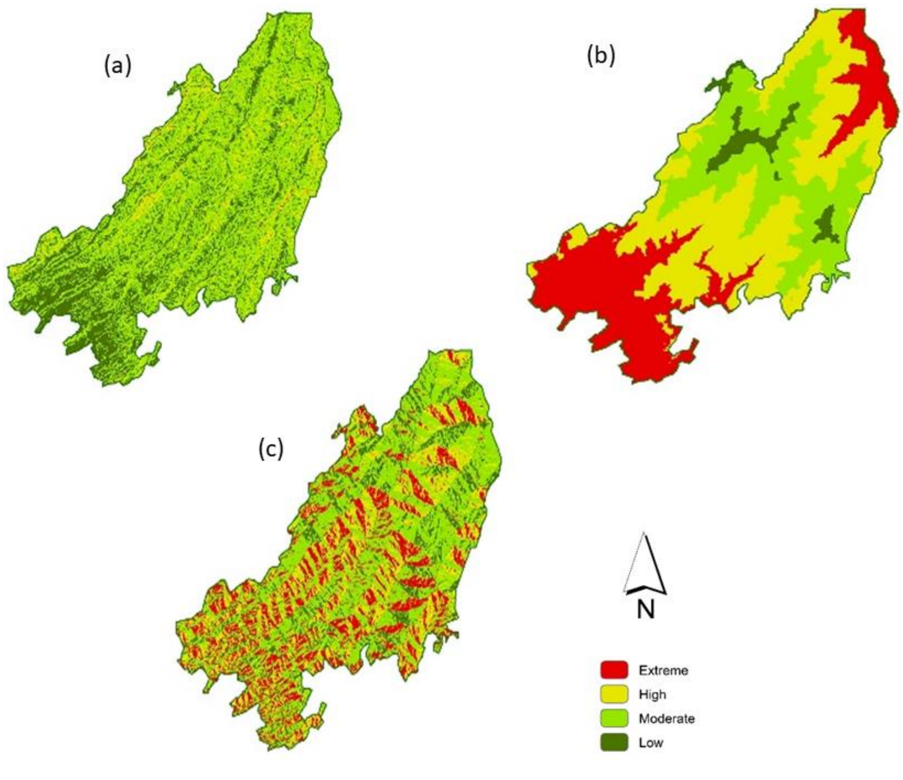

Figure 8. *Risk zones of slope (a), elevation (b), aspect (c)*

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Table 4. *Fire risk area in each variable zone*

	Extreme		High		Moderate		Low		No risk	
	ha	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**	**ha**	**%**
Land-use	10285.73	21.9	14737.65	31.4	0	0	3123.31	6.6	18742.96	39.9
Forest type	23987.15	51.14	17246.80	36.7	5664.83	12	0	0	0	0
Aspect	10339.83	22.04	7982.24	17.02	23759.13	50.66	4817.587	10.27	0	0
Slope	122.96	0.26	5871.3	12.51	27875.08	59.43	13029.41	27.78	0	0
Elevation	13520.57	28.82	18621.54	39.7	12941.61	27.6	1814.96	3.86	0	0
Roads	9947.98	21.21	7471.46	15.93	5945.14	12.68	23520.23	50.16	0	0
Settlement	122.99	0.26	5871.3	12.51	13029.41	27.78	27875.08	59.43	0	0
FRZM	4376.93	9.33	21202.32	45.20	2443.60	5.21	18670.43	39.81	0	0

Human factors such as roads and settlements initiate forest with significant effects on forest ecosystem, land and water quality. The roads and settlement corridor map 0-100 m is rated as extreme risk zone, similarly 100-200 m, 300-400 m, and 400-500 m rated as high, moderate and low fire risk zones. However, 21.21% of the area of roads is located in the extreme risk zone, 15.93% in high risk and 12.68% in moderate risk zone (see *Fig. 7c*). Similarly, settlements map showed 0.26% to be located in the extreme risk zone, 12.51% in the high-risk zone and 27.78% in the moderate as shown in *Figure 7b*. Studies have also showed that the forest fires caused by human are often located in the proximity of roads and settlement corridors (Brosofske et al., 2007; Maingi and Henry, 2007; Syphard et al., 2007). Road edges are also served as the habitat for some exotic plant species (Parendes and Jones, 2000). These species provide more combustible material fuel wood that are more easily ignited (Arienti et al., 2009). While it was also reported by Abdi et al. (2012) optimum roads network can decrease the chances of fire occurrence.

The visibility analysis showed that 4.67% of the study area is visible from the Ban fire tower, while 14.15% is visible from the Charihan fire tower. The combined visibility of the two towers is 17.70% and the total invisible area is 82.29%. The visibility of Charihan fire tower is 14.15% and Ban fire tower is 4.67% of the study area (see *Table 3* and *Fig. 9*). The earlier detection of forest fire can minimize the forest damage up to 70%, however watch towers plays an important role for emergency response.

The Final Risk Zone Map (FRZM) (*Fig. 10*) was prepared by overlaying all the layers together according to the assigned weights in weighted overlay tool. FRZM showed 8.46% to be located in the extreme risk zone while the highest 46.30% area is in the high risk of the fire zone and the moderate risk zone has 5.22% and 40.02% in low risk zone, as shown in *Table 5*.

Table 5. *Extent of fire risk zones*

S No	Risk class	Area (ha)	Percent area (%)
1	Extreme	3998.513	8.46%
2	High	22059.487	46.30%
3	Moderate	2489	5.22%
4	Low	19053	40.02%

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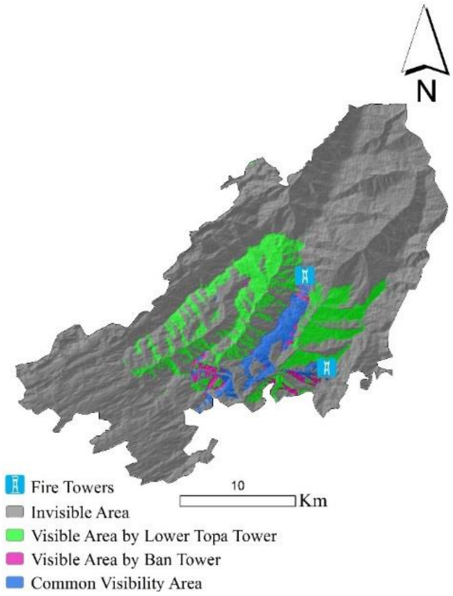

Figure 9. *Visibility analysis map of fire watch towers*

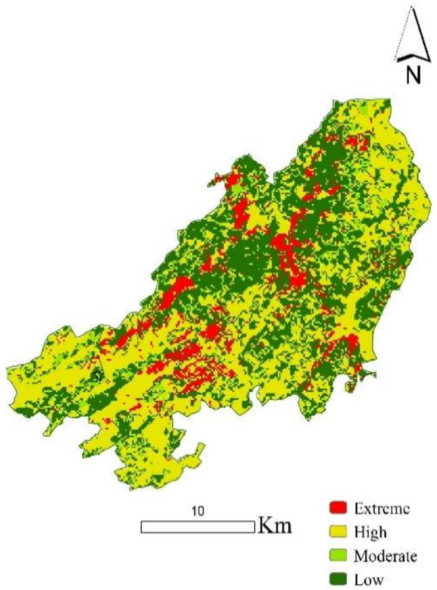

Figure 10. *Land use fire risk zones*

The FRZM shows fire vulnerability to be in more than half of the area is directly under the risk of fire. Finally, our FRZM was compared with actual fire-starting points, the majority of fire-starting points are in extreme and high fire risk zones.

Conclusion

The importance of forest fire risk zone assessment is well known but less attention has been paid towards the forest fire, CO_2 emission trend in subtropical and moist temperate forests of Pakistan.

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In this study, we have calculated the CO_2 emission tend and forest fire risk zones. We found the CO_2 emission in the last three decades has been increased at accelerated rates. This increasing trend of CO_2 is directly linked with forest fire, and our study found that increase in forest fire due to more settlements, roads construction and human disturbance in the forest. Lightening caused forest fires are 2.6%, on other hand accidental fires 85% of the total fires. Although roads and settlements act as barriers to forest fires but 80% fires initiated from the corridors of roads and settlements.

This study integrates the historic fire record, ground sampling, remote sensing and GIS tools for fire risk zone assessment. The forest fire risk zone analysis showed that more than half of our study area is under extreme and high fire threat. This study provides risk assessment, factors and trends of forest fire, but further study on risk zone mapping of forest fire under predicted climate change is required, similarly forest fire trends and its effect on local climate is requited, as well as the role of emergency response team and watch towers in fire management is required. This risk zone analysis will provide a broad picture to management authorities for combating this increasing fire trend and also suggest the future management.

Acknowledgements. This research was supported by the National Natural Science Foundation of China (No. U1710123), the Natural Science Foundation of Beijing (Key Program, No.6161001), and the medium and long-term project of the “Precision Forestry Key Technology and Equipment Research” (No. 2015ZCQ-LX-01).

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DOI: http://dx.doi.org/10.15666/aeer/1702_29833002

THE EFFECT OF HERBICIDE USE ON THE OCCURRENCE OF ENTOMOPATHOGENIC FUNGI IN THE SOIL OF BLACKCURRANT PLANTATIONS

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(Received 26th Nov 2018; accepted 4th Feb 2019)

Abstract. The aim of the paper was to present species composition and density of colony-forming units of entomopathogenic fungi (EPF) in the soil sampled from the herbicide fallow and sward strips of four blackcurrant (*Ribes nigrum* L.) fields in Poland. Samples collected in spring and autumn were used as study material. The fungi were isolated with the method of scattering diluted soil onto the selective medium. In order to protect the plants against pests, conventional plant protection was applied to all fields. In the soil taken from the blackcurrant fields three species of entomopathogenic fungi were found: *Beauveria bassiana* s.l. (Bals.-Criv.) Vuill., *Isaria fumosorosea* (Wize) and *Metarhizium anisopliae* s.l. (Metschn.) Sorokin. Both in spring and autumn isolated fungi produced more colony-forming units (CFU) in soil collected from the sward strips than from the herbicide fallow of the blackcurrant fields. In spring *B. bassiana* s.l. and *M. anisopliae* s.l. produced more CFU in the soil under sward than in the herbicide strip with intense chemical protection. In the case of *I. fumosorosea* in spring there was a tendency for CFU to be more numerous in the soil of the herbicide fallow.
Keywords: *herbicide fallow, colony forming units, Beauveria bassiana, Isaria fumosorosea, Metarhizium anisopliae*

Introduction

For many years Poland has been a leader in the production of blackcurrant fruit, which is an effect of a favourable economic climate and well developed fruit processing industry (Piotrowski et al., 2016). Chemical protection of blackcurrants is based primarily on a large number of chemical treatments, also used to maintain herbicide fallow under bushes and grass between rows of currants. Blackcurrants are perennial plants, which contributes to an increased possibility of pesticide accumulation and results in their residues in the soil. Within the framework of integrated pest management programme, it is recommended to limit significant amounts of chemicals, replacing them with agricultural and biological methods.

Constituting a large group of microorganisms colonizing the soil habitat, EPF have an ability to infect insects, and their development leads to the disruption of physiological processes in the host and to its death in the end (Zimmermann, 2007; Tkaczuk, 2008; Augustyniak-Kram et al., 2013). The anamorphic hypocrealean fungal genera *Beauveria*, *Metarhizium*, and *Isaria* are ubiquitous components of the soil microbiota in temperate regions including agroecosystems, undisturbed/natural landscapes and forests (Chandler et al., 1997; Klingen et al., 2002; Keller et al., 2003). Their basic advantage is the potential to reduce pest populations that infest agricultural and horticultural crops, fruit trees, as well as plants grown under cover (Vänninen et al., 2000; Zimmermann, 2007; Ropek et al., 2014). EPF are the basis for the production of bioinsecticides used increasingly in biological plant protection against pests (Faria and Wright, 2007; Zimmerman, 2007). Occurrence and biological activity of EPF are dependent on many factors affecting processes in the soil environment, like: type of soil, air temperature, its humidity, time of year, but also on the availability of hosts and methods of agricultural treatment (Vänninen et al., 2000; Krysa et al., 2012; Tkaczuk et al., 2014). Reports by many authors (Vänninen and Hokkanen, 1988; Miętkiewski et al., 1997; Tkaczuk, 2008; Tkaczuk et. al., 2012; Pelizza et al., 2015; Fiedler and Sosnowska, 2017) indicate that chemical substances in plant protection products can affect EPF and their natural occurrence, species composition, sporulation, colony growth, and infectiousness potential. With a wide range of pesticides used in chemical plant protection, fungicides and herbicides have the most negative effect on the population of fungi and their impact largely depends on the amount of the active substance and on the species of fungus (Andalo et al., 2004; Tkaczuk et al., 2012).

The purpose of this paper is to determine the species composition and density of colony-forming units (CFU) of EPF in soils taken from the grass strip and herbicide fallow of four blackcurrant fields.

Materials and methods

Soil sampling

Soil collected in 2012 in two periods (spring and autumn) from four blackcurrant fields located in two localities in Poland: Łuzki (Łosice County) and Żabokliki Kolonia (Siedlce County) was used as research material. Conventional plant protection against pests was used in the fields. Soil samples were taken from two habitats, i.e. from the herbicide fallow under blackcurrant bushes and from the sward between the rows. From 15-20 randomly selected points, soil material was sampled at a depth of 15-20 cm using a metal soil stick. Characteristics of the studied plantations are shown in *Table 1*.

Table 1. *Characteristics of the blackcurrant plantations*

Plantation/variety	Locality	Area of plantations (ha)	Age of plantation years	Herbicides used
I - Tisel	Łuzki	3	3	Glyphosate + MCPA
II - Ben Lomond		3	3	Glyphosate + MCPA
III - Ores	Żabokliki Kolonia	1	3	Glyphosate + MCPA
IV - Ruben		1	3	Glyphosate + MCPA

Soil samples were co-mingled into plastic bags and held at 4 °C. The soil samples were sieved with 3 mm mesh to separate debris, such as roots and pebbles, and then dried to a moisture content of approximately 25-30%. This is the optimal moisture content for fungal growth and is limiting for the development of entomopathogenic nematodes (Inglis et al., 2012).

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DOI: http://dx.doi.org/10.15666/aeer/1702_30033011

Fungal isolation

To assess the density of EPF in tested soils, the number of colony forming units (CFU) per gram of dry soil were determined using a selective medium, developed by Strasser et al. (1996). Two grams of soil from each site were added to 18 ml of distilled water with addition of 0.05 ml of Triton X-100. Two serial dilutions were made (1:100 and 1:1000) per sample with a solution of autoclaved 0.10% Tween 80 surfactant, and four replicates of both dilutions were spread on separate 9 cm Petri dishes The solution was agitated for 35 seconds before 0.1 ml was applied to media using a sterile pipette. A sterile glass spatula was used to evenly spread the solution on media plates. The selective media consisted of: 20 g of glucose, 18 g of agar, 10 g of peptone, and the selective components: 0.6 g of streptomycin sulfate, 0.005 g of chlortetracycline, 0.05 g of cycloheximide and 0.1 g of dodine dissolved into 1 L of distilled deionized water. The supplements streptomycin sulfate, chlortetracycline, cycloheximide, and dodine were added after the media cooled to approximately 30 °C to avoid degradation. These selective supplements inhibit the growth of saprophytic bacteria and fungi and favour the growth of EPF. Four replicates per sample were prepared and incubated at 22 °C and after 8-10 days colonies of each fungus species were counted.

EPF were identified on the basis of morphological characteristics, using standard identification keys (Humber, 2012; Inglis et al., 2012). Given that only morphological methods were applied during the identification of fungi, the species *Beauveria bassiana* and *Metarhizium anisopliae* were defined *sensu lato*, because, as demonstrated by the latest phylogenetic studies based on DNA sequence (Bishoff et al., 2006, 2009; Rehner et al., 2011), there are numerous fungus species, both within the genus of *Beauveria* and *Metarhizium*, which are impossible to distinguish from each other without the application of molecular methods. The experiment was replicated three times for each soil sample. The findings were presented as the number of colony-forming units (CFU) of each species of EPF in 1 g of dry soil.

Data analyses

Since the main aim of the study was to show differences in the occurrence of CFU of particular fungal species between the investigated habitats (sward and herbicide fallow) of each of the four plantations, regardless of the season and location, therefore data on density of EPF CFUs in the field survey were analysed using a 1-way analysis of variance (ANOVA) and means were separated using Tukey's honest significant difference (HSD) test. Values of $P < 0.05$ were considered statistically significant.

Results and discussion

The main habitat of EPF is soil biomass, of which they constitute an essential part (Ritz and Young, 2004). Their occurrence as well as quantity is affected by a number of biotic and abiotic factors, and also by a wide range of agronomic treatments. The widespread use of pesticides destroying pests in plantations of soft fruit plants is the primary way to reduce their numbers and has a direct impact on the population of EPF in the soil.

This experiment studying the density of colony-forming units (CFU) of EPF in soil sampled from four fields of blackcurrants in two locations in spring and the autumn showed the existence of three species: *Beauveria bassiana* s.l., *Isaria fumosorosea,* and

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DOI: http://dx.doi.org/10.15666/aeer/1702_30033011

Metarhizium anisopliae s.l. (*Tables 2* and *3*). Density of colony-forming units (CFU) of each species was varied depending on the time of year and on the habitat from which the soil was sampled.

Table 2. *The density of colony forming units of entomopathogenic fungi (CFU × 10^3 g^{-1}) in soil from blackcurrant plantations in spring*

Fungal species	Plantation							
	I		II		III		IV	
	A	B	A	B	A	B	A	B
Beauveria bassiana s.l.	0.0	0.0	0.2 a	0.0 a	15.9 a	0.7 b	0.3 a	0.2 a
Isaria fumosorosea	0.5 a	0.8 a	0.3 a	9.2 b	2.7 a	2.5 a	0.0	0.0
Metarhizium anisopliae s.l.	4.7 a	0.2 b	1.0 a	1.7 a	4.7 a	1.7 b	0.7 a	0.2 a

A - sward; B - herbicide fallow
I, II - Łuzki; III, IV - Żabokliki Kolonia; a,b - significance calculated for α < 0.05

In spring time, in soil sampled from the first blackcurrant field in the village of Łuzki (Łuzki I), *M. anisopliae* s.l. constituted the largest share of the fungi. There were more of its colony-forming units in the soil from the sward than from herbicide fallow, respectively, 4.7 and 0.2 × 10^3 g^{-1}. Another species present in this location was *I. fumosorosea*, which was more common in the herbicide strip than in the grass strip. In spring in the soil collected in the other blackcurrant field located in the same village a different relationship was found, where *I. fumosorosea* and *M. anisopliae* s.l. produced more colony-forming units in the soil of herbicide fallow than in the sward, respectively, 9.2 and 1.7 × 10^3 g^{-1}. In Łuzki I there was no the presence of the *B. bassiana* s.l. fungus in either of the strips, and in Łuzki II there was a small amount of it in the sward soil. Vänninen and Hokkaken (1988) found that in a field with soft fruit bushes the *B. bassiana* fungus was the most sensitive to herbicides commonly used to keep herbicide fallow. In the soil collected from both blackcurrant fields in Żabokliki, there were significantly more colony-forming units in the grass between the rows of bushes than in the herbicide fallow. The dominant species in the soil of Żabokliki Kolonia III turned out to be *B. bassiana* s.l., which in the grass alleys produced 15.9 × 10^3 g^{-1} colony-forming units (*Table 2*). Sapieha-Waszkiewicz et al. (2005) by examining the soil taken from blackcurrant and chokeberry fields found a clear domination of the *B. bassiana* fungus, while in the soil taken from an adjacent field there was a greater volume of the *M. anisopliae* fungus.

When considering all blackcurrant fields with all soil samples collected in spring *B. bassiana* s.l. and *M. anisopliae* s.l. produced significantly more colony-forming units in the soil taken from the sward area than in the soil of herbicide fallow, while in the case of the *I. fumosorosea* fungus the opposite tendency was reported (*Table 2*).

In soil samples taken in autumn in the Łuzki I field *B. bassiana* s.l. and *M. anisopliae* s.l. occurred to a greater volume in the soil of the fallow strip than in the sward, producing, respectively, 2.3 and 0.7 × 10^3 CFU in 1 gram of soil (*Table 3*). In the soil taken from the herbicide strip, with intense chemical protection, in the other field (Łuzki II) there were no colony-forming units of *B. bassiana* s.l. or *I. fumosorosea*, while in the sward strip they produced, respectively, 0.5 and 5.0 × 10^3 g^{-1} of them. The results

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demonstrated a negative impact of intensive chemical protection, especially herbicides used under blackcurrant bushes, on the density of colony–forming units of EPF. Kozanecka et al. (1996) reported that long-standing use of herbicides in an orchard in order to keep land fallow contributed significantly to the reduction of soil biological activity. On the other hand insects, plants and microbial populations will interact at the soil–root interface to affect EPF populations. Some EPF like *M. anisopliae* and *B. bassiana* are ubiquitous insect pathogens and possible plant symbionts, as some strains are endophytic or colonize the rhizosphere (St. Leger, 2008; Pava-Ripoll et al., 2011).

Table 3. *The density of colony forming units of entomopathogenic fungi (CFU × 10^3 g^{-1}) in soil from blackcurrant plantations in autumn*

Fungal species	**Plantation**							
	I		II		III		IV	
	A	B	A	B	A	B	A	B
Beauveria bassiana s.l.	0.2 a	2.3 b	0.5 a	0.0 a	0.3 a	0.0 a	2.0 a	0.3 b
Isaria fumosorosea	4.5 a	1.0 b	5.0 a	0.0 b	0.2 a	0.5 b	0.5 a	0.0 a
Metarhizium anisopliae s.l.	0.2 a	0.7 a	0.0 a	0.2 a	1.2 a	0.0 b	0.5 a	0.5 a

Explanations as for *Table 2*

I. fumosorosea clearly dominated in the soil of the sward in samples from both blackcurrant fields in Łuzki. In autumn in soil samples collected from the field located in Żabokliki Kolonia (III) *B. bassiana* s.l. and *M. anisopliae* s.l. were in greater quantities in the soil of the grass strip between the rows of shrubs, and *I. fumosorosea* produced much more colony-forming units in the soil of the herbicide fallow. In the case of the other field located in the village, *B. bassiana* s.l. and *I. fumosorosea* were in greater abundance in the soil collected from the sward, while the *M. anisopliae* s.l. species produced the same number of colony-forming units in the soil of both types of habitats.

The use of herbicide fallow in the rows of trees or bushes and grass in the inter-rows as a mechanism for maintaining soil in a good condition, creates specific environment for the development of soil microorganisms. Grassy vegetation roots in sward enable the rhizosphere microflora to develop. Under conditions of herbicide fallow, the biological activity of the soil depends primarily on multiannual, cumulative effect of herbicides (Akopyan and Avetisyan, 1990; Ehle and Laermann, 1991).

The main herbicide used on investigated blackcurrant plantations was glyphosate. A few herbicides, including glyphosate, have been shown to be fungistatic despite their use for weed control. Formulations of glyphosate have been shown to be fungicidal to plant pathogens including *Fusarium solani* (Martius) Sacardo and *Phytophthora* spp., but not detrimental to species like *Pythium* spp. (Kassaby, 1985; Kawate et al., 1992). A study by Morjan et al. (2002) investigated the effects of seven glyphosate formulations on EPF mycelial growth in solid media and found that the fungicidal properties of glyphosate formulations varied among fungal species but that *M. anisopliae* was susceptible to all glyphosate formulations. The fungicidal effects of herbicides like glyphosate may not occur under field conditions where the chemical can bind to clay

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30033011

particles in most soil types and reduce its bioavailability and movement through the soil substrate (Morjan et al., 2002).

Many authors point out that the way the soil is cultivated is a factor significantly affecting the species composition of EPF. Tkaczuk (2008) showed in his studies that long lasting use of chemical pesticides might cause resistance reactions of some species of EPF. The phenomenon of the formation of fungal strains with increased resistance to pesticides was also observed by Sapieha-Waszkiewicz et al. (2005). Poprawski and Majchrowicz (1995) holds that EPF probably have a specific system detoxing herbicides. According to the authors, it is a proven fact that some colonies of fungi start growing later, only after a few days of contact with a medium containing herbicides. According to Domsch (1972) soil microorganisms, including fungi, create a series of enzymatic reactions that help them in the process of pesticide degradation.

In all soil samples collected in autumn from all blackcurrant fields it was found that *I. fumosorosea* created on average significantly more colony-forming units in the sward than in the herbicide fallow. Density of colony-forming units of *M. anisopliae* s.l. was slightly higher in the soil of the grass strips than the herbicide fallow. The occurrence of *B. bassiana* s.l., both in the herbicide fallow and sward was on average at similar level.

The experiment showed that in the samples taken in spring and as well in autumn from all the fields, EPF produce together on average significantly more colony-forming units in the soil of the sward between rows of blackcurrant bushes, than in the herbicide fallow strips (*Fig. 1*). These values were lower in autumn than in spring. Hummel et al. (2002) after many years of carrying out field experiments found that pesticide application significantly limited the occurrence of EPF in the soil. This was confirmed by the research of Sapieha-Waszkiewicz et al. (2005), in which *G. mellonella* larvae in the soil of a blackcurrant field without any chemical protection against pests were infected by EPF to more than double degree compared to intensively protected field.

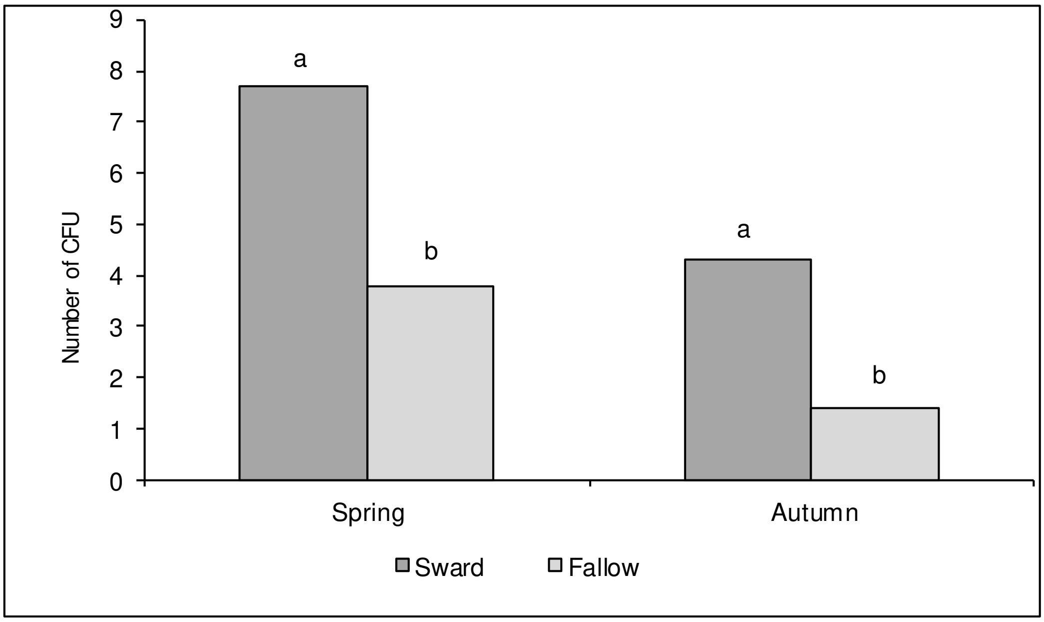

Figure 1. *The average number of colony forming units of entomopathogenic fungi in soil (CFU × 10^3 g^{-1}) from sward and herbicide fallow of black currant plantations (spring and autumn). Different letters above columns in each habitat indicate significant differences in the values at α < 0.05*

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According to the Karg and Bałazy (2009) simplified crop rotation, chemical pest and weed control, and periodically repeated tillage treatments, aimed at raising crop yield, eliminate, directly or indirectly, arthropod pathogens together with the parasites and predators as a trophic group with special environmental and nutrient requirements. However, most fungi, including EPF, are characterized by large adaptive capacity to adverse environmental conditions, usually also having high reproductive and migration potential. They may settle back in a habitat during a short period of time, if, as a result of agricultural treatments, they have been eliminated, on condition that the fields or surrounded areas are suitable for their habitat.

Conclusions

With the method of spreading soil solution on the selective medium of the soil taken from the blackcurrant fields three species of entomopathogenic fungi were identified: *B. bassiana* s.l., *I. fumosorosea*, and *M. anisopliae* s.l.. The density of colony-forming units of individual species was varied and dependent on the habitat from which the soil was taken and on the time of year. Both in spring and autumn isolated entomopathogenic fungi produced totally more colony-forming units (CFU) in the soil under grass than in the herbicide strip of blackcurrant fields. When considering all blackcurrant fields, it was found that in spring *B. bassiana* s.l. and *M. anisopliae* s.l. produced more CFU in the soil under sward than in the herbicide strip with intense chemical protection. In the case of *I. fumosorosea* in spring there was a tendency for CFU to be more numerous in the soil of the herbicide fallow under the bushes. It meant that this species could be used together with pesticides in integrated protection of blackcurrants against pests. Further studies on pesticide resistance of different isolates of EPF can be performed to search of virulent strains to be applied simultaneously as biopesticides.

Acknowledgements. The research was carried out under research theme No. 360/13/S and were financed from the science grant granted by the Ministry of Science and Higher Education.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30033011

INHERITANCE OF GRAIN YIELD AND ITS RELATED CHARACTERS FOR 5×5 DIALLEL CROSS OF F1 BREAD WHEAT

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(Received 26th Nov 2018; accepted 4th Feb 2019)

Abstract. A full Diallel cross among five cultivars of common wheat (*Triticum aestivum* L.) with their twenty F_1 progeny were evaluated at Kurdistan Reign-Iraq at two different locations, Kanipanka and Qlyasan, during the winter season of 2017-2018 using Completely Randomized Block Design (CRBD) with three replications. The mean squares of genotypes were highly significant for all studied characters. Parent Iba-95 at Kanipanka location produced highest value for grain yield/plant, while at Qlyasan location the highest value was provided by the Kauz parent. Maximum heterosis value for grain yield/plant at Kanipanka location produced by the reciprocal cross Klal×Kauz was 7.425%, while at Qlyasan location the diallel cross Hasad×Iba-95 reached 71.402%. Klal parent at both locations was the best general combiner for grain yield/plant and some yield components. The cross Aras×Iba-95 was found to be the best specific combiner for most characters at both locations. The inheritance of most characters is controlled by non-additive gene effect. Heritability in broad sense for most characters was moderate to high, while for narrow sense it was low to moderate. Grain yield/plant exhibited positive and significant correlation with most its important components at both locations. Maximum positive direct effect in grain yield recorded by weight of spikes/plant and biological yield/plant at both locations respectively.
Keywords: *common wheat, genetic analysis, combining ability, heterosis, heritability*

Introduction

Wheat species of the genus *Triticum* L. are members of the *Triticeae* Dum. Tribe of the Poaceae Barn family and represent the world's most important monocotyledenous cereals (Anonymous, 2015). The *aestivum* species is one of the most important crops all over the world. Increasing production per unit area seems to be one of the great factors for narrowing the hole between wheat production and consumption (Ismail, 2015). Bread wheat is the most important food crop in our country and is the main source of protein and energy. Among the cereals, bread wheat is commonly identified as a species with a higher requirement of nutrients (Al-Naggar et al., 2015). According to the FAO statistics, 349 million tons of wheat was produced in 2016 and more than 220 million hectare of the world farmlands were under wheat cultivation (FAO, 2016). Due to the persistent increase of the world population, wheat plays a key role in the national economy of developing countries. Wheat production can be increased either by bringing more area under cultivation or vertically by increasing per unit yield. It is not possible horizontally to increase area under wheat due to other competing crops and shortage of irrigation water. Therefore, the only alternative left is to increase yield/ha, which is possible by introducing genetically superior new high yielding cultivars that are adapted to a wider range of environments. Wheat breeders, all over the world, have been utilizing the existing genetic resources to modify the wheat varieties in order to meet the requirement of an ever increasing population (Sprague and Tatum, 1942). Most of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

wheat planted areas are located in arid or semi-arid regions where a biotic stresses, especially drought stress, are a major constraint for crop production (Tahmasebi et al., 2014). Wheat breeding in the semi-arid region aims to develop new cultivars combining high productivity and good a biotic stress tolerance. Thus, it is important to understand the genetic control of these traits (Zine El Abidine et al., 2017). Genetic improvement of wheat yield is the trait breeders aim to achieve the most to promote wheat production and face the request of a continuous population expansion. This goal can be achieved either directly by selecting for high yield or indirectly by improving yield components and morphological traits (Hannachi et al., 2013). Knowledge of the genetic control of these traits related to wheat grain yield is requisite in a breeding program to design a selection strategy and manage the progeny. Several authors have attempted to impose the genetic basis of traits interested in yield determination. The results are often inconsistent and scarce; however, a predominance of additive gene action has been noticed with dominance effects for most traits studied (Saad et al., 2010; Rashid et al., 2012; Nazir et al., 2014). The diallel cross designs are frequently used in plant breeding research to obtain information about genetic properties of parental lines or estimates of general and specific combining abilities and heritability (Baker, 1978; EL-Maghraby et al., 2005; Iqbal et al., 2007). In addition, the diallel cross technique was laid to supply early genetic information in the first generation (Chowdhry et al., 1992; Topal et al., 2004; Ataei et al., 2017). Understanding and knowing about gene action in breeding programs could improve the accuracy of selection and decrease the breeding cost and time. Therefore, the aims of this study were to investigate combining ability, gene action, heritability of some quantitative traits, correlation and path analysis in bread wheat.

Materials and methods

The current study was carried out at two locations in Kurdistan Region-Iraq. First Kanipanka Nursery Station (Lat 35° 22'; N, Long 45° 43'; E, 550 masl) in Sharazoor Valley 35 km East of Sulaimani City, and second was at Qlyasan Agricultural Research Station, College of Agricultural Sciences-University of Sulaimani located (Lat 35° 34' 307"; N, Long 45° 21' 992"; E, 765 masl) 2 km North West of Sulaimani City. Five varieties of common wheat (*Triticum aestivum* L.) were used namely (Aras, Hasad, Kauz, Klal and Iba-95). The varieties were selected according to the prior experience on some of them regarding the efficiency of these varieties and their suitability to the prevailing environmental conditions in the region and creating new combinations with other varieties that have not yet been tested in the region.

Name, pedigree and origin of parental genotypes

No.	Genotypes	Pedigree	Origin
1	Aras	(Sonora 64×Lerma Rojo 64) × Sentaelena	Mexico
2	Hasad	SNB//CMH79A955/3*CNO79/3/ATTILA	Iraq
3	Kauz	PVN/5*SUPER KAUZ	CIMMYT-Veery
4	Klal	KLEIN RECORD/38 MA//KLEIN PALANTELEN	CIMMYT
5	Iba-95	Veery "S"	Iraq

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

All possible crosses including reciprocals were perfected from April 24 to May 8, 2017 to generate the 20 F_1s crosses at Qlyasan location. Seeds of 20 F_1s with their parents were sown on December 12, 2017 at Kanipanka location and on December 6, 2017 at Qlyasan location, according to Complete Randomize Block Design with three replications. Each treatment was one row of 2 m long, 40 cm between rows and 15 cm between plants within row. Five competitive plants (excluding border plants) were tagged, and data were recorded for:

1. Morphological traits:

Number of spikes/plant, Weight of spikes/plant (g), Average spike weight (g), Spike length (cm), Number of grains/spike, Weight of grains/spike (g), 1000-grain weight (g), Biological yield/plant, Grain yield/plant and Harvest index.

2. Genetic parameters:

General Combining Ability (gca) variances and effects, Specific Combining Ability (sca) variances and effects, Heterosis % as a deviation of F_1s from their mid parents, Broad Sense Heritability, Narrow Sense Heritability, Average Degree of Dominance (ā).

3. Association analysis and path coefficient analysis.

Statistical analysis

Combining ability analysis

The (gca) and (sca) were estimated using the general linear model for the analysis which takes the formula of (Singh and Chaudhary, 2007).

$$Y_{ijk} = \mu + g_i + g_j + s_{ij} + R_{ij} + r_k + \frac{1}{bc}\sum\sum e_{ij}$$

where:
Y_{ijk}: observed value of the experimental unit,
μ: populations mean,
g_i: general combining ability (gca) effect for the i^{th} parent,
g_j: general combining ability (gca) for the j^{th} parent,
s_{ij}: specific combining ability (sca) for the diallel crosses involving parent i and j,
R_{ij}: specific combining ability (rca) for the reciprocal crosses involving parent i and j,
r_k: replication (block) effect, and
$\frac{1}{bc}\sum\sum \varepsilon_{ijk}$: means error effect.

Estimation of general, specific combining ability and reciprocal effects

$$gi = \frac{1}{2p}\left(Yi + Yj\right) - \frac{1}{p^2}Y..$$

$$si = \frac{1}{2}(Yij + Yji) - \frac{1}{2p}\left(Yi + Yi + Yj + Yj\right) + \frac{1}{p^2}Y..$$

$$ri = \frac{1}{2}\left(Yij - Yji\right)$$

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where:
gi: effect of expected general combining ability for parents *I,*
si: effect of expected specific combining ability for single diall crosses
ij when $i = j$,
ri: effect of expected specific combining ability for reciprocal crosses *ij* when $I = j$,
Yij: F1s mean as a result of crossing parent *i* with parent *j*,
Y..: sum of the means of all parents and F1s hybrids, and
P: parents number

Estimation of heterosis

The percent increase (+) or decrease (-) of F1 cross over mid- parent was calculated to determined heterotic values for all characters (AGB301, 2004).

$$\text{Heterosis } (H)\ \% = [\frac{F'1 - M.P}{M.P}]\times 100 \qquad M.P = \frac{(P1+P2)}{2}$$

where:
F’1: mean of hybrid,
P1: parent one, and
P2: parent two.

Estimation of heritability

The term heritability has been further divided into broad sense and narrow sense, broad sense heritability was calculated by dividing genotypic variance by total variance and narrow-sense heritability was calculated by dividing additive genetic variance by total variance (Singh and Chaudhary, 1985).

$$h^2 b.s = \frac{\sigma^2 G}{\sigma^2 P} = \frac{\sigma^2 A + \sigma^2 D}{\sigma^2 A + \sigma^2 D + \sigma^2 e} = \frac{2\sigma^2 gca + \sigma^2 sca}{2\sigma^2 gca + \sigma^2 sca + \sigma^2 e}$$

$$h^2 n.s = \frac{\sigma^2 A}{\sigma^2 P} = \frac{\sigma^2 A}{\sigma^2 A + \sigma^2 D + \sigma^2 e} = \frac{2\sigma^2 gca}{2\sigma^2 gca + \sigma^2 sca + \sigma^2 e}$$

where:
$h^2{}_{b.s}$: heritability in broad sense,
$h^2{}_{n.s}$: heritability in narrow sense,
$\sigma^2{}_{gca}$: the variance of general combining ability,
$\sigma^2{}_{sca}$: the variance of specific combining ability,
$\sigma^2\ e$: the variance of experimental error, i.e. environmental variance,
$\sigma^2{}_A$: additive genetic variance,
$\sigma^2{}_D$: non-additive (dominance and epistasis) genetic variance,
$\sigma^2{}_G$: total genetic variance, and
$\sigma^2{}_P$: phenotypic variance (genetic and environmental variance).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Estimation of average degree of dominance

$$\bar{a} = \sqrt{\frac{2\sigma^2 D}{\sigma^2 A}} = \sqrt{\frac{2\sigma^2 sca}{2\sigma^2 gca}} = \sqrt{\frac{\sigma^2 sca}{\sigma^2 gca}}$$

If:
$\bar{a}$ = *zero* denote no dominance,
$\bar{a}$ < 1 denote partial dominance,
$\bar{a}$ = 1 denote complete dominance,
$\bar{a}$ > 1 denote over dominance.

Analysis of variance for full diallel cross according to (Griffing, 1956b), Method I, Model II (parents + diallel crosses + reciprocal crosses)

S.O.V	d.f	SS	MS	EMS
Block	b - 1 = 2	$\frac{\sum Y^2..k}{p^2} - \frac{Y^2...}{bp^2}$	MSb	
Genotype	$P^2 - 1 = 24$	$\frac{\sum Yij^2.}{b} - \frac{Y^2...}{bp^2}$	MSg	
gca	p – 1 = 4	$\frac{1}{2p}\sum(Yi.+Y.j)^2 - \frac{2}{p^2}Y^2...$	MSgca	$\sigma^2 e + 2p(\frac{1}{p-1})\sum gi^2$
sca	$\frac{p(p-1)}{2} = 10$	$\frac{1}{2}\sum Yij(Yij+Yji)^2 \frac{1}{2p}\sum(Y.j+Yj.)^2 + \frac{1}{p^2} - Y^2...$	MSsca	$\sigma^2 e + \frac{2}{p(p-1)kj}\sum\sum_{1<j} Sij^2$
rca	$\frac{p(p-1)}{2} = 10$	$\frac{1}{2}\sum(Yij - Yji)^2$	MSrca	$\sigma^2 e + (\frac{2}{p(p-1)})\sum\sum_{1<j} rij^2$
Error	$(b - 1)(p^2 - 1) = 48$	SST-SSb-SSg	MSe	$\sigma^2 e$
Total	$bp^2 - 1 = 74$	$\sum Yijk^2 - \frac{Y^2...}{bp^2}$		

Climate conditions of Sulaimani Governorate

The climate of Sulaimani governorate is semi-arid environment: hot and dry in summer; cold and wet in winter. During July and August, the average temperature is between 39-43 °C, and often reaching nearly 50 °C. Autumn means high temperatures are 20-30 °C in October, cooling slightly in November. Precipitation is limited to winter and spring months, and the overall average annual rainfall of 550-700 mm was at Sulaimani city. An overview of experimental conditions is given in (*Table 1*).

Table 1. *The meteorological data of the two locations*

Month	Kanipanka location				Qlyasan location			
	Mini. Temp.(°C)	Maxi. Temp.(°C)	Avg. Temp.(°C)	Rainfall (mm)	Mini. Temp.(°C)	Maxi. Temp.(°C)	Avg. Temp.(°C)	Rainfall (mm)
October	22.6	30.0	15.1	-	10.4	33.1	21.2	10.0
November	14.4	20.0	8.8	71	7.6	23.9	14.2	114.6
December	10.2	16.1	4.4	18.5	-2.5	17.8	7.0	22.2
January	7.8	12.5	3.1	60	1.4	15.6	7.8	72.4
February	10.3	14.9	6.1	281	-2.3	20.9	8.7	323.0
March	14.7	21.3	8.1	19	1.0	24.4	13.0	44.6
April	17.1	24	10.5	90.5	2.2	31.6	17.4	98.6
May	22.2	29.5	15.0	68	13.0	38.1	24.7	70.4
Total rainfall				608				755.8

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Soil analysis

Soil samples belonging to both locations were taken from experimental sites in Sulaimani governorate in Kurdistan region, Iraq. The samples were taken from surface (0-30 cm), the soil samples were air dried and ground to pass through a 2-mm sieve prior to analysis, shown in (*Table 2*).

Table 2. *Some physicochemical properties of the soil samples for locations of the experiment*

Location	Physical properties of the studied soil Particle size distribution (PSD) (g/kg)			
	Sand	**Silt**	**Clay**	**Texture class**
Kanipanka	214.00	540.00	246.00	Salty loam
Qlyasan	90.40	508.40	401.20	Salty clay

	Chemical properties of the studied soil						
	pH ($dS\ m^{-1}$)	**EC_e ($g\ kg^{-1}$)**	**OM ($Cmol_c\ kg^{-1}$)**	**CEC ($mg\ kg^{-1}$)**	**Available P**	**$CaCO_3$ equivalent ($g\ kg^{-1}$)**	
						Total	**Active**
Kanipanka	8.05	0.16	22.03	22.10	7.44	195.00	100.00
Qlyasan	7.80	0.38	16.06	29.76	9.61	230.00	117.00

	Soluble ions ($mmol\ L^{-1}$)						
	Ca^{2+}	**Mg^{2+}**	**Na^{+}**	**K^{+}**	**HCO_3^{-}**	**Cl^{-}**	**SO_4^{2-}**
Kanipanka	1.20	1.05	0.19	0.05	3.20	0.90	0.91
Qlyasan	2.20	1.80	0.10	0.13	2.34	0.80	0.88

	Available micronutrients ($mg\ kg^{-1}$)		
	Zn	**Cu**	**Fe**
Kanipanka	1.563	5.07	5.15
Qlyasan	0.450	4.96	3.23

Results

The analysis of variance represent in *Table 3* was carried out according to Fisher (1918) to estimate the significant differences among genotypes. General and specific combining ability variances and effects were determined. The analysis of variance revealed highly significant differences among the genotypes for all characters. This signified the presence of wide diversity among the genotypes at both locations. The estimates of gca and sca variances represented in the same table, confirmed that both additive and non additive gene effects were important for the inheritance of different characters. The mean squares for gca were highly significant for all characters except weight of spikes/plant, and grain yield/plant which were significant, and not significant for spike length, biological yield/plant and harvest index at first location, but at second location were highly significant for all characters except spike length and biological yield/plant which were significant and it was not significant for weight of spikes/plant and grain yield/plant. The mean squares for sca was highly significant for weight of spikes/plant, grain yield/plant and harvest index, and it is significant for spike length, and biological yield/plant at first location, while at second location it was highly significant for weight of spikes/plant, biological yield/plant and grain yield/plant, but it was significant for spike length, and harvest index.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

Table 3. *Mean squares of variance analysis in common wheat genotype for studied characters at both locations (Kanipanka upper value and Qlyasan lower value)*

S.O.V	d.f	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
Blocks	2	47.284	51.486	0.032	0.968	13.117	0.193	29.528	221.224	54.650	0.004
		1.040	30.225	0.109	3.011	98.436	0.169	31.004	2437.631	16.342	0.003
Genotypes	24	16.458^{**}	241.469^{**}	0.657^{**}	1.589^{**}	242.121^{**}	0.438^{**}	164.082^{**}	1493.674^{**}	102.266^{**}	0.008^{**}
		19.803^{**}	108.637^{**}	1.263^{**}	1.172^{**}	181.974^{**}	0.926^{**}	132.428^{**}	2554.647^{**}	87.388^{**}	0.020^{**}
gca	4	16.257^{**}	115.806^{*}	0.756^{**}	$0.533^{N.S}$	168.977^{**}	0.435**	168.998^{**}	$462.408^{N.S}$	43.154^{*}	$0.001^{N.S}$
		18.175^{**}	$17.142^{N.S}$	1.123^{**}	0.475^{*}	115.406^{**}	0.641^{**}	117.569^{**}	1247.252^{*}	$29.534^{N.S}$	0.013^{**}
sca	10	$3.808^{N.S}$	99.349**	$0.094^{N.S}$	0.567^{*}	$43.078^{N.S}$	$0.073^{N.S}$	$25.904^{N.S}$	569.758^{*}	42.352^{**}	0.004^{**}
		$3.433^{N.S}$	43.350^{**}	$0.196^{N.S}$	0.326^{*}	$52.450^{N.S}$	$0.191^{N.S}$	$15.128^{N.S}$	1165.362^{**}	41.366^{**}	0.006^{*}
rcs	10	$2.855^{N.S}$	$47.504^{N.S}$	$0.129^{N.S}$	0.491^{*}	83.028^{**}	$0.103^{N.S}$	$37.763^{N.S}$	440.218^{*}	$22.199^{N.S}$	0.003**
		5.140^{**}	36.703^{**}	0.365^{*}	0.422^{**}	$46.967^{N.S}$	0.293^{**}	43.787^{**}	$379.455^{N.S}$	$16.730^{N.S}$	$0.004^{N.S}$
Exp. Error	48	6.917	103.188	0.254	0.697	79.763	0.194	59.967	641.146	42.442	0.003
		5.660	37.696	0.494	0.412	81.289	0.287	33.381	1136.154	35.093	0.008
MSe¯		2.306	34.396	0.085	0.232	26.588	0.065	19.989	213.715	14.147	0.001
		1.887	12.565	0.165	0.137	27.096	0.096	11.127	378.718	11.698	0.003

Data in *Table 4* illustrate the performance of the genotypes at both locations. At the first location the cross Hasad×Aras produced maximum grain yield/plant reached 51.852 g, but for biological yield/plant it was 161.159 produced by the cross Iba-95×Klal. Iba-95 parent showed the best value for grain yield/plant 60.724 g, and some components such as weight of spikes/plant, number of grains/spike, and biological yield/plant reached 86.160, 80.733 and 170.901 g respectively. At the second location maximum grain yield/plant was 38.962 g recorded by the cross Hasad×Aras, and maximum weight of 1000-grain was 59.403 g produced by the same cross. Kauz parent at the same location produce the highest value for grain yield/plant, harvest index and weight of spikes/plant reached 33.902 g, 0.384 and 43.252 respectively.

The estimation of heterosis value represent in *Table 5* determined as the percentage of F_1s deviation from mid parental value. For all characters positive and negative heterosis values were present. Maximum positive heterosis value for the grain yield/plant was 7.425% recorded by the cross Klal×Kauz at the first location, while at the second location reached 71.402% for the cross Hasad×Iba-95, which recorded the highest positive heterosis for some components such as average spike weight and weight of grains/spike. All characters had shown considerable amount of heterosis over mid parents.

Table 6 illustrates the general combining ability effect of parents. Klal parent was the best general combiner for grain yield/plant and number of spikes/plant, while Hasad parent and Iba-95 was the best combiner for most components at the first location. At the second location Klal was the best general combiner for grain yield/plant, number of spikes/plant and weight of spikes/plant, while Hasad parent was the best general combiner for average spike weight, weight of grains/spike, 1000-grain weight and biological yield/plant. Present findings are in confirmation with Kumar et al. (2011), Singh et al. (2013), Raj and Kandalkar (2013), Aslam et al. (2014), Ismail (2015), Kalhoro et al. (2015) and Kandil et al. (2016); they found high positive value due to gca for these characters.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

***Table 4.** The mean values of studied characters for F_1 diallel, F_1 reciprocal crosses and parents at both locations (Kanipanka upper value and Qlyasan lower value)*

Crosses and parents	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
1 x 2	19.666	59.596	4.274	11.500	51.067	3.027	59.318	112.599	44.941	0.438
	10.222	34.019	3.410	9.533	47.100	2.371	50.403	113.788	27.400	0.209
1 x 3	14.889	50.877	4.441	11.900	62.467	3.315	53.441	126.058	42.692	0.343
	8.778	31.545	5.040	11.367	64.333	3.524	54.685	95.562	24.839	0.259
1 x 4	16.667	58.492	4.484	12.700	60.600	3.270	53.718	103.250	44.257	0.428
	11.889	34.126	3.760	11.133	54.467	2.879	52.838	92.327	26.460	0.302
1 x 5	15.000	51.079	4.357	11.100	65.667	3.333	50.530	128.945	37.617	0.293
	8.000	26.848	4.091	10.833	59.400	2.935	50.295	96.009	20.754	0.213
2 x 3	19.778	59.383	3.883	11.333	58.933	2.863	47.109	140.202	40.110	0.367
	11.778	36.113	4.165	11.100	67.533	3.195	47.847	109.226	26.040	0.240
2 x 4	19.222	55.395	3.969	11.720	76.533	3.148	43.460	104.553	42.956	0.445
	14.444	49.852	4.447	12.067	78.867	3.537	44.826	140.021	36.413	0.290
2 x 5	15.667	54.476	5.057	13.400	75.400	3.979	52.981	102.906	43.124	0.446
	12.778	42.841	3.939	11.667	63.400	3.154	49.609	138.604	32.231	0.240
3 x 4	16.111	51.150	4.555	12.767	82.200	3.283	39.952	104.458	39.819	0.496
	12.111	43.454	4.433	12.700	78.267	3.153	40.087	149.018	32.904	0.235
3 x 5	16.000	61.627	4.548	12.800	63.467	3.341	52.936	149.095	46.659	0.312
	9.778	38.168	5.479	11.833	71.467	4.134	58.403	119.730	29.387	0.243
4 x 5	14.555	50.805	5.618	12.800	76.133	4.352	57.093	118.479	47.978	0.418
	11.444	39.752	4.041	10.600	64.200	3.015	47.247	132.275	29.841	0.231
2 x 1	17.445	63.583	4.866	12.300	61.733	3.557	57.533	154.662	51.852	0.354
	13.222	38.171	4.767	11.933	61.933	3.523	59.403	154.194	38.962	0.255
3 x 1	21.000	60.014	4.531	13.000	69.433	3.355	48.318	141.322	44.034	0.368
	16.111	41.122	4.222	11.567	74.600	3.201	42.910	185.791	36.180	0.197
4 x 1	15.222	53.967	4.985	12.600	64.400	3.595	56.165	120.627	38.772	0.364
	10.111	44.181	5.843	11.400	75.200	4.534	54.852	96.908	34.502	0.366
5 x 1	17.111	61.796	4.346	13.733	91.133	3.362	40.245	136.119	42.877	0.325
	12.333	42.437	4.826	12.000	75.933	3.053	39.476	123.645	31.998	0.259
3 x 2	18.667	53.242	4.583	11.387	71.067	3.415	47.960	119.454	48.534	0.403
	17.555	42.795	4.193	10.800	72.733	3.244	44.592	69.486	33.985	0.568
4 x 2	18.222	65.807	4.628	12.967	74.067	3.389	45.588	143.652	50.111	0.348
	11.889	29.316	4.125	10.885	67.350	2.865	42.603	81.353	28.900	0.365
5 x 2	15.222	60.208	5.082	11.833	65.267	3.785	57.935	140.471	46.352	0.336
	9.889	35.662	4.476	11.167	67.733	3.702	54.880	83.053	30.105	0.375
4 x 3	17.778	66.105	4.931	12.500	78.133	3.525	45.423	153.638	47.878	0.317
	12.333	38.984	4.188	12.140	67.400	2.979	45.168	126.621	28.289	0.231
5 x 3	16.000	46.294	4.038	12.278	66.678	3.042	45.839	93.517	34.396	0.378
	15.999	45.983	4.267	11.767	69.000	3.134	46.810	143.065	33.559	0.251
5 x 4	18.445	66.502	4.805	13.633	73.000	3.516	48.947	161.159	51.463	0.324
	11.111	29.283	3.413	11.667	63.933	2.684	42.340	95.348	21.557	0.240
1	19.889	56.031	3.942	11.633	59.600	2.923	49.350	151.563	41.829	0.283
	8.889	30.507	3.583	11.567	56.067	2.591	46.744	93.904	24.160	0.262
2	15.667	76.871	5.522	12.033	63.067	4.071	64.525	164.526	53.585	0.316
	7.222	35.093	5.756	11.533	74.533	4.653	62.230	124.757	22.689	0.194
3	14.000	49.221	5.140	12.600	61.533	3.991	67.306	112.131	41.088	0.365
	10.111	43.252	4.895	11.300	65.600	3.823	58.428	87.180	33.902	0.384
4	22.667	67.503	4.045	12.333	66.133	2.884	43.286	155.342	52.208	0.372
	13.778	41.162	3.991	11.000	68.933	3.019	44.090	105.669	31.690	0.304
5	21.111	86.160	4.471	11.533	80.733	3.137	39.120	170.901	60.724	0.366
	9.445	30.351	4.016	11.633	75.600	2.948	39.122	62.808	17.570	0.300
$LSD_{(p\leq 0.05)}$	4.318	16.676	0.828	1.371	14.662	0.724	12.713	41.569	10.695	0.089
	3.906	10.079	1.154	1.054	14.801	0.879	9.485	55.336	9.725	0.146

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

***Table 5.** Heterosis percentage of F_1 diallel and reciprocal crosses for studied characters at both locations (Kanipanka upper value and Qlyasan lower value)*

Crosses	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
1 x 2	10.624	-10.317	-9.679	-2.817	-16.739	-13.458	4.180	-28.755	-5.798	46.132
	26.899	3.716	-26.970	-17.460	-27.871	-34.530	-7.496	4.077	16.971	-8.254
1 x 3	-1.639	11.147	-1.255	4.814	0.055	-5.418	-7.902	-21.690	6.749	31.860
	25.144	-7.467	-11.293	-2.624	-10.466	-10.217	0.479	1.971	-8.857	-6.502
1 x 4	-7.050	-3.859	-2.763	-5.424	-6.257	-1.389	1.709	-8.635	-14.693	12.049
	3.922	0.779	9.990	-1.625	8.053	13.916	5.350	9.459	-6.751	-15.362
1 x 5	-23.577	-23.377	20.222	15.683	7.458	31.295	19.772	-36.175	-15.899	37.339
	39.394	40.790	3.667	0.575	-3.696	13.890	15.551	76.890	54.473	-14.692
2 x 3	7.863	-2.251	-14.688	3.924	1.873	-17.132	-19.691	7.783	-1.432	-8.370
	12.821	-2.565	2.892	3.650	1.998	-2.462	-3.193	12.986	3.856	-15.868
2 x 4	-8.984	-11.919	1.721	0.958	-4.438	2.291	6.728	-3.296	-1.975	3.004
	25.927	0.114	-2.195	5.917	-13.662	-8.176	11.743	33.834	43.298	2.410
2 x 5	-17.222	-33.796	-0.240	6.931	-10.431	-0.250	8.378	-28.076	-32.162	6.849
	21.336	35.019	19.593	-1.583	0.178	19.295	8.241	3.332	71.402	48.213
3 x 4	1.818	-8.772	-0.218	-8.663	11.332	-0.669	-13.266	-10.679	4.043	9.263
	46.974	1.394	-5.619	-3.139	8.127	-5.183	-13.008	-27.938	3.626	65.293
3 x 5	-13.292	-11.054	5.750	-1.934	-8.247	6.182	8.874	-0.738	-8.945	-8.162
	1.137	-3.096	0.464	-2.616	-4.060	9.343	12.517	10.746	16.974	9.552
4 x 5	-26.903	-39.747	-5.166	2.885	-9.200	1.041	11.252	-42.671	-39.086	2.349
	37.788	28.600	6.586	3.976	-4.520	5.039	12.508	69.834	36.253	-16.823
S.E Diallel crosses	4.014	4.791	2.956	2.189	2.725	4.123	3.827	5.255	4.737	5.978
	4.745	5.627	3.999	2.043	3.412	5.020	3.060	10.170	8.586	9.160
2 x 1	16.249-	23.438-	6.157-	0.563	1.848	5.204-	6.140-	20.239-	10.512-	14.636
	8.965	3.825-	7.938	1.587-	1.480-	2.706-	0.364	12.593-	6.038	13.367
3 x 1	11.473-	2.940-	4.045-	8.391-	8.420	3.606-	13.369-	2.201-	9.266-	9.764-
	15.789-	27.200-	3.492-	5.248-	2.356-	8.492-	4.357-	6.038	28.511-	34.056-
4 x 1	9.660-	10.316-	0.626-	2.197-	21.739	8.415	6.171-	31.866-	8.641-	35.841
	27.448	39.117	17.437	6.942	26.187	26.108	1.301-	40.321	30.396	2.295
5 x 1	21.409-	28.055-	8.288	10.216	17.150	8.327	9.682-	35.213-	22.345-	52.851
	32.121	42.806	16.687	9.483	18.886	13.842	6.628-	90.180	57.700	16.469-
3 x 2	1.874-	19.416-	5.384	3.924	22.204	7.954	13.384-	14.350-	1.354	22.663
	32.053	1.479	24.111-	7.153-	8.373-	28.871-	21.685-	24.825	5.461	19.908-
4 x 2	9.565	16.863-	5.275-	6.703	7.482	3.537-	10.365-	11.637-	16.754-	7.074
	53.438	7.853	13.371-	2.663	3.996	16.570-	19.282-	61.259	33.066	20.884-
5 x 2	6.947-	24.192-	13.029-	16.549	26.750	6.723-	22.342-	18.839-	24.981-	4.501-
	48.000	29.687	1.228-	3.597	1.155	19.663-	22.101-	31.842	58.961	4.922
4 x 3	0.607-	12.756	0.769	4.011	16.031	1.406-	17.557-	7.414	7.425	5.648-
	0.465-	30.542-	7.165-	2.377-	0.124	16.251-	16.887-	15.630-	11.880-	6.253
5 x 3	1.266	2.343-	2.615	3.591	9.841	1.094-	14.640-	8.566	5.948-	13.269-
	26.136	5.929	6.000-	5.872	4.533-	12.002-	7.394-	68.842	9.919	32.554-
5 x 4	15.735-	13.444-	12.830	14.246	0.590-	16.785	18.794	1.203-	8.859-	12.105-
	4.309-	18.104-	14.761-	3.093	11.531-	10.044-	1.763	15.630-	12.478-	20.684-
S.E Reciprocal crosses	2.992	3.947	2.408	2.366	2.881	2.464	3.512	4.791	3.118	7.114
	7.222	8.256	4.276	1.718	3.715	5.163	2.958	68.842	9.361	5.388

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

Table 6. *Estimation of general combining abilities effect of parents for studied characters at both locations ($\hat{g}_{ii}$) (Kanipanka upper value and Qlyasan lower value)*

$\hat{g}_{ii}$	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
1	0.238	-4.196	-0.314	-0.367	-3.531	-0.212	-0.802	-9.775	-3.517	0.014
	-0.871	-1.820	-0.330	-0.054	-4.873	-0.281	-0.388	-0.577	-1.836	-0.029
2	-0.718	2.153	0.261	0.135	-2.141	0.186	4.687	6.416	1.263	-0.013
	-1.004	0.157	0.539	-0.078	0.960	0.392	4.388	14.327	0.476	-0.040
3	-1.429	-2.867	0.247	-0.007	-0.991	0.221	3.856	-4.261	-0.278	-0.010
	-0.349	-0.585	0.040	-0.209	-1.828	0.066	2.493	-15.292	0.180	0.049
4	1.971	1.075	-0.265	-0.007	-0.367	-0.213	-3.590	4.536	1.353	0.005
	2.318	1.695	-0.216	-0.029	1.958	-0.132	-2.844	6.168	2.525	0.021
5	-0.062	3.834	0.070	0.246	7.030	0.018	-4.150	3.084	1.179	0.003
	-0.093	0.552	-0.033	0.370	3.783	-0.045	-3.649	-4.626	-1.344	-0.001
S.E	0.679	2.623	0.130	0.216	2.306	0.114	1.999	6.538	1.682	0.014
	0.614	1.585	0.181	0.166	2.328	0.138	1.492	8.703	1.530	0.023

Data represent in *Table 7* illustrate the estimation of sca effects for crosses. The cross Kauz×Klal was the best specific combiner for grain yield/plant, but the cross Aras×Iba-95 was the best specific combiner for most traits including average spike weight, spike length, number of grains/spike, weight of grains/spike and harvest index in the first location. At the second location the cross Aras×Iba-95 was the best specific combiner for grain yield/plant, number of spikes/plant, weight of spikes/plant and spike length, while the cross Aras×Klal recorded the best specific combiner for average spike weight, number of grains/spike and weight of grains/spike, similar results reported previously by Kapoor et al. (2011), Singh et al. (2013), Raj and Kandalkar (2013), Desale et al. (2014) and Kandil et al. (2016).

Table 7. *Estimation of specific combining abilities effect for the diallel crosses at both locations ($\hat{s}_{ij}$) (Kanipanka upper value and Qlyasan lower value)*

$\hat{s}_{ij}$	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
1 x 2	0.318	-2.168	-0.194	-0.403	-6.299	-0.222	1.772	-9.697	0.636	0.021
	-0.273	-3.356	-0.359	-0.826	-7.793	-0.437	-0.252	-21.888	-1.893	0.023
1 x 3	-0.593	-1.836	-0.075	-0.253	1.371	-0.026	-2.031	-11.801	-1.902	0.0002
	0.304	-4.794	-0.678	-0.498	-5.568	-0.517	-2.747	-15.091	-4.288	0.003
1 x 4	-0.149	1.063	-0.100	-0.435	2.893	0.012	-1.046	-4.767	-1.737	0.019
	0.016	5.306	0.478	0.259	8.692	0.505	0.773	6.218	1.165	-0.007
1 x 5	-1.727	-6.272	0.446	0.869	6.563	0.406	0.696	-22.012	-1.625	0.085
	1.760	6.614	0.174	0.460	4.500	0.205	0.089	36.201	6.376	-0.013
2 x 3	-0.016	-2.518	-0.029	0.337	4.194	0.021	-4.251	-0.754	0.899	0.019
	0.316	1.587	-0.193	0.095	1.278	-0.158	-2.852	14.154	-0.415	-0.053
2 x 4	0.529	-0.877	0.098	0.187	-0.647	0.064	1.106	4.655	-0.107	0.001
	1.705	-0.007	-0.203	0.449	-2.075	-0.173	0.817	36.683	5.197	-0.036
2 x 5	-0.493	-7.554	-0.270	0.451	4.140	-0.144	-3.055	-13.513	-7.052	-0.014
	0.671	4.799	0.454	0.0003	3.400	0.173	-2.371	-12.239	4.746	0.073
3 x 4	0.462	1.868	0.019	-0.145	5.187	-0.024	-4.215	-1.107	2.813	0.012
	1.105	-2.855	-0.039	-0.328	2.488	-0.153	-4.846	-28.270	-0.635	0.116
3 x 5	0.551	2.741	0.085	-0.408	-3.077	-0.002	1.250	15.846	0.780	-0.036
	-0.095	-0.445	-0.049	0.084	-1.812	0.046	2.385	11.942	0.989	-0.026
4 x 5	-2.127	-7.959	0.012	0.381	-5.562	0.056	4.411	-12.667	-5.036	-0.026
	-0.318	-2.415	-0.285	-0.032	-6.698	-0.188	2.273	4.850	-2.996	-0.055
S.E	1.358	5.246	0.260	0.431	4.612	0.228	3.999	13.076	3.364	0.028
	1.229	3.171	0.363	0.332	4.656	0.277	2.984	17.406	3.059	0.046

The estimation of specific combining abilities for reciprocal crosses represent in *Table 8*. At first location the cross KauzxAras produced maximum rca value for grain yield/plant, spike length and harvest index, while at second location the reciprocal cross Iba-95xKlal exhibited the highest rca value for grain yield/plant, weight of spikes/plant and biological yield/plant, while the cross KauzxHasad was the best specific combiner for average spike weight, spike length and number of grains/spike.

Table 8. *Estimation of specific combining abilities effect for the reciprocal crosses at both locations ($\acute{r}_{ij}$) (Kanipanka upper value and Qlyasan lower value)*

$\hat{s}_{ij}$	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
2 x 1	2.389	4.360	0.083-	0.200-	5.700-	0.144-	2.938	6.729-	1.125	0.047
	0.722	1.237	0.815-	0.917-	8.617-	0.576-	2.141-	9.113	1.281	0.025-
3 x 1	0.833	3.707	0.063	0.800	2.533-	0.031-	1.594	12.847-	3.320	0.068
	1.944	3.639	0.165-	0.150	2.467-	0.028-	1.272	1.841-	2.853	0.045
4 x 1	0.278	1.994	0.043-	0.193-	8.800-	0.142-	1.825	17.825	1.423-	0.039-
	1.333-	6.869-	0.141-	0.483-	5.667-	0.171-	1.510	15.398-	5.187-	0.025-
5 x 1	0.222-	1.663	0.251	0.317	3.400-	0.348	6.515	0.776-	1.653	0.025-
	0.333	0.307-	0.247-	0.517-	7.433-	0.001	4.761	5.207-	0.337-	0.003
3 x 2	0.722	5.411	0.535-	0.000	6.333-	0.506-	2.079-	15.308	0.659-	0.053-
	0.833-	0.792-	0.719	0.617	3.633	0.560	5.578	6.273-	0.227-	0.006
4 x 2	1.778-	1.784	0.167	0.350-	3.850-	0.101	4.607	6.670	3.909	0.007-
	1.444-	1.476-	0.272	0.183	6.333-	0.161	8.246	15.798-	1.391	0.029
5 x 2	0.945-	3.914-	0.320	0.567-	13.367-	0.117	7.960	7.746-	2.052-	0.019
	1.111-	0.872	0.509	0.300-	0.367-	0.740	7.688	13.369-	1.252	0.054
4 x 3	0.222	6.282-	0.023-	0.790-	1.500-	0.013	1.186	12.099-	0.789-	0.028
	2.833	6.740	0.034	0.043-	2.692	0.189	0.994	5.934-	2.543	0.102
5 x 3	1.278-	2.949-	0.075	0.333-	6.433-	0.130	6.256	6.584-	0.763-	0.009
	1.222-	1.661-	0.144	0.487-	0.167	0.361	4.856	21.784-	0.908	0.072
5 x 4	1.222-	10.104-	0.383-	0.678-	3.161-	0.237-	1.554-	33.821-	8.534-	0.027
	2.444	8.350	0.427	0.050	2.533	0.225	2.235	23.859	6.001	0.006
S.E	1.519	5.865	0.291	0.482	5.156	0.255	4.471	14.619	3.761	0.0313
	1.374	3.545	0.406	0.371	5.205	0.309	3.336	19.461	3.420	0.051

Data in *Table 9* revealed that both additive and non additive gene effects were most important in the inheritance of different characters at both locations. The estimated value of σ^2gca for the characters average spike weight, weight of grains/spike and 1000-grain weight was higher than its σ^2sca, which indicates the predominance of additive gene effect as the ratio of σ^2gca/σ^2sca was more than unity, while the rest showed the predominance of non-additive gene action at first location. At the second location the estimated value of σ^2gca was higher than its σ^2sca for number of spikes/plant, average spike weight and 1000-grain weight. The average degree of dominance value for the characters average spike weight, weight of grains/spike and 1000-grain weight indicated partial dominance, and the rest showed over dominance at the first location, while at the second location the characters number of spikes/plant, average spike weight and 1000-grain weight showed partial dominance. The estimation of heritability in broad sense represent in the same table was found to be moderate to high for almost all characters, while in narrow sense it was found to be low to moderate for all characters at both locations except number of spikes/plant, average spike weight and 1000-grain weight at the first location which was found to be high.

***Table 9.** Estimation of some genetic parameters for the studied characters at both locations (Kanipanka upper value and Qlyasan lower value)*

Parameters	No. of spikes/plant	Weight of spikes/plant (g)	Average spike weight (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Grain yield/plant (g)	Harvest index
Mse^{-}	2.306	34.396	0.085	0.232	26.588	0.065	19.989	213.715	14.147	0.001
	1.887	12.565	0.165	0.137	27.096	0.096	11.127	378.718	11.698	0.003
σ^2gca	1.395	8.141	0.067	0.030	14.239	0.037	14.901	24.869	2.901	0.00003
	1.629	0.458	0.096	0.034	8.831	0.055	10.644	86.853	1.784	0.001
σ^2sca =σ^2D	1.502	64.953	0.009	0.334	16.490	0.008	5.915	356.043	28.204	0.003
	1.546	30.784	0.031	0.188	25.354	0.096	4.001	786.644	29.669	0.003
σ^2gca / σ^2sca	0.929	0.125	7.485	0.090	0.863	4.446	2.519	0.070	0.103	0.010
	1.053	0.015	3.089	0.179	0.348	0.570	2.660	0.110	0.060	0.306
σ^2A	2.790	16.282	0.134	0.060	28.478	0.074	29.802	49.738	5.801	0.0001
	3.258	0.915	0.192	0.067	17.662	0.109	21.288	173.707	3.567	0.002
σ^2Dr	0.275	6.554	0.022	0.129	28.220	0.019	8.887	113.251	4.026	0.001
	1.627	12.069	0.100	0.142	9.935	0.099	16.330	0.368	2.516	0.001
$\bar{A}$	1.038	2.825	0.366	3.337	1.076	0.474	0.630	3.784	3.118	10.004
	0.974	8.202	0.569	2.363	1.694	1.324	0.613	3.010	4.078	1.808
h^2 b.s	0.651	0.703	0.628	0.629	0.628	0.560	0.641	0.655	0.706	0.729
	0.718	0.716	0.575	0.651	0.614	0.682	0.694	0.717	0.740	0.679
h^2 n.s	0.423	0.141	0.589	0.096	0.398	0.503	0.535	0.080	0.120	0.014
	0.487	0.021	0.495	0.172	0.252	0.363	0.585	0.130	0.079	0.258
$\bar{A}$r	0.444	0.897	0.574	2.076	1.408	0.720	0.772	2.134	1.178	5.847
	0.999	5.135	1.023	2.052	1.061	1.346	1.239	0.065	1.188	0.939
h^2 bsr	0.571	0.399	0.649	0.449	0.681	0.590	0.659	0.433	0.410	0.488
	0.721	0.508	0.639	0.604	0.505	0.685	0.772	0.315	0.342	0.537
h^2 nsr	0.520	0.284	0.557	0.142	0.342	0.469	0.508	0.132	0.242	0.027
	0.481	0.036	0.420	0.194	0.323	0.359	0.437	0.314	0.201	0.372

The simple correlation coefficient among studied characters for both locations represent in *Table 10*. At first location number of spikes/plant correlated positively and significantly with weight of spikes/plant, biological yield/plant and grain yield/plant 0.498, 0.416 and 0.407 respectively, while it correlated negatively and high significantly with weight of grains/spike -0.670, but negative and highly significant correlation was recorded between number of spikes/plant and 1000-grain weight -0.543. Positive and highly significant correlation was recorded between weight of spikes/plant with biological yield/plant and grain yield/plant 0.802, 0.853 respectively. Spike length correlated positively and significantly with number of grains/spike 0.477. Number of grains/spike showed negative and highly significant with 1000-grain weight -0.644, but weight of grain/spike associated positively and high significantly with 1000-grain weight 0.610. Biological yield/plant recorded negative and highly significant correlation with harvest index -0.691, whilst it correlated positively and highly significant correlation with grain yield/plant. At second location number of spikes/plant showed positive and highly significant correlation with weight of spikes/plant and grain yield/plant 0.618 and 0.675 respectively, but it correlated negatively and significantly with 1000-grain weight -0.444. Weight of spikes/plant recorded positive and highly significant correlation with number of grains/spike and grain yield/plant 0.538 and 0.820 respectively, whilst it correlated positively and significantly with biological yield/plant 0.508. Number of grains/spike recorded positive and significant correlation with weight of grains/spike 0.417. Weight of grains/spike recorded positive and highly significant correlation with biological yield/plant 0.731. Negative and highly significant

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DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

correlation was recorded between biological yield/plant and harvest index -0.616, while positive and highly significant correlation was recorded between biological yield/plant and grain yield/plant 0.543. Previously it could be noticed that harvest index was significantly and positively correlated with number of spikes/plant, plant height, spike length, 1000-grain weight and grain weight/spike. Number of spikes/plant was significantly and positively correlated only with plant height, while it was negatively associated with spike length and1000-grain weight as well as positively correlated with grain weight/spike. Moreover, plant height was significantly and positively correlated with 1000-grain weight and grain weight/spike. In the same time, spike Length was significantly and positively correlated with 1000-grain weight and grain weight per spike.1000-grain weight were significantly and positively correlated with grain weight/spike (Mohsin et al., 2009; Fellahi et al., 2013; Motawea, 2017).

Table 10. *The simple correlation coefficient among all pairs of traits at both locations (Kanipanka upper value and Qlyasan lower value)*

Characters	No. of spikes/plant	Weight of spikes/plant (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Harvest index	Grain yield/plant (g)
No. of spikes/plant	1.000								
Weight of spikes/plant (g)	0.498	1.000							
	0.624								
Spike length (cm)	-0.190	-0.001	1.000						
	0.137	0.356							
No. of grains/spike	0.021	0.167	0.477	1.000					
	0.280	0.517	0.610						
Weight of grains/spike (g)	-0.670	-0.070	0.374	0.170	1.000				
	-0.226	0.342	0.275	0.513					
1000-grain weight (g)	-0.543	-0.177	-0.069	-0.644	0.610	1.000			
	-0.447	-0.047	-0.149	-0.268	0.669				
Biological yield/plant (g)	0.416	0.802	-0.035	0.033	-0.056	-0.092	1.000		
	0.392	0.503	0.395	0.195	0.074	-0.019			
Harvest index	0.024	-0.298	0.081	0.156	0.020	-0.115	-0.691	1.000	
	0.316	0.193	-0.187	0.187	0.148	-0.025	-0.607		
Grain yield/plant (g)	0.407	0.853	0.031	0.178	0.159	-0.041	0.712	-0.141	1.000
	0.677	0.820	0.223	0.291	0.257	0.072	0.543	0.266	

Data in *Table 11* illustrate the direct and indirect effects of grain yield components in grain yield/plant at both locations. At first location maximum positive direct effect recorded by weight of spikes/plant 0.504 and followed by biological yield/plant 0.493, while maximum negative direct effect was -0.079 recorded by spike length. Maximum positive indirect effect in grain yield/plant was 0.405 recorded by weight of spikes/plant via biological yield/plant, and followed by 0.395 for biological yield/plant via weight of spikes/plant. At second location biological yield/plant recoded maximum positive direct effect in grain yield/plant 1.094 and followed by harvest index 0.983. Maximum negative direct effect was -0.252 recorded by number of grains/spike and followed by -0.208 for number of spikes/plant. Maximum positive indirect effect in grain yield/plant was 0.551 recorded by biological yield/plant via weight of spikes/plant, and followed by 0.433 for biological yield/plant via spike length. Maximum negative indirect effect was -0.664 recorded by biological yield/plant via harvest index, and followed by -0.597 for harvest index via biological yield/plant.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

Table 11. *Path coefficient analysis illustrates direct effect (diagonal values) and indirect effect of studied characters in grain yield at both locations (Kanipanka upper value and Qlyasan lower value)*

Characters	No. of spikes/plant	Weight of spikes/plant (g)	Spike length (cm)	No. of grains/spike	Weight of grains/spike (g)	1000-grain weight (g)	Biological yield/plant (g)	Harvest index
No. of spikes/plant	0.164	0.251	0.015	-0.001	-0.262	0.026	0.205	0.008
	-0.208	0.200	0.004	-0.070	-0.004	0.016	0.429	0.311
Weight of spikes/plant (g)	0.082	0.504	0.0001	-0.007	-0.028	0.008	0.395	-0.103
	-0.130	0.321	0.011	-0.131	0.006	0.002	0.551	0.190
Spike length (cm)	-0.031	-0.001	-0.079	-0.019	0.146	0.003	-0.017	0.028
	-0.029	0.114	0.032	-0.154	0.005	0.005	0.433	-0.184
No. of grains/spike	0.003	0.084	-0.038	-0.040	0.067	0.031	0.016	0.054
	-0.058	0.167	0.020	-0.252	0.009	0.010	0.213	0.183
Weight of grains/spike (g)	-0.110	-0.036	-0.030	-0.007	0.391	-0.029	-0.028	0.007
	0.047	0.110	0.009	-0.129	0.018	-0.024	0.081	0.145
1000-grain weight (g)	-0.089	-0.089	0.005	0.026	0.239	-0.048	-0.045	-0.040
	0.093	-0.015	-0.005	0.068	0.012	-0.036	-0.021	-0.024
Biological yield/plant (g)	0.068	0.405	0.003	-0.001	-0.022	0.004	0.493	-0.238
	-0.082	0.162	0.013	-0.049	0.001	0.001	1.094	-0.597
Harvest index	0.004	-0.150	-0.006	-0.006	0.008	0.006	-0.340	0.345
	-0.066	0.062	-0.006	-0.047	0.003	0.001	-0.664	0.983

Discussion

The analysis of variance confirmed highly significant genotype effect for all characters under the study. This provides evidence of the presence of sufficient genetic variability among genotypes at both locations. The mean squares due to genotypes were highly significant for all characters at both locations except number of spikes/plant and average spike weight which was significant at the second location. The results indicated that the data for all traits studied can be analyzed further to estimate general and specific combining ability effects. Similar results reported previously confirmed that both gca and sca variances were significant for most of the characters indicating importance of both additive as well as non-additive components of genetic variance in the control of these traits (Kumar et al., 2011; Burungale et al., 2011; Singh et al., 2013; Mandal et al., 2016; Rahul, 2017; Ljubičić et al., 2017). Combining ability describes the breeding value of parental lines to produce crosses (Romanus et al., 2008). The general combining ability has been equated with additive gene action and specific combining ability with non-additive gene action (Griffing, 1956b).

All characters had shown considerable amount of heterosis over mid parents. These results are in confirmation with Devi et al. (2013) and Rahul and Kandalkar (2018). To improve any character, plant breeders heavily rely on the availability of genetic variability generated from different matting designs. It is also well known phenomena that in a hybridization program, certain crosses pass on more favorable genes than the others. Thus, some cross associations may be superior as compared to their parents for improving any economic traits (Baloch et al., 2016).

Combining ability plays a major role for estimation of inbred in terms of their breeding value, which help to decide suitable breeding method to be used in segregating generation. The gca is primarily a function of additive genetic variance it helps in the selection of good general combiner parents for hybridization (Rahul and Kandalkar, 2018).

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DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

The estimation of sca effects for crosses is mainly a function of dominance variance, helps in the identification of superior cross combination for commercial exploitation of heterosis.

The non-additive gene effect controlled the inheritance of most characters. Several researchers reported the predominance of non-additive gene effect (Bhowmik et al., 1991; Khan et al., 1995; Ajmal et al., 2000; Subhani and Chowdhry, 2000; Singh, 2003; Chaman et al., 2005; Heidari et al., 2006; Kumar et al., 2011; Singh et al., 2012a, b), while some workers exhibited the importance of additive type of gene action (Rhahman and Krons, 1991; Bhutta et al., 1997; and Tawfiq et al., 2008).

Beche et al. (2013) and Baloch et al. (2016) reported high values of heritability for grain yield/plant. Grain yield/plant is the prime objective of plant breeders. High estimates of variability and heritability for this trait would be helpful for the breeders to select for the best combinations and to reach at the desirable level of yield potential.

Grain yield/plant exhibited positive and significant correlation with most its important components at both locations. These results are agreement with those reported by Tofiq (2004), Hama-Ali (2006), Hama-Ameen (2008), Mohsin et al. (2009) and Fellahi et al. (2013). Previous researches indicated the positive correlation between grain yield and yield component traits in wheat such as spike number/plant (Mondal and Khajuria, 2001), grains number/spike (Kashif and Khaliq, 2004), 1000-grain weight and biological yield/plant (Akbar et al., 1995). Estimation of the correlation between yield and its components alone is not sufficient to understand the importance of each one of these components in determining the grain yield reported by Bhutta et al. (2005), Anwar et al. (2009) and Ali and Shakor (2012).

Unlike the correlation coefficient, which measures the extent of the relationship, the path coefficient measures the magnitude of direct and indirect contribution of a component character to a complex character and it has been defined as a standardized regression coefficient which splits the correlation coefficient into direct and indirect effects (Arbuckle, 2009). Path coefficients have been used to develop selection criteria for complex traits in several crop species of economic importance such as wheat (Larik, 1979; Aydin et al., 2010). Path analysis grains/spike followed by 1000-grain weight, spikes/plant and harvest index had positive direct effects on grain yield of bread wheat obtained from Majumder et al. (2008). The path coefficient analysis provides more information among variables than do correlation coefficients since this analysis provides the direct effects of specific yield components on yield and indirect effects via other yield components (Arshad et al., 2006). In agricultural, path analysis has been used by plant breeders to assist in identifying traits that are useful as selection criteria to improve crop yield (Dewey and Lu, 1959; Milligan et al., 1990). In a study of path analysis it was indicated that 1000-grain weight had the highest positive direct effect on yield followed by spike length, while plant height and grains/spike had a negative direct effect on yield (Iftikhar, 2012).

Conclusion

It can be stated that enough genetic variation existed among the studied genotypes for most of the studied traits. Additive and non-additive gene effects were involved in the expression of all traits. Thereafter, it is recommended that breeding methods, which make the best use of additive effects such as direct selection, are applied for those traits where dominant effects are negligible. However, using hybrid vigor will be more

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

efficient than selection for those traits that show high levels of dominant effects. Proportion of variances due to sca confirmed the predominance of dominant genes in the expression of majority of the traits. Among the parents, the Klal parent at both locations were the best general combiner for grain yield and some of its components, and thus can reliably be used in a hybridization program so as to select the desirable plants from segregating populations. So, it is concluded that this parent may be used in breeding program to develop high yielding wheat varieties. The cross Kauz×Klal was the best specific combiner for grain yield/ plant, but the cross Aras×Iba-95 was the best specific combiner for most grain yield components at the first location, while at the second location the cross Aras×Iba-95 was the best specific combiner for grain yield and most its components that can be used in developing cross varieties. The characters with high estimates of heritability indicated the presence of additive genes effect in their inheritance, and suggested reliable wheat improvement through selection.

Recommendation

Klal parent was a good general combiner for grain yield/plant, biological yield/plant, number of spikes/plant, weight of spikes/plant and number of grains/spike, which could be used in the development of high-yielding varieties using selection from promising segregating cross generations.

It was recommended to conduct a favourable selection method to the cross Aras×Iba-95 in the future to develop new common wheat variety.

The varieties should be tested at different locations along with growth seasons to evaluate these traits, so that these traits are more inconsistent in different growth seasons.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30133032

MODELING FLOOD SHOCK WAVE PROPAGATION WITH THE SMOOTHED PARTICLE HYDRODYNAMICS (SPH) METHOD: AN EXPERIMENTAL COMPARISON STUDY

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(Received 30th Nov 2018; accepted 4th Feb 2019)

Abstract. The applicability of experimental and numerical models used for the solution of dam-break flows is vital for better dam projects and also in preventing related accidents. The high cost and the time-consuming nature of laboratory studies require consistency in the investigation of numerical models. In this study, the propagation of a flow using a fluid with a different density from that of normal water in the reservoir was investigated both experimentally and numerically. Salt water was preferred as a Newtonian fluid in order to observe the propagation of flows in different density after a sudden break. A small-scale channel was constructed and laboratory data were obtained using image processing techniques. For the numerical model, Smoothed-Particle Hydrodynamics (SPH) method and Reynolds Averaged Navier-Stokes (RANS) equations solved by Flow-3D software, were applied. Flow depth changes were observed in the reservoir and the downstream. The data obtained from all methods were compared with each other. The results of two numerical simulations point out that the disagreements on graphs in the time evolutions of the fluid levels in the SPH increase due to turbulence effects, whilst, these differences decrease in the RANS equations solved by Flow-3D software. Consequently, since the SPH provides taking the measures and developing intervention strategies to reduce the risks connected to the evolution of dam-break flows, it is thought that future validation studies of the model will be require with the use of data observed in this field.
Keywords: *dam-break, density effect, image processing, particle method, volume of fluid*

Introduction

Some environmental flows can be simulated like dam-break induced shock waves. The prediction of these flows is one of required elements in the design of a dam. Dams are constructed for specific purposes such as water supply, flood control, irrigation, navigation, sedimentation control and hydropower (Lee et al., 2018). Past examples show us that the dams can be destroyed due to various reasons. The reasons of failure can be different for each dam and each case. While rockfill dams usually fail after a certain amount of time elapses, concrete dams fail a lot more suddenly. In practice the release of dam-break flows will be more gradual than this idealization. However, the sudden release may be expected to give the worst scenario. The shock wave propagation, caused by a dam-break, produces damaging effects in the downstream. It can be seen common that the propagation of water mixture which of different density from water can be formed in the downstream after torrential rain or a dam collapse. Hence, the flood wave can damage both living species and the environment (Yenigun et

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

al., 2016). For the process of the dam construction and the evaluation of a demolition situation after the construction, it is important to simulate the flow characteristics on a dam-break problem with laboratory experiments and numerical models. Various dam-break flow problems, which use water as the fluid in the reservoir, was experimentally and numerically examined in the literature. Ritter was the first to examine this flow problem in 1892. He used Saint-Venant equations to solve the sudden dam-break problem in a horizontal, rectangular, frictionless channel and he considered the downstream as a dry bed and the reservoir in an infinite length (Vischer and Hager, 1998; Chanson, 2005). There are numerous studies investigating a dry and/or wet bed downstream (Stoker, 1957; Bellos et al., 1992; Mohapatra and Bhallamudi, 1996; Stansby, 1998; Janosi et al., 2004; Bukreev and Gusev, 2005; Gao et al., 2010; Oertel and Bung, 2012; Ozmen-Cagatay et al., 2014; Kocaman and Ozmen-Cagatay, 2015; Turhan, 2017; Turhan et al., 2019). It is possible to see many studies in which dam-break flow problem has been examined experimentally using image processing techniques in the literature. (Spinewine and Zech, 2007; Ozmen-Cagatay and Kocaman, 2010; Aureli et al., 2011; Yang et al., 2011; Kocaman and Ozmen-Cagatay, 2012; Lobovsky et al., 2013; Kunugi et al., 2013; Albano et al., 2014; Aureli et al., 2014; Amaral et al., 2016; Hui et al., 2017; Liu, 2018). There are a limited number of studies that include different density Newtonian fluids in the reservoir (Janosi et al., 2004; Ancey and Cochard, 2009; Shakibaeini and Jin, 2011; Li et al., 2013; Furuya et al., 2014). Concerning the research in this flow problem, using salt water as a Newtonian fluid in a dry channel in rapidly unsteady flow can be interpreted an original study. Since laboratory experiments are costly and time-consuming processes, using software program to solve these problems is essential (Karaer et al., 2018). With recent developments in computer technology, using particle methods has become more widespread and also, their sensitivity in modeling applications requires further testing. There are several software programs in which the dam-break flow problem is numerically simulated. In this study, Smoothed-Particle Hydrodynamics (SPH) method used as a particle method and it was first developed by Gingold and Monaghan (1977), Lucy (1977), Liu and Liu (2003) for astrophysical problems. Subsequently, it has been applied to various problems in fluid dynamics (Dalrymple and Rogers, 2006; Gomez-Gesteira et al., 2012; Dominguez et al., 2013; Crespo et al., 2015; Gu et al., 2017).

Dam-break shock waves usually affect large downstream regions and the common tendency is to comment flow characteristics in the horizontal scale rather than along the flow depth (Miao et al., 2011; Luo et al., 2017; Akbari, 2018). Recently, due to the increase in computer technology, Flow-3D, which is computational fluid dynamics (CFD) software based on volume of fluid (VOF) method, has been used widely to analyze free-surface flows among the other commercially available software programs (An et al., 2012; Ozmen-Cagatay et al., 2014; Robb and Vasquez, 2015; Hu et al., 2018). In the literature, the SPH model was applied to predict a dam-break flood propagation, which might occur in a specific living area. The studies in the literature represents the significant potentials of numerical and laboratory modeling in practical engineering applications. Moreover, the results obtained from all methods assisted to validate the results of each other in terms of SPH, RANS and laboratory experiments. (Chen et al., 2005; Albano et al., 2016; Gu et al., 2017; Xie et al., 2017).

In this paper, the sudden dam-break flow propagation of a different density Newtonian fluid in a small-scale, rectangular and horizontal channel was experimentally and numerically investigated. The purpose of this study is to model the flow movement

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

in an idealized dam-break configuration in case of a Newtonian fluid in the reservoir. Normal water is a Newtonian fluid which of that the dynamic viscosity value is 0.001 kg/m.s and density value is 1000 kg/m^3 at the constant temperature of T_0 = 23.7 °C. Salt water was preferred as a Newtonian fluid to investigate the effect of a fluid with a different density to normal water. Thus, the behavior of a high density fluid has been tried to be analyzed. Experimental data were digitized using image processing techniques. Fluid free-surface profile graphs and snapshots were obtained at four downstream locations and at various specified distances in the reservoir and downstream. The SPH method and RANS equations, solved by the Flow-3D software program, were employed for the numerical models. The results obtained from the SPH were compared to laboratory experiments and RANS equations solutions.

Materials and methods

Experimental set-up

The reservoir and the downstream lengths of the rectangular and horizontal channel, in which the experiments employed, were designed to be 0.30 m and 0.916 m, respectively, as can be seen in *Figure 1*. In order to observe the sudden and unsteady flow behavior, the channel was selected in a small-scale. The channel was made of plexiglas and a gate of 0.35 m height was performed to simulate the dam-break flow problem. Furthermore, a pulley system, connected to the gate, was utilized to observe the sudden break situation. Moreover, a metal component with approximately 2 kg weight, which was attached to the hole on the gate with a rope, was designed. The rope was passed through two pulleys and it was bound to a load of about 12 kg filled with sand.

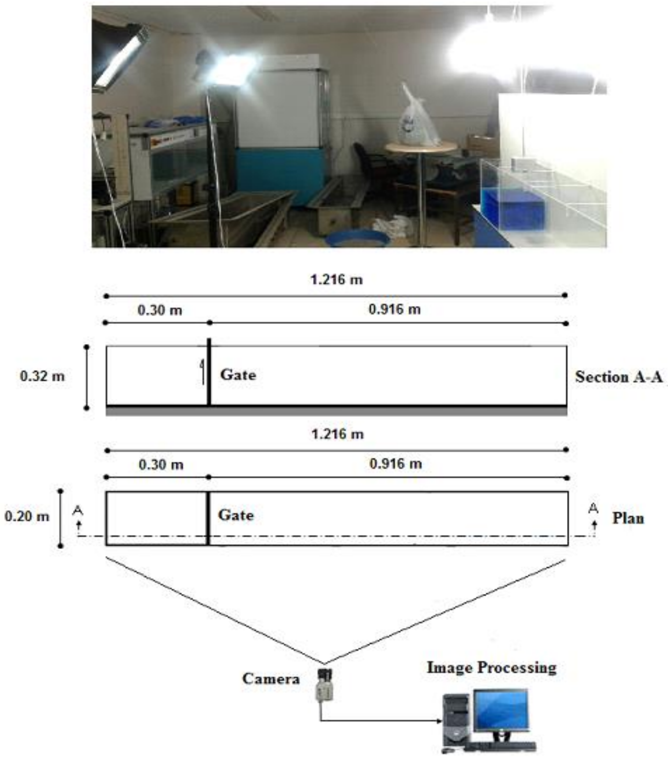

Figure 1. *Experimental set-up and gate mechanism (Turhan et al., 2018)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

The temperature of the laboratory was measured at 23.7 °C during the experiments, hence this value was used in the numerical models. The viscosity was measured to be 18 cP (0.018 kg/m.s) at this temperature with the help of a viscometer. The mixing ratio of salt water was evaluated to be 20% and a homogeneous mixture was obtained by a mixer. As a result, the fluid density was specified to be 1200 kg/m^3.

The reservoir contained salt water at a height of 0.15 m (h_o) and the downstream have a dry bed. In order to create a sudden dam-break condition, the time of gate lifting should be less than $1.25(h_o/g)^{1/2}$ (h_o: initial height and g: gravitational acceleration) as suggested by Lauber and Hager (1998). This limit value has been used in many studies (Chanson, 2005; Spinewine and Zech, 2007; Ozmen-Cagatay et al., 2014; Kocaman and Ozmen-Cagatay, 2012, 2015; Aureli et al., 2014; Turhan et al., 2019). In order to obtain approximate value of data, ten experiments were performed and the average of the values was calculated. As h_o is 0.15 m, 0.155 s becomes the limit value for sudden dam-break and in this study lifting time was under approximately 0.10 s in the video images.

Flow measurement and camera calibration

The digital image processing is produced by transferring real images to digital media in different formats such as .jpg, .bmp, .png, etc. (Yıldırım et al., 2003; Aureli et al., 2011, 2014; Liu, 2018). In this technique, as seen in *Figure 2,* the image was divided into pixels and grid view was obtained. Normal water was used as the Newtonian fluid for camera adjustments and blue food dye was preferred to provide color.

The lighting in the laboratory, where the experiment was conducted, was improved to use image processing techniques effectively before starting laboratory experiments. In order to obtain better images from the experiment, continuous lamp source of approximately 500 W was employed in the laboratory. The optimum shooting option was evaluated to be 1/125 snapshot, 85 mm, f3.5 lens and 1250 ISO proof.

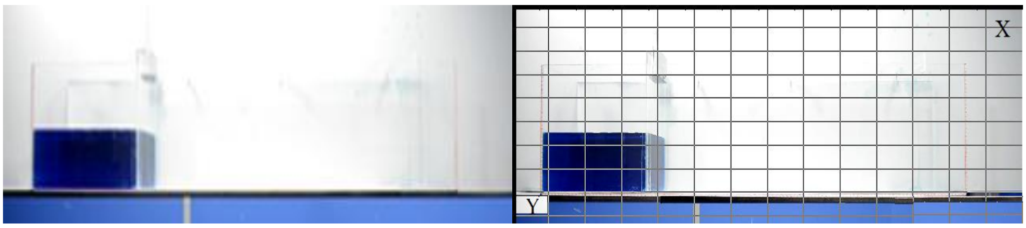

Figure 2. *Grid view in image processing (pixel)*

The frame rate and the resolution were set to 60 frames per second (fps) and 1280*720 pixels, respectively. The camera calibration process was performed with the images taken at different angles and the image distortions were eliminated. Camera shooting at the same coordinate significantly reduced the calibration error rate. The channel measurement points can be seen in *Figure 3*. Depth changes in the images were measured using a virtual wave probe (Kocaman and Ozmen-Cagatay, 2015). (x, y) coordinates were obtained by using an edge detection function at the specified locations, with the help of different colors. The simulation time and time-interval was assumed to be 3 s and approximately 0.017 (1/60 fps), respectively.

The level changes graphs are dimensionless (H-T) for all methods. For the dimensionless analysis, the values were calculated from the following equations

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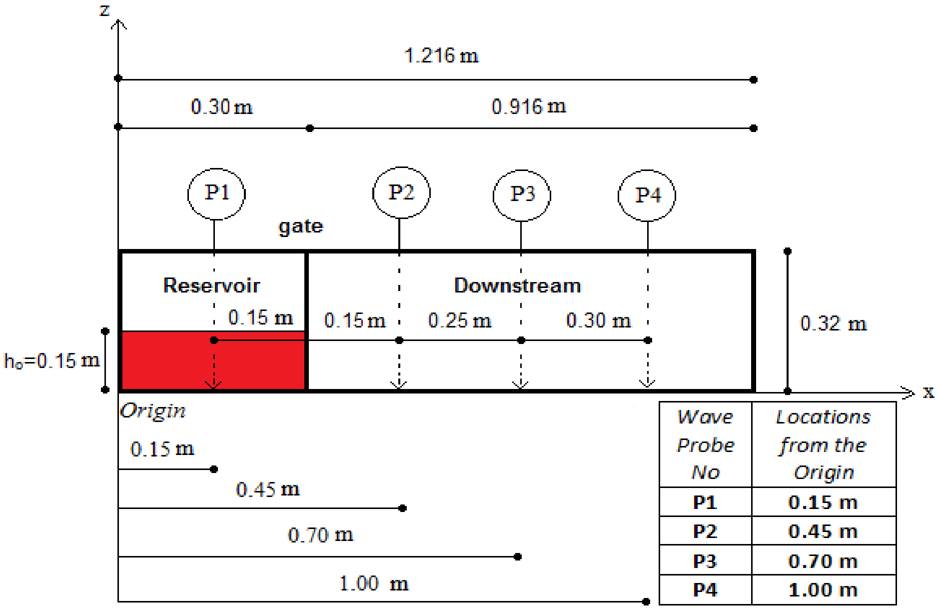
DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

$T = t(g/h_o)^{1/2}$ and $H = h/h_o$ where H and T are dimensionless terms and h denotes the changes in the fluid height over time. The use of dimensionless parameters can reduce the number of variables and therefore, dimensional effect-independent assessments become possible.



Wave Probe No	Locations from the Origin
P1	0.15 m
P2	0.45 m
P3	0.70 m
P4	1.00 m

Figure 3. *Channel measurement points*

Numerical modeling

Reynolds averaged Navier–Stokes (RANS) equations

RANS equations for an incompressible Newtonian fluid are shown in *Equations 1* and *2* (Luo et al., 2017):

$$\frac{\partial u_i}{\partial x_i} = 0 \qquad \text{(Eq.1)}$$

$$\rho\frac{\partial u_i}{\partial t} + \rho\frac{\partial (u_i u_j)}{\partial x_j} = -\frac{\partial p}{\partial x_i} + \mu\frac{\partial}{\partial x_j}\left(\frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i}\right) - \rho\frac{\partial\left(\overline{u_i' u_j'}\right)}{\partial x_j} + \rho g_i \qquad \text{(Eq.2)}$$

where ρ is density, u_i and u_j indicate the velocity component in the defined direction, t is time, p is pressure, μ is dynamic viscosity and g_i is gravitational force in the defined direction. When *Equation 2* is compared with the Navier–Stokes equations, it can be seen that there is an extra term, namely $\partial(\rho\overline{u_i' u_j'})/\partial x_j$ and it characterizes turbulence-induced stresses or Reynolds stresses. In the RANS equations, scalar values for all the physical properties of flow are evaluated as mean magnitudes. For laminar flow, the numbers of equation are equal to the unknown parameters. In the RANS, distinct terms have to be adjoined to the available equations. Hence, turbulence closure models are utilized to solve these problems. The term μ_t is used for turbulence viscosity, and it is found in the solution steps for the time varying RANS equations. Furthermore, the solution of this problem can be obtained with the help of many different turbulence

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

models (Wilcox, 2000). The k-ε turbulence model is generally preferred for the final step of the process (Kocaman and Guzel, 2011; Souders et al., 2013; Ozmen-Cagatay et al., 2014; Heydari and KhoshKonesh, 2016; Bayon et al., 2016; Ozdil et al., 2017).

The numerical solutions for the RANS were computed using Flow-3D software CFD program based on the VOF method. This program contains a mesh–grid system. It comprises a fractional area/volume obstacle representation method (FAVOR) as cell porosity technique (Flow Science Inc., 2017). All the surfaces of the channel were presumed to be smooth. The channel sidewalls were considered to be symmetric, which implies no flux and shear of any property across it. Tangential and normal velocities were accepted as zero at the solid boundary according to the no-slip condition (Rostami and Siosemarde, 2015; Kocaman and Ozmen-Cagatay, 2015). For this study, a grid size was adopted as 5 mm after executing accuracy analysis for 2 mm and 10 mm. The time step Δt was specified according to the Courant–Friedrichs–Lewy (CFL) criterion and also, it was determined automatically with the help of the Flow-3D software.

Smoothed particle hydrodynamics (SPH) method

The SPH method is described as a technique that includes numerical solutions for fluid dynamics equations using a set of particles and fluid displacement (Dalrymple and Rogers, 2006). One of the most important features of this technique is that SPH is a Lagrangian mesh-free particle method. The Navier–Stokes equations, which are discretized in fluid dynamics problems, can be unified by the physical properties of the particles surrounding each particle position (Vacondio et al., 2012). The average smoothing length is calculated by *Equation 3:*

$$F(r) = \int F(r')\, W(r - r', h)\, (dr') \qquad \text{(Eq.3)}$$

where "W" is the kernel function and "h" is the smoothing length. "W" employed in the smoothing process, can involve several features. It is possible to consider some of these properties as being positive in a specified interaction region. Thus, it maintains gradually decreasing result values owing to the normalization and distance. The function is applied to a set of particles. Also, this function is called as application regions when defined by smoothing length. Therefore, a summation is applied to all particles within these application regions of the kernel (*Eq. 4*) (Ozbulut et al., 2014).

$$F(r_a) \approx \sum_b F(r_b)\, W(r_a - r_b, h)\Delta v_b \qquad \text{(Eq.4)}$$

In *Equation 4,* "a" and "b" symbolize individual particles. Furthermore, Δv_b points out the volume of the neighboring particle "b". As the volume is defined as $\Delta v_b = m_b/\rho_b$, where "m" is the mass and "ρ" is the density of particle, *Equation 4* can be modified as shown in *Equation 5* (Crespo et al., 2015):

$$F(r_a) \approx \sum_b F(r_b)\, \frac{m_b}{\rho_b} W(r_a - r_b, h)\Delta v_b \qquad \text{(Eq.5)}$$

The momentum equation is used to define the acceleration of a particle as a consequence of interaction with a neighboring particle (*Eq. 6*) (Altomare et al., 2015):

$$\frac{dV_a}{dt} - \sum_b m_b \left(\frac{P_b}{{\rho_b}^2} + \frac{P_a}{{\rho_a}^2} + \prod_{ab}\right) \nabla_a W_{ab} + g \qquad \text{(Eq.6)}$$

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In *Equation 6,* v is velocity, P is pressure, m is mass and W_{ab} is the kernel function. The kernel function also depends on the distance between two particles. The term Π_{ab} is called the artificial viscosity (Dominguez et al., 2012). The mass for each particle is constant and the differences in the fluid density are calculated by solving the conservation of mass or continuity equation (*Eq. 7*). Furthermore, Cubic spline was selected as the kernel function type for this study (Cunningham et al., 2014).

$$\frac{d\rho_a}{dt} = \sum_b m_b v_{ab.} \Delta_a W_{ab} \quad \text{(Eq.7)}$$

The pressure value of the flow is calculated using Tait's equation of state as can be seen in *Equation 8* (DualSPHysics Team, 2016):

$$P = b\left[\left(\frac{\rho}{\rho_0}\right)^{\gamma} - 1\right] \quad \text{(Eq.8)}$$

where $\gamma = 7$, $b = c_0^2 \rho_0/\gamma$, ρ_0: reference density, c_0: the speed of sound. The speed of sound value is calculated from *Equation 9* (Vacondio et al., 2012):

$$c_0 = c(\rho_0) = \sqrt{\left(\frac{\partial P}{\partial p}\right)}\,|_{\rho_0} \quad \text{(Eq.9)}$$

For this study, the Verlet algorithm was used in order to prepare the time stepping process. A variable time-step process was performed, which relies on applying the CFL condition, forcing terms and a viscous diffusion term (Barreiro et al., 2013). Moreover, a boundary condition, where the fluid–boundary interactions can be computed inside the same loops like the fluid particles, is suitable to fulfill due to computational simplicity in this paper. Seeing determine the optimum value of the artificial viscosity, different artificial viscosity values were tested and the optimum value obtained as 0.10. There are many studies in the literature about the use of various artificial viscosity values in the SPH (Gomez-Gesteira et al., 2012; Dominguez et al., 2013; Cunningham et al., 2014; Crespo et al., 2015; Gu et al., 2017; Zhu et al., 2017; Tantekin et al., 2017). SPH parameters can be seen in *Table 1*.

Table 1. *SPH parameters (Turhan et al., 2019)*

Property	Options
Version	DualSPHysics v4.0
Dimension	2D
Type of the kernel function	Cubic spline
Time-stepping	Verlet
Density filter	Shepard filter
Viscosity treatment	Artificial viscosity (α = 0.10)
Equation of state	Tait equation
Boundary conditions (BC)	Dynamic
Distance between particles (dp)	0.001
Smoothing length	1.0
The number of fluid particles	46857

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Results and discussion

In this study, the fluid wave propagation was investigated over a dry bed. Experimental video records were digitized using image processing techniques and the SPH method and RANS equations were used for the numerical models. The propagation of salt water, in a small-scale channel under a sudden dam-break situation, was researched and the variation of fluid depth at four specified locations were examined. In order to show the variation in the free-surface profile of the flow for sudden dam-break flow conditions, the following time values, which represent the initial stages after suddenly lifting the gate, were selected as $t_0 = 0.00$ s, $t_1 = 0.017$ s, $t_2 = 0.083$ s, $t_3 = 0.134$ s, $t_4 = 0.184$ s and $t_5 = 0.234$ s. Moreover, time values, which represent the change of waves reflecting from the wall at the end of the channel, were selected as $t_6 = 1.002$ s, $t_7 = 1.252$ s, $t_8 = 1.670$ s, $t_9 = 2.004$ s, $t_{10} = 2.338$ s and $t_{11} = 2.989$ s (see *Figs. 4* and *5*).

According to the selected time values, the snapshots and level changes in selected locations were compared and the solution sensitivity of the SPH method was analyzed to better understand the unsteady flow behavior. With a sudden lifting of the gate, the fluid starts to move in the downstream direction due to gravitational force. At the initial stages of the propagation, all methods are in good agreement with each other (An et al., 2012; Albano et al., 2014; Bayon et al., 2016; Gu et al., 2017; Zhu et al., 2018). The shock wave front has a parabolic shape for the three methods at t = 0.083 s. At time t = 0.134 s, the wave front is subjected to the deformation and it becomes concave shape. As can be understood from the literature, a similar fluid behavior can be seen in the sudden wave propagation where the fluid is selected as water (Bellos et al., 1992; Stansby et al., 1998; Janosi et al., 2004; Spinewine and Zech, 2007; Ozmen-Cagatay and Kocaman, 2010; Gao et al., 2010; Girolami et al., 2012; Ye et al., 2016; Bayon et al., 2016; Hui et al., 2017; Turhan et al., 2019). However, in the numerical models, the wave front has a convex shape. This difference can be explained by the fact that the experimental channel has minor surface roughness compared to the numerical models.

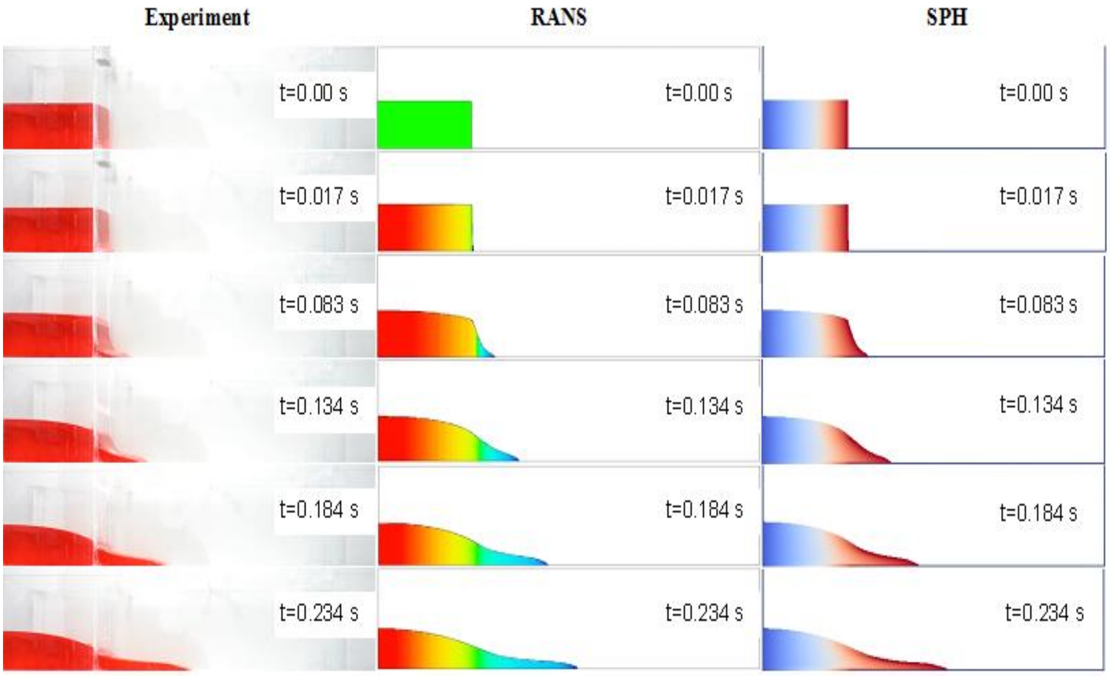

Figure 4. *Evolution of free-surface profiles with time at the initial stages after sudden lifting from t = 0.00 s to t = 0.234 s for experiment, RANS and SPH*

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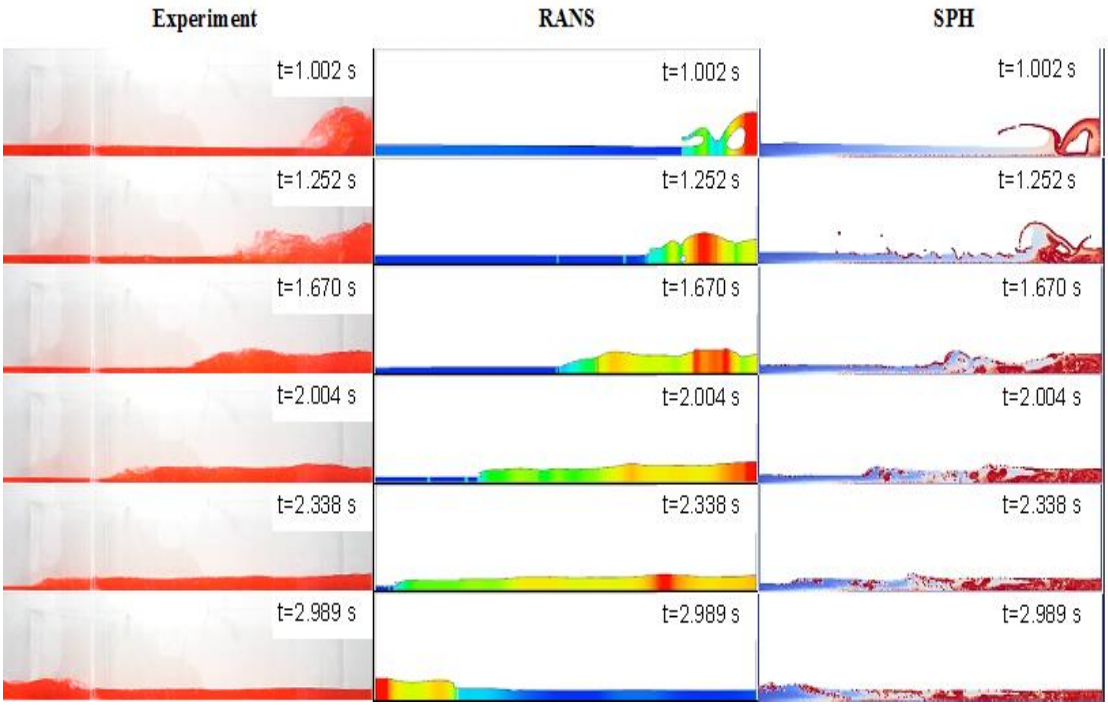

Figure 5. *Evolution of free-surface profiles of reflecting wave with time from t = 1.002 s to t = 2.989 s for experiment, RANS and SPH*

A dam-break flood shock wave over a dry bed can be separated two parts as an initial wave and a dynamic wave after the initial stages (Lauber and Hager, 1998; Chanson, 2005; Ozmen-Cagatay and Kocaman, 2010; Kocaman and Ozmen-Cagatay, 2015; Hui et al., 2017; Turhan et al., 2018). Nsom (2002) defines a convex shape of the wave front for highly viscous fluids. *Figure 5* shows the change in free-surface profiles in the experimental and numerical models for the selected locations in the case of a reflected wave. At t = 1.002 s, the wave rises on the channel surface for a while and the formation of air bubbles is observed in the wave front. When the wave is breaking in the RANS example, the wave jumps to a further point due to disintegration in the SPH. High energy losses in the experiments cause a decrease in the velocity because of the turbulence effect and the fluid level is observed at higher position (Kocaman and Ozmen-Cagatay, 2012; Souders et al., 2013; Rostami and Siosemarde, 2015; Gu et al., 2017; Turhan et al., 2019). At t = 1.252 s, the wave front has a different shape for each methods. In general, RANS results are compatible with the experimental results in terms of the wave front velocity (Ozmen-Cagatay and Kocaman, 2010; Bayon et al., 2016; Heydari and KhoshKonesh, 2016; Hu et al., 2018).

In *Figure 6,* for P1 = 0.15 m, the numerical and experimental results are in good agreement with each other except for T = 20 to 25. For P2 = 0.45 m, except for a few differences, the numerical methods show similar results to each other until T = 15. In the SPH method, between T = 15 and 20, it is seen that the fluid height increased at the initial stages but later, it decreased with time and also it is constant at H = 0.20. However, in the RANS method, the depth was observed to be higher than with the SPH (Turhan et al., 2019). At T = 20, the front of wave became vertical shape for the SPH due to the artificial viscosity coefficient. For P4 = 1.00 m and T = 8 to 10, some fluctuations were seen in the experimental results and the fluid height is lower

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

compared to the SPH. Until the end of the propagation, the maximum H value is occurred for T = 10 in the particle method. For P4 = 1 m, the obtained results in RANS and experiments are quite similar except for T = 8 to 15.

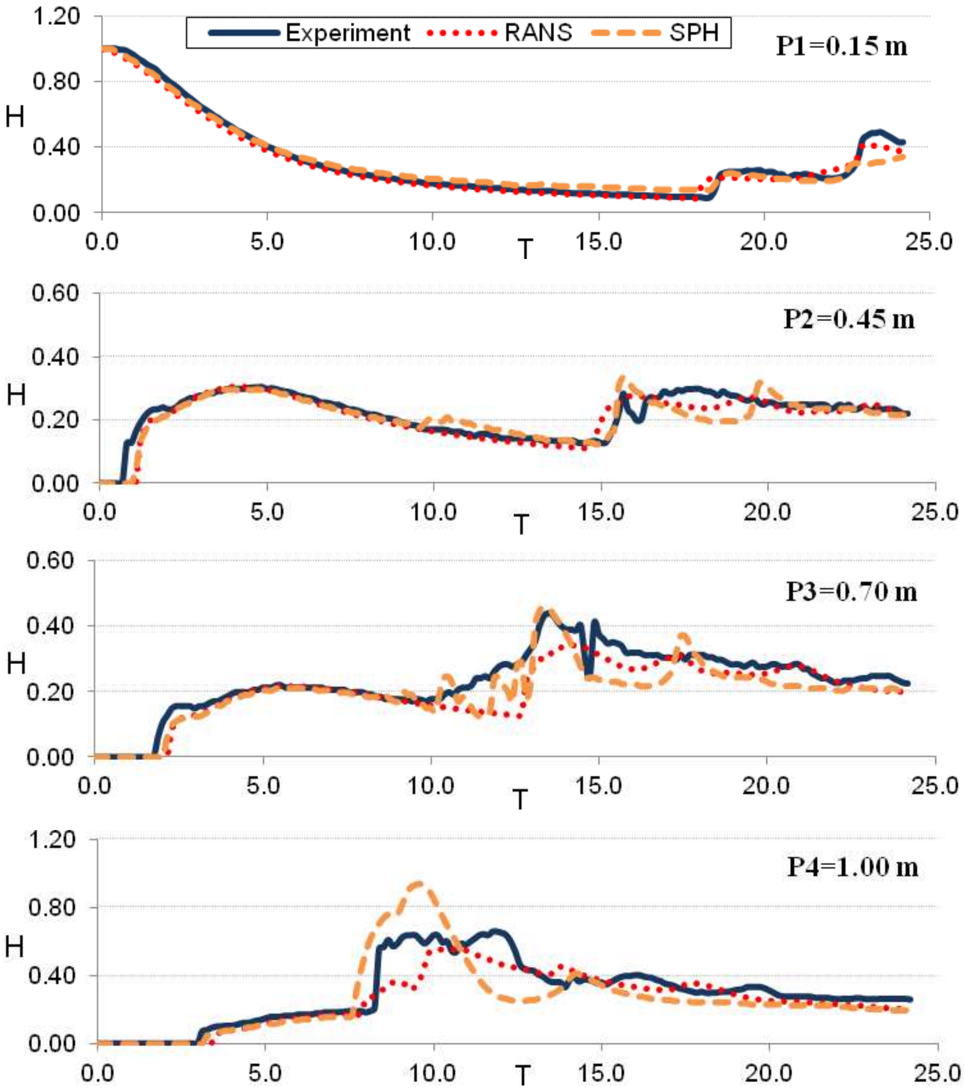

Figure 6. *Flow depth variations with time at P1, P2, P3 and P4*

At T = 10 to 15, the SPH results have been observed to differ from the experimental results. The disintegration of the reflected waves can be mentioned as one of the reasons for the differences due to particle collision in the flow. At T = 25, the experimental and SPH results in the graphs began to meet each other. Furthermore, a more sensitive analysis of the SPH parameters and modeling the wave motion with high-technology-aided computers will greatly increase the capability of the numerical solution (Dominguez et al., 2013; Valdez-Balderas et al., 2013; Turhan, 2017; Akbari, 2018). While the numerical model can generate highly compatible data in the initial stages of the dam-break, the differences between the results for various methods at P4 can be clearly observed due to the wall at the end of the channel. As a result, the propagation of flow in a Newtonian fluid with a different density, such as salt water for this study, can

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

yield similar results with the cases in which water was used as a fluid in the initial stages for both experimental and numerical results.

In addition, the reflected waves after hitting the wall become more vertical shape in the propagation of water and the formation of bubbles on the front face of the wave draws more attention (Zhu et al., 2018; Turhan et al., 2018). For fluid propagation, wave breaking, jet and bubble formations can be observed for some time due to the turbulence effect (Janosi et al., 2004; Li et al., 2013; Hu et al., 2018; Liu et al., 2018).

Conclusion

Difficulties in the application of dam-break flow problems require from utilizing numerical methods because of rough real case conditions. In recent years, the advances in computer technology have enabled computer-aided software programs to easily solve these problems. Therefore, the validation of numerical methods can considerably provide to test the sensitivity of the experimental solutions. In this study, the dam-break flow problem, in which the liquid is salt water, was investigated both experimentally and numerically over a dry bed to gain a better understanding of the effects of different density fluid. According to the results, the values obtained from both numerical methods and experiments are close to each other, but when the turbulence effect is high, the free-surface profiles and graphs show some differences. In these periods, major changes are observed using the SPH method. However, RANS results are in good agreement with the experiments. The artificial viscosity and particle approach in the SPH cause differences in the results in terms of profiles and graphs. The use of artificial viscosity in the SPH increases the differences between the results in each case. Although the results obtained from the SPH is usually considered to be good in agreement with the results obtained from other methods, studies performing more sensitive analyses can make greater contributions with more consistency. It can be stated that SPH, a mesh-free particle based Lagrange method, may be adopted as a useful numerical method for solving complex open channel flow problems.

As further studies, force and velocity measurements in dam-break problems, the effect of channel base roughness and channel slope on flow characteristics can be examined. In addition, several artificial viscosity and smoothing length values can be investigated to observe the performance of the numerical model under different parameters.

Acknowledgements. This study was supported by Çukurova University Scientific Research Projects Unit with the project code of *FDK-2015-4887*. The authors would like to thank Çukurova University Scientific Research Projects Unit on account of providing and helping in this research.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30333047

POTENTIAL DISSEMINATION OF ANTIMICROBIAL RESISTANCE FROM SMALL SCALE POULTRY SLAUGHTERHOUSES IN PAKISTAN

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(Received 3rd Dec 2018; accepted 4th Feb 2019)

Abstract. The importance of environmental reservoirs as a source from which antimicrobial resistance emerges and subsequently transferred is already established. To evaluate the role of small scale poultry slaughterhouses in resistance dissemination in Pakistan, a comparative genomic analysis of antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs) in the gut microbiome of broiler and household chickens (*Gallus gallus domesticus*) was performed. An array of 52 qPCR primers targeting the 16S rRNA gene, ARGs and MGEs was used in the experiment to analyse the abundance of selected genes in collected samples. A total of 22 ARGs and 7 MGEs were detected in all samples. The detection frequency of specific gene classes and diversity of genes was found to be higher in broiler as compared to household chicken. *Sul1* was the most abundant ARG with the highest percent relative abundance (2.4%). Total percent relative abundance of ARGs in broiler chicken was found to be significantly ($p < 0.05$) greater than household chicken fecal samples. A significant linear correlation ($R^2 = 0.89$) was found between relative abundances of *int1* and total ARGs. The clustering and correlation of selective ARGs with MGEs has implied that small scale poultry slaughterhouses can be a potential source for the dissemination of ARGs to other non-resistant environmental and/or clinical bacteria.
Keywords: *ARGs, MGEs, qPCR, AMR, microbiome*

Introduction

Antimicrobial resistance genes (ARGs) are emerging environmental contaminants causing serious public health concerns (Sanderson et al., 2016). The annual number of human deaths due to antimicrobial resistance is expected to reach up to 10 million by 2050 (de Kraker et al., 2016). Accumulating scientific evidence have suggested that the abuse of antibiotics in clinical and agricultural settings is mainly responsible for the emergence of antimicrobial resistance in microorganisms (Rather et al., 2017; York, 2017). Antibiotic resistant bacteria and associated ARGs can be transported from different environmental reservoirs contributing to the ever increasing global problem of antimicrobial resistance (Waseem et al., 2017a). The scientific community is already exploring new directions for evaluating the spread of the antimicrobial resistance from environmental routes to clinical pathogens (Andersson, 2015; Waseem et al., 2019; Williams et al., 2017). Fecal microflora from animals and birds harbours a vast variety

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DOI: http://dx.doi.org/10.15666/aeer/1702_30493063

of ARGs that can be transferred into other bacteria including human pathogens (Bonnedahl and Järhult, 2014; Zhao et al., 2018).

The poultry industry is one of the fastest growing agricultural sectors of the world. Use of antibiotics at therapeutic and sub-therapeutic levels as growth promoters have been employed in poultry for over half a century (Castanon, 2007). Although antibiotics utility for growth promotion has already been ostracized in many developed countries, but the situation is disquieting in developing countries like Pakistan and Egypt (Mund et al., 2017). In Pakistan, over the counter availability of antibiotics and less implementation of health and safety regulations in the poultry industry makes the challenge of antibiotic abuse and subsequent resistance dissemination far more intriguing. By 2030, the global consumption of antibiotics for cattle, chicken, and pigs is expected to reach 105,596 ± 3,605 tons (Van Boeckel et al., 2015). The consumption of antibiotics is continuously putting increasing pressure on environmental and clinical bacteria to acquire antimicrobial resistance. Apart from the resistance development under selection pressure for antibiotics, bacteria can also develop resistance by acquiring resistance genes via mobile genetic elements (MGEs) mediated horizontal gene flow (Oliveira et al., 2017).

One of the most useful strategies against the global antimicrobial resistance is to mitigate the spread of ARGs from environmental reservoirs. Surveillance and monitoring of such reservoirs are thus essential for curbing the dissemination of ARGs into the environment. Small scale poultry slaughterhouses are one of the potential reservoirs for antibiotic resistance. They are widely distributed throughout Pakistan. Chicken fecal waste from these small scale slaughterhouses, a source of resistant bacteria and ARGs, is generally being disposed of in the environment without any proper treatment. Chances of meat contamination with the antimicrobial resistant bacteria at the time of slaughtering in unhygienic conditions are also there (Amir et al., 2017). Additionally, ARGs can also be disseminated into the environment when poultry feces are used as organic fertilizers for fruits and vegetables.

Antibiotics can also influence ARGs to which they are not explicitly related (Foxman, 2012; Guo et al., 2018). Therefore, instead of antibiotic specific screening of ARGs in chicken *(Gallus gallus domesticus)* gut microbiota, analysis of ARGs from all major classes is recommended. Additionally, most of the research on antimicrobial resistance was focused on individual bacteria mainly foodborne pathogens (Lee et al., 2017; McMahon et al., 2007), which represents only a small fraction of the gut microbiome. Evaluation of resistance determinants from gut microbiome community can give a comprehensive analysis of dissemination and/ or fate of the ARGs into the environment.

To the best of our knowledge the status of AMR in gut microbiome of chicken in Pakistan yet remains elucidative to a greater extent. The primary purpose of the current study was to evaluate and compare the detection frequency, diversity, and abundance of selected ARGs from chicken fecal samples collected from small scale poultry slaughterhouses. The abundance and occurrence of selected MGEs have also been evaluated as they are believed to be involved in the dispersal of ARGs into non-resistant pathogenic bacterial strains. The possible correlations between ARGs and MGEs were also determined. Present research is a pilot scale study with only a limited sample set but it will likely provide first-hand information regarding ARGs dissemination from the small scale poultry slaughterhouses.

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Materials and methods

Sample collection and DNA extraction

Sample collection was performed in early spring of 2018 from a small scale poultry slaughter house in Johar Town, Lahore, Pakistan. Pools of fresh fecal samples from different cages of broiler and household chickens were collected (*Fig. A1* in the *Appendix*). A total of 6 fecal samples 3 from broiler chickens and 3 from household chickens were collected in 30 ml falcon tubes using a sterile spatula. Samples were thoroughly mixed and homogenized before DNA extraction (*Fig. 1*). DNA was extracted from collected samples by using QIAGEN DNeasy PowerSoil kit (QIAGEN Inc., MD, USA) as per manufacturer's instruction. Extracted DNA was stored at -20 °C until the amplification was performed. All the experiments in our study were performed at Environmental Microbiology Laboratories, Department of Microbiology, Quaid-i-Azam University, Islamabad. The concentration of DNA from each sample was normalized to 10 ng/µl before using the DNA for qPCR reactions.

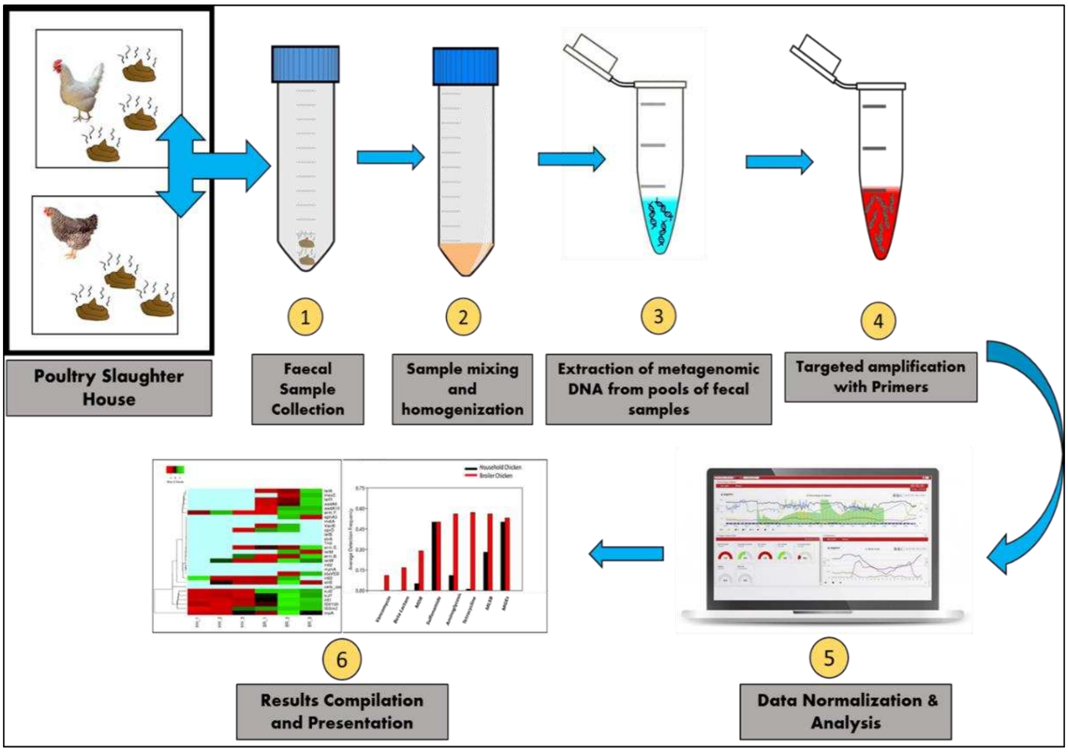

***Figure 1.** Diagrammatic flowchart of the whole experimental design*

Primers and qPCR

A total of 52 primers were used in this study for the evaluation of ARGs and MGEs abundance in fecal samples. The primers used in our study are a subset of qPCR Primer set 2.0 (Stedtfeld et al., 2018). The sequences of the qPCR primers can be found in the *Appendix* (*Table A1*). The primers were designed, validated and assayed as described in previous studies (Johnson et al., 2016; Karkman et al., 2016; Stedtfeld et al., 2017, 2018). The design protocol and parameters of each primer set are to target sequence diversity within a gene to analyse the environmental resistome. The array covered seven

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DOI: http://dx.doi.org/10.15666/aeer/1702_30493063

major antibiotic classes including MDR (7 genes), tetracyclines (7 genes), aminoglycosides (6 genes) beta-lactams (6 genes), macrolide-lincosamide-streptogramin B (MLSB) (6 genes), Vancomycin (6 genes) sulfonamides (3 genes) involved in different resistance mechanisms (*Fig. 2*).

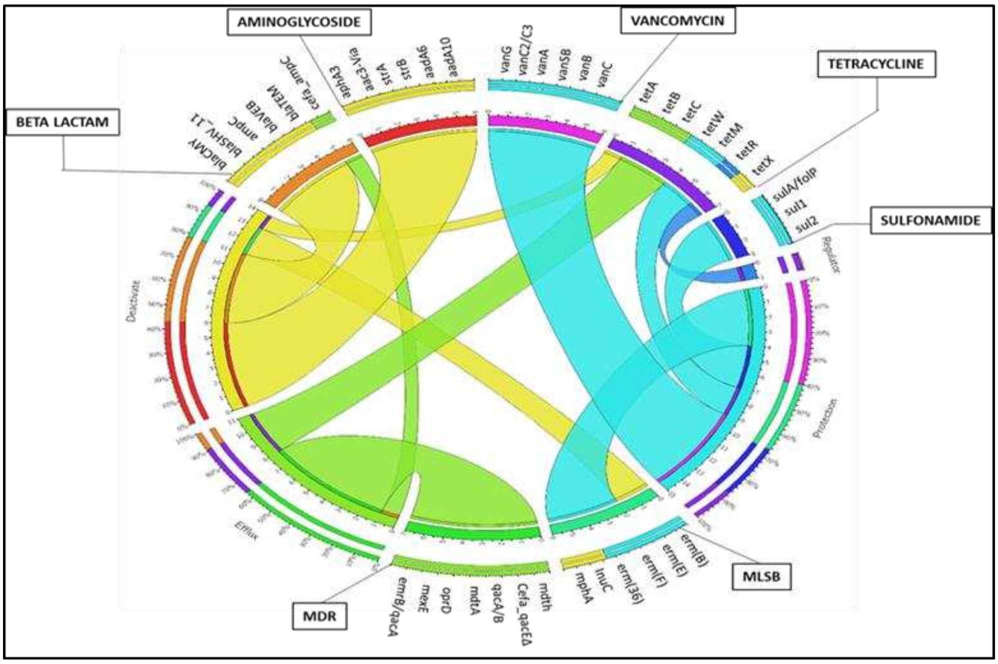

Figure 2. *Circos plot showing the antibiotic resistance class and mode of resistance mechanism of all 41 ARGs included in our array. The plot is made by using Cir-cos software (Krzywinski et al., 2009)*

A real-time qPCR thermocycler (Mastercycler® ep *realplex;* Eppendorf) was used to perform DNA amplification in 25 µl reaction volume. The contents used in qPCR are as follows: 2x Power SYBR Green PCR Master Mix (12.5 µl), 10 µm primer mix (1.25 µl), sample DNA (1 µl), and water (10.25 µl). Cycling conditions for qPCR reactions were as follow: an initial activation step at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Gene copy number was estimated from the Cycle threshold (Ct) values based on the method previously described (Looft et al., 2012). A Ct value of 29 was used as the cut-off limit. All sample-primer assays were performed in triplicates. A negative control without sample DNA was also included for every assay. Genes detected from at least two out of three technical replicates were considered as true positive and all false positive results were removed. Overall a total of 1248 qPCR reactions were performed during the whole study.

Data analysis

Relative abundance was calculated by dividing the estimated gene copy number of the targeted gene with the gene copy number of 16S rRNA. A multivariate principal component analysis (PCA) of ARG profiles between sample types was performed in Paleontological Statistics Software Package for Education and Data Analysis (PAST: v3.20) (Hammer et al., 2001). The scale of the principal components was transformed into eigenvalues. Basic data processing of qPCR results was performed on Microsoft

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Excel 2010. A student t-test was applied to the sum of total ARGs and MGEs abundance in order to test the significant differences between two different types of chicken. Shannon diversity calculations and scatter plots were also made in PAST v3.20 using percent relative abundance of genes in order to see the diversity of the ARGs in different samples. A heat map based on the natural log-transformed values of the relative abundance of ARGs and MGEs was made by using heat mapper software (Babicki et al., 2016) to evaluate the presence of individual genes in fecal samples. Complete linkage clustering method and Euclidean distance measurement method was used to cluster the rows representing genes in the heat map (Yeung et al., 2003).

Results

Detection and diversity of ARGs and MGEs

For evaluating the ARGs of 6 fecal samples from 2 different types of chicken (broiler vs. household), a qPCR sub-array of 52 primer sets targeting 16S rRNA gene, antibiotic resistance genes (ARGs) and transposase genes related to horizontal gene transfer (HGT) were used in the present study (*Fig. 2*). Out of 52 targeted genes, a total of 22 ARGs were detected in the fecal samples. The detected ARGs represented every targeted class of antibiotics (*Fig. 3*). In addition to ARGs 7 MGEs were also detected in both groups. The detected MGEs belonged to insertion sequences, transposase, and integrase groups are believed to play a role in HGT. Out of 29 detected ARGs and MGEs 8 genes were detected all (6/6) samples while 6 genes were detected in only 1 out of 6 samples. Two genes conferring resistance to aminoglycosides (*aadA6*, *aadA10*) and two tetracycline resistant genes (*tetA*, *tetR*) were absent in all three samples from household chicken but were detected in all three samples from broiler chicken. Overall more genes were detected in broiler chicken samples as compared to household chicken samples.

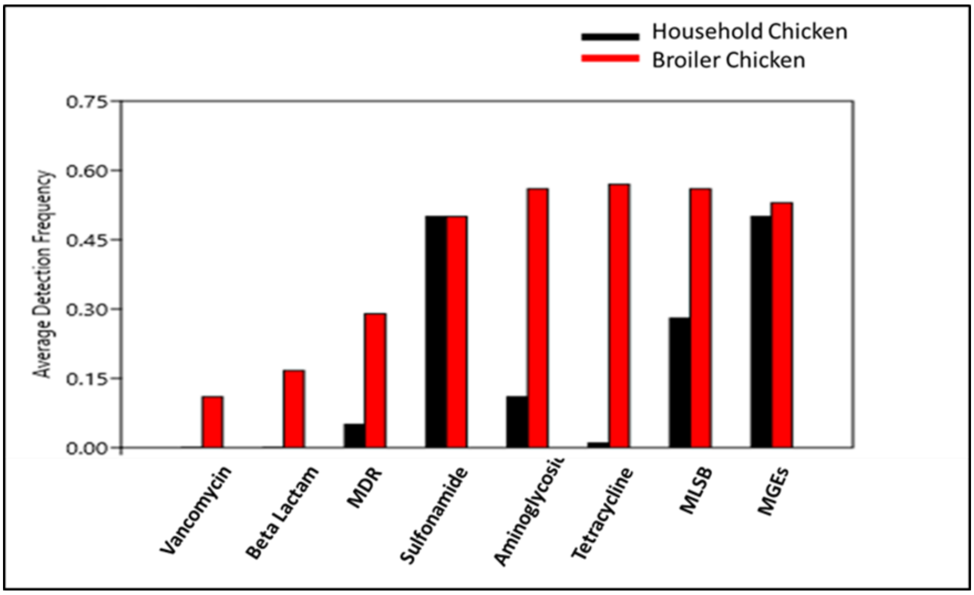

Figure 3. *Antibiotic resistance class wise average detection frequency. Average detection frequency is an average number of samples in which genes within a class is detected divided by total number of samples against which the genes are tested for one or more genes conferring resistance to that AR class*

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The number of detected genes ranged from 8 to 13 in fecal samples from household chicken whereas the genes detected in fecal samples of broiler chicken ranged from 18 to 24. Shannon diversity indicating the richness and abundance of genes in the samples was calculated by taking an average of the relative abundance of the three technical replicates for each of the 6 samples. Shannon diversity of the broiler chicken samples was found to be higher than the household chicken (*Fig. 4*).

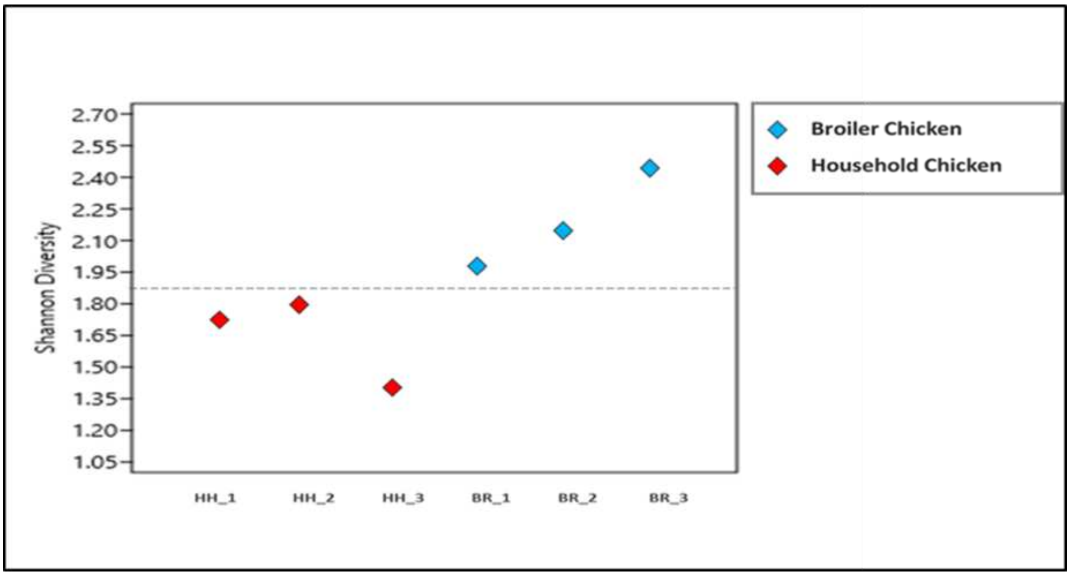

Figure 4. *Scatter plot showing the Shannon diversity of the ARGs and MGEs in 6 fecal samples*

The abundance of the ARGs and MGEs

To minimize the sampling variations, the estimated gene copy number of ARGs and MGEs was normalized to 16S rRNA gene copy number to achieve the relative abundance. The relative abundance of different genes varied significantly among the samples (*Fig. 5a*). The most abundant gene was *tnpA,* a transposase from IS-6 family detected in all 6 samples with percent relative abundance ranging from 0.52 to 4.68 whereas the least abundant gene was an ARG *vanB* from vancomycin group, the only gene detected from this group, ranged from 2.4×10^{-3} to 1.3×10^{-2}. Overall the summed relative abundance of ARGs ($p < 0.05$) and MGEs was higher in broiler chicken fecal samples than in broiler chicken samples (*Fig. 5b*).

Gene cluster patterns were observed by using ordination analysis. Relative abundances of the ARGs were used to perform multivariate PCA analysis in order to assess the distribution of ARGs among two chicken types (broiler vs. household). The PCA plot has revealed that the ARGs profile of fecal samples from two chicken groups is different from each other (*Fig. 6*).

Correlations among different gene classes were also evaluated in our study. Significant correlations were observed between total abundances of aminoglycosides, sulphonamides and tetracycline with *int1* and/ or total MGEs. The highest coefficient of determination (R^2) values was observed for aminoglycosides with MGEs and int1 (*Table 1*). The abundance of *int1* was also considerably correlated to the total relative abundance of the ARGs based on the linear regression analysis (*Fig. 7*).

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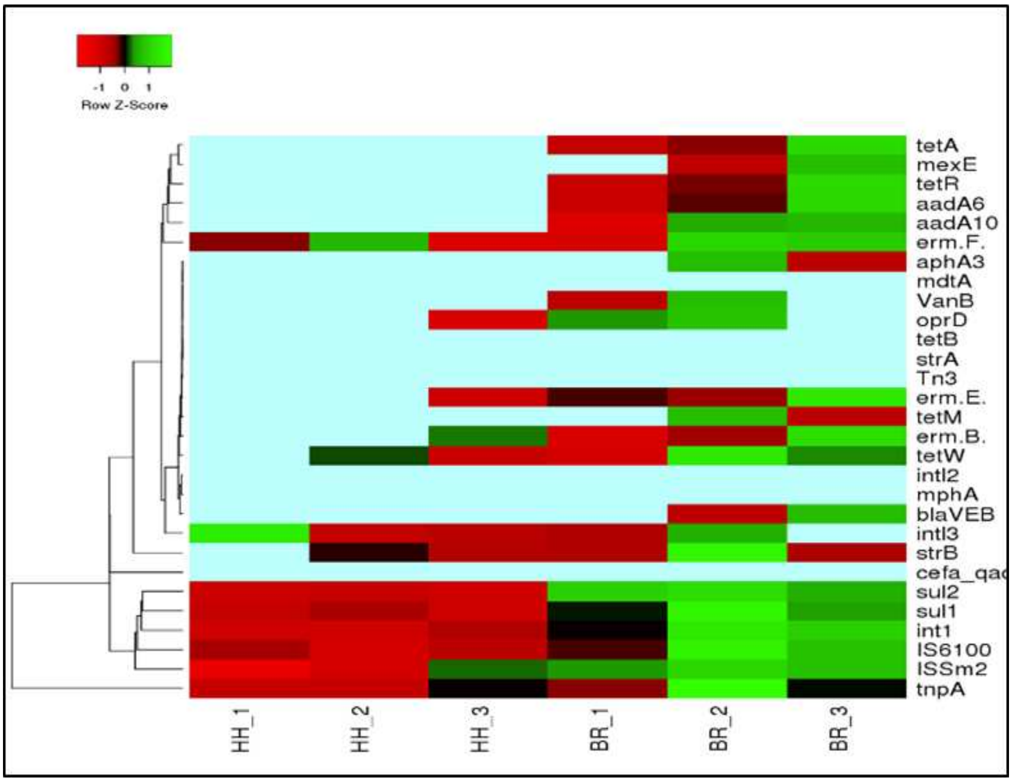
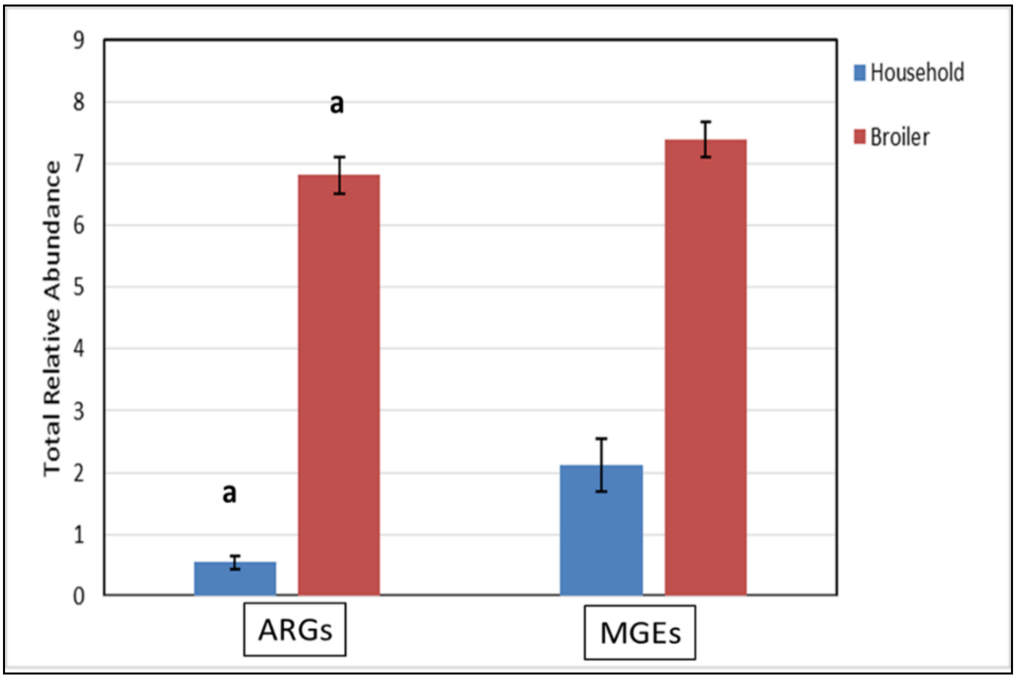


A



B

***Figure 5. a** Heat Map showing the ARGs and MGEs detected in 6 fecal samples (3 technical replicates each). Relative abundance (log transformed) was used for the formation of the heat map. Complete linkage clustering is showing that the sul genes are clustered with many MGEs including int1. **b** Sum of percent relative abundances of ARGs and MGEs in two types of chicken measured by 6 pools of fecal samples (3 technical replicates each). Letter (a) shows that the difference is significant at 95% confidence of interval. Error bars are showing the standard error of the calculated mean*

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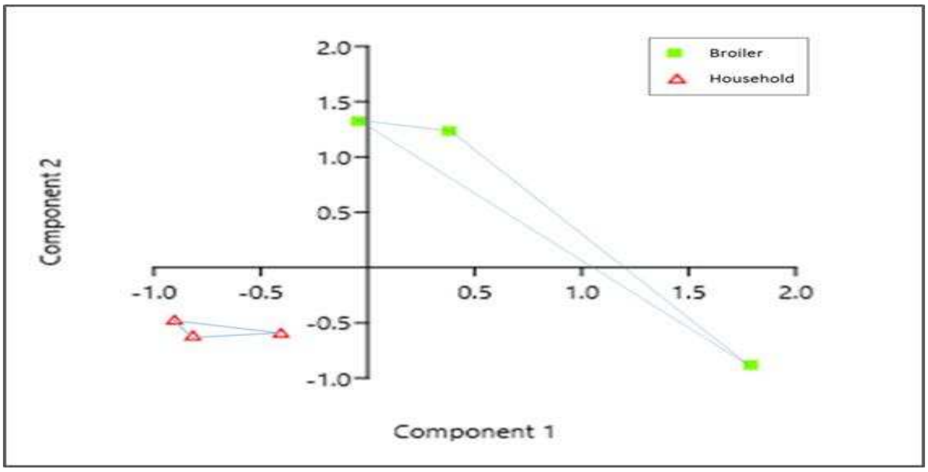

Figure 6. *Ordination of ARGs with 6 fecal samples from two different chicken types. Red triangles represented fecal samples of household chickens. Green squares represented fecal samples from broiler chickens*

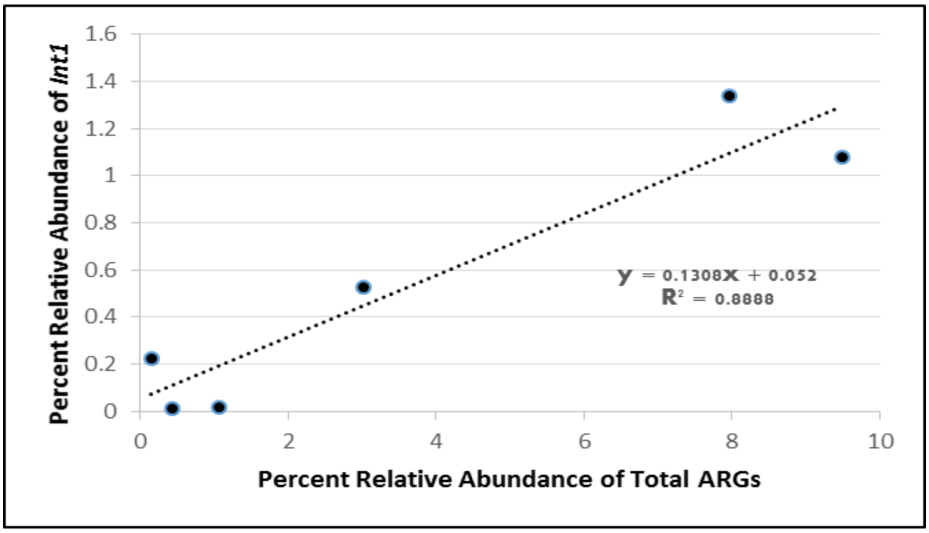

Figure 7. *Linear regression curve is showing a significant correlation between int1 and total ARGs abundances in 6 samples*

Table 1. *Coefficient of determination R^2 values between different gene classes*

Sr. no.	Gene class 1	Gene class 2	R^2 value
1	Total ARGs	Total MGEs	0.67
2	Aminoglycoside	int1	0.93
3	Aminoglycoside	MGEs	0.88
4	Sulfonamide	Int1	0.85
5	Sulfonamide	MGEs	0.78
6	Tetracycline	int1	0.53
7	Tetracycline	MGEs	0.29
8	MDR	int1	0.26
9	MDR	MGEs	0.08
10	MLSB	Int1	0.4
11	MLSB	MGEs	0.25

Discussion

A large body of literature has provided information regarding the threats of antimicrobial resistance using conventional methods (Álvarez-Fernández et al., 2013; Khalili et al., 2012). In recent years, dissemination of ARGs via environmental routes has gained much attention. Characterization of mobile genetic elements along with other genes is becoming essential in antimicrobial resistance research (Waseem et al., 2017b). In our study, we have opted a relatively broader and comprehensive approach of targeting multiple genes (ARGs and MGEs) in the gut metagenomes of broiler and household chickens (*Gallus gallus domesticus*). Our data is useful for assessing the role of small scale poultry slaughterhouses, which are widely distributed throughout the country, in ARGs dissemination. Most of the conventional small scale slaughterhouses have improper blood and wastewater drainage system (Sohaib and Jamil, 2017) which further intensifies the threat of ARGs dissemination into the environment.

Fecal samples from broiler chickens, which get routine antimicrobial treatments, were found to have higher abundance and diversity of ARGs compared to the household chickens which had been on organic antibiotic-free feed. Our results are in accordance with many different studies where the resistance abundance or detection frequency of resistant genes was significantly higher in antibiotics treated chickens, pigs and cattle as compared to the controls (Cameron and McAllister, 2016; Guo et al., 2018; Looft et al., 2012). Our findings demonstrated that the detection frequency of MGEs was almost equal in two sample types but the total percent relative abundance was higher in broiler chicken. Similar results of elevated MGEs were also observed in small scale production birds as compared to household birds during a study of small scale production poultry operations in rural Ecuador (Moser et al., 2018). This signifies that apart from the selection of ARGs under the selection pressure of antibiotics, higher abundance of MGEs could also be a critical factor in the transfer of genes within chromosomes, across bacteria and even in between different species (Stokes and Gillings, 2011).

The most frequently detected ARGs (at least 5/6 samples) in our study were *sul1, sul2, ermF, strB, tetW.* The presence of ARGs in household chicken type is not surprising. The same genes were also detected in *Gallus gallus domesticus* feces and many other environmental matrices without antibiotic treatment (Berglund, 2015; Sui et al., 2016; Zhang et al., 2013). This implies some resistance genes could be ubiquitously present in the environment without antibiotic's selection pressure. For example, a diverse intrinsic antibiotic resistome was recently studied in bacteria isolated from a cave (Pawlowski et al., 2016). Such genes can provide a competitive advantage to environmental bacteria harbouring them over others.

Sulfonamide resistance in bacteria usually arises by acquiring sul genes, encoding dihydropteroate synthase that is not inhibited by the antibiotics (Antunes et al., 2005). Sul genes have been most consistent targets in poultry and animal live stocks studies (Kozak et al., 2009; McKinney et al., 2010). Both of these genes were detected in all 6 samples. Considerable differences were found in the relative abundance of these genes within each sample. The difference in the abundance of these closely related genes can be ascribed to the differences in their dissemination mechanism. *Sul2* is normally located on Inc. Q family of plasmids whereas *sul1* is believed to be distributed with the help of integrons (He et al., 2014). Similar observation of different relative abundance of these related genes has been reported in other environmental matrices as well (Cheng et al., 2013; Sui et al., 2016; Zhang et al., 2013). A study had reported the prevalence and distribution of sulfonamide resistance genes in soils around poultry and livestock

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farms in Jiangsu Province, Southeastern China. Distribution frequency of *sul* genes and most frequent combination of *sul*1 and *sul*2 was studied (Wang et al., 2014). Our results are in close agreement with this study as the type of animal and waste can affect the distribution of ARGs in the environment.

Transposon specific gene *tnpA*, integron specific gene *int1* along with two other MGEs have been clustered together with sulphonamide resistant genes (*Fig. 4a*). Transposons and integrons have been investigated in-silico (Loot et al., 2017) and also in environmental matrices (Stokes and Gillings, 2011) to evaluate the possible enrichment of ARGs. *TnpA* association with antibiotic resistance is also highlighted in many studies (Carnelli et al., 2017; Feng et al., 2017). The *int*1 gene is believed to be responsible for the evolution and dissemination of multiple antibiotic resistant bacteria and the spread of mobile genetic elements in the environments (Moura et al., 2012). In our recent publication *int*1 gene as a proxy for environmental surveillance of ARGs has also been reconfirmed (Stedtfeld et al., 2017). An important observation in our present study is the strong correlation among the relative abundance of *int*1 gene and total ARGs. The reason behind this correlation could be co-occurrence of *int*1 and ARGs on a single plasmid and/ or chromosome. Our results are supported by the finding of a recent study about distribution and characterization of class 1 integrons where it was reported that these integrons harboured genes belonging to class aminoglycosides (Sung and Oh, 2014).

*tnp*A and *int*1 genes were two of the most abundant genes in broiler chicken which is in accordance with the results from a study of ARGs analysis in production chicken in rural Ecuador where the relative abundance of these two genes were highest among others (Guo et al., 2018). The clustering of *intI*1 and *tnp*A with ARGs has implied that broiler chickens in small scale slaughterhouses can negatively play their role by spreading ARGs in clinically important strains and thus can produce multiple antibiotic resistant strains which can cause serious public health concerns. For example, Talebiyan and colleagues have isolated 318 multiple resistant *E. coli* from commercial broiler flocks that have been given different doses of antibiotics (Talebiyan et al., 2014). This signifies that the broiler chickens in small scale slaughterhouses can provide an appropriate environment for the proliferation of ARGs through horizontal and/ or vertical gene transfer. Our results about the abundance and detection of ARGs in chicken fecal samples are in accordance with the trends of ARGs abundance observed in other gut microbiomes (Waseem et al., 2019).

Concrete measures should be opted in order to mitigate the threat of antimicrobial resistance. In this regard, strict AMR centric environmental strategies should be implemented on commercial poultry farms so that the use of antibiotics in the poultry industry should be regulated. Small scale poultry slaughterhouses, especially located in clusters, should be encouraged to develop their own waste and water management systems. Use of untreated chicken feces and litter as fertilizer should be discouraged. Apart from formulating and implementing rules and regulations, general public awareness campaigns about antimicrobial stewardship should be launched. This can also increase the demand for organic meat and/ or household chicken thus forcing the poultry farm owners to restrict antimicrobials use as growth promoters.

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Conclusion

Our study has provided an effective assessment and potential insight into the role and potential risk of small scale poultry slaughterhouses with the perspective of antimicrobial resistance. More clinically important ARGs were detected from the samples collected from cages of broiler chicken. Broiler chicken mostly raised in large scale commercial farms are usually located in outskirts of communities. Their transport poses a risk in the introduction of exogenous ARGs in the environment since such small scale poultry slaughterhouses are distributed widely throughout the country. Our research will aid in curbing the dissemination of antimicrobial resistance into the environment from such environmental reservoirs. Further research with larger sample size, more ARG and MGE targets along with bacterial community analysis will be helpful for validating the trends of ARGs spread in the environment.

Acknowledgements. The authors are very grateful to Dr. Maggie Williams from Michigan State University, East Lansing, Michigan, USA and Dr. Robert Stedtfeld from Swift Biosciences, Ann Arbor, Michigan, USA for their continuous support and assistance.

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APPENDIX

Figure A1. a & b *Collection and transfer of household chicken fecal samples in centrifuge tubes;* ***c & d*** *Collection and transfer of broiler chicken fecal samples in centrifuge tubes*

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DOI: http://dx.doi.org/10.15666/aeer/1702_30493063

Table A1. *Primer sequences of 16S rRNA gene, 41 ARGs and 10 MGEs*

Sr. no.	Targeted gene	Forward sequence	Reverse sequence
1	16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG
2	vanG	TGTTTCGCAGAACCGTGTCAA	CCCTGCACTGTTCCATCTTCTC
3	vanC2/vanC3	TGACTGTCGGTGCTTGTGA	GATAGAGCAGCTGAGCTTGTTC
4	vanA	GGGCTGTGAGGTCGGTTG	TTCAGTACAATGCGGCCGTTA
5	vanSB	GAAGATAAAGAGGGAAGCGTACTC	CCGAATTGTCAGCCCTTGATAA
6	VanB	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT
7	vanC	CCTGCCACAATCGATCGTT	CGGCTTCATTCGGCTTGATA
8	blaCMY	AAAGCCTCATGGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA
9	cefa_ampc	CAGGATCTGATGTGGGAGAACTA	TCGGGAACCATTTGTTGGC
10	blaSHV-11	TTGACCGCTGGGAAACGG	TCCGGTCTTATCGGCGATAAAC
11	ampC	CTGGCGCATACCTGGATTAC	GCCAGTTCAGCATCTCCCA
12	blaVEB	CCCGATGCAAAGCGTTATG	GAAAGATTCCCTTTATCTATCTCAGACAA
13	blaTEM	CGCCGCATACACTATTCTCAG	GCTTCATTCAGCTCCGGTTC
14	mdth	ATGCTGGCTGTACAAGTGATG	CACTCCAGCGGGCGATA
15	cefa_qacEdelta	TAGTTGGCGAAGTAATCGCAAC	TGCGATGCCATAACCGATTATG
16	qacA/B	AAGGGCCACTGCATTAGCTG	CCAGTCCAATCATGCCTGCA
17	mdtA	ACAAGCCCAGGGCCAAC	CCTTAATGGTGCCTTCGGTTTC
18	oprD	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA
19	mexE	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC
20	sulA/folP	CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT
21	sul2	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT
22	sul1 NEW	GCCGATGAGATCAGACGTATTG	CGCATAGCGCTGGGTTTC
23	aphA3	AAAAGCCCGAAGAGGAACTTG	CATCTTTCACAAAGATGTTGCTGTCT
24	aac3-Via	GTGTCCGTCGCCAAGGA	GGTGACGGCCTTGTCGA
25	strA	CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG
26	strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT
27	aadA6	CCATCGAGCGTCATCTGGAA	CCCGTCTGGCCGGATAAC
28	aadA10	ACAGGCACTCAACGTCATCG	CGCGGAGAACTCTGCTTTGA
29	tetA	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG
30	tetB	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA
31	tetW	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC
32	tetX	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT
33	tetC	ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG
34	tetR	CCGTCAATGCGCTGATGAC	GCCAATCCATCGACAATCACC
35	tetM	GGAGCGATTACAGAATTAGGAAGC	TCCATATGTCCTGGCGTGTC
36	lnuC	GGGTGTAGATGCTCTTCTTGGA	CTTTACCCGAAAGAGTTTCTACCG
37	emrB/qacA	CTTTTCTCTAACCGTACATTATCTACGATAAA	AGAACGTAGCGACTGATAAAATGCT
38	erm(B)	GAACACTAGGGTTGTTCTTGCA	CTGGAACATCTGTGGTATGGC
39	erm(E)	GTCACGCAGCTGGAGTTCG	CGGTGAAGCACAGCTCGAC
40	mphA	TCAGCGGGATGATCGACTG	GAGGGCGTAGAGGGCGTA
41	erm(F)	TCTGATGCCCGAAATGTTCAAG	TGAAGGACAATTGAACCTCCCA
42	erm(36)	GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC
43	intl3	CAGGTGCTGGGCATGGA	CCTGGGCAGCATCACCA
44	ISCR1	ATGGTTTCATGCGGGTT	CTGAGGGTGTGAGCGAG
45	Tn3	GCTGAGGTGTTCAGCTACATCC	GCTGAGGTAGTCACAGGCATTC
46	IS6/257	ATATCGTGCCATTGATGCAGAG	ACCATTGCTACCTTCGTTGAAG
47	tnpA	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC
48	int1	CGAAGTCGAGGCATTTCTGTC	GCCTTCCAGAAAACCGAGGA
49	intl2	TGCTTTTCCCACCCTTACC	GACGGCTACCCTCTGTTATCTC
50	ISPps1	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC
51	ISSm2	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT
52	IS1111	GTCTTAAGGTGGGCTGCGTG	CCCCGAATCTCATTGATCAGC

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30493063

ECOLOGICAL RISK ASSESSMENTS OF HEAVY METALS IN SURFACE SEDIMENTS COLLECTED FROM HAQAL COASTAL WATERS (TABUK REGION), SAUDI ARABIA

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(Received 6th Dec 2018; accepted 28th Jan 2019)

Abstract. Haqal is a small city located in north-western part of Arabian Peninsula. The anthropogenic activities in this region are evident. This region is targeted for future development by Saudi government. In this study, the surface sediment of Haqal coastal waters were analysed for heavy metals (Cd, Cu, Fe, Ni, Pb, and Zn). The total concentration (mg/kg dry weight) ranged from 0.012-0.186 for Cd, 0.582-1.13 for Cu, 0.51-2.18 for Ni, 0.68-2.64 for Pb, 1.97-4.52 for Zn while for Fe, it ranges from 0.155 to 0.254%. Based on ecological risk assessment results, the values of PERI were categorised as 'low ecological risk', thus all sampling sites were unpolluted with heavy metals. Despite that, this monitoring study had a positive result for non-heavy metal pollution, future mitigation of the heavy metal pollution in coastal areas of Tabuk should be given priority by the authorities. The present study can be considered as the first effort to monitor the pollution of heavy metals in Haqal. This provides baseline information for future ecotoxicological studies which can involve application of bioindicators to assess the quality of the marine environment in this region.
Keywords: *The Red Sea, marine environment, Arabian Peninsula, pollution*

Introduction

Sediment is an important sink where pollutants are accumulated from the water body, and a secondary pollution source which might impact the surrounding aquatic ecosystem (Birch and Taylor, 2002; Wang et al., 2011; Hahladakis et al., 2016; Kumwimba et al., 2017; Wong et al., 2017). Thus, determining the ecological risks to aquatic ecosystems posed by heavy metal toxicity is crucial to be determined (Kumwimba et al., 2017; Wong et al., 2017). In this study, two approaches were deployed to assess ecological risks for heavy metals in sediments, i.e. 1) sediment total metal concentrations, and 2) metal GFs in sediment. The sub-approaches that are based on total metal concentrations are i) comparisons with sediment quality guidelines (SQGs), ii) geochemical pollution indexes (Igeo), and enrichment factor (EF), iii) contamination factor (Cf), potential risk of individual metal (Er) and potential ecological risk index (PERI), proposed by Hakanson (1980). These SQGs are developed for marine and estuarine ecosystems (Long et al., 1995) via (a) the effect range low

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

(ERL)/effect range median (ERM)/interim sediment quality value-low (ISQV-low) and (b) the threshold effect level (TEL)/probable effect level (PEL)/interim sediment quality value-high (ISQV-high) values.

Originating from Long and Morgan (1990), the SQG is an informal tool to evaluate sediment chemical data in relation to possible adverse effects on aquatic biota. The SQGs were proposed to be used as benchmark for evaluating sediment chemistry information to identify situations that are potentially harmful to aquatic organisms associated with bad sediments; as well as the benchmark to help set targets for sediment quality for the broader management strategy of long term aquatic ecosystem health sustainability management (CCME, 2002).

Among the basics of SQGs, SQGs based on ERL and ERM are used extensively in assessment of the pollutants' impact on environment. It has been tested against a large dataset gained in US EPA and US NOAA (National Oceanic and Atmospheric Administration, US). These datasets consisted of synchronized measurements of chemical and toxicological variables (O'Connor, 2004). However, O'Connor (2004) has made a clarification that SQG's ERL is not a threshold chemical toxicity in sediment and there is no basis for the assumption of pollutant concentration above ERL increase the probability of toxicity. However, there are still multiple recent heavy metal risk assessment studies that are employing ERL-ERM-based SQG at their assessment (Long and Morgan, 1990; Yap, 2010; Yap et al., 2002; Amin et al., 2009; Garcia et al., 2011; Ali et al., 2015). Therefore, the ERL and ERM approach of risk assessments are still viable approaches of heavy metal risk assessment and will still be used to evaluate the heavy metal risk in this study. In this study, the heavy metal levels in sediments were compared SQGs with multiple approaches (ERL, ERM, TEL and PEL) to assess and deduce the possible environmental impact of the sediment's heavy metals level. Hakanson (1980) has proposed a series of indexes as diagnostic tools for pollution control purposes, i.e. the contamination factor (Cf), risk index of individual metal (Er) and Total Risk Index (RI), and degree of contamination.

Ecological risk assessment (ERA) using above-mentioned approaches have been widely used in ERA-based studies. These studies reported have wide geographical distribution. In Asia region, there are such reports from Malaysia (Yap et al., 2002 and Yap, 2010), Khuzestan coastal waters, Iran (Madiseh et al., 2009), Northern Bohai and Yellow Seas, China (Luo et al., 2010), Dongjiang Harbor, China (Guo et al., 2010), Yangtze Estuary, China (Zhao et al., 2012), Lake Cıldır, Turkey (Kukrer et al., 2014), Ulsan Bay, Korea (Ra et al., 2014), and mangrove sediments of Peninsular Malaysia (Cheng and Yap, 2015).

Tabuk coastal areas including Haqal city supported high floral and faunal diversity. On the other hand, the environment of the coastal areas of Haqal is threatened by several human activities which may increase the pollution burden to the area. Studies on monitoring of heavy metals in these areas are scarce. On the other hand, this area has a promising future as it is targeted for future development with the country development plan. Thus, this study can be considered as pioneer in the assessment of heavy metal contamination of the coastal environment of Haqal. The present study aims to estimate the ecological risk assessments in the surface sediment collected from Haqal coastal waters based on 1) SQGs, 2) two geochemical pollution indexes, and 3) PERI.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

Materials and methods

Study site

Haqal is a small city belongs to Tabuk region and is located in the north-western part of Arabian Peninsula. It is located within geographical coordinates of 29° 17’ 39” North, 34° 57’ 4” East. It is situated in the northwest of Saudi Arabia near the head of the Gulf of Aqaba adjacent to Aqaba across the Jordanian border (lies about 5 km from Jordanian border). This is coastal area is not a port used for the Red Sea shipping. However, coasts of the region are scenic. The climate of this city is arid with annual rainfall of less than 50 mm. The rainy season is in the winter and the annual mean temperature is 24° C. This region is targeted for future development under the NEOM project. Therefore, it is expected that this region will witnessed huge infrastructure development and the environmental assessment is a necessary tool at this stage.

Collection of sediment samples

The surface sediments (3-5 cm from surface sediment) in 15 sites of Haqal coastal areas were collected on early summer of 2018 (*Fig. 1*). The plastic spoon was used to collect the top-sediment to minimize the metal contamination. Sufficient amount of the sediment was collected from each site. The collected sediment samples were transferred into an acid-washed polyethylene bag and brought back to laboratory for temporary storage and analysis. The samples taken back to laboratory were frozen (-20 °C) in a freezer prior to analysis.

Figure 1. *Map showing sampling area in Haqal coastal waters in Tabuk, Saudi Arabia*

Before the analysis, sediment samples were oven-dried at 60 °C for at least 16 h until constant dry weights were achieved. Then the dried sediment particles were sieved

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

through a 0.50 mm stainless steel sieve. During the sieving process, the samples were also shaken vigorously to produce homogeneity. Only sediment particles with size below 63 µm in diameter are considered for metal analysis.

The analyses of total Cd, Cu, Fe, Ni, Pb and Zn concentrations in sediment samples were done according to direct aqua-regia method. After sieving, about one gram of each dried sample (Particle size < 63 µm) was weighed and digested in a aqua-regia solution, which is a combination (ratio 4:1) of concentrated HNO_3 (AnalaR grade, BDH 69%) and $HClO_4$ (AnalaR grade, BDH 60%). The digestion was first conducted at low temperature (40 °C) for 1 h and then the temperature was elevated to 140 °C for a minimum 3 h until the samples have been fully digested. The resulting solutions of the digestion were than diluted to a fixed volume of 40 ml using double distilled water. The sample was then filtered filter paper (Whatman no. 1, pore size 11 µm) and the filtrates were stored until metal determination. Flame-Atomic Absorption Spectroscopy (FAAS; Perkin Elmer Model AAnalyst 800) was used to determine the concentration of Cd, Cu, Fe, Ni, Pb and Zn. The resulting data were expressed as mg/kg of samples' dry weight (dw).

To ensure the accuracy and credibility of the result, some pre-caution steps have been done. During metal analysis, a quality control sample was routinely analysed to ensure the accuracy of the analysis. As the result, the metal recovery of the quality control samples was acceptable at 90-110%. All glassware and equipment used during sample processing and metal analysis were soaked in 10% HNO_3 to wash away the possible metal contaminant. Certified Reference Materials (CRM) for Soil (International Atomic Energy Agency, Soil-5, Vienna, Austria) was used to assure the quality of direct aqua-regia method. The CRM was treated exactly the same as the other samples. The result of metal analysis was compared to the certified reference value of the respective metals. The recovery of the CRMs was found to be satisfactory.

Data treatment

Geoaccumulation index (I_{geo})

The values of I_{geo} were calculated according to Muller's (1969) formula (*Eq. 1*):

$$I_{geo} = log_2 \frac{C_n}{1.5 \times B_n} \quad \text{(Eq.1)}$$

where C_n is the sediment heavy metal concentrations and B_n is the "preindustrial reference values" (PRV; unit = mg/kg dw; Hakanson, 1980) which act as geochemical background values for each metal. The PRV for Cd, Cu Pb and Zn was 1.0, 50, 70 and 175, respectively (Hakanson, 1980). Owing to the absence of the PRV of Fe and Ni was taken from "upper continental crust" from Wedepohl (1995) and Rudnick and Gao (2003), in which the values were 3.09% and 47.0 mg/kg dw, respectively (*Table 2*).

To minimize the possible variation in background metal concentrations which might be contributed by lithogenic variations, a factor of 1.5 was introduced into the formula (Al-Haidarey et al., 2010; Hasan et al., 2013). It permits the content fluctuation of the metals in sediment and also some negligible anthropogenic influences (Loska et al., 1997; Hurley et al., 2017). Muller (1969) has classified the resulting Igeo values into six classes according to the extent of the metal geoaccumulation.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

Enrichment factor (EF)

The EF in this study was determined using a formula defined by Buat-Menard and Chesselt (1979), with Fe as a normaliser (*Eq. 2*):

$$EF = \frac{\left(\frac{C_n}{C_{Fe}}\right)_{sample}}{\left(\frac{C_n}{C_{Fe}}\right)_{crust}} \quad \text{(Eq.2)}$$

where (C_n/C_{Fe}) sample is the metal to Fe ratio in the sediments; (C_n/C_{Fe}) crust is the metal to Fe ratio in the earth crust which considered as pre-industrial unpolluted metal value of a sediment.

The normalisation using Fe are necessary to correct for differences in sediment grain size and mineralogy (Schiff and Weisberg, 1999). The use Fe as normaliser was made based on the fact that Fe is a major sorbent phase for trace metals and is a quasiconservative tracer of the natural metal-bearing phases in fluvial and coastal sediments (Schiff and Weisberg, 1999; Hurley et al., 2017). According to Hasan et al. (2013), natural resources (98%) vastly dominated the input of Fe. In this expression, the normaliser (reference element) is assumed to have little variability of occurrence, and is present in trace concentration in the examined environment (Loska et al., 1997). The degrees of EF are categorised by Taylor (1964) and Birth (2003).

Ecological risk assessments

The contamination factor (C_f) was calculated to describe the contamination status of metals in the industrial drainages studied (Hakanson, 1980). C_f was calculated as *Equation 3:*

$$C_f = \frac{C_{sed}}{C_{ref}} \quad \text{(Eq.3)}$$

where C_f is the contamination factor; C_{sed} is the mean metal concentration in the sediment; C_{ref} is the PRV of metals in the sediments. Hakanson (1980) has classified the C_f values into 4 categories.

According to Hakanson (1980), the potential risk for individual metal (E_r) can be calculated using *Equation 4:*

$$E_r = TR \times C_f \quad \text{(Eq.4)}$$

where TR is the toxic-response factor for a metal (*Table 2*) Due to the fact that the absence of TR value for Fe, the E_r for Fe was not calculated. C_f is the contamination factor for the same substance. The E_r for each metals were defined in accordance of Hakanson’s (1980) standard.

The total risk index (RI) or potential ecological risk index (PERI) was calculated as *Equation 5:*

$$RI = \sum_{i=1}^{m} E_r = \sum_{i=1}^{m} (TR \times C_f) \quad \text{(Eq.5)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

where E_r is the risk index of individual metal, TR is the toxic-response factor and C_f is the contamination factor. The PERI can be described according to categories suggested by Hakanson (1980).

The classification of SQGs along with its effects and comparison results are presented in *Table 2*. It is important to determine whether the total concentrations of heavy metals in sediments found pose a threat to aquatic life. In the present study, the sediment risk of each metal investigated is assessed by three sets of SQGs namely (1) Effects Range Low (ERL) and Effects Range Medium (ERM) by Long et al. (1995), (2) lowest effect level (LEL) and severe effect level (SEL) by NYSDEC (1999), and (3) threshold effects level (TEL) and probable effects level (PEL) by MacDonald et al. (1996) and MacDonald (2003). These three sets of numerical SQGs were directly applied (without normalization) to assess possible risk arises from the heavy metal contamination in sediments of the study area.

Results and discussion

The concentrations (mg/kg dry weight; except for Fe in %) of surface sediments collected from Haqal coastal waters are shown in *Table 1*. The surface sediment of Tabuk coastal waters from Saudi Arabia were analysed for Cd, Cu, Fe, Ni, Pb, and Zn. The metal concentrations (mg/kg dry weight) ranges for total concentration are 0.012-0.186 for Cd, 0.582-1.13 for Cu, 0.51-2.18 for Ni, 0.68-2.64 for Pb, 1.97-4.52 for Zn while for Fe, it ranges from 0.155-0.254%. These values of heavy metals are remarkably lower compared to those reported in Jeddah coastal surface by Badr et al. (2009).

Table 1. *Concentrations (mg/kg dry weight; except for Fe in %) of heavy metals of surface sediments collected from Haqal coastal waters (Tabuk, Saudi Arabia)*

Location	Ni	Pb	Cu	Zn	Cd	Fe%
1	0.589	1.996	0.602	4.504	0.081	0.196
2	0.506	1.738	0.873	2.699	0.012	0.216
3	2.179	1.404	0.582	2.172	0.024	0.174
4	1.668	1.330	0.641	1.967	0.032	0.220
5	0.971	1.478	0.885	2.336	0.090	0.168
6	1.042	1.508	0.691	3.955	0.138	0.176
7	1.569	1.502	0.826	1.979	0.064	0.178
8	2.083	2.162	0.802	2.897	0.031	0.205
9	1.966	2.643	1.076	3.398	0.071	0.178
10	1.747	1.727	0.898	2.006	0.125	0.186
11	1.744	1.443	0.955	3.294	0.044	0.206
12	0.704	1.428	0.822	2.407	0.118	0.213
13	0.863	1.206	0.988	2.528	0.186	0.254
14	0.854	0.675	1.094	4.522	0.124	0.172
15	1.306	1.329	1.130	3.364	0.167	0.155

All the values of metals are below ERL, indicating that adverse effects on aquatic biota should rarely occur. There should be no toxicological effects of Cd, Cu, Ni, Pb

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

and Zn in the present study area and that may not occasionally be associated with adverse biological effects.

When compared to the LEL–SEL SQGs, among the metals, Cd, Cu, Ni, Pb and Zn concentrations are also below LEL in all sites. According to Thompson et al. (2005), the LEL represents the contaminant concentration below the harmful effects on benthic invertebrates. However, by comparison to the TEL–PEL SQGs, 100% of the concentrations of Cd, Cu, Pb and Zn in all the samples are below TEL. It is interpreted that TEL as the concentrations, below which adverse biological effects rarely occurs. Hence, it is considered to provide a high level of protection for aquatic organisms. This requires a lower level of protection for aquatic organisms.

In comparison to reference values (RVs) (*Table 2*), all the metals levels of all sites are below the levels of pre-industrial reference (PIR) level (Hakanson, 1980), and the upper continental crust (UCC) values proposed by Taylor and McLennan (1995), Wedepohl (1995) and Rudnick and Gao (2003).

Table 2. *Comparisons between total heavy metal concentrations (mean, µg/g dw; except for Fe in %) with those cited from sediment quality guidelines (SQGs) and reference values (RVs). The values of toxic-response factors, employed in this study are also presented*

Site no.	Cd	Cu	Fe (%)	Ni	Pb	Zn	References
Haqal coastal sediment	0.012-0.186	0.582-1.13	0.155-0.254	0.51-2.18	0.68-2.64	1.97-4.52	This study
Jeddah (Saudi Arabia)	3.08-3.51	17.47-23.77	20.32-26.71	67.78-85.50	80.30-98.77	52.74-76.36	Badr et al. (2009)
Mangrove area of Peninsular Malaysia	1.11-2.00	5.59-28.7	1.29-4.89	-	25.36-172.6	29.35-130.3	Cheng and Yap (2015)
SQGs	**Cd**	**Cu**	**Fe (%)**	**Ni**	**Pb**	**Zn**	
Effects range low (ERL)	1.20	34.0	-	-	46.7	150	Long et al. (1995)
Effects range median (ERM)	9.60	270	-	-	218	410	Long et al. (1995)
LEL	0.6	16	2	16	31	120	NYSDEC
SEL	9	110	4	50	110	270	NYSDEC
Treshold effect level (TEL)	0.68	18.7	-	-	30.2	124	MacDonald et al. (1996)
Probable effect level (PEL)	4.21	108.2	-	-	112.2	271	MacDonald et al. (1996)
RVs	**Cd**	**Cu**	**Fe (%)**	**Ni**	**Pb**	**Zn**	
Pre-industrial reference level	1.00	50.0	-	68.0*	70.0	175	Hakanson (1980)
Upper continental crust	0.098	25.0	-	44.0	17.0	71.0	Taylor and McLennan (1995)
Upper continental crust	0.102	14.3	3.09	18.6	17.0	52.0	Wedepohl (1995)
Upper continental crust	0.09	28.0	-	47.0	17.0	67.0	Rudnick and Gao (2003)
Toxic-response factor (*Tr*)	30.0	5.00	-	2.00	5.00	1.00	Hakanson (1980)

All concentrations are presented in µg/g dw except for Fe in % and *Tr* values are unitless

The values of enrichment factor (EF), geoaccumulation index (Igeo), contamination factor (CF), ecological risk (ER) and potential ecological risk index (PERI) of heavy metals based on the surface sediments from all sampling sites are presented in *Table 3*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

All the values of EF for Cd, Cu, Ni, Pb, and Zn are below 2.0, indicating ‘depletion of mineral enrichment’ (Sutherland, 2000; Hsu et al., 2016), except for 5 sites of Cd levels where the EF values are between 2 and 3, indicating ‘moderate enrichment’. Fro Igeo, all values are below 0.0 (in negative values), indicating ‘practically unpolluted’ (Muller, 1969). For CF for individual metal, all values are below 1.00, indicating ‘low contamination factor’ (Hakanson, 1980). For ER, all values are below 40.0, indicating ‘low potential ecological risk’ (Hakanson, 1980). Lastly, for PERI, all values are below 150, indicating ‘low ecological risk’ (Hakanson, 1980).

Table 3. *Summary results (mean) enrichment factor (EF), geoaccumulation index (Igeo), contamination factor (CF), ecological risk (ER) and potential ecological risk index (PERI) of heavy metals based on the surface sediments from the present study*

	Ni				Pb				Cu				Zn				Cd				Total ER/ PERI
Sites	**EF**	**Igeo**	**CF**	**ER**	**EF**	**Igeo**	**CF**	**ER**	**EF**	**Igeo**	**CF**	**ER**	**EF**	**Igeo**	**CF**	**ER**	**EF**	**Igeo**	**CF**	**ER**	
1	0.14	-7.44	0.01	0.02	0.45	-5.72	0.03	0.14	0.19	-6.96	0.01	0.06	0.41	-5.86	0.03	0.03	1.28	-4.21	0.08	2.44	2.68
2	0.11	-7.66	0.01	0.01	0.36	-5.92	0.02	0.12	0.25	-6.42	0.02	0.09	0.22	-6.60	0.02	0.02	0.17	-6.98	0.01	0.36	0.60
3	0.57	-5.55	0.03	0.06	0.36	-6.22	0.02	0.10	0.21	-7.01	0.01	0.06	0.22	-6.92	0.01	0.01	0.42	-5.99	0.02	0.71	0.94
4	0.34	-5.93	0.02	0.05	0.27	-6.30	0.02	0.09	0.18	-6.87	0.01	0.06	0.16	-7.06	0.01	0.01	0.45	-5.56	0.03	0.96	1.17
5	0.26	-6.71	0.01	0.03	0.39	-6.15	0.02	0.11	0.32	-6.41	0.02	0.09	0.24	-6.81	0.01	0.01	1.66	-4.05	0.09	2.71	2.95
6	0.27	-6.61	0.02	0.03	0.38	-6.12	0.02	0.11	0.24	-6.76	0.01	0.07	0.40	-6.05	0.02	0.02	2.42	-3.44	0.14	4.14	4.37
7	0.40	-6.02	0.02	0.05	0.37	-6.13	0.02	0.11	0.29	-6.50	0.02	0.08	0.20	-7.05	0.01	0.01	1.10	-4.56	0.06	1.91	2.15
8	0.46	-5.61	0.03	0.06	0.47	-5.60	0.03	0.15	0.24	-6.55	0.02	0.08	0.25	-6.50	0.02	0.02	0.47	-5.58	0.03	0.94	1.26
9	0.50	-5.70	0.03	0.06	0.65	-5.31	0.04	0.19	0.37	-6.12	0.02	0.11	0.34	-6.27	0.02	0.02	1.23	-4.40	0.07	2.14	2.51
10	0.43	-5.87	0.03	0.05	0.41	-5.93	0.02	0.12	0.30	-6.38	0.02	0.09	0.19	-7.03	0.01	0.01	2.09	-3.58	0.13	3.76	4.04
11	0.39	-5.87	0.03	0.05	0.31	-6.19	0.02	0.10	0.29	-6.29	0.02	0.10	0.28	-6.32	0.02	0.02	0.66	-5.10	0.04	1.31	1.58
12	0.15	-7.18	0.01	0.02	0.30	-6.20	0.02	0.10	0.24	-6.51	0.02	0.08	0.20	-6.77	0.01	0.01	1.71	-3.67	0.12	3.54	3.76
13	0.15	-6.89	0.01	0.03	0.21	-6.44	0.02	0.09	0.24	-6.25	0.02	0.10	0.18	-6.70	0.01	0.01	2.26	-3.02	0.19	5.57	5.79
14	0.23	-6.90	0.01	0.03	0.17	-7.28	0.01	0.05	0.39	-6.10	0.02	0.11	0.46	-5.86	0.03	0.03	2.22	-3.60	0.12	3.71	3.92
15	0.38	-6.29	0.02	0.04	0.38	-6.30	0.02	0.09	0.45	-6.05	0.02	0.11	0.38	-6.29	0.02	0.02	3.34	-3.17	0.17	5.02	5.28

Conclusion

Based on the monitoring survey of the metal concentrations in the surface sediments of Haqal coastal waters of Saudi Arabia, the values of PERI were categorised as ‘low ecological risk’, thus all sampling sites were unpolluted by heavy metals. Nevertheless this monitoring study showed a positive outcome of non-heavy metal pollution, future mitigation of the heavy metal pollution at the study area should be given priority by the enforcement. Further studies concerning the heavy metal assessment in this region are needed to better understand the accumulation of heavy metals and their transfer in the food web of the living organisms.

Acknowledgements. We would like to express our sincere gratitude to the farms’ owners for facilitating the data collection. This study was supported by the Research Grant (S-1439-0036) provided by The Deanship of Scientific Research, University of Tabuk. This project was also partially supported by the Research Centre, College of Science, King Saud University, Riyadh.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30653075

SIMULATION OF INFLUENCE OF THE TRAFFIC NETWORK UPON THE ENVIRONMENT WITHIN THE TECHNOLOGICALLY LOADED ECOSYSTEM

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(Received 7th Dec 2018; accepted 4th Feb 2019)

Abstract. Traffic networks within megapolises is the factor of acceleration and modernisation of the socio-economic environment. However, there exist a number of problems, which restrict the possibilities of development of a city transport medium. Particularly, road design will only begin when the living environment of urban residents is completed. In this case, formation of the road network and making decisions in respect of its modernisation are performed in accordance with the actual workload of the city network. Authors of this article state that such planning is an inconsistent process, because it cannot predict the decline of the socio-economic environment. The article expressly demonstrates that use of traditional parameters in respect of quality of design of a road network does not comply with the modern requirements to the quality of the socio-economic environment, which must be in line with the already designed road network. Authors of this article believe that it is absolutely necessary to have clear definitions of those criteria, which will be universal ones for design and management of the road network within a modern megapolis. As concerns possible solution of this problem, authors of this article propose to use ecological indicators, as well as content of toxic elements and motor vehicle emissions near the road network.
Keywords: *road network, management, loaded system, socio-economic environment, simulation methods*

Introduction

Influence of the motor transport upon the ecological situation in Republic of Kazakhstan has achieved a critical limit already. Various environmental pollution sources exist, however motor vehicle systems (motor roads and motor vehicles) are the most dangerous ones. Analysis of the previous experience of ecological investigations in the road traffic sphere demonstrates that in many cases these investigations are performed separately for traffic flows and motor roads (Goniewicz et al., 2016). Environmentally destructive influence of the motor vehicle systems is widespread one due to the great mileage and branching of the motor road network, as well as due to high mobility of traffic flows along with the ever-growing quantity of the transportation facilities (Duduta, 2015; Ghahramani and Hemmatyar, 2017). Scales of contamination of the environment are also under the influence of irregularity of motor vehicle traffic flows within the cities, particularly in the places of delays before traffic lights and in the cases of road traffic congestions on the motor roads.

The aim of this investigation is the performance of ecological monitoring in the urban agglomerations to develop the relevant methods to decrease harmful influence of transport upon the environment with the help of the proper strict organisation of the road traffic. The presented research will help to ensure a sufficient level of ecological safety in the urban agglomerations, as well as determine the priority and volume of the environmental protection measures and provision of the ecological monitoring points with software applications, organisation and technical means and facilities for performance of such monitoring. Scientific novelty of the work: a general method has been developed for the integrated assessment of the technogenic impact of a road-transport complex on an ecosystem that considers the state of the ecosystem taking into account all the essential components of the object of study and the relationship between them in the "driver-car-road-environment" system; a number of indicators characterizing the impact of road transport on the environment were identified; the factors and spatial features of the impact of road transport on the environment were identified.

The results of the study can be applied in justification of targeted regional environmental and economic programs aimed at improving the ecological status of the region; in the study of the interaction of road transport systems and the environment in other regions and countries.

Materials and methods

Depending on the goal of an investigation, various methods can be used in order to define the road traffic characteristics: documentary methods, natural full-scale methods, as well as simulation methods (Goniewicz et al., 2016).

Documentary methods are based on the study and analysis of the planning, reporting, statistical materials, as well as design-and-technical materials. In addition, this group of methods includes questionnaire surveys in respect of the traffic flows and passenger flows. Such documentary methods use dependences between traffic volumes and volumes of production, density of population within traffic areas, travel behaviour of population etc. As a rule, these documentary methods are characterized as the labour-intensive techniques, which ensure low accuracy of final results (Kureckova, 2017). Methods of the natural full-scale surveys are based on the performance of direct measurements of the road traffic characteristics in various investigative areas. It is possible to obtain proper information with the help of direct observations or with the help of the automatic registration facilities (Chang et al., 2014).

Such natural full-scale surveys include local, zonal, and regional surveys. Local surveys are performed in order to study intensities, speeds, and composition of flows at the road intersections, as well as at certain sections of roads and streets (Chang, 2011).

Zonal surveys are performed in order to obtain spatial and temporal characteristics within a certain zone. These surveys are performed as the sample surveys. Regional surveys are performed in order to obtain summary values of parameters of traffic flows within a district, city, and region. These parameters are used for predicting trends of changes in the flow characteristics in the course of construction and reconstruction of objects. Advantages of methods of the natural full-scale surveys are connected with their simplicity and high accuracy. Their disadvantages are connected with high labour-

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intensity of these surveys, as well as with the impossibility of utilisation of these methods for the objects that were designed earlier.

In addition, methods of simulation are often used. These methods are based on the use of mathematical and nonmathematical models (physical and analogous models) of changes of the traffic flow parameters. For example, main equation of the transportation flow is derived from the mathematical model, which describes interconnection between intensity, speed, and density of the traffic flow. Simulation methods ensure lower accuracy as compared with the methods of the natural full-scale surveys. However, these methods are simple in the course of their utilisation and they do not require engagement of many recorders. In addition, simulation methods can be applied for the already designed objects (Svensson et al., 2014).

In order to perform investigations of ecosystems, it is necessary to estimate physical level of emissions, degree of influence of motor vehicle flows upon the environment in the specific conditions of certain cities, as well as to define the most essential factors. These estimations and definitions must be made in accordance with the results of the natural full-scale observations (Marisamynathan and Vedagiri, 2018).

These investigations were performed on the basis of the reports on organisation of the traffic scheme of the transportation facilities within a city and its surroundings, as well as on the basis of the results of observations in respect of levels of physical and chemical pollution of the atmospheric air. Experimental investigations have been included the following areas for collection of the initial material: results of measurements of the meteorological data at the regime observation points; information on the nature of the transport and passenger flows; disposition of routes in respect of various tracks, scheme of their disposition on the city map; traffic charts over various routes.

To study the complex dependence of concentration of the summary emissions of the nitrogen dioxide, carbon oxides, sulphur dioxide, and hydrocarbons, the quantity and type of the motor vehicles passing through the relevant road intersection (*Fig. 1*, Shymkent, Republic of Kazakhstan) were calculated. And the multiple-factor correlation-and-regression analysis was used.

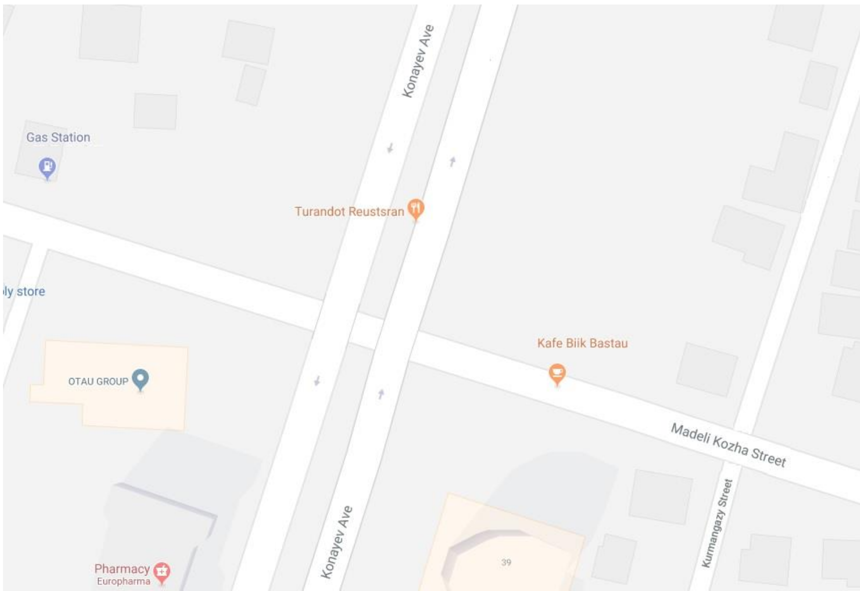

Figure 1. *Investigated road intersection (Shymkent, Republic of Kazakhstan)*

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In order to define characteristics of the motor vehicle flows in respect of the selected section of the certain street-and-road network, the following groups of motor vehicles were recorded (these vehicles moved in both directions):

- Gasoline-powered passenger cars (GPPC);
- Diesel-powered passenger cars (DPPC);
- Gasoline-powered lorries (GPL);
- Diesel-powered lorries (DPL);
- Gasoline-powered buses (GPB);
- Diesel-powered buses (DPB).

Calculation of the transportation facilities, which have been gone through the relevant road intersection, has been performed during 20 min on an hourly basis. In the cases of high traffic intensity (more than 2-3 thousand motor vehicles per hour), calculation of motor vehicles has been performed synchronously and separately in respect of each direction of traffic.

In order to define the maximum traffic load, observations have been performed during peak hours. Two maximums were assumed for the relevant road intersection: morning and evening maximums (7-11 a.m. and 4-7 p.m. respectively). In accordance with the results of this experimental investigation, which are presented in *Table 1*.

Table 1. *Experimental results of the investigation with the purpose to define content of the polluting substances in the atmospheric air at the section 1 in 2017*

Month	DPPC, pcs.	GPPC, pcs.	DPL, pcs.	GPL, pcs.	GPB, pcs.	DPB, pcs.	Total, pcs.	CO, mg/m^3	NO_2 mg/m^3	CH mg/m^3	SO_2 mg/m^3
1	218.952	246.929	57.431	26.742	251.967	125.975	927.996	3.13	0.09	0.025	0.026
2	326.562	312.786	84.329	54.285	311.734	216.853	1,306.549	4.4	0.12	0.03	0.031
3	339.567	324.128	87.532	58.621	362.749	239.524	1,412,121	4.9	0.136	0.04	0.032
4	336.873	368.354	83.218	54.671	372.961	218.496	1,434,573	5.1	0.14	0.039	0.031
5	367.561	389.572	71.643	41.866	343.451	206.781	1,420.874	5.4	0.126	0.039	0.031
6	332.986	378.612	75.328	37.549	327.693	198.669	1,350.837	4.76	0.114	0.037	0.03
7	298.456	369.532	67.589	26.431	263.651	177.533	1,203,192	4.6	0.102	0.027	0.02
8	321.568	384.523	74.873	32.187	311.678	185.462	1,310,291	5.07	0.113	0.03	0.028
9	373.467	386.673	79.003	42.622	312.621	226.548	1,420.934	6	0.13	0.04	0.029
10	327.855	371.220	70.455	39.781	366.720	194.331	1,370,362	5.46	0.12	0.033	0.031
11	341.879	322.853	78.451	51.439	344.112	223.471	1,362,205	4.62	0.128	0.035	0.031
12	210.547	223.731	40.571	22.760	237.622	103.651	838,882	2.4	0.071	0.018	0.023

Results and discussion

Multiple-factor correlation-and-regression analysis

In order to investigate complex dependence of concentration of the summary emissions of the nitrogen dioxide (*Fig. 2*), carbon oxides (*Fig. 3*), sulphur dioxide (*Fig. 4*), and hydrocarbons (*Fig. 5*) from the quantity and type of the motor vehicles, which have gone through the relevant road intersection, we will use the multiple-factor correlation-and-regression analysis. This kind of analysis will make it possible to estimate degree of influence of each parameter upon concentration of the exhaust gases provided that average level of other parameters is fixed. In this case, the following

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DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

condition is important: absence of any functional relationships between these parameters.

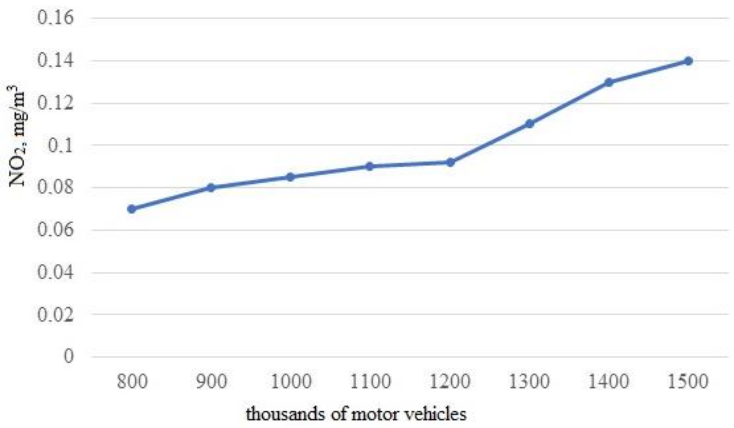

Figure 2. *Graphical dependence of the nitrogen dioxides concentration from quantity of motor vehicles at the experimental area 1 in 2017*

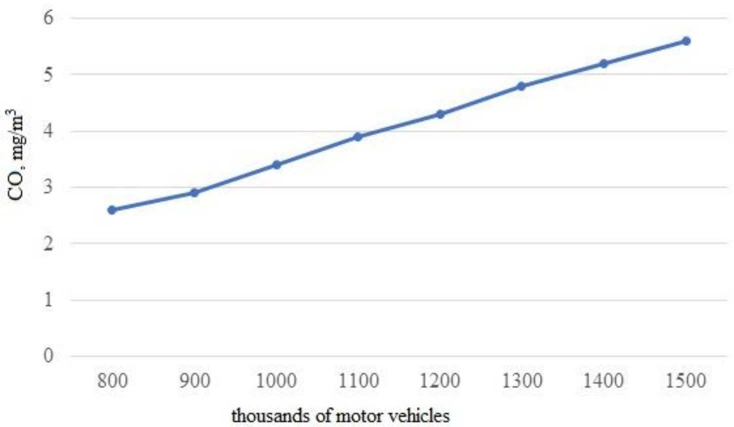

Figure 3. *Graphical dependence of the carbon oxides concentration from quantity of motor vehicles at the experimental area 1 in 2017*

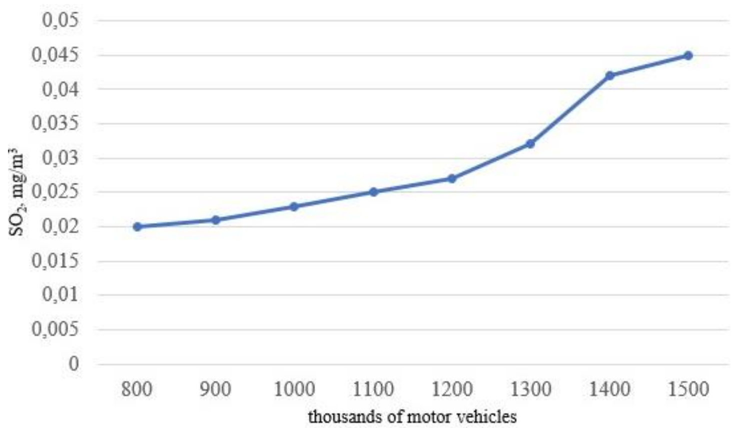

Figure 4. *Graphical dependence of the sulphur dioxides concentration from quantity of motor vehicles at the experimental area 1 in 2017*

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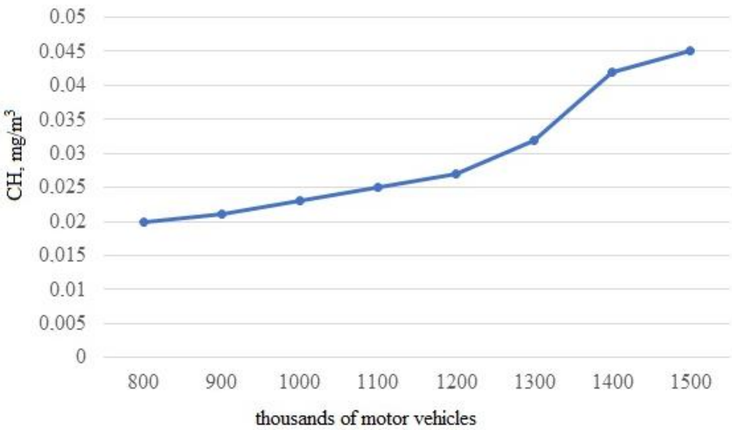

Figure 5. *Graphical dependence of the hydrocarbon concentration from quantity of motor vehicles at the experimental area 1 in 2017*

From a mathematical standpoint, the problem reduces to finding the analytic expression, which would describe relationships of the factorial characteristics with the resulting characteristics in the best way possible, that is, we have to find the following function (*Eq. 1*):

$$y = f(x_1.x_2,...,x_p,a_0,a_1,...,a_p) \qquad \text{(Eq.1)}$$

where $a_0, a_1,..., a_p$ are coefficients of the regression equation, which would ensure the least scatter of empirical values in respect of the surface, which is described by this function.

The most difficult problem is connected with selection of the form of interconnection, selection of the analytic expression. On the basis of this selection, it is possible to define the resulting characteristic, that is, the function, which is connected with the existing factors. This mass function describes actual interconnections between the parameter to be investigated and other relevant factors better than other possible functions. The empirical substantiation of the function type with the help of the graphical analysis of the interconnections for the multiple-factor models is almost impractical. Therefore, it is possible to define proper form of interconnection through the fitting of functions of various types, but it is connected with the great quantity of the unnecessary calculations. Despite of the fact that it is possible to transform any function of multiple variables (through logarithmation or with the help of replacement of variables) to the linear form, it is also possible to present a multiple regression equation in the linear form as follows (*Eq. 2*):

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + ... + a_nx_n \qquad \text{(Eq.2)}$$

In our case, we will assume concentrations of the relevant exhaust gases as the resulting characteristics. In addition, we will assume quantities of the relevant motor vehicles as the factorial characteristics. Therefore, the sought-for equation of the linear six-factorial regression will be presented in the following form (*Eq. 3*):

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 \quad \text{(Eq.3)}$$

where *y* are calculated values of the resulting characteristics – functions (in our case), that is, concentrations of the exhaust gases; x_1, x_2, x_3, x_4, x_5, x_6 are factorial characteristics on the condition that: x_1 is the quantity of the gasoline-powered passenger cars; x_2 is the quantity of the diesel-powered passenger cars; x_3 is the quantity of the gasoline-powered lorries; x_4 is the quantity of the diesel-powered lorries; x_5 is the quantity of the gasoline-powered buses; x_6 is the quantity of the diesel-powered buses; a_0, a_1, a_2, a_3, a_4, a_5, a_6 are equation parameters, which are calculated in accordance with the least square method. Therefore, it is necessary to solve the system of normal equations (*Eq. 4*):

$$\begin{cases} na_0 + a_1\sum x_1 + a_2\sum x_2 + a_3\sum x_3 + a_4\sum x_4 + a_5\sum x_5 + a_6\sum x_6 = \sum y \\ a_0\sum x_1 + a_1\sum x_1^2 + a_2\sum x_1x_2 + a_3\sum x_1x_3 + a_4\sum x_1x_4 + a_5\sum x_1x_5 + a_6\sum x_1x_6 = \sum x_1y \\ a_0\sum x_2 + a_1\sum x_1x_2 + a_2\sum x_2^2 + a_3\sum x_2x_3 + a_4\sum x_4x_4 + a_5\sum x_2x_5 + a_6\sum x_2x_6 = \sum x_2y \\ a_0\sum x_3 + a_1\sum x_1x_3 + a_2\sum x_2x_3 + a_3\sum x_3^2 + a_4\sum x_3x_4 + a_5\sum x_3x_5 + a_6\sum x_3x_6 = \sum x_3y \\ a_0\sum x_4 + a_1\sum x_1x_4 + a_2\sum x_2x_4 + a_3\sum x_3x_4 + a_4\sum x_4^2 + a_5\sum x_4x_5 + a_6\sum x_4x_6 = \sum x_4y \\ a_0\sum x_5 + a_1\sum x_1x_5 + a_2\sum x_2x_5 + a_3\sum x_3x_5 + a_4\sum x_4x_5 + a_5\sum x_5^2 + a_6\sum x_5x_6 = \sum x_5y \\ a_0\sum x_6 + a_1\sum x_1x_6 + a_2\sum x_2x_6 + a_3\sum x_3x_6 + a_4\sum x_4x_6 + a_5\sum x_5x_6 + a_6\sum x_6^2 = \sum x_6y \end{cases} \quad \text{(Eq.4)}$$

Each coefficient of this equation determines degree of influence of the relevant factor upon the resulting parameter on the condition of the fixed values of the rest factors, that is, each coefficient determines degree of change of the resulting parameter in the case of change of a certain factor by one unit. The sample correlation coefficient and sample estimates a_0, a_l, a_2, a_3, a_4, a_5, a_6 of the regression parameters (which were calculated on the basis of the restricted quantity of the experimental data) always contain elements of randomness, and they themselves are random values in essence. In this connection, there occurs a necessity to check meaningfulness of these sample characteristics.

The criterion for testing adequacy of the regression function envisages testing of meaningfulness of the multiple correlation coefficient R. In this connection, the hypothesis “H0: R2 = 0” is proposed in respect of the fact that coefficient of determination R^2 of the general totality (from which this sample was obtained) is equal to zero. This hypothesis is equally matched with the hypothesis H_0: $a_0 = a_l = a_2 = a_3 = a_4 = a_5 = a_6 = 0$ in respect of the fact that none of those factors, which were included to this regression, has no essential (meaningful) influence upon the response *y*. Let us consider the hypothesis H1: $R^2 \neq 0$ as an alternative, that is, situation, when at least one of the regression coefficients $a_i \neq 0$. The following statistics (*Eq. 5*) is used in the course of testing this hypothesis:

$$F = \frac{R^2}{1-R^2} \times \frac{n-p}{p-1} \quad \text{(Eq.5)}$$

where *n* is quantity of those observations, on the basis of which value R has been calculated, while *P* is quantity of the regression parameters. In addition, in the course of

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testing the hypothesis H_0, the right-side critical region $\Omega[f(\alpha; p-1; n-p), \infty)]$ is used, where $F_{кр} = f(\alpha; p-1; n-p)$ is the critical value of α order of the Fisher-Snedecor distribution with (p - 1) and (n - p) degrees of freedom.

Then the right-side critical region is defined by the inequality f > F_{crit}, while acceptance region of the zero hypothesis is defined by the inequality. If the calculated value of the f-statistics exceeds its critical value F_{crit}, that is, f < F_{crit}, then the zero hypothesis H_0 is rejected and the alternative hypothesis H_1 is assumed. In this case, the coefficient of determination R^2 differs from zero essentially, while the regression function is the statistically meaningful function, which describes the original data adequately.

We will estimate degree of influence of those characteristics, which are included in (3), in accordance with the results of testing the hypothesis concerning statistical meaningfulness of the coefficient of the relevant regression equation, which complies with proper characteristics.

With this aim in view, the hypothesis H_0: $a_i = 0$ is proposed in respect of the fact that factor x_i has no essential influence upon the response *y*. In addition, the hypothesis H_1: $a_0 \neq 0$ is proposed as an alternative. In the course of testing of this hypothesis the two-sided region $(|T| \geq t(\frac{\alpha}{2}; n-p))$ is used, where $T = t(\frac{\alpha}{2}; n-p)$ is the critical value of the order $\frac{\alpha}{2}$ of the Student distribution with (n - p) degrees of freedom. If $t_i \leq T_{cr}$, then the hypothesis H_0: $a_i = 0$ is assumed, otherwise it is thought that this coefficient is other than zero.

Concentration of the summary emissions of NO_2, CO, SO_2, CH

Taking into account the fact that volume of the data is a comparatively great one in order to find coefficients of the regression equation, it is possible to apply *Mathlab* application software, because it includes the built-in statistical functions, which make it possible to perform calculations quickly. We will perform these calculations separately for the carbon oxides (CO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and for the hydrocarbons (CH). Results of these calculations are presented in *Table 2*.

Table 2. *Carbon oxides (CO) (2017)*

$3.12524 \cdot 10^{-5}$	$1.58101 \cdot 10^{-5}$	-0.000108872	$1.44834 \cdot 10^{-5}$	$-1.40348 \cdot 10^{-5}$	$6.46997 \cdot 10^{-6}$	4.141839001
$1.58577 \cdot 10^{-5}$	$4.87854 \cdot 10^{-6}$	$3.67525 \cdot 10^{-5}$	$3.16138 \cdot 10^{-5}$	$6.99962 \cdot 10^{-6}$	$9.99331 \cdot 10^{-6}$	1.01508043
0.9542	0.241512841	N/A	N/A	N/A	N/A	N/A
17.36	5	N/A	N/A	N/A	N/A	N/A
6.076024405	0.291642262	N/A	N/A	N/A	N/A	N/A

Therefore, we will obtain the following numerical data (*Eq. 6*):

Coefficients of regression: $a_0 = 4.141839001$; $a_l = 6.46997 \times 10^{-6}$; $a_0 = -1.40348 \times 10^{-5}$; $a_3 = 1.44834 \times 10^{-5}$; $a_4 = -0.000108872$; $a_5 = 1.58101 \times 10^{-5}$; $a_6 = 3.12524 \times 10^{-5}$; coefficient of determination: $R^2 = 0.9542$; calculated value of statistics F = 17.36; quantity of observations n = 12; quantity of the regression parameters with degrees of freedom of the denominator p = 7; quantity of degrees of freedom of the denominator p - 1 = 6; quantity of degrees of freedom of the numerator

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n - p = 5; root-mean-square deviations of the normal random variable εi: SE_{y0} = 1.01508043; SE_{y1} = 9.99331 × 10^{-6}; SE_{y2} = 6.99962 × 10^{-6}; SE_{y3} = 3.16138 × 10^{-5}; SE_{y4} = 3.67525 × 10^{-5}; SE_{y5} = 4.87854 × 10^{-6}; SE_{y6} = 1.58577 × 10^{-5}; dispersion ε: SE_{y2} = 0.241512841; sum of squares that is caused by regression: SS_{regr} = 6.076024405; residual sum of squares: SS_{resid} = 0.291642262.

Equation of regression will be written in the following form:

$$C_{CO} = 4.141839001 + 6.46997 \times 10^{-6} x_1 - 1.40348 \times 10^{-5} x_2 + 1.44834 \times 10^{-5} x_3 - \\ - 0.000108872 x_4 + 1.58101 \times 10^{-5} x_5 + 3.12524 \times 10^{-5} x_6 \quad \text{(Eq.6)}$$

Let us test meaningfulness of the coefficient of determination R^2. Let us test it in accordance with the meaningfulness level α = 0.05. Let us find critical value with the help of tables of F-distribution with quantity of degrees of freedom p - 1 = 6 and n - p = 5, that is, we will have F_{crit} = f(0.05;6;5) = 4.95. We will obtain the calculated value F = 17.36. Let us compare it with the table critical value F_{crit} = f(0.05;6;5) = 4.95 ≤ F = 17.36, then $F_{crit} \leq F$ means that coefficient of determination R^2 differs from zero substantially. Therefore, hypothesis H_0: $a_0 = a_l = a_2 = a_3 = a_4 = a_5 = a_6 = 0$ concerning absence of the regression dependence between variables C_{CO} and x_1, x_2, x_3, x_4, x_5, x_6 is rejected because this fact does not comply with actual data of observations.

Let us estimate meaningfulness of the sample regression coefficients a_0, a_l, a_2, a_3, a_4, a_5, a_6. Let us assume the following level of meaningfulness α = 0.05. Let us calculate the critical value of the Student distribution with n - p = 5 degrees of freedom (of the order α/2 = 0.025), that is, quantity T_{crit} = t(0.025;5) = 2.57. Let us use formula $t = \frac{a_i}{SE_i}$ and calculate value of statistics for all sample regression coefficients a_i. Results are presented in *Table 3*.

Table 3. *Values of regression coefficients for CO*

a_i	a_0	a_1	a_2	a_3	a_4	a_5	a_6
t	4.080306228	0.647429763	-2.005075304	0.458135006	-2.962295561	3.240750483	1.970797859

Not all calculated values exceed their critical values in absolute magnitude. That is, these values are lesser for the coefficients a_1, a_2, a_3, and a_6, that is, the parameters, near which these coefficients stand (namely: quantity of the gasoline-powered passenger cars; quantity of the diesel-powered passenger cars; quantity of the gasoline-powered lorries, and quantity of the diesel-powered buses), do not have any essential influence upon the resulting parameter (upon the concentration of the carbon oxide C_{CO}) (*Table 4*).

Table 4. *Nitrogen oxide (NO_2) (2017)*

-2.10713×10^{-6}	1.10808×10^{-7}	-3.49816×10^{-7}	3.11257×10^{-6}	5.882×10^{-7}	-3.53302×10^{-7}	0.146914115
4.71704×10^{-7}	2.3661×10^{-7}	7.27917×10^{-8}	5.48378×10^{-7}	1.0444×10^{-7}	1.49108×10^{-7}	0.015145818
N/A	0.9146	0.003603566	N/A	N/A	N/A	N/A
N/A	8.9263	5	N/A	N/A	N/A	N/A
N/A	0.000695488	6.49284×10^{-5}	N/A	N/A	N/A	N/A

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DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

Therefore, we will obtain the following numerical data (*Eq. 7*):

Coefficients of regression: $a_0 = 0.146914115$; $a_l = -3.53302 \times 10^{-7}$; $a_2 = 5.882 \times 10^{7}$; $a_3 = -2.10713 \times 10^{-6}$; $a_4 = 3.11257 \times 10^{-6}$; $a_5 = -3.49816 \times 10^{-7}$; $a_6 = 1.10808 \times 10^{-7}$; coefficient of determination: $R^2 = 0.9146$; calculated value of statistics $F = 8.9263$; quantity of observations $n = 12$; quantity of the regression parameters with degrees of freedom of the denominator $p = 7$; quantity of degrees of freedom of the denominator $p - 1 = 6$; quantity of degrees of freedom of the numerator $n - p = 5$; root-mean-square deviations of the normal random variable εi: $SE_{y0} = 0.015145818$; $SE_{y1} = 1.49108 \times 10^{6}$; $SE_{y6} = 2.3661 \times 10^{-7}$; dispersion ε: $SE_{y2} = 0.003603566$; sum of squares that is caused by regression: $SS_{regr} = 0.000695488$; residual sum of squares: $SS_{resid} = 6.49284 \times 10^{-5}$.

Equation of regression will be written in the following form:

$$C_{NO2} = 0.146914115 - 3.53302 \times 10^{-7} x_1 + 5.882 \times 10^{-7} x_2 - 2.10713 \times 10^{-6} x_3 + 3.11257 \times 10^{-6} x_4 - 3.49816 \times 10^{-7} x_5 + 1.10808 \times 10^{-7} x_6 \quad \text{(Eq.7)}$$

Let us test meaningfulness of the coefficient of determination R^2. Let us assume the following level of meaningfulness $\alpha = 0.05$. Let us find critical value with the help of *Table 4* of F-distribution with quantity of degrees of freedom $p - 1 = 6$ and $n - p = 5$ that is, we will have $F_{crit} = f(0.05;6;5) = 4.95$. We will obtain the calculated value $F = 8.9263$. Let us compare it with the table critical value $F_{crit} = f(0.05;6;5) = 4.95 \leq F = 8.9263$, then $F_{crit} \leq F$ means that coefficient of determination R^2 differs from zero substantially. Therefore, hypothesis H_0: $a_0 = a_l = a_2 = a_3 = a_4 = a_5 = a = 0$ concerning absence of the regression dependence between variables C_{NO2} and x_1, x_2, x_3, x_4, x_5, x_6 is rejected because this fact does not comply with actual data of observations.

Let us estimate meaningfulness of the sample regression coefficients a_0, a_l, a_2, a_3, a_4, a_5, a_6. Let us assume the following level of meaningfulness $\alpha = 0.05$. Let us calculate the critical value of the Student distribution with $n - p = 5$ of degrees of freedom (of the order $\alpha/2 = 0.025$), that is, quantity $T_{crit} = t(0.025;5) = 2.57$. Let us use formula $t = \frac{a_i}{SE_i}$ and calculate value SE_i of statistics for all sample regression coefficients a_i. Results are presented in *Table 5*.

Table 5. *Values of regression coefficients for NO_2*

a_i	a_0	a_l	a_2	a_3	a_4	a_5	a_6
t	9.699978901	-2.369433996	5.631947074	-4.46706903	5.675968749	-4.805713687	0.468313082

Not all calculated values exceed their critical values in absolute magnitude. That is, these values are lesser for the coefficients a_1 and a_6, and parameters, near which they stand (namely: quantity of the gasoline-powered passenger cars and quantity of the diesel-powered buses), do not have any essential influence upon the resulting parameter (upon the concentration of the nitrogen dioxide (NO_2)).

Therefore, we will obtain the following numerical data (*Eq. 8*):

Coefficients of regression: $a_0 = 0.039613$; $a_l = -2.03592 \times 10^{-7}$; $a_2 = 7.72122 \times 10^{-8}$; $a_3 = -4.72374 \times 10^{-7}$; $a_4 = 3.05224 \times 10^{-7}$; $a_5 = -1.68538 \times 10^{-8}$; $a_6 = 3.63821 \times 10^{-7}$; coefficient of determination: $R^2 = 0.9609$; calculated value of statistics $F = 20.4988$;

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

quantity of observations n = 12; quantity of the regression parameters with degrees of freedom of the denominator p = 7; quantity of degrees of freedom of the denominator p - 1 = 6; quantity of degrees of freedom of the numerator n - p = 5; root-mean-square deviations of the normal random variable εi: $SE_{y0} = 0.006553$; $SE_{y1} = 6.45084 \times 10^{-8}$; $SE_{y2} = 4.51836 \times 10^{-8}$; $SE_{y3} = 2.04072 \times 10^{-7}$; $SE_{y4} = 2.37244 \times 10^{-7}$; $SE_{y5} = 3.14917 \times 10^{-8}$; $SE_{y6} = 1.02364 \times 10^{-7}$; dispersion ε: $SE_{y2} = 0.001559004$; sum of squares that is caused by regression: $SS_{regr} = 0.000298934$; residual sum of squares: $SS_{resid} = 1.21525 \times 10^{-5}$. Equation of regression will be written in the following form:

$$SO_2 = 0.039613 - 2.03592\times10^{-7}x_1 + 7.72122\times10^{-8}x_2 - 4.72374\times10^{-7}x_3 + \\ + 3.05224\times10^{-7}x_4 - 1.68538\times10^{-8}x_5 + 3.63821\times10^{-7}x_6 \quad \text{(Eq.8)}$$

Let us test meaningfulness of the coefficient of determination R^2. Let us assume the following level of meaningfulness α = 0.05. Let us find critical value with the help of tables of F-distribution with quantity of degrees of freedom p - 1 = 6 and n - p = 5 that is, we will have $F_{crit} = f(0.05;6;5) = 4.95$. We will obtain the calculated value F = 20.4988. Let us compare it with *Table 6* critical value $F_{crit} = f(0.05;6;5) = 4.95 \le F = 20.4988$, then $F_{crit} \le F$ means that coefficient of determination R^2 differs from zero substantially. Therefore, hypothesis H_0: $a_0 = a_l = a_2 = a_3 = a_4 = a_5 = a_6 = 0$ concerning absence of the regression dependence between variables C_{NO} and x_1, x_2, x_3, x_4, x_5, x_6 is rejected because this fact does not comply with actual data of observations.

Table 6. *Sulphur dioxide (SO_2)*

3.63821×10^{-7}	-1.68538×10^{8}	3.05224×10^{7}	-4.72374×10^{-7}	7.72122×10^{-8}	-2.03592×10^{-7}	0.039613
1.02364×10^{-7}	3.14917×10^{-8}	2.37244×10^{-7}	2.04072×10^{-7}	4.51836×10^{-8}	6.45084×10^{-8}	0.006553
0.96094	0.001559004	N/A	N/A	N/A	N/A	N/A
20.4988	5	N/A	N/A	N/A	N/A	N/A
0.000298934	$1.21525\cdot10^{-5}$	N/A	N/A	N/A	N/A	N/A

Let us estimate meaningfulness of the sample regression coefficients a_0, a_l, a_2, a_3, a_4, a_5, a_6. Let us assume the following level of meaningfulness α = 0.05. Let us calculate the critical value of the Student distribution with n - p = 5 of degrees of freedom (of the order α/2 = 0.025), that is, quantity $T_{crit} = t(0.025;5) = 2.57$. Let us use formula $t = \frac{a_i}{SE_i}$ and calculate value of statistics for all sample regression coefficients a_i (*Table 7*).

Table 7. *Values of regression coefficients for SO_2*

a_i	a_0	a_l	a_2	a_3	a_4	a_5	a_6
t	6.045499	-3.156060558	1.708852297	-2.314738779	1.2865452	-0.535182576	3.554182534

Not all calculated values exceed their critical values in absolute magnitude. That is, these values are lesser for the coefficients a_2, a_3, a_4 and a_6, and parameters, near which they stand (namely: quantity of the diesel-powered passenger cars, quantity of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

gasoline-powered lorries, quantity of the diesel-powered lorries, and quantity of the diesel-powered buses), do not have any essential influence upon the resulting parameter (upon the concentration of the sulphur dioxide SO_2).

Therefore, we will obtain the following numerical data (*Eq. 9*):

Coefficients of regression: $a_0 = 0.031884$; $a_l = 2.2113 \times 10^{-7}$; $a_2 = 2.12811 \times 10^{-7}$; $a_3 = -7.6196 \times 10^{-7}$; $a_4 = 1.99258 \times 10^{-6}$; $a_5 = -2.02801 \times 10^{-7}$; $a_6 = -3.85243 \times 10^{-7}$; coefficient of determination: $R^2 = 0.9745$; calculated value of statistics $F = 31.8623$; quantity of observations $n = 12$; quantity of the regression parameters with degrees of freedom of the denominator $p = 7$; quantity of degrees of freedom of the denominator $p - 1 = 6$; quantity of degrees of freedom of the numerator $n - p = 5$; root-mean-square deviations of the normal random variable εi: $SE_{y0} = 0.009114$; $SE_{y1} = 8.97233 \times 10^{-8}$; $SE_{y2} = 6.28449 \times 10^{-8}$; $SE_{y3} = 2.8384 \times 10^{-7}$; $SE_{y4} = 3.29977 \times 10^{-7}$; $SE_{y5} = 4.38011 \times 10^{-8}$; $SE_{y6} = 1.42376 \times 10^{-7}$; dispersion ε: $SE_{y2} = 0.002168383$; sum of squares that is caused by regression: $SS_{regr} = 0.000898877$; residual sum of squares: $SS_{resid} = 2.35094 \times 10^{-5}$. Equation of regression will be written in the following form:

$$C_{CH} = 0.031884 + 2.2113\times10^{-7}x_1 + 2.12811\times10^{-7}x_2 - 7.6196\times10^{-7}x_3 + \\ + 1.99258\times10^{-6}x_4 - 2.02801\times10^{-7}x_5 - 3.85243\times10^{-7}x_6 \quad \text{(Eq.9)}$$

Let us test meaningfulness of the coefficient of determination R^2. Let us assume the following level of meaningfulness $\alpha = 0.05$ Let us find critical value with the help of *Table 8* of F-distribution with quantity of degrees of freedom $p - 1 = 6$ and $n - p = 5$ that is, we will have $F_{crit} = f(0.05;6;5) = 4.95$. We will obtain the calculated value $F = 31.8623$. Let us compare it with *Table 9* critical value $F_{crit} = f(0.05;6;5) = 4.95 \le F = 31.8623$, that is, $F_{crit} \le F$ means that coefficient of determination R^2 differs from zero substantially. Therefore, hypothesis H_0: $a_0 = a_l = a_2 = a_3 = a_4 = a_5 = a_6 = 0$ concerning absence of the regression dependence between variables C_{CH} and x_1, x_2, x_3, x_4, x_5, x_6 is rejected because this fact does not comply with actual data of observations.

Table 8. *Hydrocarbons (CH)*

-3.85243×10^{7}	0.031884	-2.02801×10^{7}	1.99258×10^{6}	-7.6196×10^{7}	2.12811×10^{-7}	2.2113×10^{-7}
1.42376×10^{-7}	0.009114	4.38011×10^{-8}	3.29977×10^{-7}	2.8384×10^{7}	6.28449×10^{-8}	8.97233×10^{-8}
0.9745	N/A	0.002168383	N/A	N/A	N/A	N/A
31.8623	N/A	5	N/A	N/A	N/A	N/A
0.000898877	N/A	$2.35094\cdot10^{-5}$	N/A	N/A	N/A	N/A

Let us estimate meaningfulness of the sample regression coefficients a_0, a_l, a_2, a_3, a_4, a_5, a_6. Let us assume the following level of meaningfulness $\alpha = 0.05$. Let us calculate the critical value of the Student distribution with $n - p = 5$ of degrees of freedom (of the order $\alpha/2 = 0.025$), that is, quantity $T_{crit} = t(0.025;5) = 2.57$. Let us use formula $t = \frac{a_i}{SE_i}$ and calculate value of statistics for all sample regression coefficients a_i (*Table 9*).

The calculated values exceed their critical values in absolute magnitude, that is, all coefficients have essential influence upon the resulting parameter (concentration of hydrocarbons (CH)). In order to ensure formal characterization and development of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

sustainability, it is necessary to define the model of correlation not only in respect of the ecological traffic safety, but also in respect of the possibilities for connection of the ecological safety parameters with the road traffic safety.

Table 9. *Values of regression coefficients for hydrocarbons (CH)*

a_i	a_0	a_1	a_2	a_3	a_4	a_5	a_6
t	3.498505	2.464577608	3.386289809	-2.684472656	6.038556981	-4.630032638	-2.705815029

Influence of the road conditions upon the traffic safety

In accordance with the traffic terminology, "road conditions" term is meant as the totality of geometrical parameters, road service qualities of roads, road pavements, and components of the engineering equipment, which have direct influence upon conditions of traffic (performance of relevant operations by drivers in the course of transportation of passengers and freight).

The above definition does not describe interconnections between the road conditions and traffic safety. At the same time, road conditions (which are the constant component of the complex system – "road conditions-transport flows-environment") have positive or negative influence upon the traffic safety in accordance with the quality state of the road conditions. Therefore, it is necessary to define this term more exactly and we will interpret road conditions as the totality of those factors, which have positive or negative influence upon the road traffic safety of transportation facilities and pedestrians. Such road factors include all the factors that exist on the roads except for the traffic participants and environment conditions.

It is worthy of note that road conditions are not constant/stationary ones in respect of a driver in the course of traffic: they are not changing all the time in themselves, they are changing in a driver's perception in the course of traffic. If a vehicle moves with the speed of 90 km/h (25 m/s), components of road conditions (road signs, guideposts, delineators, traffic dividers), bridges, pedestrian crossings, road intersections, and so on occur and change during several seconds only. During a short period of time, a driver acquires information, analyses it, makes a decision, and performs this decision. In the course of a time, the driver forgets previous information because he/she acquires new information without interruptions.

It is possible to reveal direct influence of the road conditions upon the traffic safety with the help of analysis of the actual mileage distribution of road traffic accidents (RTA) in respect of the road length.

If RTAs are uniformly distributed (without any obvious aggregations) over the length of the road sections that are analysed, then road conditions comply with the requirements of the traffic, which was organised in one way or another, and it is possible to describe these road conditions as the conditions that are supportive to the road traffic safety. If road conditions on a certain road section do not comply with the traffic safety requirements, and there exists an aggregation (concentration) of the RTAs, this fact suggests that there is a negative influence of one road factor or several road factors upon the road traffic safety. In order to ensure the road traffic safety of the on-the-road vehicles and pedestrians on the motor roads in operation, we have to analyse various factors of the road conditions (Dj).

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DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

Only strictly determined factors of the road conditions can be treated as the road conditions of a specific road section.

For example, the following factors can be treated as the road conditions of the one-level interchange with the jughandle left turns: vertical and horizontal road markings of the traffic way, metal traffic dividers, stopping lanes on the shoulders of roads, non-skid properties and friction factor of the traffic way, as well as road signs.

It is possible to describe the entire assembly of the specific road factors (complex of the road factors (K_d)), which are necessary in order to ensure the road traffic safety, with the help of the following formula (*Eq. 10*):

$$K_d = \left\{ D_1, D_2, ..., D_j, ..., D_k \right\}$$
$$j = \overline{1,h} \quad \text{(Eq.10)}$$

where K_d – complex of the road factors, which are conductive to the road traffic safety; Dj – j-th road factor, which has influence upon the road traffic safety.

Let us connect each specific road factor (Dj) with relevant probabilistic event (Aj) and vice versa (*Eq. 11*):

$$D_j \leftrightarrow A_j$$
$$j = \overline{1,h} \quad \text{(Eq.11)}$$

then *Equation 10* (taking into account *Eq. 11*) will be written in the following form (*Eq. 12*):

$$K_d = \left\{ A_1, A_2, ..., A_j, ..., A_k \right\} \quad \text{(Eq.12)}$$

where A_j – j-th probabilistic event, which corresponds to (Dj) road factor.

We will treat a specific road factor on the specific section of a road as the probabilistic event in respect of the road conditions. For example, availability of the road markings of the traffic way, specific road sign, and the speed change lane, that is, availability of the specific kinds of the installed road elements, which are conductive to the road traffic safety.

We will treat each specific section of the roads in operation as the probabilistic object with probabilistic factors. In accordance with the probabilistic point of view, we will understand “observation” as the analysis of the mileage distributions of the RTAs, as well as analysis of the road conditions over the specific sections of the motor roads in operation. Certain road conditions (such as physical reality, for example) contain the probabilistic essence, which is an imperceptible one for a driver in the course of traffic and which is connected with the improvidence of the pre-determined change in the existing road conditions, elements of roads, quality state of roads, in the geometrical dimensions in plan view, as well as in the longitudinal and cross profiles.

In the course of traffic, driver does not estimate length of the straightforward or curved sections, radiuses of bends in plan view and in the longitudinal profile, clear vision distance of the road surface, sight distance to the on-coming motor vehicle, he/she does not estimate width of the traffic way, width of the traffic lanes, tire-to-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

surface friction coefficient of the road pavement, road pavement smoothness and all the rest circumstances. It is this factor that can become the direct or indirect reason of occurrence of the error, which results in the incorrect selection of the traffic condition by the driver in the case of occurrence of a conflict situation that is transformed into an emergency situation and results in occurrence of the RTA.

In the connection with the above-stated conclusions, one may state that there is a necessity in the permanent and targeted monitoring over the state of the road conditions. It is possible to perform such monitoring with the help of results of investigations and analysis of actual distributions of the RTA within the one-level road junctions, including road junctions with the jughandle left turns. Development of the prognostic stochastic models makes it possible to ensure operational finding the places or sections of possible occurrence of various RTAs, estimate existing conditions, and prevent accidents within these sections through the object-oriented improvement of the road conditions, which include the entire complex of interventions into relevant factors, as well as influence upon the traffic flow modes.

As concerns practice of the operational maintenance of roads, the road traffic safety is in direct dependence on the quality state of the road conditions because this state is one of the controllable components of the entire system.

Road junctions impact on the environment

The most difficult conditions for traffic of the transportation facilities occur on the motor roads junctions (Bevrani and Chung, 2012). These road junctions determine traffic capacity of the entire network, and they have influence upon the density and speed of traffic (Cao et al., 2016). Road junctions are the places, where one can always wait for occurrence of traffic congestions, increased risk of accidents, as well as growth of the emission rate of harmful components by motor engines to the environment (Yue et al., 2016).

Road junctions (highway crossings and road junctions) of motor roads are usually designed and constructed in one level or in several levels (Ghahramani and Hemmatyar, 2017). The one-level road junctions are the most dangerous ones. Intensity of traffic on any highway crossing is equal to the sum of intensities on the roads, which joint to the highway (Chaudhary et al., 2017). Therefore, motor roads near a highway crossing have greater loads as compared with other road sections (Cacciabue, 2018). Turns of certain motor vehicles from the right side and (particularly) from the left side create obstacles for traffic of those transportation facilities, which go without changes in the traffic direction (Lo and Tsai, 2009).

As concerns the one-level road junctions, there occur points of crossing, branching, as well as points of influence of the traffic trajectories of the transportation facilities (Tian et al., 2018). A simple four-side highway crossing includes 16 highway crossing points, 8 branching points, and 8 points of influence (Nagler and Ward, 2016). Such points are called the conflict points (Mendoza, 2017). The higher traffic intensity and the greater share of the transportation facilities, which make right and particularly left turns, the higher danger of occurrence of traffic congestions, which ensure growth of the increased pollution of the environment by the components, which are discharged along with the exhaust gases, as well as which ensure propagation of noise and vibrations (Wattana and Nishio, 2017).

As concerns the channelized road junctions, width of the traffic lane on the main road is determined at the level of 3.75 m (Bartoková et al., 2015). The least length of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

such lane in both sides from an interchange on the main road is equal to 150 m if traffic intensity does not exceed 2,000 vehicle/day; 200 m if traffic intensity is equal to from 2,000 to 3,000 vehicle/day; 500 m if traffic intensity is equal to from 3,000 to 4,000 vehicle/day, and 900 m if traffic intensity is more than 4,000 vehicle/day. Width of the traffic lane of the secondary highways at the distance of no lesser than 50 m in both sides from an interchange is determined at the level of no lesser than 7 m for all categories of motor vehicles on the two-lane road. Width of the traffic lane on the ramps/exits of the channelized road junctions is determined at the level of from 4 to 5.8 m depending on the ramp/exit radius, which must be no lesser than 10 m (FISITA, 2011). It is also necessary to strengthen shoulders within the road junctions, while their width is to be defined at the level of 3.75 m for the main road and at the level of 2.5 m for the secondary road. At the same time, it is necessary to construct special speed change lanes (overtaking lanes) being no lesser than 3.75 m in width at all channelized and partially channelized one-level road junctions. It is recommended to design channelized road junctions at the summary consolidated traffic intensity from 2,000 to 8,000 consolidated transportation facilities per day (Duduta, 2015).

It is also recommended to design roundabout (traffic circle) one-level road junctions in the cases, if traffic intensities on the roads, which cross each other, are the same or if difference between these intensities does not exceed 20%, while quantity of flows of motor vehicles within the left-turn flows is equal to no lesser than 40% on both roads. A roundabout (circle) intersection with a big radius of the centre (rotary) island is the most dangerous one as compared with all other kinds of one-level road junctions. All manoeuvres of the transportation facilities on the roundabout road junctions are restricted to inclusion of motor vehicles into the traffic flow and subsequent exit from the flow (traffic convergence and divergence). Therefore, there exist interlacing of flows on the roundabout road junctions, but there are no any highway crossings. However, big quantity of interlacements of the traffic trajectories of the transportation facilities causes decrease of speed as compared with the speed on the approach roads. In addition, great areas are required for construction of the roundabout intersections. The one-level road junctions result in decrease of the traffic speed and traffic capacity of a road, as well as they cause growth of quantity of the road traffic accidents.

The authors have compaired the results, which were obtained in 2018, with relevant results for 2017 (the year, when organisation of traffic was changed). It is possible to state the following: quantity of harmful emissions (carbon oxides (CO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and hydrocarbons (CH)) has decreased at the expense of the changes in the traffic organisation within relevant road intersection, as well as at the expense of decrease in the standing time of motor vehicles on the road intersection, and these factors have caused decrease of emissions of relevant gases. It is obvious from the investigation results that quantity of the gasoline-powered and diesel-powered buses has the biggest influence upon concentration of harmful emissions (this is the case for all groups of harmful emissions). Diesel-powered buses and lorries have essential influence upon emissions of the nitrogen dioxide (NO_2). High value of the correlation coefficient (within the limits from 0.88 to 0.97) confirms the great strength of interconnection between relevant variables.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30773095

Conclusions

Through analyzing the obtained results, it is possible to make the following conclusions:

1. Quantity of the diesel-powered lorries and quantity of the gasoline-powered buses (respectively) have the biggest influence upon concentration of the polluting substances in the atmospheric air. The diesel-powered passenger cars and diesel-powered lorries have somewhat less influence, while quantity of the gasoline-powered buses has almost insufficient influence upon concentration of the polluting substances.

2. All groups of motor vehicles have essential influence on hydrocarbons. The gasoline-powered buses have essential influence on the concentration of the carbon oxides (CO), while diesel-powered buses and lorries have influence on the concentration of the nitrogen dioxide (NO_2).

3. Not all calculated values exceed their critical values in absolute magnitude. That is, these values are lesser for the coefficients a_2, a_3, a_5, a_6, that is, the parameters, near which they stand, do not have any essential influence upon the resulting parameter (upon the concentration of the carbon oxides and concentration of hydrocarbons (CH)). Only quantity of the gasoline-powered passenger cars has essential influence upon this component of the atmosphere pollution, while quantity of the diesel-powered passenger cars has somewhat less influence in respect of this factor.

4. Having analysed the results obtained, the authors have proposed the following changes in organisation of the traffic on the road intersection under consideration: partial traffic decrease at the expense of organisation of the jughandle right turn and installation of additional sections of the traffic light. It was found that quantity of harmful emissions (carbon oxides (CO), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and hydrocarbons (CH)) has decreased at the expense of the changes in the traffic organisation within relevant road intersection, as well as at the expense of decrease in the standing time of motor vehicles on the road intersection, and these factors have caused decrease of emissions of relevant gases.

5. It was found that the development of the prognostic stochastic models makes it possible to ensure operational finding the places or sections of possible occurrence of various RTAs, estimate existing conditions, and prevent accidents within these sections through the object-oriented improvement of the road conditions, which include the entire complex of interventions into relevant factors, as well as influence upon the traffic flow modes.

6. The analysis of the drivers' specific features of perception of the road conditions let us to make the conclusion in respect of the fact that we must treat road conditions as the dangerous ones in the situations, where the sudden change in the traffic modes of the transportation facilities occurs as compared with the previous conditions and when drivers must work with the increased attention. These road conditions cause occurrence of the road traffic situations, which transform into accidents, and the RTA aggregation is observed.

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PATTERNS AND DRIVERS OF SOIL RESPIRATION AND VEGETATION AT DIFFERENT ALTITUDES IN SOUTHERN CHINA

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(Received 29th Aug 2018; accepted 7th Nov 2018)

Abstract. The objective of the present study was to evaluate soil organic carbon stock and mineralization characteristics in forest ecosystems in subtropical China. We explored the factors underlying soil respiration and vegetation in 12 soils at different altitudes in Jiangxi province, Wuyi mountain national nature reserve. Soil organic carbon was applied to analysis field survey and indoor cultivation techniques. Our results showed uneven distribution of four perpendicular bands: meadow grassland, subalpine elfin, mixed coniferous and broad-leaved forests. Differences in total carbon (TC) and total nitrogen (TN) content in the soil were not significant. Soil pH was acidic. The crown density representing relative soil water content suggested fewer differences in low dissolved organic carbon (DOC) with altitude. The soil microbial biomass carbon (MBC) was the lowest in the meadow grassland soil, and in that of the mixed needle leaved forest. The NO_3^--N levels decreased with altitude and the changes in NH_4^+-N were not obvious. The respiratory rate of the meadow grassland soil was the lowest, and the respiratory rate of the subalpine elfin forest was high. Cumulative emissions without soil CO_2 increased with altitude showing regular changes related to vegetation. In mixed needle the soil respiration was significantly stronger than in the meadow grassland and broad-leaved forest soil. Carbon emissions produced by soil respiration and total carbon (TC) and C: N were positively correlated ($P < 0.05$), with no obvious correlation with MBC. The soluble and microbial carbon levels in the soils at different altitudes were positively but not significantly related. Soil pH and TC were negatively correlated with TN and NO_3^--N. The effect of soil total carbon, total nitrogen and crown density partially controlled soil respiration.
Keywords: *Wuyi mountain, altitude, soil organic carbon, soil respiration, vegetation*

Introduction

Climate change is a major environmental challenge. The carbon cycle plays a key role. Soil organic carbon dynamics underlying soil carbon cycle is a hot issue of climate change (Williams et al., 2004). Soil respiration is an important component of terrestrial ecosystem comprising soil and atmospheric CO_2 exchange (Pierce et al., 2004). Global climate change due to increased temperature accelerates mineralization of soil organic carbon (Schlesinger et al., 2000; Zerva et al., 2005). The CO_2 released from soil respiration into the atmosphere further aggravates global warming. Therefore, soil respiration is an important factor underlying global climate change (Schlesinger et al., 1997).

Soil respiration is a complex process, which is affected by a variety of factors including hot soil water, texture, vegetation types, and land use practice (Wang et al., 2005; Maljanen et al., 2006; Yashiro et al., 2008; Yang et al., 2010). Changes in altitude affect vegetation composition and alter the soil environment, resulting in a significant impact on the size and dynamics of soil respiration and sensitivity to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_30973106

temperature (Maljanen et al., 2006; Yashiro et al., 2008). Wuyi mountain national nature reserve is the central Asian tropical forest ecosystem, representing a vertical spectrum of vegetation distribution. It includes subtropical evergreen broad-leaved forest, mixed needle leaf and subalpine elfin forest and alpine meadow. The complete vegetation zone provides convenient conditions to study spatial and temporal heterogeneity of soil respiration. Wuyi mountain is a tourist destination. Human interference has tourists altered the original land use pattern and forest vegetation composition, and accordingly changed the soil carbon composition. Altered dynamics of soil respiration with altitudinal gradient is a key factor in predicting global climate change (Williams et al., 2004; Norby et al., 2001). Current studies mainly focus on differentiation of soil carbon, nitrogen and organic matter, and biodiversity due to the complexity of soil composition of organic carbon and factors influencing diversity (Lu et al., 2012a). Studies reported soil respiration in subalpine thickets of monsoon forest, coniferous and broad-leaved mixed forest soil with altitude (Xu et al., 2015). Studies have also investigated the trends in temperature and moisture levels of broad-leaved Korean pine, spruce and fir forest and *Betula ermanii* in Changbai mountain with changes in altitude (Wang et al., 2003). However, these studies have not systematically analyzed the relationship between altitudinal gradient and soil respiration. Data pertaining to relative changes in dynamic and quantitative description of soil carbon mineralization with altitude are lacking. These studies cannot reflect the dynamic balance in carbon levels between tropical and subtropical biota. In this study, the soil samples had been analysed in Wuyi mountain nature reserve at different altitudes. We measured the soil nutrient and respiration levels in laboratory, to determine the mechanisms underlying altered soil properties and respiration with altitude.

Materials and methods

Study site

This study was conducted in Wuyi mountain county, Jiangxi province national nature reserve, located in the transition zone of the subtropical marine and inland climate, southern China. Local meteorological data (2001-2010) show a mean annual precipitation of 2583 mm and a mean annual temperature of 15 °C, annual average relative air humidity of about 85%. The natural environment is unique, with a complex geological structure, and various types of landforms. Altitude in the area increases from 700 m in the northwest to 2100 m in the southeast. Huanggang mountain at 2160.8 m is the highest peak. Jiangxi province represents the main distribution area of virgin forest, with a wide variety of wild fauna and flora, and lush vegetation. The distribution of vegetation presents a perpendicular band spectrum, with vertical differentiation: evergreen broadleaf forest (EBF), 117°44.183’-117°44.183’E, 27°51.082’-27°51.082’N (altitude of 800-1100 m); coniferous broadleaf forest (CBF), 117°44.806’-117°44.806’E and 27°51.037’-27°51.133’N (altitude of 1100-1500 m); dwarf forest (DF), 117°45.447’-117°45.984’E, 27°50.329’-27°51.038’N (altitude of 1500-1900 m); and alpine meadow (AM), 117°46.214’-117°46.987’E and 27°50.362’-27°51.636’N (altitude above 1900 m), respectively. We tested selected samples representing the four vegetation types, respectively, at 1500 to 1900 m and random samples above 1900 m.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30973106

Experimental design and sampling

Soil samples were obtained in October 2013. In this study, we selected four different vegetation types across an altitude gradient to examine *Rs* variation, in soils of EBF, CBF, DF and AM, respectively. Samples of each vegetation type were tested in triplicate. There were totally twelve 20 × 20 m plots. Five soil sample replicates using a 7.5 cm diameter soil auger were mixed together at a depth of 0-20 cm, sieved using a 2 mm mesh, and stored at 4 °C in a refrigerator and until further laboratory analysis.

Soil respiration and properties

The soil samples were divided into two subsamples. One subsample was used to measure soil respiration using the alkali absorption method. In brief, we initially weighted 30 g of fresh soil and adjusted to 60% of the field water holding capacity (Chen et al., 2004). The adjusted soils were aerobically incubated at 22 °C in a 1L sealed glass jar. Soil respiration was trapped in 0.1 M NaOH and measured with 0.1 M HCl titration after 1, 3, 7, 14, 21, 28, 35, 42, 49 and 56 days. The total soil respiration was estimated by calculating the cumulative production of soil respiration from soils during the incubated 56 days.

Another subsample was used to measure soil properties: total carbon (TC), total nitrogen (TN), organic matter content (OM), dissolved organic matter (DOC), microbial biomass carbon (MBC), soil water content (SM) and pH. Briefly, TC and TN were measured using an elemental analyzer (Isoprime- EuroEA3000, Milan Italy). OM, DOC, MBC, SM and pH were measured by potassium dichromate oxidation-outer heating, high temperature catalytic oxidation, chloroform fumigation extraction, oven drying and potentiometric, respectively (Brookes et al., 1985; Vance et al., 1987).

Data analysis

Statistical analysis was carried out using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). Two-way ANOVA followed by Fisher's least significant difference (LSD) test was used to examine the effect of land use practice, soil depth and their interaction on soil properties. As *Rs* were measured repeatedly across time, we also used repeated measures ANOVA (RMANOVA) to examine variation in *Rs* with time. Simple linear analyses were used to examine the relationship of soil respiration with soil properties.

Results

Plant diversity

Climate conditions affected plant species as a dominant factor of vegetation distribution. Changes in terrain redistribution of precipitation and heat affected plant community and vertical zoning. Elevation was the primary factor controlling the pattern of mountain species distribution. Our results suggested that the distributions of vegetation types varied with altitude (*Table 1*).

Effect of different altitudes on soil physicochemical properties

No significant differences were found among soil TC and TN concentrations along altitudinal gradient (*Table 2*). The soil in the alpine meadow was acidic, but had a

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significantly higher pH than the dwarf forest ($P < 0.01$). Bulk density in coniferous broadleaf forest was significantly lower than that in alpine meadow ($P < 0.01$). Forests in lower altitudes, including evergreen and coniferous broadleaf forests, had significantly higher crown densities than the other two vegetations in higher altitudes ($P < 0.01$). Soil moisture in evergreen and coniferous broadleaf forest was significantly lower than that in the other two vegetations (*Table 2*). In addition, there was an increasing trend in soil moisture along altitudinal gradient. Soil DOC concentrations were 1.693-2.196 mg g^{-1} and increased with increasing altitude. Soil MBC content was the highest in meadow grassland and the lowest in coniferous broadleaf forest. The NO_3^--N content decreased with an increase in altitude.

Table 1. *The distributions of vegetation types varied with altitude*

Altitude	Species	Vegetation types
1900 m	*Calamagrostis brachytricha*, *Stranvaesia davidiana*, *Berberis julianae*, *Rhododendron fortune*, *Pyrus calleryana*, *Pinus tanwanensis*, *Rhododendron latoucheae*, *Rhododendri Daurici*, *Symplocos paniculata*, *Clethra cavaleriei*, *Spiraea chinensis*	Alpine meadow
1500-1900 m	*Symplocos paniculata Cyclobalanopsis multinervis*, *Eurya saxicola*, *Eurya muricata*, *Stewartia sinensis*, *Illicium simonsii*, *Tsuga chinensis*, *Cryptomeria fortune*, *Erythroxylum sinensis*, *Toxicodendron succedaneum*, *Carpinus viminea*, *Lauraceae. Obtusiloba*, *Greyblue Spicebush*, *Actinidia chinesis*, *Fargesia spathacea*, *Rubus lambertianus*, *Symplocos wikstroemiifolia*, *Eurya loquaiana*, *Camellia fraternal*, *Pieris Formosa*, *Hydrangea chinensis*, *Daphniphyllum macropodum*, *Schima superba*, *Clethra cavaleriei*, *Rhododendri Daurici*, *Rhododendron fortune*, *Rhododendron latoucheae*, *Pinus tanwanensis*	Elfin and coniferous broadleaf forests
1100-1500 m	*Rhododendron ovatum*, *Betula luminifera*, *Liquidambar formosana*, *Glochidion wilsonii*, *Lindera erythrocarpa*, *Indocalamus latifolius*, *Schima superba*, *Camellia fraternal*, *Illicium simonsii*, *Rhododendron latoucheae*, *Cyclobalanopsis multinervis*, *Pinus tanwanensis*, *Symplocos Wikstroemiifolia, Eurya loquaiana*, *Eurya muricata*, *Tsuga chinensis*, *Symplocos chinensis*	Coniferous and coniferous broadleaf forests
800-1100 m	*Castanopsis carlesii*, *Cunninghamia lanceolata*, *Caulis Sargentodoxae*, *Pueraria lobata*, *Oligostachyum oedogonatum*, *Phyllostachys heterocycla*, *Glochidion wilsonii*, *Actinidia chinesis*, *Eurya loquaiana*, *Liquidambar formosana*, *Schima superba*, *Indocalamus latifolius*, *Lindera erythrocarpa*, *Cryptomeria fortunei*	Evergreen broad-leaved forests

Table 2. *Basic properties of soil along an altitudinal gradient*

Vegetation types	TC%	TN%	pH	BD g.cm^{-3}	Canopy density %	Moisture %
Alpine meadow	6.25±0.87a	0.46±0.05a	5.13±0.13a	0.99±0.03a	15±8c	65.74±1.97a
Dwarf forest	8.56±0.90a	0.57±0.04a	4.70±0.17b	0.89±0.04ab	50±11b	57.79±2.16b
Coniferous broadleaf forest	9.20±1.94a	0.54±0.10a	4.85±0.12ab	0.87±0.05b	85±3a	44.47±1.92c
Evergreen broadleaf forest	7.72±1.36a	0.58±0.12a	4.88±0.11ab	0.93±0.02ab	90±0a	37.45±3.09c

TC: total soil C concentrations; TN: total soil N concentrations; BD: bulk density. Differences are significant when lowercase letters are different

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Effect of different altitudes on soil efflux

Soil respiratory rate

The patterns of soil respiratory rate were similar among different vegetations, with a decrease with incubation time (*Fig. 1a*). The respiratory rate sharply decreased after 7 days of incubation time and kept stable from the 14th days (*Fig. 1a*). It was the lowest in alpine meadow and highest in dwarf forest. Soil respiration in broad-leaved and coniferous broadleaf forests decreased faster after cultivation for 14 days. After 14 days, it decreased as follows: subalpine elfin forest > coniferous broadleaf forest > needle forest > meadow grassland. The soil respiration rate in subalpine elfin forest was significantly higher than that in meadow grassland ($P < 0.01$).

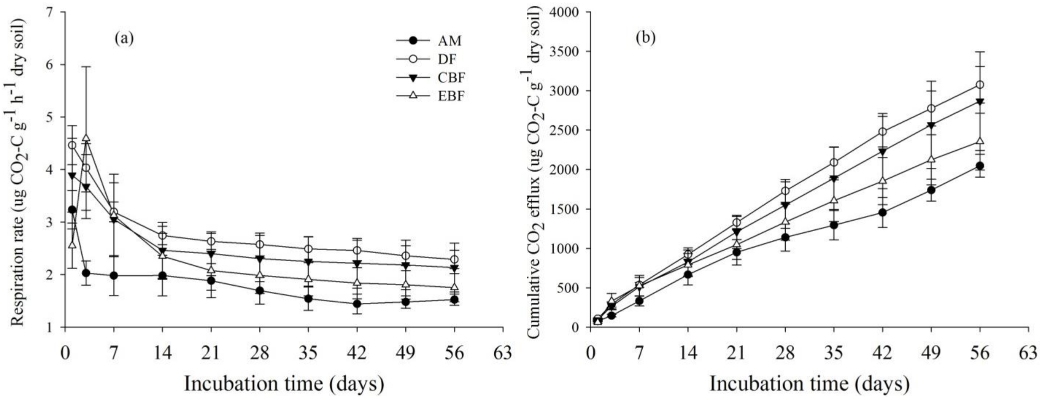

Figure 1. *Changes in (a) the rate of respiration, and (b) cumulative CO_2 efflux during 56-day incubation in soils of different treatments. The abbreviations of vegetation types are the same as Figure 1*

Soil cumulative CO_2 efflux

The soil respiratory emissions of CO_2 rapidly increased within 7 days of culture. Soil organic matter rapidly decomposed in soils regulated to 60% water holding capacity, due to microbial activity. The soil carbon emissions slowly increased after incubation for 7 days at different altitudes. No significant differences were seen between soil CO_2 accumulation emissions and altitude. However, soil CO_2 emissions were related to vegetation. The soil respiration in coniferous broadleaf forest was significantly stronger than in meadow grassland and broad-leaved forest soil ($P < 0.01$).

Correlation between soil carbon emissions and vegetation types

The vegetation types and soil nutrients were analyzed by principal component analysis (PCA) of soil at different altitudes. Our results showed that the differences in soil physiochemical properties were complex at different altitudes, and the first and second principal components explained 40.95% and 20.27% of the variation, respectively (*Fig. 2*). The soil carbon emissions and TC, and C: N were positively correlated ($P < 0.05$), but not between soil carbon emissions and MBC. No significant difference between DOC and MBC was observed at different altitudes. The soil pH and TC, TN and NO_3^-—N were negatively correlated ($P < 0.05$). In this study, we found that TC, TN, and crown density also played important roles in regulating *Rs* under different altitude gradients.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30973106

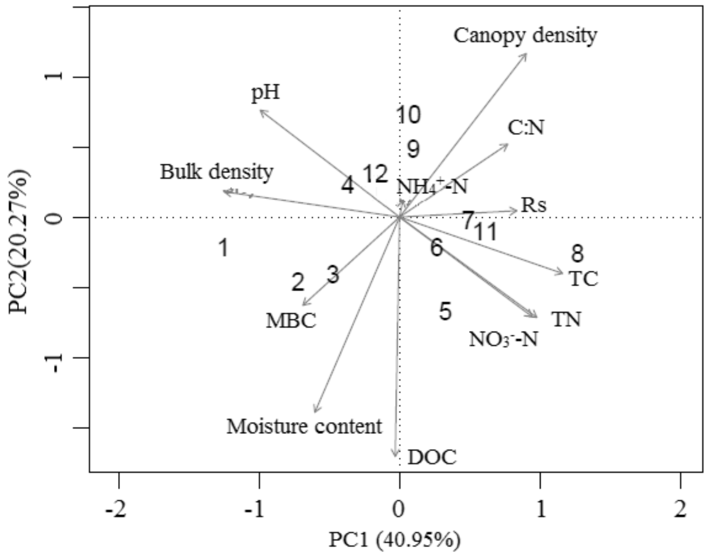

***Figure 2.** Principal component analysis (PCA) of physicochemical properties of soils from forests at different altitudes*

Discussion and conclusion

Vegetation type is heterogeneous in the Nature Reserve, dominated by the evergreen broad-leaf and coniferous broadleaf forests. The variation coefficients of different vegetation types were larger along the altitudinal gradient. The vegetation types along the altitudinal gradient are: artificial vegetation, bamboo forest, evergreen broad-leaved forest, coniferous broadleaf forest, coniferous forest, broadleaf needle elfin wood and mid-mountain meadow. The distribution of various vegetation types overlapped substantially at each altitudinal zone.

Zonal climate condition is the dominant factor of vegetation distributions by affecting plant species compositions. The temperature decreased with increasing altitude. The precipitation, solar radiation and atmospheric composition also changed in altitude. Changes in terrain redistribution of precipitation and heat affected plant community and vertical zoning. Altitude was the primary factor governing the pattern of mountain species distribution. However, the terrestrial factors affecting vegetation type and the contribution of soil nutrients, water availability and altitude alone and their interactive effect on *Rs* variation was still unknown. The zonal climate was the dominant factor for the distribution of plant species within the Wuyi Mountain Nature Reserve (Rouget et al., 2015). A vertical variation in hydrothermal factor along with decreased temperature with increases altitude was observed (Sarrazin et al., 2015). Terrain changes redistribution of precipitation and energy, affecting vegetation and vertical zonal distribution. Altitude is the primary factor controlling the pattern of mountain species distribution (Lou et al., 2000; Dolezal et al., 2002). Our results also demonstrated four perpendicular bands, and lack of typical and alpestrine mountain vegetation perpendicular band. The order of perpendicular bands with increase in altitude included evergreen broad-leaved forest, coniferous broadleaf forest, elfin wood and mid-mountain moss, and mid-mountain meadow vegetation types. Zonal warm coniferous forests were present at 1650-1700 m. The main vegetation types included evergreen broad-leaved and coniferous broadleaf forests, with an uneven distribution,

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and an obvious vertical band with change of altitude in sites. The vegetation was limited by growing conditions above 1900 m. The vegetation was replaced with elfin forest or meadows for hilltop effect, elfin forest and coniferous broadleaf forest at 1500-1900 m and coniferous forest and coniferous broadleaf forest at 1100-1500 m. The hydrothermal condition was mainly observed at 800-1100 m, and the vegetation included evergreen broad-leaved forest.

Soil respiration is one of the important indicators of soil quality and reflects the strength of soil biological activity and metabolism. Soil respiration represents an index of soil biological activity, fertility and permeability (Li et al., 2010). Soil microbial competition for nutrients, however, is likely to drive the microbial community structure and corresponding alteration in nutrient mineralization (Yang et al., 2017). Our study showed that the level of CO_2 emissions due to soil respiration increased rapidly after cultivation for 7 days. Soil microbes were the most active and decomposed soil organic matter when soil moisture was adjusted to 60% water-holding capacity (Lu et al., 2012b). Soil carbon emissions slowly increased after cultivation for 7 days. The difference in soil CO_2 emissions with altitude was not obvious (Bai et al., 2008). However, soil CO_2 emissions were related to vegetation. Soil respiration of coniferous broadleaf forest was significantly stronger than that of meadow grassland and broad-leaved forest ($P < 0.01$), probably due to the microbial activity. There was no obvious influence of soil moisture on soil respiratory. Soil respiratory rate suggested the ability to release CO_2. The soil respiratory rate decreased with altitude (Lu et al., 2011; Gao et al., 2011). Soil respiratory rate decreased sharply within 7 days, and slowed down or remained steady, which was consistent with previous findings (Zhou et al., 2012). Soil respiration rate in meadow grassland was the lowest, while that of the subalpine elfin forest was the highest. Soil respiratory rate of broad-leaved and coniferous broadleaf forests decreased rapidly after cultivation for 14 days. The soil respiratory rate decreased as follows: subalpine elfin forest > coniferous broadleaf forest > needle forest > meadow grassland. The soil respiratory rate of subalpine elfin forest was significantly higher than that of meadow grassland ($P < 0.01$). No significant effect of soil moisture on the respiratory rate was observed, which was inconsistent with previous findings (Liu et al., 2009). These results suggest the combined effect of plant types, microbes and soil moisture. The change of soil moisture was minimal in plant growth, with less effect of soil moisture on the respiratory rate (Fang et al., 2001).

Soluble organic matter had an important effect on soil physicochemical and biological characteristics (Lu et al., 2012b). Soil organic matter is mostly composed of DOC from plant litter (Shen et al., 2015). The ground vegetation types were closely related to decomposition of humus, microbial biomass and root secretion. Our results suggest that soil DOC ranged from 1.693 to 2.196 mg g^{-1}, and gradually increased with increase in altitude, with a small amplitude. Our results were consistent with previous findings in that soil carbon of biological communities was high at low temperature and humidity. Soil organic matter accumulated at high altitude, low temperature and humidity. The organic type, quantity and quality of the soil influenced the properties of MBC. Meadows improved soil granular structure and activity to increase MBC (Wang et al., 2007). Our studies suggest that MBC increases with altitude, resulting in a significant positive correlation between soil organic carbon and MBC. The soil MBC was the highest in meadow grassland and the lowest in coniferous broadleaf forest. NO_3^--N decreased with increased altitude, and the change of NH_4^+-N was not obvious with altitude. DOC and MBC did not show similar correlation, which was consistent

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DOI: http://dx.doi.org/10.15666/aeer/1702_30973106

with Liu's finding (Liu et al., 2012) and inconsistent with Han Lin's results (Han et al., 2010). Plants absorb nutrients from the soil organic matter by microbial decomposition. MBC reflects soil nutrient condition and activity of microorganisms. MBC was an effective index of evaluation of soil fertility and soil quality.

The differences and similarities in vegetation type and soil nutrient comprehensive status were analyzed by PCA of soil physicochemical properties at different altitudes (Wu et al., 2014). In our studies, the difference of soil physicochemical properties varied with altitude. The first and second principal components explain 40.95% and 20.27% of the variation, respectively.

Soil CO_2 emissions were positively correlated with TC, C:N ($P < 0.05$) by PCA (Liu et al., 2009), but not with MBC. There was no significant difference between DOC and MBC. The pH was negatively correlated with TC, TN and NO_3^--N. TC, TN. Crown density had a significant impact on soil respiration, and was used as a regulatory factor of soil respiration. Soil respiration showed significant negative correlation with organic matter and the correlation coefficient was 0.871 ($P < 0.05$) (Zhang et al., 2012). Soil moisture was low, and its effect on soil microbial activity reduced the soil organic carbon mineralization at low altitudes.

Acknowledgements. The work was funded by National Natural Science Foundation of China (31560168, 31660072, 31760136), Jiangxi Province Ministry Education Foundation of China (GJJ160312, GJJ170198). The authors take this opportunity to thank all for support extended for this study.

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DOI: http://dx.doi.org/10.15666/aeer/1702_30973106

SOIL AND PLANT RELATIONS OF SOME *ORNITHOGALUM* (ENDEMIC/NON ENDEMIC) SPECIES

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(Received 31st Oct 2018; accepted 25th Jan 2019)

Abstract. The study was carried out to determine the soil properties of the Ornithogalum L. species in the West Mediterranean Region of Turkey and their effects on plant morphology. Plant and soil samples concerning species *Ornithogalum umbellatum* L., *O. oligophyllum* E.D. Clarke, *O. narbonense* L., *O. pyrenaicum* L., *O. lanceolatum* Labill., *O. pamphylicum* (endemic) O.D. Düşen & Sümbül, *O. montanum* Cirillo, *O. armeniacum* Baker, *O. neurostegium* Boiss. & Blanche, *O. orthophylum* Ten. and *O. chetikianum* (endemic) Uysal, Ertugrul & Dural were collected from 32 different populations between March and June in 2012. The soils of the plant taxa were textured with sandy loam, loam, silty loam, and sandy clay loam; their EC values were in the range 71-741 µmhos cm^{-1}; and their pH values varied between 6.9 and 8.3. The highest organic matter content was in *O. orthophylum*, whereas the lowest value was in *O. chetikianum* (endemic) and *O. pamphylicum* (endemic). According to the means, the highest values of phosphorus, potassium, calcium, and magnesium were determined in the soil samples belonging to *O. lanceolatum* (106.0 mg kg^{-1}), *O. lanceolatum* (657.5 mg kg^{-1}), *O. montanum* (6552 mg kg^{-1}) and *O. pyrenaicum* (746.8 mg kg- 1), respectively.
Keywords: *Ornithogalum, ecology, habitats, soil properties, morphological characters, Turkey*

Introduction

Asparagaceae is a family of flowering plants containing 143 plant genera and 3632 species. The genus *Ornithogalum* L. is in the family *Asparagaceae* in the major group Angiosperms. *Ornithogalum* L. is a perennial plant and is widely distributed in Europe, Asia, Africa and Madagascar. The genus *Ornithogalum* in tribe Ornithogaleae (Caurel) J.C. Manning & Goldblatt of subfamily Ornithogaloideae Speta (Hyacinthaceae *Batsch*) consists of about 200 species of bulbous herbs native to Southern Europe, the Mediterranean basin, and South Africa (Manning et al., 2009; Andric et al., 2015; Aykurt et al., 2016).

The West Mediterranean Region is one of the richest places of Turkey in terms of plant diversity. According to the latest research, there are 54 known *Ornithogalum* species in the world, with 17 of them being endemic to Turkey. The West Mediterranean Region possesses 18 *Ornithogalum* species and 5 of them are endemic to the region (Davis, 1988; Düşen and Deniz, 2005; Uysal et al., 2005; Mutlu and Karakuş, 2012).

Ornithogalum species is used for ornamental or medicinal plant and can also be consumed as vegetable (Kamanestsky and Okubo, 2012). There are a number of studies on the taxonomy, cytology, ecology, seed micromorphology (Goldblatt and Manning, 2011; Yılmaz, G., 2014; Rat et al., 2016; Corominas et al., 2017), and in vitro propagation of *Ornithogalum* (Yanagawa and Ito, 1988; Nayak and Sen, 1995; Ziv and Lilien-Kipnes, 2000; Kariuki and Kako, 2003; Malabadi and van Staden, 2004; Naik and Nayak, 2005; Ozel and Khawar, 2007).

Ornithogalum species in Turkey have distribution from 0 to 2600 m and in the Mediterrenean, Euro Siberian and Iranian Turan phytogeographical region.

To the best of knowledge, there is no study on the natural populations of Ornithogalum with the physical and chemical characteristic concentrations of the soils convenient.

Soil is a key environmental factor for wild plant populations since plant development depends largely on the nutrients of soil. Therefore, soil analyses of wild plant populations may be the means for understanding soil and its possible correlations with plants (Li and Mazza, 1999; Roca-Perez et al., 2004). Several studies showed that microelement contents in plants were affected by not only soil microelement concentrations but also the differences in chemical properties (i.e. pH and carbonate content) of the soil (Brun et al., 1998; Rapp et al., 1999; Saatçi and Yağmur, 2000; Roca-Perez et al., 2004).

Soil is known to have a significant impact on plants. Knowledge of the physical and chemical properties of soil helps to keep and grow plants effectively, and the use of the type of soil required for a plant will be essential for its faster cultivation and development (Reed, 2011).

The main aim of our study was aimed to determine the interactions between soil and plant properties in different *Ornithogalum* species growing in the West Mediterranean Region of Turkey, in order to provide information to the future cultivation studies in Ornithogalum species.

Materials and methods

The study was conducted in 32 populations of 11 Ornithogalum species (*O. umbellatum* L., *O. oligophyllum* E.C. Clarke, *O. narbonense* L., *O. pyrenaicum* L., *O. lanceolatum* Labill., *O. pamphylicum* O.D. Düşen & Sümbül 'endemic', *O. montanum* Cyr., *O. armeniacum* Baker, *O. neurostegium* Boiss. & Blanche, *O. orthophylum* Ten., and *O. chetikianum* Uysal, Ertugrul & Dural 'endemic' located in the West Mediterranean Region of Turkey. Soil samples were collected by means of a garden spade in a depth of 30 cm after removing the surface litter between March and June in 2012. Details of original locations of collected samples are shown in *Figure 1*.

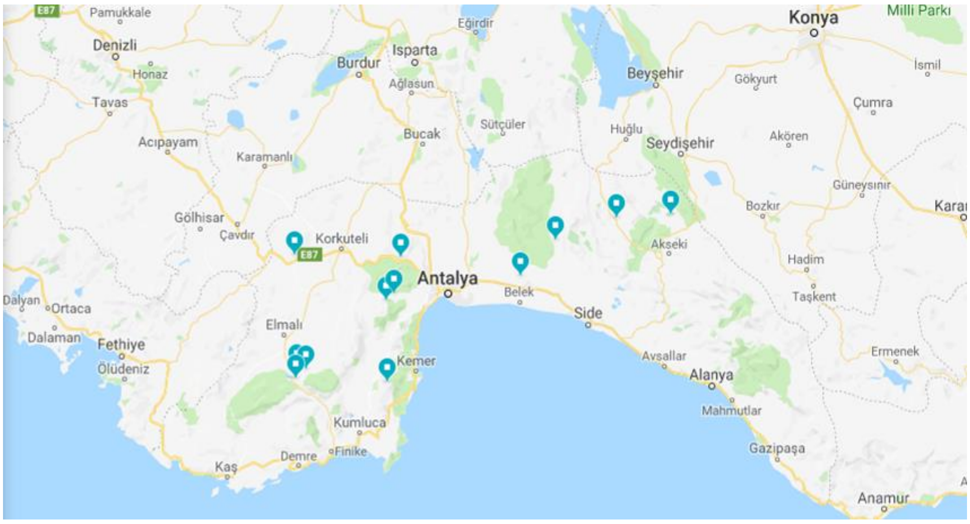

Figure 1. *The location of sampling site of Ornithogalum species*

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Their appearances of flowering stage of the Ornithogalum species are presented in *Figure 2*.

Figure 2. *Some different genotypes of Ornithogalum species plant sampling flowering period.* ***a*** *O. pamphyllicum (endemic),* ***b*** *O. lanceolatum,* ***c*** *O. orthophyllum,* ***d*** *O. pyrenaicum,* ***e*** *O. narborense,* ***f*** *O. montanum,* ***g*** *O. umbellatum,* ***h*** *O. oligophyllum,* ***i*** *O. armeniacum*

At every site, four 1000-g soil samples were collected at three randomly selected points from an area of 25 m^2, placed in clear plastic bags (35 × 25 cm), sealed with a rubber band, and brought to the laboratory. Then, the air-dried soil samples were prepared for the analyses by sifting them through a 2-mm mesh sieve. The analyses were conducted in the soil analysis laboratory of the Batı Akdeniz Agricultural Research Institute (BATEM). The pH (potential of hydrogen-soil reaction) and EC (The Electrical Conductivity) values of the soils were analyzed in a 1:2.5 soil and water mixture, as recommended by Jackson (1967). The organic matter was determined using

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the Walkley-Black method, as suggested by Black (1965). The available P was found with the Olsen method (Olsen and Sommers, 1982). $CaCO_3$ was determined with the Scheibler calcimeter method (Kacar, 2009). The texture of soil was tested with the hydrometer method by Bouyoucos (1951), and the available K, Ca and Mg were found by using 1 N ammonium acetate extraction suggested by Kacar (2009). All the collected data were subjected to the analysis of variance, and the mean values were compared using Duncan's multiple range tests at the 0.05 level (Gomez and Gomez, 1984).

In the study, morphological characters of the plants such as flowering time (day), length of flower stems (mm/plant), bulb diameter (number/plant) and the number of flowers (number) were determined according to Martinez-Azorin et al. (2007). Taxonomic description was made according to Davis (1988). Morphologic characters of the five plants belong to each species were measured in natural habitats when the soil samples were collected. All the measurements were done by a compass and analytical balance.

The number of the soil samples varied from 2 to 6, because the number of location was limited for some species. The number of the soil sample was collected according to the number of location.

Results

Thirty-two soil samples were collected from different geographical sites distributed throughout the West Mediterranean, Antalya between March and June in 2012. *Table 1* presents the soil numbers, localities, habitats, and altitudes, while *Table 2* provides some properties of the soils (texture, pH, EC, CaCO3, organic matter, potassium, phosphorus, calcium and magnesium).

Table 1. *Soil samples, localities, altitudes and habitats of the Ornithogalum species*

Species	Soil number	Date of collection	Locality	Coordinates	Altitude (m)	Habitat
O. pamphylicum	1	17.04.2012	The Feslikan Plateau Sakarpınarı	N 36.50492 E 30.24494	1787	Stony, rocky, and limestone slopes
	2	12.04.2012	Çığlıkara Forest, Elmalı	N 36.52530 E 29.82927	1782	Stony and rocky areas
O. lanceolatum	3	14.04.2012	Sedir Research Forest Road, Elmalı	N 36.35215 E 30.01264	1593	Ground water withdrawal, meadows
	4	10.04.2012	Sedir Research Forest, Elmalı	N 36.36023 E 29.57353	1350	Rocky slopes
O. orthophyllum	5	14.04.2012	Entrance of the Yenice Village, Old Korkuteli Road	N 37.00433 E 30.29021	402	Road side and the rocky and limestone area
	6	20.03.2012	Akdeniz University Health Sciences School	N 36.53497 E 30.39151	35	Road side and macquis groves
O. armeniacum	7	09.04.2012	The Çakıllı Passage, Cevizli, Akseki	N 37.10287 E 37.04368	1210	Macquis groves as well as Rocky and limestone areas

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	8	21.04.2012	The Ormana Village, İbradı	N 37.04368 E 31.35383	978	Rocky and limestone areas
	9	22.05.2012	Köprülü Canyon, Manavgat	N 37.14490 E 31.06435	1468	Rocky areas
	10	14.04.2012	Sedir Research Forest, Elmalı	N 36.36023 E 29.57353	1089	Macquis groves and stony area
	11	01.05.2012	The Serinyaka Village, Gündoğmuş	N 36.47351 E 31.49572	620	Stony and rocky slopes
	12	15.04.2012	Hisarönü, İbradı	N 37.05503 E 31.36025	1075	Rocky and humid area
O. neurostegium	13	14.04.2012	6 km from Korkuteli to Kızılcadağ	N 37.01023 E 29.58256	663	Limestone and stony slopes
	14	14.04.2012	Between Korkuteli and Elmalı Road	N 36.480172 E 30.007571	1231	Road side and open field
O. umbellatum	15	21.04.2012	The Burmahancı Village, Serik	N 36.56288 E 31.03501	15	Humid meadows
	16	09.04.2012	Akseki Ömer Duruk Facility	N 36.998694 E 31.759739	1100	Road side humid area
O. montanum	17	14.04.2012	Sedir Research Forest Road, Elmalı	N 36.35285 E 29.58350	1273	Macquis groves and limestone and stony area
	18	14.04.2012	Between Elmalı and Finike Road	N 40.46678 E 36.23239	849	Rocky and stony area as well as under the Juniperus
O. chetikianum	19	24.05.2012	The Feslikan Plateau	N 36.48588 E 30.22266	2016	Road side, slopes as well as sandy and open area
	20	15.05.2012	Above Feslikan Plateau, Sakarpınarı	N 36.49413 E 30.24328	1850	Open slopes
O. pyrenaicum	21	14.04.2012	The Göltarla Village, Elmalı	N 36.33197 E 29.58208	1172	Slope and macquis groves
	22	20.04.2012	Çıralı Kemer	N 40.21440 E 36.27147	10	Sandy and macquis groves
	23	17.04.2012	Mt. Tahtalı, Tekirova	N 36.32274 E 30.25091	649	Road side and under the pine forest
	24	14.04.2012	Yukarı Karaman, Korkuteli	N 37.06781 E 30.04358	1500	Field side
O. narbonense	25	17.04.2012	Entrance of the Altınbeşik Cave, Manavgat	N 37.02188 E 31.37312	645	Rocky and limestone slopes
	26	09.04.2012	The Çakıllı Passage, Cevizli, Akseki	N 37.10287 E 31.47583	1210	Roadside, humid, limestone area
	27	20.04.2012	The Ovacık Plateau, Kemer	N 36.38153 E 30.25331	1080	Open flat meadows

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	28	24.05.2012	Mountain Tahtalı, Tekirova	N 36.32274 E 30.25091	645	Road side and under the pine forest
	29	14.05.2012	Akdeniz University Health Sciences School	N 36.53528 E 30.39529	49	Road side and macquis groves
	30	15.05.2012	Yukarı Karaman, Korkuteli	N 37.06781 E 30.04358	1500	Field side
O. oligophyllum	31	15.04.2012	The Gembos Plateau (Karamuklu), İbradı	N 37.12381 E 31.29384	1478	Humid, open slopes
	32	27.04.2012	The Ormana Village, İbradı	N 37.04368 E 31.35383	978	Rocky slopes

Table 2. *Some physical and chemical properties of the soils where the Ornithogalum species were grown*

Species	Soil number	Texture	pH (1:2.5)	EC (µmhos/cm)	CaCO3 (%)	Organic matter (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mgkg^{-1})	Mg (mgkg^{-1})
O. pamphylicum	1	Sandy loam	8.1	210	47.0	4.2	19	85	1.870	689
	2	Sandy loam	8.0	214	52.0	4.5	22	75	1.780	695
O. lanceolatum	3	Loam	7.8	216	1.8	5.9	104	661	4.426	365
	4	Loam	7.8	225	1.9	5.2	108	654	4.342	380
O. orthophyllum	5	Sandy loam	7.8	363	13.8	11.6	89	481	5.089	430
	6	Sandy loam	7.9	380	11.2	10.8	84	456	5.123	445
O. armeniacum	7	Loam	7.9	108	2.6	3.9	5	152	4.032	327
	8	Loam	8.0	104	2.6	4.8	53	205	5.880	112
	9	Sandy loam	6.9	741	1.8	13.2	211	799	4.241	1.232
	10	Loam	7.6	196	5.4	5.1	21	515	9.030	226
	11	Sandy loam	8.3	96	2.0	2.1	3.2	62	2.320	93
	12	Loam	7.9	147	23.1	4.8	53	205	5.880	112
O. neurostegium	13	Sandy loam	8.2	187	4.1	4.1	24	436	5.698	542
	14	Loam	7.8	419	16.4	12.6	118	607	4.978	391
O. umbellatum	15	Sandy loam	8.2	256	33.3	1.8	16	178	2.899	265
	16	Loam	7.6	310	10.2	7.6	145	718	5.429	336
O. montanum	17	Loam	7.8	419	16.4	12.6	118	607	4.978	391
	18	Loam	7.8	142	10.0	1.6	28	275	8.127	210
O. chetikianum	19	Sandy	8.0	187	2.6	2.4	16	92	4.468	391

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		loam								
	20	Sandy loam	8.1	212	2.8	2.9	25	100	4.325	386
O. pyrenaicum	21	Silty loam	7.9	196	1.8	8.3	25	453	4.735	420
	22	Loam	7.1	143	1.7	10	7	178	3.381	1.690
	23	Silty loam	7.9	120	2.3	4.3	7	257	8.190	470
	24	Silty loam	7.9	108	2.9	1.4	40	723	7.878	407
O. narbonense	25	Sandy loam	7.3	685	5.2	13.1	111	276	4.736	381
	26	Loam	7.3	71	2.3	3.9	5	152	4.032	327
	27	Loam	7.3	78	2.4	3.6	8	165	4.535	342
	28	Sandy clay	7.9	120	2.3	4.3	7	257	8.195	470
	29	Sandy loam	8.1	160	22.2	3.6	13	183	4.970	427
	30	Silty loam	7.9	108	2.9	1.2	40	723	7.880	407
O. oligophyllum	31	Sandy loam	7.2	709	2.3	13.3	48	288	5.020	515
	32	Sandy clay	8.0	104	2.6	4.8	53	205	5.880	112

Ornithogalums are usually grown in open meadows, roadside or limestone rocky slopes. Their altitude ranges from 15 m to 2016 m. The flowers are generally starched, petals are green striped and there are 2-20 flowers. Flower stem lengths vary between 2-45 cm. Leaves are thin or long strip, linear or thick-fleshy, with bright dark green or matte gray-green colors, bulbs of different sizes (small to medium-large) are round or ovoid (*Table 3*).

***Table 3**. Morphological characters of natural Ornithogalum species*

Species	Bulb size-average bulb diameter (mm)	Leaf characteristics	Average number of flowers per inflorescence (number)	Stem length (mm/plant)	Flowering time
O. pamphylicum	Small, long-ovoid-6.5	Thin long strip	2-2.5	70-80	Mid May
O. lanceolatum	Big ovoid-26.7	Thick fleshy bright dark green	2	20-30	Mid April
O. orthophyllum	Small round-10.8	Quite thin	2.67-3.00	50-100	March
O. armeniacum	Medium round-17.0	Thin	4.5-6.00	100-250	April-early May
O. neurostegium	Medium long ovoid-18.7	Thin	5.63-6.63	200-250	Mid May
O. umbellatum	Numerous number of tooth shaped bulblet, Medium Round-18.3	Thin	12.00-18.00	100-200	Mid March

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O. montanum	Big round-24.3	Thick fleshy matte gray-green	9.79-13.00	100-250	End of April
O. chetikianum	Numerous bulblet, Medium, Round-18.8	Thick	7.17-9.13	200-300	Early May
O. pyrenaicum	Small ovoid-17.2	Thin long strip	17.93-21.75	300-450	Mid May-end of May
O. narbonense	Big round-22.8	Thin linear matte gray-green	52.00-57.33	250-350	End of April-end of May
O. oligophyllum	Small round-12.7	Thick	8.00-9.25	100-150	Mid March-end of March

Statistical analyses of the results for the soil analyses and the soil classes of the *Ornithogalum* species are shown in *Tables 4* and *5,* respectively.

The soil analyses results are also shown in graphs (*Fig. 3a-h*). Some evaluations and interpretations of the soil analysis results are provided in detail below.

Table 4. *Results of the statistical analyses (Mean ± SE and minimum–maximum) on the soils of Ornithogalum species*

Species	N	pH (1:2.5)	EC (µmhos/cm)	$CaCO_3$ (%)	Organic matter %	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})
		Mean±SE (Min.-Max.)							
O. pamphylicum	2	8.1^{ns}±0.1 (8.0-8.1)	212.0^{ns}±2.0 (210.0-214.0)	49.5a**±2.5 (47.0-52.0)	4.4^{ns}±0.2 (4.2-4.5)	20.5^{ns}±1.5 (19.0-22.0)	80.0b**±5.0 (75.0-85.0)	1825.0b**±45.0 (1780.0-1870.0)	692.0^{ns}±3.0 (695.0-689.0)
O. lanceolatum	2	7.8±0.0 (7.7-7.8)	220.5±4.5 (216.0-225.0)	1.9c±0.1 (1.8-1.9)	5.6±0.4 (5.2-5.9)	106.0±2.0 (104.0-108.0)	657.5a±3.5 (654.0-661.0)	4384.0ab±42.0 (4342.0-4426.0)	372.5±7.5 (365.0-380.0)
O. ortophyllum	2	7.9±0.1 (7.8-7.9)	371.5±8.5 (363.0-380.0)	12.5bc±1.3 (11.2-13.8)	11.2±0.4 (10.8-11.6)	86.5±2.5 (84.0-89.0)	468.5ab±12.5 (456.0-481.0)	5106.0ab±17.0 (5089.0-5123.0)	437.5±7.5 (430.0-445.0)
O. armeniacum	6	7.8±0.2 (6.9-8.3)	232.0±102.9 (96.0-741.0)	6.3c±3.4 (1.8-23.1)	5.7±1.6 (2.1-13.2)	57.7±32.0 (3.2-211.0)	323.0ab±113.8 (62.0-799.0)	5230.5ab±933.7 (2320.0-9030.0)	350.3±180.1 (93.0-1232.0)
O. neurostegium	2	8.0±0.2 (7.8-8.2)	303.0±116.0 (187.0-419.0)	10.3bc±6.2 (4.1-16.4)	8.4±4.3 (4.1-12.6)	71.0±47.0 (24.0-118.0)	521.5ab±85.5 (436.0-607.0)	5338.0ab±360.0 (4978.0-5698.0)	466.5±75.5 (391.0-542.0)
O. umbellatum	2	7.9±0.3 (7.6-8.2)	283.0±27.0 (256.0-310.0)	21.8b±11.6 (10.2-33.3)	4.7±2.9 (1.8-7.6)	80.5±64.5 (16.0-145.0)	448.0ab±270.0 (178.0-718.0)	4164.0ab±1265.0 (2899.0-5429.0)	300.5±35.5 (265.0-336.0)
O. montanum	2	7.8±0.0 (7.7-7.8)	280.5±138.5 (142.0-419.0)	13.2bc±3.2 (10.0-16.4)	7.1±5.5 (1.6-12.6)	73.0±45.0 (28.0-118.0)	441.0ab±166.0 (275.0-607.0)	6552.5a±1574.5 (4978.0-8127.0)	300.5±90.5 (210.0-391.0)
O. chetikianum	2	8.1±0.1 (8.0-8.1)	199.5±12.5 (187.0-212.0)	2.7c±0.1 (2.6-2.8)	2.7b±0.3 (2.4-2.9)	20.5±4.5 (16.0-25.0)	96.0b±4.0 (92.0-100.0)	4396.5ab±71.5 (4325.0-4468.0)	388.5±2.5 (386.0-391.0)
O. pyrenaicum	4	7.7±0.2 (7.1-7.9)	141.8±19.5 (143.0-196.0)	2.2c±0.3 (1.7-2.9)	6.0±1.9 (8.3-10.0)	19.8±8.0 (7.0-40.0)	402.8ab±121.4 (178.0-723.0)	6046.0a±1182.3 (3381.0-8190.0)	746.8±314.7 (407.0-1690.0)
O. narbonense	6	7.6±0.2 (7.3-8.1)	203.7±97.1 (71.0-685.0)	6.2c±3.2 (2.3-22.2)	5.0±1.7 (1.2-13.1)	30.7±16.9 (5.0-111.0)	292.7ab±88.5 (152.0-723.0)	5723.5ab±742.4 (4032.0-8190.0)	392.3±21.9 (327.0-470.0)
O. oligophyllum	2	7.6±0.4 (7.2-8.0)	406.5±302.5 (104.0-709.0)	2.5c±0.2 (2.3-2.6)	9.1±4.3 (4.8-13.3)	50.5±2.5 (48.0-53.0)	246.5ab±41.5 (205.0-288.0)	5450.0ab±430.0 (5020.0-5880.0)	313.5±201.5 (112.0-515.0)
General	32	7.8±0.3 (6.9-8.3)	241.7±39.6 (71.0-741.0)	9.7±2.6 (1.7-52.0)	6.0±0.9 (1.2-13.3)	50.8±10.5 (3.2-211.0)	350.7±52.0 (62.0-799.0)	5135.6±484.9 (1780.0-9030.0)	437.1±68.5 (93.0-1690.0)

Mean seperation within columns by Duncan's multiple range test, at 0.05 level
N.S, *, **, ***: Non significiant or significiant at $P < 0.05$, 0.01 and 0.001 alpha level respectively
[a]Capitals show the comparison between the averages given vertically (along the column)

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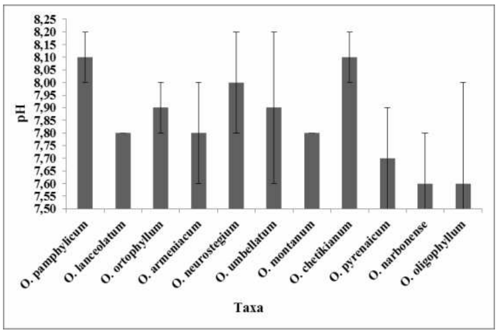
DOI: http://dx.doi.org/10.15666/aeer/1702_31073121

Table 5. *Soil classes of the Ornithogalum species*

Species	pH (1:2.5)	EC (µmhos/cm)	CaCO3 (%)	Organic matter (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg g^{-1})	Mg (mg kg^{-1})
O. pamphylicum	Alkaline	Non-saline	Excess	Medium	Medium-high	Medium	Medium	High
O. lanceolatum	Slightly alkaline	Non-saline	Low	High	High	Very high	High	High
O. ortophyllum	Slightly alkaline	Non-saline	Very high	High	High	Very high	High	High
O. armeniacum	Neutral-alkaline	Non-saline-very slightly saline	Low-excess	Medium-high	Low-high	Low-very high	Medium-high	High
O. neurostegium	Slightly alkaline-alkaline	Non-saline-very slightly saline	Medium-very high	Medium-high	High	Very high	High	High
O. umbellatum	Slightly alkaline-alkaline	Non-saline	Very high-excess	Low-high	Medium-high	Low-very high	High	High
O. montanum	Slightly alkaline	Non-saline-very slightly saline	Very high	Low-high	High	High-very high	High	High
O. chetikianum	Alkaline	Non-saline	Medium	Medium	Medium-high	Low	High	High
O. pyrenaicum	Slightly alkaline-alkaline	Non-saline	Medium	Low-High	Low-high	Low-very high	High	High
O. narbonense	Neutral-alkaline	Non-saline-very slightly saline	Low-excess	Low-high	Low-high	Low-very high	High	High
O. oligophyllum	Slightly alkaline-alkaline	Non-saline-very slightly saline	Low-medium	Medium-high	High	Medium-high	High	High
General (average)	Slightly alkaline	Non-saline	High	High	High	Very high	High	High



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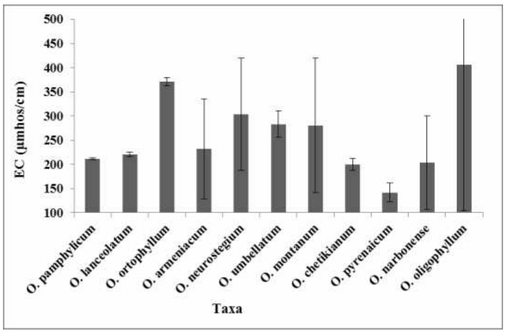

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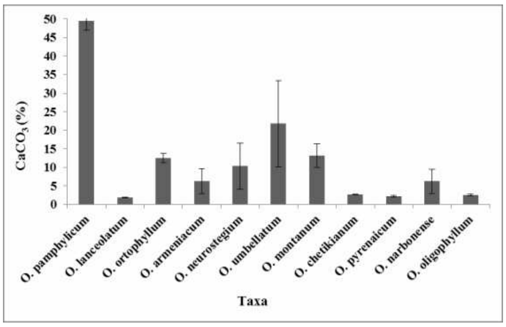

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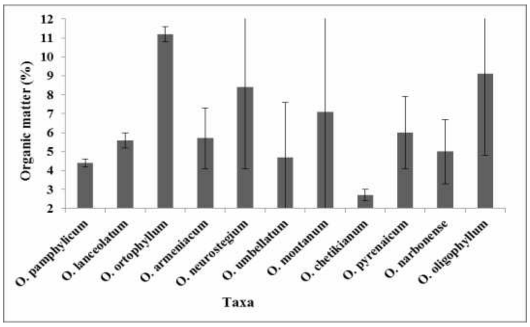

d

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DOI: http://dx.doi.org/10.15666/aeer/1702_31073121

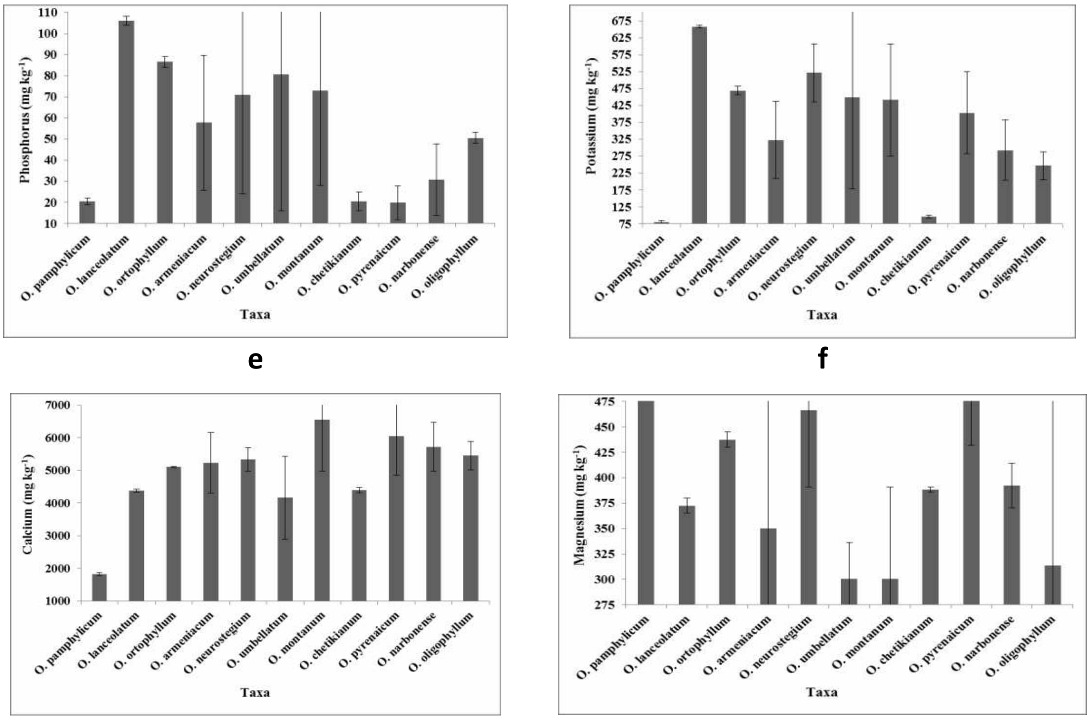

Figure 3. ***a*** *pH values,* ***b*** *EC values,* ***c*** *CaCO3 contents,* ***d*** *organic matter contents,* ***e*** *phosporus contents,* ***f*** *potassium contents,* ***g*** *calcium contents,* ***h*** *magnesium contents*

The results indicated that there were no significant differences in pH, EC (µmhos/cm), organic matter (%), phosphorus (mg kg^{-1}) or magnesium (mg kg^{-1}) values among the statistical averages of the soil samples ($P > 0.05$). Nevertheless, there were significant differences in the $CaCO_3$ (%), potassium (mg kg^{-1}), and calcium (mg kg^{-1}) values ($P < 0.001$) (*Table 4*). Regarding the soil texture, the species generally grew in the medium-textured types of soil. The soil samplesof the species were classified as 'loam', 'sandy loam', and 'silty loam' (*Table 2*). However, Soil Samples 28 (C3: Mt. Tahtalı, Tekirova, Antalya) and 32 (C3: The Ormana Village, İbradı, Antalya) were in the 'sandy clay' soil class. Furthermore, there were no differences in salinity among the values of the statistical test results. All soil samples were classified as 'non-saline' and 'very slightly saline'. An aggregation was present among the soils in terms of salinity. The EC values of the soil samples in the present study were low and varied between 71 and 741 µmhos cm^{-1}, while the highest EC value (741 µmhos cm^{-1}) was in *O. armeniacum* (C3: Mt. Termessos, Antalya) but the lowest EC value (71 µmhos cm^{-1}) in *O. narbonense* (C3: The Çakıllı Passage, Cevizli, Akseki, Antalya). Most *Ornithogalum* species grew in slightly alkaline soils. In the present study, the pH values were in the range 6.9-8.3, and both the highest pH values (C: Mt. Güllük, Termessos, Antalya) and the lowest (C3: The Serinyaka Village, Gündoğmuş, Antalya) pH values were determined in *O. armeniacum.* No significant difference in soil reaction (pH) existed among the species. The results demonstrated that the *Ornithogalum* species grew in soils with low to excess $CaCO_3$ levels. There was a significant difference among the species ($P < 0.05$). In terms of $CaCO_3$, the soils of *O. pamphylicum* (49.5%) were statistically quite different from the others ($P < 0.01$). The averages of the $CaCO_3$ contents of the species were very close to each other. *O. montanum* (13.2%)*, O. neurostegium* (10.3%)*, O. umbellatum* (21.8%) and

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O. o*rthophyllum* (12.5%) grew in soils with a very high CaCO3 content. The soils, where *O. lanceolatum* (1.9%), *O. chetikianum* (2.7%), *O. pyrenaicum* (2.2%) and *O. oligophyllum* (2.5%) are grown had low $CaCO_3$ values (*Table 4*). According to the average $CaCO_3$ values, the soils belonging to *O. pamphylicum* were regarded as excess but the soils belonging to *O. narbonense* as low. In addition, the averages of the other species were in the 'medium' and 'very high' soil classes (*Table 5*). The species grew in soils mostly with high organic matter. There was no significant difference in organic matter among the soils of the species ($P > 0.05$). However, the evaluation of the organic matter contents showed that the soils of *O. oligophllum* and *O. orthophyllum* contained high organic matter. Additionally, the soils of the other species had low to high organic matter contents (*Table 4*), and there were great variations among the organic matter contents (1.2 and 13.3%) of the soil samples studied (*Table 2*), with the highest and the lowest organic matter contents recorded in *O. oligophllum* (C3: The Gembos Plateau, İbradı, Antalya) and *O. narbonense* (C3: Yukarı Karaman, Korkuteli, Antalya), respectively. No differences in phosphorus were present among the averages of the statistical test results. The phosphorus values of the soil samples were in the range 3.2-211.0 mg kg^{-1} (*Table 2*) and high in most of the soil samples (*Table 4*). In addition, both the highest (C3: Mt. Termessos, Antalya) and the lowest (C3: The Serinyaka Village, Gündoğmuş, Antalya) phosphorus values were found in *O. armeniacum* and the phosphorus values showed great variations (*Tables 1* and *2*). The *Ornithogalum* species grew in soils with high concentrations of potassium. According to the statistical tests, there was a significant difference in potassium among the species ($P < 0.05$). According to the values, the species were included in various classes. For instance, potassium was medium and low in the soils of *O. pamphylicum* and *O. chetikianum,* whereas the soils of *O. lanceolatum* (657.5 mg kg^{-1}) had the highest potassium values (*Tables 3* and *4*). The *Ornithogalum* species grew in soils with high concentrations of calcium. As the statistical tests showed, a significant difference in calcium was present among the species ($P < 0.05$). In terms of calcium, the soils of *O. montanum* (6552 mg kg^{-1}) and *O. pyrenaicum* (6046 mg kg^{-1}) were statistically quite different from those of the others. The averages of the calcium contents of the species were very close to each other, and the soil of *O. pamphylicum* (1825 mg kg^{-1}) had a medium concentration of calcium (*Table 4*). The *Ornithogalum* species grew in soils with high concentrations of magnesium. There was no significant difference in magnesium among the species according to the statistical tests ($P > 0.05$). As a result of the evaluation of the averages, magnesium was found high in most of the species (*Table 5*). Moreover, the highest and the lowest averages of magnesium were determined in *O. pyrenaicum* (746.8 mg kg^{-1}) and *O. umbellatum* (300.5 mg kg^{-1}), respectively (*Table 4*).

Discussion

As a result, physical and chemical properties of the soil of natural Ornithogalum species have been determined, and the relationship between the plant and soil was evaluated in the present study. It is thought that the results will provide significant contributions to the cultivation studies regarding of Ornithogalum in its natural habitat.

The soil samples of the Ornithogalum species were mostly medium-textured (classified as 'loam' and 'sandy loam). Their salt contents were very low (non-saline); they were generally slightly alkaline; and their $CaCO_3$ values ranged from low to excess. The organic matter contents showed great variability in the types of locality and varied

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DOI: http://dx.doi.org/10.15666/aeer/1702_31073121

between the ‘low’ and ‘high’ classes. Additionally, the P, K, Ca and Mg concentrations of the soil samples were generally high.

Soil texture affects many physical properties of soil such as the water-holding capacity, and the water infiltration rates. Sandy soils generally have high infiltration rates but poor water-holding capacity. All soil samples in the study were medium-textured; therefore, they had poor water infiltration rates and poor water-holding capacity. Soil texture influences the inherent fertility of soil as well. More nutrients can be absorbed by a gram of clay particles than by a gram of sand or silt particles, for the clay particles provide a much greater surface area for adsorption. Soil Samples 23, 24, 28, 30 and 32 had high Ca concentrations (8190, 7878, 8195, 7880, and 5880). According to Havlin et al. (2013), the pH values of soil samples are slightly alkaline. The level of acidity or alkalinity in a soil affects the availability of soil nutrients and the activity of soil micro-organisms and can affect the level of exchangeable nutrients. Soil Sample 9 had a lower pH value. pH affects the availability of nutrients to plants and can therefore be used to determine the production potential of the soil. pH preferences by plants can vary, though for most plants the desirable pH range is 6.0-6.5. At this level, microbial growth was also at the maximum level.

According to Dellavalle (1992), the EC levels become non-saline in the soil samples. Soil Sample 9 with a lower pH value had the highest EC level. Soil salinity affects a plant’s uptake of water and nutrients and limits plant growth. The salt contents of all soil samples analyzed were low; thus, it was hard to evaluate the *Ornithogalum* species in terms of resistance to salt. The resistance of plants to calcium carbonate ($CaCO_3$) is one of the parameters which limit expansion and cultivation of natural plant species. Differences in the $CaCO_3$ contents of the soils where the *Ornithogalum* species were grown were found among the varieties. The $CaCO_3$ contents were high in the soils of the *O. pamphylicum, O. armeniacum, O. umbellatum* and *O. narbonense* varieties, low and medium in the soils of the *O. lanceolatum, O. chetikianum, O. pyrenaicum* and *O. oligophyllum* varieties, and very high in the soils of the *O. orthophylum*, *O. neurostegium* and *O. montanum* varieties.

Organic matter is a source of nutrients for plants besides being effective on the physical and chemical properties of soils. In general, organic matter content of soils that Ornithogalum species are grown was found to be high. The most interesting situation in organic matter content results is that the endemic species have the lowest organic matter content. *O. pamphylicum* (endemic) and *O. chetikianum* (endemic) were found to be grown in soils with low organic matter content, *O. orthophylum* in soils with high organic matter content, *O. umbellatum, O. montanum*, *O. pyrenaicum* and *O. narbonense* in soils with low to high organic matter content, *O. armeniacum*, *O. neurostegium* and *O. oligophyllum* in soils with medium to high organic matter content.

Phosphorus is essential in photosynthesis, and it is involved in energy transfer. It improves root development and rapid growth; encourages blooming; and increases resistance to diseases. An excess amount of phosphorus does not damage plants. Phosphorus also improves microbial activity in the soil. All soil samples had a high P value. Khan et al. (2016) showed that phosphorus significantly affected bulb volume and number of bulblets per plant, except the number of bulbs per plant in *Polianthes tuberosa*. Moreover, high phosphorus supply increased tuber biomass; however, it decreased the number of flowers per plant in Zantedeschia (Scagel and Schreiner, 2006). Furthermore, high phosphorus content reduced the number of flowers. This situation can be explained

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by the fact that phosphorus is extremely important as a structural element of many components, particularly nucleic acids and phospholipids.

Potassium plays a vital role in the physiological and biochemical functions of plants. It is absorbed in large amounts by plants. Moreover, it is used to build proteins; increases resistance to diseases by strengthening stalks and stems; increases the cuticle (waxy layer) to prevent water loss; helps prevent wilting; and enhances fruit size and development. According to Olsen and Sommers (1982), the potassium levels in the soil are adequate for the plant. According to Claassens (1990), the potassium requirements of Ornithogalum are low and the highest potassium levels do not significantly reduce the yield, but low potassium levels may reduce the probability of inconsistent yields. Bulbs of *O. lanceolatum* have a bigger size than the other species's bulbs. In our study, the highest phosphorus and potassium contents were obtained from the soils of the *O. lanceolatum.*

Calcium, which is an essential part of the plant cell wall structure, provides for normal transport and retention of other elements as well as strength in the plant. It is also considered to counteract the effect of alkali salts and organic acids within a plant. Ghoname et al. (2007) reported that calcium treatment to the plant from the soil significantly affected the plant length, fresh and dry weight however, in the vegetative stage, there was no significant effect on the diameter of the bulb. According to the results, *O. pyrenaicum*, *O. narborense*, *O. montanum*, species had the maximum flower stem lengths. Calcium contents of these species were also found to be high.

Magnesium is part of the chlorophyll in all green plants and essential for photosynthesis. It also helps activate many plant enzymes needed for growth. Gerandas and Fuhrs (2013) reported that the degradation of anthocyanins at high temperature decreased by Mg applications in ornamental plants. Anthocyanins are important constituents for specific colouration to flowers in ornamentals. Ornithogalum species are white in color. In this study, there was no significant difference between the Ornithogalum species and Mg contents.

Conclusions

The bulbs of *O. nutans* species are collected from the natural habitat and exported for ornamental and medicinal purposes in Turkey. In some cases, harvesting from nature is illegal for the species; nevertheless, the populations are in decline and are not cultivated. Therefore, cultivation and breeding studies are urgently needed. Natural distribution area should be taken into consideration when *Ornithogalum* sp. is cultivated. Understanding the relationship between certain soil properties and plant relation for Ornithogalum species, which have an economic value, can act as a guide for florists and botanists for a particular purpose.

Acknowledgements. This study was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) (Project number: TOVAG 104 O 327).

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FUSION OF SENTINEL-1A AND LANDSAT-8 IMAGES FOR IMPROVING LAND USE/LAND COVER CLASSIFICATION IN SONGKLA PROVINCE, THAILAND

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(Received 3rd Nov 2018; accepted 12th Feb 2019)

Abstract. The objective of this study is to compare the performance of different data fusion techniques for improving the land use/land cover types classification accuracy in Hat Yai district, Songkla province, Thailand. SAR Sentinel-1A and optical Landsat-8 satellites are used as standalone inputs as well as to perform a data fusion based on the resolution merge and LMVM techniques. The four input datasets are classified with a supervised maximum likelihood algorithm and compared against base land cover maps; the results indicate that resolution merge of optical and SAR satellite images can significantly improve the interpretation and classification accuracy of land cover and land use types at the area of interest.
Keywords: *multi-source data fusion, resolution merge, SAR, optical, LULC*

Introduction

Mapping land use/land cover of large cities is a main component of detecting rapid changes from non-industrial to industrial areas. Hat Yai district hosts the fourth largest city in Thailand undergoing rapid and fundamental landscape changes leading to increased urbanization. Therefore, mapping land use/land cover holds an important role in monitoring and assessing environmental changes, land use planning and decision-making activities (Dengsheng et al., 2012). Remote sensing is an effective technology for analysing land use/land cover change in attributes such as crop, forest and agriculture. South Thailand is located in the tropical climatic zone and is frequented by cloud coverage which is an impediment for passive microwave and optical sensors; active microwave sensors, such as Synthetic Aperture Radar (SAR), on the other hand, are weather independent systems. A combination of radar and optical data integrates complimentary information from two fundamentally different systems and improves land use/land cover classification accuracy (Sukawattanavijit, 2015). Image fusion is an active field of research in remote sensing (for a practical guide the reader is directed to Pohl and Genderen, 2016) and other application areas such as medical imaging and night vision (Li et al., 2017).

Several studies have attempted to fuse satellite data. For instance, Sukawattanavijit (2015) classified land cover in Wang Muang district located at the northern part of Saraburi Province, central Thailand by using RADARSAT-2 and Landsat-8 satellite

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

images with wavelet-based fusion. The results showed that the fused image had higher accuracy compared to the single radar data. Dimov et al. (2016) found that the Ehlers fusion is the best technique when looking at crop areas in Fergana Valley in Uzbekistan. In another study Chen et al. (2017) investigate the performance of multi-source remotely sensed data fusion in the context of improving land cover classification; they found that the fused data, which integrated temporal, spectral, angular, and topographic features, achieved a higher mapping accuracy for land cover classification in comparison to the original data sources. Salman et al. (2017) demonstrated that fused dataset provided accuracy 99.6% of land use/land cover classification using machine vision. Similarly, Sukawattanavijit et al. (2017) suggested that genetic algorithms and Support Vector Machines (SVMs) lead to improve classification of up to 95% of different land cover types when fusing SAR RADARSAT-2 data and optical Thaichote (THEOS) data. In another case study, Puttinaovarat and Horkaew (2017) investigated the extraction of buildings from fused satellite images and on the basis of spectral indices instead of reflectance bands. It is worth noting, however, that there are other studies which mention that combining optical and SAR data does not guarantee improvement compared with using single data source for urban land cover classification (e.g. Zhang and Xu, 2018).

The objective of this study is to evaluate the capability of SAR Sentinal-1, optical Landsat-8 and their fusion resulting from two independent techniques for extracting land use/land cover information via supervised classification in Hat Yai district, Songkla province, Thailand.

Experimental

Study area

Hat Yai district is located in South Thailand, situated at 7°1’N latitude 100°28’E longitude, and encompasses the country’s fourth largest city. It covers a total area of approximately 852.796 km^2 and hosts a population of over 800,000 inhabitants. The climatic conditions of this tropical area are hot and humid. The dry season lasts from January to April and the wet season from May to December. Hat Yai is a commercial district with the predominant activities being tourism, fishing industries, agriculture, rubber and oil palm plantations (Appianing et al., 2016). The study area is depicted in *Figure 1*.

SAR data

Sentinel-1A is a SAR satellite providing medium and high resolution imagery at C-band (centre frequency: 5.405 GHz) under all-weather conditions. The C-SAR is useful for land and marine monitoring (ESA, 2016). In this study, a Sentinel-1A image with VV/VH dual polarization was used. The spatial resolution is 5 × 20 m and was acquired at Level-1 Ground Range Detected (GRD) mode with 250 km swath, multilook intensity only and Interferometric Wide (IW) swath mode. The specifications of Sentinel-1A are presented in *Table 1*.

Optical data

The Landsat-8 Operational Land Imager (OLI) image was acquired on 18th March 2015. Landsat-8 has 11 spectral bands with a spatial resolution of 15, 30 and 100 m for

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DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

the panchromatic, multispectral and thermal bands respectively. The specific image was selected based on the relatively low cloud coverage (<10%). Landsat-8 band specifications are presented in *Table 1*.

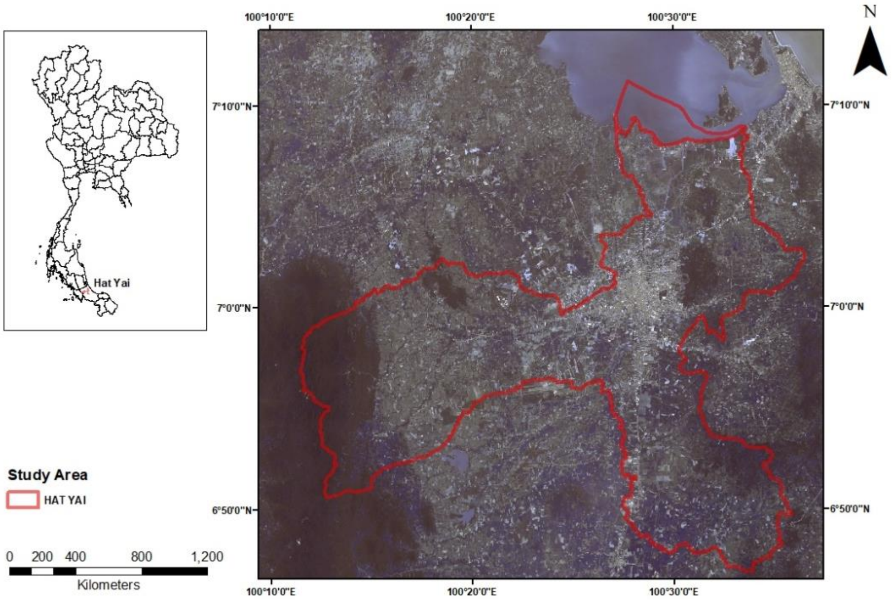

Figure 1. *Map of the location of the study area: Hat Yai district, Songkla province, Thailand*

Table 1. *Sentinel-1A and Landsat-8 OLI image specifications*

Specifications	Sentinel-1A	Landsat-8 OLI
Acquisition date	25th February 2015	18th March 2015
Acquisition time (hh:mm)	23:02 (UTC)	03:33 (UTC)
Imaging mode	IW swath mode	OLI and TIRS
Polarization	VV/VH dual polarization	-
Data product	Level-1 ground range detected mode	Level 1 Terrain
Resolution mode	20 m (full resolution)	30 m (except panchromatic (15 m) and thermal bands (100 m))
Bands	C-band (5.4 GHz)	0.43 - 0.45 (Coastal Aerosol) 0.45 - 0.51 (Blue) 0.53 - 0.59 (Green) 0.64 - 0.67 (Red) 0.85 - 0.88 (Near Infrared NIR) 1.57 - 1.65 (SWIR 1) 2.11 - 2.29 (SWIR 2) 0.50 - 0.68 (Panchromatic) 1.36 - 1.38 (Cirrus) 10.60 - 11.19 (Thermal Infrared - TIRS 1) 11.50 - 12.51 (Thermal Infrared - TIRS 2)

Ground truth data

Auxiliary data were provided by the GEO-Informatics Research Centre for Natural Resources and Environment and the Southern Regional Centre of Geo-Informatics and

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DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

Space Technology, Thailand. *Figure 2* depicts the map produced in 2015 (the same year with the two satellite image acquisitions) encompassing land use/land cover classes of urban area, paddy fields, plantations (fruits, palm oil and rubber), forests and lake. Land cover has been classified using high resolution satellite images and ground data.

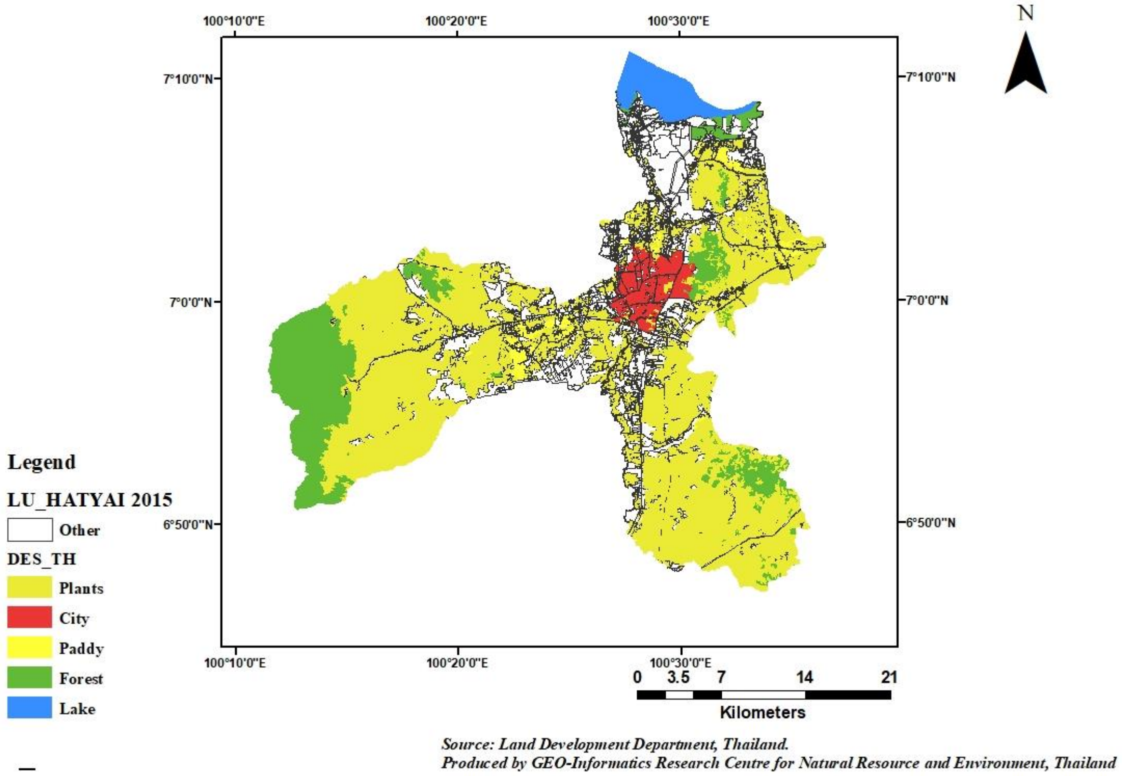

***Figure 2.** The auxiliary data from the Land Development Department, Thailand. (The data were taken with the permission of the Land Development Department, Thailand. Produced by the GEO-Informatics Research Centre for Natural Resources and Environment Southern Regional Centre of Geo-Informatics and Space Technology, Thailand 2015)*

Methodology

Pre-processing

The Sentinel-1A image at Level-1 GRD mode was first calibrated so that the pixel values represent the radar backscatter of the reflecting surface. Subsequently, the multilook of the SAR image was generated by averaging over the range and azimuth resolution cells to improve the radiometric resolution. A GammaMAP 5 × 5 window size was applied to reduce speckle noise. The Shuttle Radar Topography Mission (SRTM) 3sec Digital Elevation Model (DEM) was used for the terrain correction. Finally, the Sentinel-1A backscattered values were converted to decibel (dB) as shown in *Figure 3a*.

The Landsat-8 OLI image was converted from Digital Numbers (DN) to Top-Of-Atmosphere (TOA) reflectance with the ENVI 5.1 software. The Fast Line-of-sight Atmospheric Analysis of Spectral Hypercube (FLAASH) atmospheric correction method was used for retrieving the Top-Of-Canopy (TOC) spectral reflectance from the Landsat-8 OLI image as depicted in *Figure 3b*.

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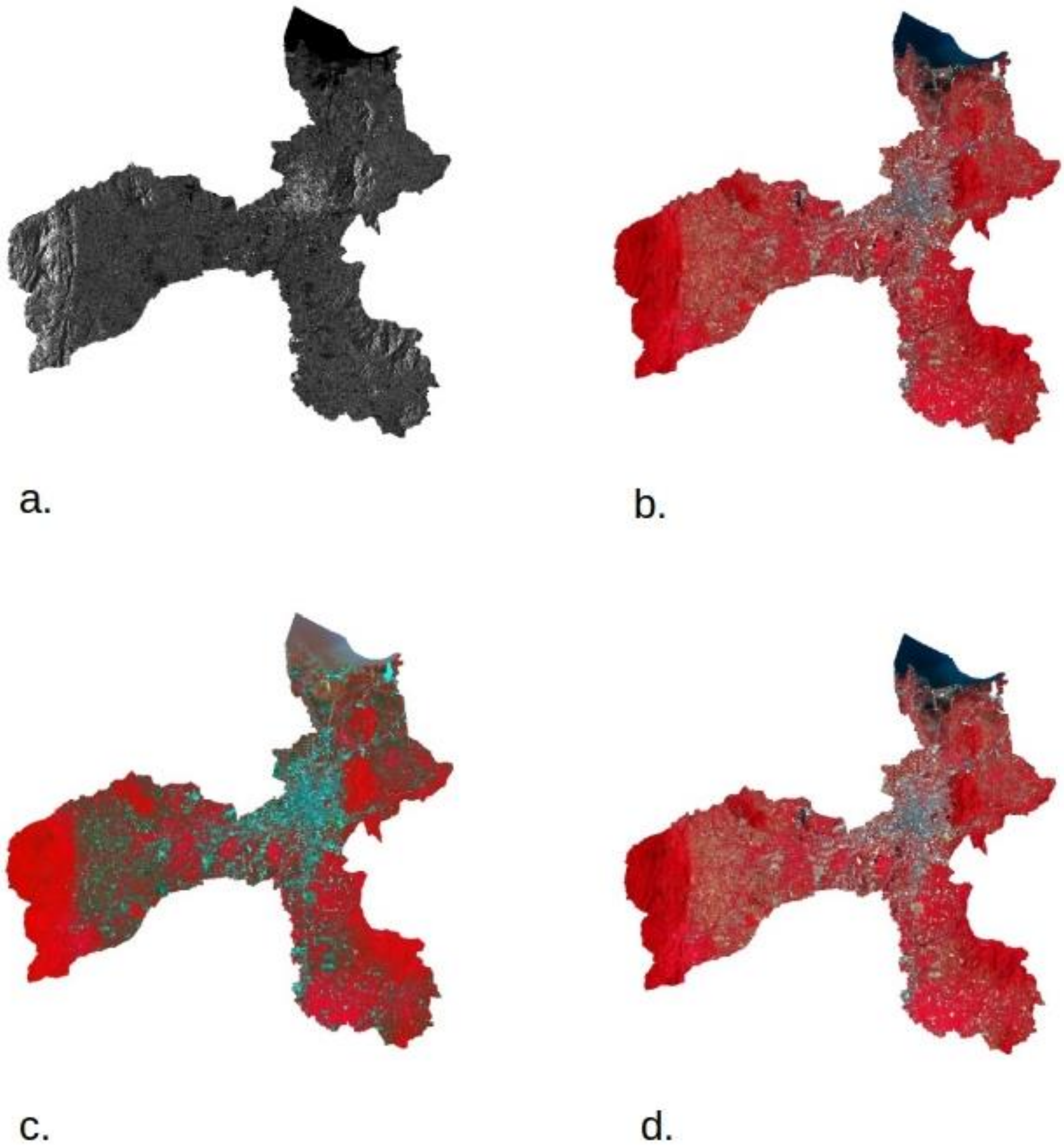

Figure 3. *The four input layers in the classification. a) Sentinel-1A, b) Landsat-8 (5-4-3), c) resolution merge, d) LMVM*

Data fusion

The VV/VH polarized C band Sentinel-1A image and a layer stack of three spectral bands (i.e. 5, 4, 3) from the Landsat-8 image were used for the two fusion methodologies. The Local Mean Variance Matching (LMVM) and the Resolution merge data fusion techniques were applied in this study and the final result is presented in *Figure 3c* and *d*.

The LMVM is a robust algorithm for fusing multispectral and panchromatic images of the same sensor (Nikolakopoulos, 2008). In this study, it was used to integrate the two images (*Eq. 1*), namely a high spatial resolution SAR Sentinel-1 A image with spectral information from a low spatial resolution multispectral Landsat-8 image (Chinsu et al., 2015; Zhang, 2004; Wald et al., 1997; Ranchin and Wald, 2000).

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$$F_{K(i,j)} = P_{(i,j)} \times \frac{\overline{M}_{k(i,j)(w,h)}}{\overline{P}_{(i,j)(w,h)}} \quad \text{(Eq.1)}$$

where:

$F_{K(i,j)}$ = the fused image

$P_{(i,j)}$ = the high spatial resolution images at pixel coordinates (i,j)

$M_{k(i,j)}$ = the low spatial resolution images at pixel coordinates (i,j)

$\overline{M}_{k(i,j)(w,h)}$ = the local means calculated inside the window of size (w,h)

$\overline{P}_{(i,j)(w,h)}$ = the local means calculated inside the window of size (w,h)

The resolution merge combines images of differing spatial resolutions. VV/VH polarized C band Sentinel-1A image and three bands (i.e. 5, 4, 3) of the Landsat-8 multispectral image were merged together into a common image file format. This step prepares the fused multi-sensor data for classifications as in *Figure 3c* (Erdogan et al., 2008; Vaiopoulos et al., 2001).

Image classification

We classified the input layers using the maximum likelihood algorithm in the 5 main land cover / land use classes of the study area; namely forest, paddy rice, plantations, urban, lake, and unclassified. Ground truth and test data were collected from the Land Development Department, Thailand. A cumulatively large training and validation set for each representative class was selected based on the ground truth dataset to train and validate the classifier (*Table 2*).

Table 2. *Training and validation sets size used in the 4 classification scenarios*

Class	Training set (number of pixels)	Validation set (number of pixels)
Lake	1345	920
Forest	569	539
City	264	934
Paddy	1247	464
Plants	232	575

Accuracy assessment

The accuracy of the image classification was assessed against the reference data with a confusion matrix (Congalton, 1991). The main measures of comparison were the overall accuracy and the kappa coefficient of agreement (κ). Overall accuracy is the total number of correctly classified samples (i.e. the diagonal cells in the confusion matrix) divided by the total number of reference pixels. In essence, it presents the probability that a sample will be correctly classified by the algorithm. κ is a measure of the correctly classified samples adjusted to the amount of agreement that could be expected only due to chance. κ utilizes all elements from the confusion matrix by taking into account both errors of commission and omission, and thus provides a more complete picture of the information comprising the confusion matrix than the overall accuracy (Napoleon et al., 2013). The Kappa statistics and the overall accuracy assessment were calculated to compare the accuracy of the classified images.

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Statistical analysis

Two statistical moments, namely the mean and the standard deviation (square root of the variance), are used as evaluation indicators to judge on the ability to use the two fusion image techniques. A low standard deviation is regarded as an indicator of higher class separability (Napoleon et al., 2013).

The mean is obtained by dividing the sum of observed values by the number of observations, N as in *Equation 2:*

$$\bar{X} = \frac{\sum fX}{N} \quad \text{(Eq.2)}$$

where f is the probability of the occurrence of the value of X.

The standard deviation is a measurement of the variation or dispersion from the average. A low standard deviation indicates that the data points tend to be very close to the mean (the so called expected value); a high standard deviation indicates that the data points are spread out over a large range of values. The standard deviation is calculated according to *Equation 3:*

$$Std = \sqrt{\frac{\sum(X - \bar{X})^2}{N}} \quad \text{(Eq.3)}$$

where:
Std is standard deviation,
X is sample,
$\bar{X}$ is sample mean,
N is number of score in sample.

Validation

Validation was engaged to assess the performance of this model. Lu et al. (2016) stated that R^2 is commonly used to validate a model. Two groups of data were chosen, namely observed and predicted. Observed data were derived from land-use ground truth data (Land Development Department, Thailand) and predicted data were obtained from the resolution merge image classification. We selected 5 land use types (city, forest, lake, paddy, plants and others) from observed data and resolution merge image classification for the validation. The linear regression was estimated to validate the model and the reliability was judged upon the high R^2.

Results and discussion

The results for the standalone classification of the Sentinel-1A SAR image indicate an overall accuracy of 58.50% and a kappa coefficient of 0.48 while for the Landsat-8 optical image an overall accuracy of 67.16% and a kappa coefficient of 0.59 is observed (*Table 3*). The individual SAR image is able to clearly extract the water class (i.e. lake). However, the extraction of other classes is not so accurate; especially at the hilly parts of the study area misclassifications occur for the vegetation classes as the forested,

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DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

plants and largely the urban areas are underestimated. Moreover, there are high overlaps with paddy and plants classes. The reason might be attributed to the fact that the speckle noise and the mixed pixel problem cannot be entirely eliminated. The Landsat-8 optical image provides a better representation of the forest class in comparison to the Sentinel-1 SAR image. However the urban class is overestimated in comparison to the base map. In the case of the classification of the fused products, the resolution merge provided an overall accuracy of 79.75% and a kappa coefficient of 0.75 which is higher than the individual satellite images while LMVM provided an overall accuracy of 59.84% and a kappa coefficient of 0.52. LMVM classification includes many unclassified pixels and its overall accuracy of 59.84% and kappa coefficient of 0.52 are similar to the performance of the standalone Sentinel-1A classification (*Table 3*). LMVM has been suggested as a robust algorithm when fusing multispectral and panchromatic images (Nikolakopoulos, 2008), however, in our case of merging multi-source data it does not offer any significant advantage. The thematic maps of the classification results are presented in *Figure 4*.

Resolution merge resulted to the most accurate representation with producer accuracy of the lake area being 83.91 while the user accuracy is 99.61%. The producer accuracies of forest, city, plants and paddy areas are 72.17%, 86.94%, 62.09% and 87.72%, respectively while the user accuracies for the above-mentioned classes are 99.49%, 88.65%, 64.67% and 57.73%, respectively. Apart from the highest classification accuracy, resolution merge is the result with the best visual agreement with the base map (*Fig. 2*). Our findings are in agreement with other studies which have concluded that the classification accuracy is increased after fusing data from separate satellite sources in comparison to stand-alone classification of a images from a single satellite sensor (e.g. Chen et al., 2016).

From overall classification of the Landsat-8, resolution merge and LMVM classified images we found that lake and forest recorded almost 100% classification accuracy while urban, plants and paddy showed the lower percentages accuracies of land use and land cover classes. This might be explained by the fact that the forest in dominated by dense evergreen and high-density trees. Plants and paddy were confused between each other and, consequently, this decreased the reliability of their accuracies when compared to other land use and land cover types classified (Nwaogu et al., 2017; Johnson et al., 2016). Another reason, might be that some areas from the secondary data were attributed another class in comparison to the classification of the satellite images in land use types (lake, forest, city, plants and paddy).

Table 3. *The overall classification accuracy of the classified images. PA and UA refer to producer's and user's accuracy, respectively. All values are in percentages (%)*

Land cover types	Sentinel-1A		Landsat-8		Resolution merge		LMVM	
	PA	UA	PA	UA	PA	UA	PA	UA
Lake	100.00	100.00	65.76	100.00	83.91	99.61	44.89	100.00
Forest	31.17	36.21	61.22	100.00	72.17	99.49	52.50	100.00
City	27.73	59.13	91.86	77.58	86.94	88.65	89.94	88.05
Plants	67.65	37.33	51.13	75.58	62.09	64.67	41.57	80.74
Paddy	58.62	49.19	46.98	39.21	87.72	57.73	60.13	42.92
Overall accuracy	58.50		67.16		79.75		59.84	
Kappa coefficient	0.48		0.59		0.75		0.52	

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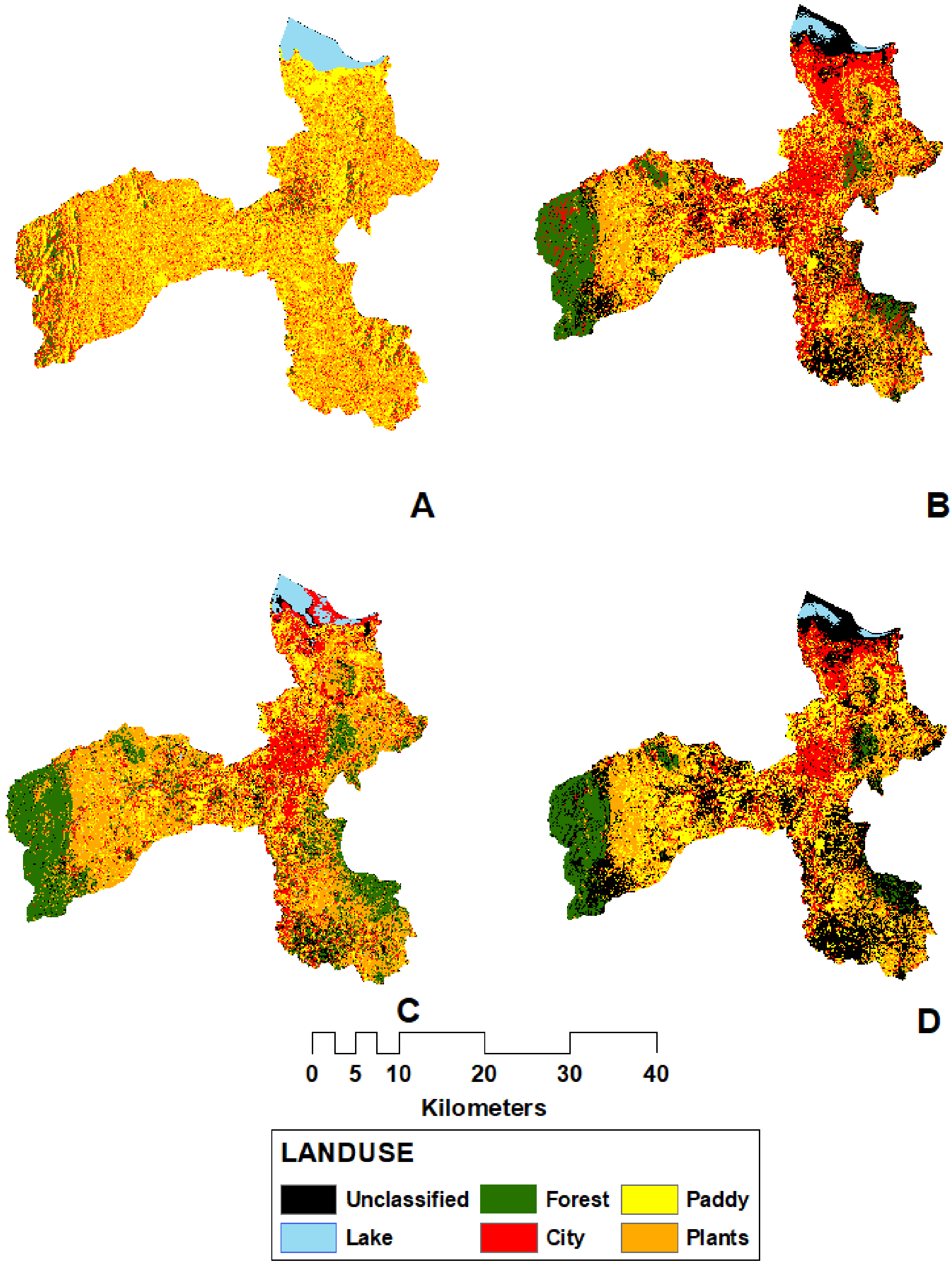

Figure 4. *Thematic maps produced from the maximum likelihood classification of the four input datasets: Sentinel-1A (A), Landsat-8 (B), Resolution merge (C) and LMVM (D)*

From the statistical analysis of the mean and the standard deviation as presented in *Table 4,* it is obvious that the highest standard deviation is 2.13 for Sentinel-1 and followed by 1.76 for Landsat-8, 1.72 for LMVM and 0.94 for resolution merge. As a good performance (i.e. higher class separability as suggested by Napoleon et al., 2013) is indicated by a low standard deviation, it is clear that resolution merge fusion technique perform the best in this study.

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Table 4. *The mean and standard deviation of the 4 input layers*

Image	Mean	Standard deviation
Sentinel-1A	1.62	2.13
Landsat-8	1.39	1.76
Resolution merge	2.75	0.94
LMVM	0.98	1.72

The validation exercise resulted to *Table 5* and *Figure 5* and suggests that the correlation between the observed and predicted model gave a moderate R^2. This means that approximately 52% of the observed values could be explained by the predicted model. The linear regression analysis and the equation describing it are presented in *Figure 5,* where y is predicted value and x is observed value. The reason R^2 value resulted to moderate level is because the land use map from the predicted model were indicated otherwise as in *Table 5*. For example, the land use type of “others” was classified around 23.22% while predicted resolution merge resulted to just 48% in this case.

Table 5. *Statistics of the observed and predicted model for the resolution merge input*

LU	Observed (area km^2)	Observed (%)	Predicted resolution merge (area km^2)	Predicted (%)
City	26	3.16	154	19.09
Forest	112	13.89	161	19.94
Lake	28	3.40	22	2.77
Paddy	15	1.86	115	14.19
Plants	441	54.47	308	38.04
Others	188	23.22	48	5.98
Total	809	100.00	809	100.00

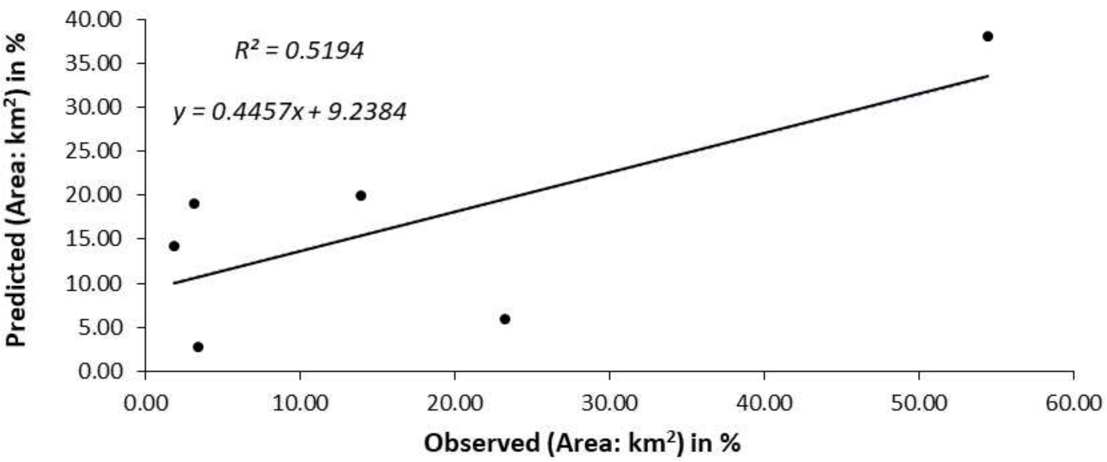

Figure 5. *Scatter plot of observed values and predicted model*

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DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

Conclusions

The present study classified land use in Hat Yai in the year 2015 using two schemes; first the dual polarization Sentinel-1A and the Landsat-8 images standalone, therefore based solely on the SAR or optical information respectively; second, the Sentinel-1A was fused with the Landsat-8 image in an attempt to combine complimentary information acquired from the SAR and optical sensors. Resolution merge and LMVM techniques were used to fuse images in the latter case. The maximum likelihood supervised classification algorithm was used for the classification of the four experiments. The outcome VV/VH dual polarization image did not result to confident results. However, when combining an optical Landsat-8 image with a dual polarized (VV/VH) Sentinel-1A image, the results indicated that the images conserved the spectral characteristics in the original Landsat-8 data and also improved the spatial information. The fusion of a Sentinel-1A dual polarization image with a Landsat-8 image with the resolution merge technique outperformed the LMVM technique according to calculated statistical results. Our study strongly suggests that the most accurate and realistic classification scheme tested is the resolution merge fusion of the optical and SAR data. We showcased that the synergistic use of SAR and optical satellite data greatly improve the land cover and land use classification accuracy, in accordance with other studies in the literature (e.g. Otukei et al., 2015). In the future, it is recommended to consider other land use and land cover areas in the analysis and also use other SAR satellite images to investigate the impacts of different SAR input in the fusion outputs. In addition to that, image classification processes could be applied to evaluate the impacts of fusion algorithms over land use map production.

Acknowledgements. The authors would like to thank GEO-Informatics Research Center for Natural Resource and Environment and Southern Regional Center of Geo-Informatics and Space Technology for their help and support by making available the auxiliary data. We would also like to thank the Faculty of Environmental Management, Prince of Songkla University for providing funding in support of this research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31233135

THE EFFECT OF ORGANIC MATTER APPLICATIONS ON THE SATURATED HYDRAULIC CONDUCTIVITY AND AVAILABLE WATER-HOLDING CAPACITY OF SANDY SOILS

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(Received 3rd Dec 2018; accepted 8th Feb 2019)

Abstract. Soils are natural resources that require preservation and good management due to their ever-changing structure. Among the soil management applications, organic material amendments are known to be the most practical soil management methods in the improvement of the chemical and physical properties of soils. Organic matter amendments are used to preserve water in coarse-textured soils and arid region soils. This study was carried out at Bingol University Research laboratory located in eastern Turkey. In the study, different doses of soil organic matter (SOM) including walnut sawdust (WS), earthworm manure (EM) and farmyard manure (FM) were applied to sandy soils to measure their effects on the saturated hydraulic conductivity (K*sat*) and available water-holding capacity (AWC) of the soils. Compared with the control application, the SOM amendments decreased the hydraulic conductivity of the sandy soils ($p < 0.01$). A comparison with the control application showed that the 8% WS application reduced the hydraulic conductivity of the soils by 51%, followed by the FM application with 35% and EM application with 39%. On the other hand, the SOM amendments increased the available water-holding capacity of the soils when compared with the control application ($p < 0.01$). The application with the most notable effect was determined to be the 1% WS application.
Keywords: *soil properties, soil management, vermicompost, agricultural wastes, soil water*

Introduction

In soil systems, saturated hydraulic conductivity (Ksat) is related to multiple soil properties and thus, is of great importance for engineers, scientists, and agriculturalists (Lal and Shukla, 2004; Gamie and De Smedt, 2018). Depending on the number and diameter of the pores in the soil, the hydraulic conductivity of a soil is a function of certain properties such as bulk density, porosity, pore size distribution and water-holding capacity (Kessler and Oosterbaan, 1974; Varallyay, 2002; Hillel, 2003).

Sandy soils are widespread around the world and have a sand content above 50%. According to the United States Department of Agriculture (USDA, 2010), they are classified as Entisols and cover 18% of the Earth's land surface. Sandy soils are described as soils comprising rocks and mineral particles and containing rocks such as limestone, quartz and granite. The shape, size, degree of weathering and geological origin of the mineral soil components can strongly affect the hydrophysical properties of the soil, especially its water-holding capacity and hydraulic conductivity (Martens and Frankenberger, 1992; Brouwer and Anderson, 2000; Cousin et al., 2003). Due to their low water-holding capacity and high permeability, soil conditioners have attracted attention in recent years for their use in soil improvement (Al-Omran and Al-Harbi, 1997; Zibilske, 1998). Various studies carried out using soil conditioners have reported aggregate development, increased water-holding capacity and decreased infiltration and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31373146

saturated hydraulic conductivity (Al-Omran et al., 1987; El-Shafei et al., 1994; Choudhary et al., 1995).

The organic soil materials used as conditioners contain an array of compounds such as proteins, fats and carbohydrates and high-molecular weight humic and fulvic acids. Today, many mineral and organic soil conditioners are used to improve the physical and chemical properties of problem soils. The increase in the water-holding capacity and decrease in the infiltration and saturated hydraulic conductivity are more evident in coarse-textured soils than in fine-textured soils.

Several studies have been carried out to determine the effect of mixing organic materials with soils on the physical properties. In their study, Unger and Stewart (1974) determined that organic matter amendment in clay soils increased the aggregate stability and soil permeability. In a similar study, Zebarth et al. (1999) reported that the most important effects of organic materials on soils were the decrease in the bulk density and increase in the water-holding capacity, aggregate stability, hydraulic conductivity and permeability. In their study, Nyamangara et al. (2001) investigated the effects of different doses of a chemical fertilizer and farmyard manure on the aggregate stability and water-holding capacity of soils. Their results showed that aggregate stability and water-holding capacity increased with the application of farmyard manure compared with the control and other applications. Asghari et al. (2009) applied 25 g/kg cattle manure to sandy loam soil and determined that, compared with the control, the application significantly increased the number of micropores and significantly decreased the number of macropores ($P \leq 0.05$) and correspondingly, led to an increase in the water holding-capacity. Eusufzai and Fujii (2012) reported that the application of compost, rice straw and sawdust to clay loam-textured soil increased the water-holding capacity, hydraulic conductivity and pore size of the soil.

The study aims to improve the physical properties of sandy soils and thus, the chemical properties of the soils were excluded from the study. There were many studies that aimed to determine the effects of worms on soil (Schrader and Zhang, 1997; Feng et al., 2001; Topoliantz et al., 2002; Panicker et al., 2007), but there is still a lack of information. The number of the studies investigating the relationship between earthworm manure and soil-water is limited. The studies have generally focused on the effects of earthworm population in manure on soil properties. On the other hand, it is not exactly known how food wastes (eg walnut shells) that are thrown or mixed after use have affected soil properties. This study investigates the effects of the application of different ratios of walnut sawdust (WS), earthworm manure (EM) and farmyard manure (FM) to sandy loam soil on the hydraulic conductivity (Ksat) and available water-holding capacity (AWC) of the soils.

Materials and method

The study was carried out under laboratory conditions to reduce the effects of environmental factors (climate, the effects of terrestrial and subterranean organisms, etc.) and prepare the conditions for the incubation of SOM. The study was conducted under laboratory conditions. During the study, mean humidity was 65 ± 5% and mean temperature was 25 ± 3 °C. The soils used in the study were collected from the Research and Application Farm of the Bingol University, Bingol, Turkey, from the depth of 0-30 cm. The general properties of the soil were determined by Demir (2016) and are given in *Table 1*. The organic matters (SOM) comprising walnut sawdust (WS),

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DOI: http://dx.doi.org/10.15666/aeer/1702_31373146

earthworm manure (EM) and farmyard manure (FM) were procured from a commercial agricultural company (https://www.ekosol.net.tr; http://www.kabukcu.com.tr). The general properties of the material were determined by the company and are given in *Table 1*.

Table 1. *The general properties of the materials*

Properties	Materials			
	Soil	**WS**	**EM**	**FM**
Clay (%)	17.4	-	-	-
Silt (%)	24.6	-	-	-
Sand (%)	58.0	-	-	-
Soil tekstural class	Sandy Loam	-	-	-
Great Soil Group	Xerorthent	-	-	-
pH	7.0	7.4*	6.7*	6.5*
EC (µS/cm)	176.4	-	-	-
$CaCO_3$ (%)	0.1	<0.1	<0.1	<0.1
Bulk density (g/cm^3)	1.49	0.57	0.38	0.32
Porosity (%)	42.7	68.0	72.0	77.0
Organic Matter (%)	2.3	77	46	69
CEC	31.6	33.4	48.2	47.1
C:N	9:1	78:1	15:1	22:1

*1:2 H_2O, WS: Walnut shell, EM: Earthworm manure, FM: Farm manure, EC: Electrical conductivity, CEC: Cation exchange capacity, C:N: Rate of carbon on nitrate

The soils were analyzed by Demir (2016) and the size distribution of the sand, silt and clay fractions of the soils was determined using the Bouyoucos hydrometer method (Gee and Bauder, 1986). The pH and electrical conductivity were measured in accordance with Mclean (1982) and Rhoades (1982) under saturated soil conditions. The organic matter content of the soil was determined following the Smith Weldon method (Nelson and Sommers, 1982). Soil lime content was determined using a Scheibler Calcimeter by following the method proposed by Nelson (1982). The cation exchange capacity was determined using sodium acetate in accordance with Chapman (1965). The bulk density values of the soils were determined by following the method proposed by Blake and Hartge (1986).

Preparation of the study materials

The SOMs were prepared by sieving with a 0.5-mm sieve and mixed with the soils at ratios of 1%, 2%, 4% and 8% on a weight/weight (w/w) basis. SOM application rates were determined according to the farmer practices (between 0 and 8% w/w) in Turkey. The mixtures were prepared in three replications and added to the test containers. In addition, a non-SOM-containing mixture was prepared to minimize the errors that may occur during the experiment. The test containers were made of plastic (pot volume: 22 L; top diameter: 420 mm; height: 310 mm). The mixtures in the test pots were irrigated under laboratory conditions at the level of field capacity and incubated for three months (*Fig. 1*). The pots were irrigated at 3-day intervals to maintain the soil moisture content at around field capacity. For this purpose, irrigation was carried out at a certain rate

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determined depending on the moisture hold by soils at field capacity (pF 2.54). The quality of the irrigation water was classified as C1S1 in accordance with the US Salinity Laboratory (USSL, 1954).

Figure 1. *Incubation of organic matter-soil mixture under laboratory conditions*

Hydraulic conductivity and water-holding capacity

The hydraulic conductivity (Ksat) of the soils under saturated conditions was determined using a permeameter by following Klute (1965). The Ksat was calculated using *Equation 1:*

$$Ksat = \frac{aL}{At}\ln\left(\frac{h0}{h1}\right) \quad \text{(Eq.1)}$$

Here, Ksat represents the hydraulic conductivity (cm/s), a represents the cross-sectional area of the burette (cm^2), A represents the cross-sectional area of the soil sample (cm^2), L represents the column length of the soil sample (cm), h0 represents the initial water height (cm), h1 represents the final water height (cm), and t represents the time between the start and end (s).

The available water-holding capacity and the moisture content at the field capacity (FC) and wilting point (WP) of the soils were measured using a pressure plate apparatus Cassel and Nıelsen (1986). Using a pressure membrane device, the field capacity of the undisturbed soil samples was determined using their moisture content at 1/3 atm and the permanent wilting point of the disturbed soil samples was determined using their moisture content at 15 atm.

The available water-holding capacity of the soils was calculated using *Equation 2:*

$$AWC = FC - WP \quad \text{(Eq.2)}$$

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Here, AWC represents the available water-holding capacity (%), FC represents the moisture content at field capacity (%) and WP represents the moisture content at wilting point (%).

The variance analysis of the results was performed using the SPSS software program (SPSS, 2015).

Results and discussion

The WS, FM and EM applications were determined to affect the hydraulic capacity of the sandy soils (*Table 2*). Compared with the control application, the SOM amendments reduced the hydraulic conductivity of the sandy soils ($p < 0.01$). The lowest mean hydraulic conductivity values were obtained with the WS and EM applications. The 8% dose of the SOM applications was more effective than the other doses. Compared with the control application, the 8% WS application decreased the hydraulic conductivity by 51%, followed by the FM application with 35% and EM application with 39%. Considering the average manure application dose, hydraulic conductivity was determined to decrease with increasing doses.

Table 2. *The effect of the organic materials on the saturated hydraulic conductivity of the soils*

Treatments	Ksat (x 10^{-4} cm/s)			
	WS	**FM**	**EM**	**Means**
%1	2.26bcd	2.69bc	2.60bc	2.51BC
%2	2.11cd	3.53a	2.17cd	2.61B
%4	2.43bcd	2.13cd	2.47bcd	2.34C
%8	1.89**d**	2.12cd	2.06cd	2.02**D**
Control	2.87ab	2.87ab	2.87ab	2.87A
Means	2.31B	2.67A	2.43B	
	Df	**Sum of squares**	**F**	**P**
Dose	2	9.8289567E-9	9.51	<0.01
SOM	4	3.5693963E-8	17.27	<0.01
Dose*SOM	8	3.5026754E-8	8.47	<0.01
Error	30	1.5498863E-8		
General	44	9.6048537E-8		

AWC: Awailable water content, WS: Walnut shell, EM: Earthworm manure, FM: Farm manure, SOM: Soil organic matter

The hydraulic conductivity of the sandy soil was also affected by the interaction between SOM and dose ($p < 0.01$). Within this context, the lowest K*sat* value was obtained with the 8% WS application. In other words, the 8% dose of the WS application was determined to decrease the hydraulic conductivity by 51%. The negative correlation between K*sat* and soil pore size results in increased water flow rate. The filling of large soil pores with fine organic materials leads to reduced K*sat* (Esmaeelnejad et al., 2017). Pulat et al. (2018) reported that the application of 1% biopolymer solution to a 70% sand-containing soil sample resulted in a 25-fold decrease in the hydraulic conductivity. In the same vein, Zhang et al. (2016) determined that the

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application of ground biochar particles to sandy soil decreased the hydraulic conductivity and attributed the decrease to the destruction of the pore structure of the sandy soil by the materials added to the soil.

Addition of organic matters to soils is one of the most commonly used methods to increase the water-holding capacity of soils in soil management applications. Water-holding capacity is defined as the amount of water retained by soils for the use of plants. This amount is largely determined by the texture of the soil and to a certain degree, by the structure and organic material content (Soıl Management Guıde, 2008). *Table 3* shows the effects of WS, FM and EM applications to sandy loam soil on the water-holding capacity. The SOM amendments resulted in increased water-holding capacity in comparison with the control application ($p < 0.01$). The results of the study showed that the 1% WS application was the most effective application, while the 1% FM application had the lowest effect. In the study, both the differences between the averages of the SOM varieties and the averages of the treatment doses were statistically significant. *Table 3* shows that the averages of the application doses were in the same significance group, while the differences between the averages of the SOM varieties were in different statistical significance groups. The data indicated that the WS application resulted in a higher increase in the AWC of the soils than other SOM applications.

Table 3. *The effect of the organic materials on the available water-holding capacity of the soils*

Treatments	**AWC (%)**			
	WS	**FM**	**EM**	**Means**
%1	11.96[a]	8.50[e]	10.17[abcde]	10.21**A**
%2	11.31[ab]	10.28[abcde]	10.83[abcd]	10.80**A**
%4	10.84[abcd]	10.06[bcde]	9.66[bcde]	10.18**A**
%8	11.26[abc]	9.40[cde]	11.33[ab]	10.66**A**
Control	9.27[de]	8.47[e]	9.60[bcde]	9.11**B**
Means	10.92**A**	9.34**C**	10.32**B**	
	Df	**Sum of squares**	**F**	**P**
Dose	2	15.91	25.06	<0.01
SOM	4	19.15	10.41	<0.01
Dose*SOM	8	11.70	3.83	<0.05
Error	30	11.46		
General	44	58.23		

AWC: Available water content, WS: Walnut shell, EM: Earthworm manure, FM: Farm manure, SOM: Soil organic matter

Considering the effect of the different doses of SOMs on the water-holding capacity, the highest effect was obtained with the 1% dose of the WS application, 2% dose of the FM application and 8% dose of the EM application. Thus, the effect of the dose varied depending on the characteristics of the SOM. Here, the most important issue is the effect of the organic matter on the moisture content both at field capacity (pF:2.54) and permanent wilting point (pF:4.2). When organic matters have the same effect at both levels, they do not have a significant effect on the water-holding capacity. However, when they have a higher effect at the field capacity than at the wilting point, they

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positively affect the water-holding capacity. Whether organic matter increases the water-holding capacity is an ongoing debate among many researchers. According to Hudson (1994), there is a positive correlation between the organic matter content and water-holding capacity of sandy soils (r = 0.79). Furthermore, Karhu et al. (2011) reported an 11% increase in the water holding capacity after biochar application to soil. Mınasny and Mcbratney (2018) analyzed the results of a total of 60 studies and reported increasing water-holding capacity with increasing amounts of organic carbon in soils and determined that this effect was more evident in sandy soils. The organic matters' retention of higher amounts of water than sandy soils is an expected outcome. This is attributable to the higher pore number of organic materials than that of sandy soils. In addition, the capillarity of organic materials is much higher than the capillarity of sandy soils. Indeed, the results of several studies agree with this theory. In various studies investigating the improvement of the water-holding capacity of soils, organic materials of different origins were used (farmyard manure, legume leaves, rice stalk, biochar) and the results have revealed that the use of organic materials led to increased available water-holding capacity (Yılmaz and Alagöz, 2008). Agreeing with previous studies, in the study, walnut sawdust was determined to increase the water-holding capacity of sandy soils.

Conclusions

The sustainable use of soils depends on knowing and preserving their properties. Soil organic matters, which have positive effects on various physical and chemical properties of soils, are also among the soil properties that require preservation and improvement. In the study, the application of walnut sawdust, earthworm manure and farmyard manure to sandy soil reduced the hydraulic conductivity of the soil. Moreover, the application of the materials to the soil increased the available water-holding capacity. In conclusion, the outcomes of the study can be summarized as follows:

1. The experiments showed that the application of the increasing doses of walnut shell, earthworm manure and farmyard manure to sandy-textured soil decreased the hydraulic conductivity.
2. Among the three organic waste types, the highest decrease in hydraulic conductivity was obtained with farmyard manure. In addition, in all applications, the highest decrease in hydraulic conductivity was obtained with the dose of 8%. This is of significance in terms of water conservation in sandy soils and especially in arid-region soils.
3. Another outcome of the study was the increased water-holding capacity due to organic matter application to soil. Furthermore, walnut sawdust increased the water-holding capacity more than other materials.
4. In this study, it is concluded that walnut shell waste decreases hydraulic conductivity in sandy soils and increases water holding capacity.
5. The use of the organic wastes that we use in our daily lives, such as walnut shell, in the improvement of the physical properties of problem soils should be investigated. If these materials are deemed suitable for this purpose, they should be used in soil management applications. They can especially be used effectively in combating erosion, which is the greatest soil problem worldwide.
6. The results of the study showed that earthworm manure improved the hydraulic conductivity and water-holding capacity of soils. However, its production in

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limited amounts and high-cost restrict its use in large agricultural areas. In these areas, solely increasing the earthworm population in the soil layer will prove more beneficial. In the current circumstances, earthworm manures can be used in ornamental plant breeding and greenhouses.

Acknowledgements. This study was produced from project (number: ZF: 2017.007) supported by The Scientific Research Projects Coordination Unit of Bingol University

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31373146

A RESEARCH ON THE FUNCTION AND USABILITY OF HISTORICAL URBAN PARKS: CASE STUDY OF SHAH GOLI PARK, TABRIZ, IRAN

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(Received 5th Dec 2018; accepted 4th Feb 2019)

Abstract. In this study, the recreational opportunities, which were created by the Shah Goli Park for the users in terms of its functionality, having a historical and unique characteristic, which is located in Iran, Tabriz, are analyzed. Accordingly, its availability for the local people is examined, as well. The data collection tool is in the form of a questionnaire. The objective of this questionnaire is to define the park availability factors for those, who visit the Shah Goli Park. The options with regards to the availability of the park were on socialization, physical activity, nature involvement and relaxation. The respondents were asked to address questions in order to show their preferences within the scope of a 5-point Likert scale (1 = Strongly disagree, 2 = Disagree, 3 = Don't know, 4 = Agree, and 5 = Strongly agree) and the park's availability preference were measured using 14 items. Survey-analysis and questionnaire processes of the study were carried out in July-2018. The gathered data was subjected to an analysis by utilization of normal statistical methods within SPSS version: A set of park availability items were defined using the factor analysis, in order for designating the main and underlying factors, followed by a group mean comparison (t-test) in order to identify the differences between the demographic information and availability factors.
Keywords: *land use, recreation area, open-green spaces, sustainability, historical parks*

Introduction

Parks and open spaces, offering significant social and environmental functions, are valued leisure and amenity resources in cities. Urban parks are critical components within the metabolism of cities as well as the core scenes for open-air leisure communal activities. Historically, the functions of urban parks have been connected to leisure and aesthetics, but with the change of the surroundings and necessities of urban centres, leisure now includes sport activities, with the consequent implementation of fitness equipment, jogging tracks and sport courts (Almidia et al., 2018). Also, urban parks play a unique and indispensable role in providing public spaces and recreation opportunities for urban populations (Chan et al., 2018). As one of the most complex processes, overcrowding and unplanned growth are experienced, which raises concerns on the cities' sustainability, thus questioning how sustainable cities could be achieved. The objective of this study is to identify the potential advantages of historical urban green spaces with regards to the urban sustainability concept. In consequence, we consider sustainability concept, along with sustainable development, heritage, urban green spaces and quality of life and well-being as the main outline of the study, which are discussed in the following chapters. Urbanization processes, gathering pace with the growth of industrialization, has led the transformation of living areas of people from rural to urban. Today, in many of our large cities, witnessing the urbanization and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31473163

industrialization movements; the structural density, increasing in parallel with the population growth, along with other unplanned developments, improper selection of settlement areas, has led the connection of urban with the rural to be ruptured, thus causing the open and green spaces in urban areas to diminish (Gürbüz and Yılmaz, 2017). Open and green spaces, in other words "parks", having the potential to freshen up the people, instill a new dynamic, particularly providing the opportunity to be involved in recreational activities in megacities, facing with physical, social, biological and environmental problems, are deemed required (Yılmaz et al., 2007).

The phenomenon "city" can be described not only as a physical, but also as social, political, economical and cultural production areas (Altuntaş, 2012) – a dynamic concept evolving from simple to complex, bearing various meanings from past to date (Topal, 2004). In this context, cities are the complex structures, comprising in the interface of a number of different subjects like land use, demographical qualifications, social and cultural structure, economical characteristics, historical and environmental subjects. In a highly dynamic and variable urban system, it is quite challenging to complete the designing and planning processes one level having the ability to come up with solutions against problems. In this system, the first time the problems became a current issue concerning the urban areas that change in time based on the socio-economical and cultural structure of the society, characterized as the complete areas used by those urbanized on the urban land and where the urban-related events occur, and where the social or personal needs are met, was in the second half of 20th century, leading the physical and social problems to come to the forefront (Oktay, 2007). However, each and every characteristic is directly related to those urbanized in and of itself, which turns into a system running after being planned and designed as a whole, focusing on people (Akyol, 2006).

Urban spaces

More than half of the world's population nowadays live in cities. With more people coming to live and work in cities, urban public open spaces become even scarcer. Urban open spaces, such as squares, green spaces, or parks can provide environmental, ecological, social and economic benefits to cities and are indispensable for healthy urban living (Chen et al., 2015).

The first communities started to put down the roots in naturally enriched areas, along with those that were protected against natural powers, which were selected to meet their nutrition and accommodation requirements, as the most fundamental needs of people. Where people started to feel committed to an area, they also started to intervene the physical environment more, which emerged the need to build relatively more permanent structures. Having been experienced for centuries, this matter has reached to its peak point in today's world and led the emergence of modern cities (Özbilen, 1991; Çınar, 2008). Yet, increasing human population and rapid development of urbanization led to the destruction of nature and green areas (Yazıcı, 2015; Özdemir, 2007). In the case of the urban environment, it is both possible to define the city as one single ecosystem and to see the city as composed of several individual ecosystems, e.g. parks and lakes. Bolund and Hunhammar (1999) identify seven different urban ecosystems which we call natural, even if almost all the areas in cities are manipulated and managed by man. The ecosystems comprise of street trees, lawns/parks, urban forests, cultivated land, wetlands, lakes/sea, and streams (Bolund and Hunhammar, 1999). Rogers' reports that there is a continuing trend towards urbanization, both in Britain (in 1991, over 80% of

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DOI: http://dx.doi.org/10.15666/aeer/1702_31473163

the population lived in towns and cities of over 10,000 people) and world-wide (today nearly 50% of the world's population live in urban settlements) (Thompson, 2002). Open spaces such as parks, playgrounds and plazas have been criticized in the past as a largely failing to serve their intended uses and users. Non-use of parks, vandalism, and outdated facilities are examples of problems commonly identified with urban open spaces. Indeed, urban open research originated from public awareness of the social failure of many urban open spaces (URL 1, 2018).

Urban green spaces play a remarkable role to reduce bad consequences of the rapid rate of urbanization (Li et al., 2017). Green spaces and other nature-based solutions offer innovative approaches to increase the quality of urban settings, enhance local resilience and promote sustainable lifestyles, improving both the health and the well-being of urban residents. Parks, playgrounds or vegetation in public and private places are a central component of these approaches and can help to ensure that:

- Urban residents have adequate opportunities for exposure to nature;
- Urban biodiversity is maintained and protected;
- Environmental hazards such as air pollution or noise are reduced;
- The impacts of extreme weather events (heatwaves, extreme rainfall or flooding) are mitigated;
- The quality of urban living is enhanced;
- The health and well-being of residents are improved (WHO, 2018).

There is evidence that rural residents experience a health disadvantage compared to urban residents, associated with a greater prevalence of health risk factors and socioeconomic differences compared to urban and suburban populations, along with the evidence of a health disadvantage associated with living in rural areas. Research in Canada has identified higher mortality rates, decreased life expectancy, greater incidence and prevalence of morbidity, and poorer self-reported health status in rural populations. For example, life expectancy at birth is at least 2 years less for men in rural areas compared to urban areas and the risks of death from circulatory disease or respiratory disease are as much as 10% higher in rural areas. This health disparity may be the result of differences in health risk factors, including health behaviours and socioeconomic status (SES). Additionally, differences in activity patterns between urban and rural populations may potentially lead to differences in exposure and risk(s) related to environmental contaminants, further contributing to the health disparity (Matz et al., 2015).

Urban parks

For the recreational life and outdoor activities of people, urban parks play a critical role. Parks are generally used by people in urban areas. As a result of the outdoor environment complexity, scarcely any steps have been taken to understand the impact of thermal environment on how people use the outdoor spaces (Thorsson et al., 2004; Çınar, 2008; Polat and Güngör, 2013). The sustainability and regeneration strategies of cities essentially focus on man-made and built components within the urban environment, when compared, attention to the natural components and urban structure's green spaces are still limited (Chiesura, 2004). However, it is discussed that the urban parks and open green spaces are of a strategic significance within the scope of the quality of our increasingly urbanized society's life. As a matter of fact, increasing

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empirical evidence shows that the presence of natural assets (i.e. urban parks and forests, green belts) and components (i.e. trees, water) within urban contexts contribute to the quality of life under quite a lot of manners. Additionally, critical environmental services like air and water purification, wind and noise filtering or microclimate stabilization, the natural areas provide social and psychological service that are of vital importance with regards to the liveability of modern cities, along with the well-being of urban dwellers. Park experience bears the potential to reduce stress, enhancing contemplativeness, rejuvenate the city dweller, thus providing a sense of peacefulness and tranquility (Kaplan, 1983; Chiesura, 2004). Parks are important recreation areas within open and green area system (Onsekiz and Emür, 2008; Aksoy and Akpınar, 2012). Providing the societies with the opportunity to perceive the nature itself, while being used for recreational purposes for centuries, the parks have gained a gradually increasing importance and value, particularly in urban environments. Formed based on cultural values, political and socio-economical structure, the urban parks have taken its form with social changes thanks to aforementioned characteristics, thus acting as a reflector for them, as well (Kurtaslan, 2017).

Urban parks constitute an important place of leisure and recreation for city residents. They are also important because they tend to be among the few places that offer urban dwellers opportunities for contact with nature and vegetation (Mahrous et al., 2018). Urban parks offer multi-dimensional urban ecosystem functions as well as spaces for human-environment connection (Chan et al., 2018). Urban open-green spaces provide many advantages like creating areas for recreational activities, reducing the air pollution, balancing the atmospheric oxygen and carbon dioxide rate, regulating the micro-climate, minimizing the floods, providing shielding against noise and wind, creating living space for wild-life, contributing positively in physical and mental health, etc. (Waterman and Wall, 2009; Yazıcı, 2015). People in industrialized countries spend on average less than 10% of their time outdoors (Lee and Kim, 2015). One vital role that urban parks play is providing space for the expression of diversity, both personal and cultural; this raises issues of democratic provision for and access to public open space. It suggests, inter alia, that the role of the urban street as public space may need to be re-thought. The social and cultural values of open space include attitudes towards nature and the desire for connection with it, as well; contemporary understandings of ecology offer new insights into ways to serve both human needs and the broader ecological framework of urban open space structures (Thorsson et al., 2004)

Urban parks are valuable urban green areas serving for citizens to keep away from the stressful city life and relax thanks to its social and environmental functions. For this reason, the importance of urban parks is obvious with its advantages like providing an aesthetical view, minimizing the noise, clearing the air, along with its micro-climate affect and pollution control (Lam et al., 2005). Urban parks are green areas that keep people out of the intense and boring impact of a city, while providing various recreational benefits (Zaloğlu, 2006). Urban parks can also be described as open-green spaces, created with the combination of natural and cultural concepts towards meeting the active and passive recreations needs of those urbanized (Kızılaslan, 2007). Planning and management of urban parks are of significance for urban development. Urban parks possess critical ecological, social and economical functions. Therefore, social life styles, values, attitudes towards the nature and sustainability will increase the demand on urban parks in the future. Parks and open areas should not be deemed as luxury within the city (Loures et al., 2007). Urban parks are public service areas that are of crucial importance in re-establishing the ruptured relations of people with the nature itself, developing as a condition to urbanization within the complex urban organization, along with undertaking various functions (Kızılaslan, 2007).

Urban park standards

The factors that designate the standards required for the park areas in line with the life style of our time can be summarized as follows:

- Population,
- City size,
- Geographical location,
- Climate,
- Distance and density of use (Polat, 2006).

According to Oğuz (1998), park and green area sizes vary depending on every country, settlement and their needs. The need for green area increases as directly proportional to the population and structure.

The changes of open-green spaces are shown in *Table 1* (Özkır, 2007) – Open Green Spaces' Sizes and Locations (Tümer, 1976; Müftüoğlu, 2008; Yazgan, and Khabbazi, 2013).

Table 1. *Open-green spaces' sizes and locations (Tümer, 1976; Müftüoğlu, 2008)*

Park areas	Population criterion da/per capita		Area size criterion (da)		Its place in recreation areas
	Minimum	Optimal	Minimum	Optimal	
Quarter Park	6	12	20	40	As a single unit or within neighborhood parks
Neighborhood park	6	14	200	400	As a single unit or within neighborhood parks
Urban park	1	2	400	1000	Main recreation area in the urban fabric
Regional park	4	12	3000	4000	1 to 2 h drive around the city

On the other hand, classifies the parks based on the number of residences to which the park serves, dwelling unit and size (*Table 2*) (Özkır, 2007; Müftüoğlu, 2008; Yazgan and Khabbazi, 2013) and also represent the national recreation and park board's standards on parks (*Table 3*).

Table 2. *Figures concerning the sizes of park areas within the city (Özkır, 2007; Müftüoğlu, 2008; Yazgan and Khabbazi, 2013)*

Parks	Number of residences addressed	Required dwelling unit	Size
Quarter Park	700-1000	Primary school	1-4 ha
Neighborhood park	1000-5000	District	4-20 ha
Urban park	5000-10000	City	20-50 ha
Regional park	20000-30000	Region	200 ha and over

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DOI: http://dx.doi.org/10.15666/aeer/1702_31473163

Table 3. *National Recreation and Park Board's Standards on Parks (Özkır, 2007; Yazgan and Khabbazi, 2013)*

Type	da/1000	Size	Population served	Accessibility
Quarter Park	10	20-80	2000-10000	400-800 m
Neighborhood park	10	80-400	10000-50000	800-4800 m
Urban park	20	Variable	Variable	30 min of drive
Regional park	80	1000 and over	The entire population in small cities	1 h of drive
Private areas	No applicable standard. It comprises trees, roads, beaches, transcendental areas, etc.			

The role of urban parks, quality of life and well-being

Urban parks and green spaces play an important role for environmental services such as air and water purification, wind and noise filtering, and microclimate stabilization. They also provide social and psychological services, which are important for public well being (Urban areas are recognized as one of the key components of sustainable cities, and accordingly, creating more sustainable urban areas is the key to sustainability (Özdemir, 2007). Sustainable areas can be described as those, which are created to support sustainable living primarily focusing on the economic, environmental and social sustainability (Huseynov, 2005). This has a unique significance for urban green areas, tending to imply and symbolize a distinct set of ideals concerning the city's identity, along with its history and culture on one side, and its local economic growth on the other. In this context, the urban landscape is recognized as quite positively linked to sustainability policies (Beck, 1992; Sachs, 1995; Ferris, et al., 2001; Loures et al., 2007). The empirical research, in addition to the theoretical study, has underlined the role of urban landscapes in the environmental, aesthetical, psychological, social and economical aspects of urban life (Grahn, 1985; Burgess et al., 1988; Conway, 2000; DeGraaf, 2003; Chiesura, 2004). Similarly, urban green spaces are recognized as the essential component within the scope of urban sustainability, for their beneficial services and also one possible step to the creation of sustainable urban environments. (Zhang et al., 2012). Hence, these aspects like "amount of urban green spaces per inhabitant," "public parks," and "recreation areas" are recognized to have strategic significance for improving the life quality (Teal et al., 1998), making the city livable, pleasant and appealing for citizens (Chiesura, 2004)

The term "well-being" has been defined in various ways using socio-economic, psychological, and psychosocial parameters as well as feelings of connectedness to nature (Bell, 2005; Dutcher et al., 2007). The World Health Organization (WHO, 2018) defines health and well-being as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity". Greenberg (1985) suggests that wellness (or well-being) differs from health; in that, "it is the integration of physical, emotional, mental, spiritual and social health at any level of health or illness". The term "well-being" includes material security, personal freedoms, good social relations and physical health, whereas the term "health" includes biological, sociological, economic, environmental, cultural, and political factors (Walter et al., 2005). Based on this definition, a person might be regarded as achieving the status of well-being within one of the domains of health, which is defined as asymmetrical health (Greenberg, 1985). Well-being is defined in this study as an inner state of wellness that

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includes physical, social, mental, spiritual, and emotional states of consonance, which exist in healthy environments (Burns, 2006) or are achieved through connection or engagement with natural environments and green spaces.

Eventually, for making cities more sustainable, urban parks have the ability to play a key role, along with providing benefits for the habitat, air and water quality, and also letting the kind of high neighbourhoods densities, which are contributive in terms of lowering the energy consumption while increasing active, human powered transportation. Surely, it is critical to possess wide open spaces, and especially natural areas which provide habitat, as well as reinforcing the compact city design. Additionally, urban dwellers can be encouraged in order to appreciate the natural processes within their neighbourhoods, providing authentic facilities in which people are provided with the opportunity to gather either informally or more formally. Parks serve to connect people to the history of both the environment that was built, and also the natural processes in the area, as well. Altogether, these areas can be rick in cultural resources, allowing people to sustain the community life over time.

The present study aims to investigating the recreational opportunities, which were created by the Shah Goli park for the users in terms of its functionality, having a historical and unique characteristic, which is located in Iran, Tabriz.

Materials and methods

Case area: location, boundaries and geographical characteristics of Tabriz City

Tabriz is located at 38° 8’ and 46° 15’ East of Greenwich with an area of about 131 km^2 (Ghorbani, 2006). The city is located in 1200 m above sea level (Rahimi, 2006)’. In the winter, the average temperature is 12.4 °C (88.4 °F) and in the summer the average temperature can be up to 34.1 °C (110.1 °F). Lack of water resources is the most important climatic problem in the city; meanwhile the annual rainfall (snow and rain) is only about 321 m^2 and most of the rains occur during winter and spring. Despite harsh weather, the population of this city is 1,579,312, according to the Census Central Organization of Iran (*Fig. 1*).

Data collection instrument

The data collection tool is in the form of a questionnaire. The objective of this questionnaire is to define the park availability factors for those, who visit the Shah Goli Park. The options with regards to the availability of the park were on socialization, physical activity, nature involvement and relaxation. The respondents were addressed questions in order to show their preferences within the scope of a 5-point Likert scale (1 = Strongly disagree, 2 = Disagree, 3 = Don’t know, 4 = Agree, and 5 = Strongly agree) and the park’s availability preference were measured using 14 items.

Data analysis

The gathered data was subjected to an analysis by utilization of normal statistical methods within SPSS version: The set of park availability items were defined using the factor analysis, in order for designating the main and underlying factors, followed by a group mean comparison (t-test) in order to identify the differences between the demographic information and availability factors.

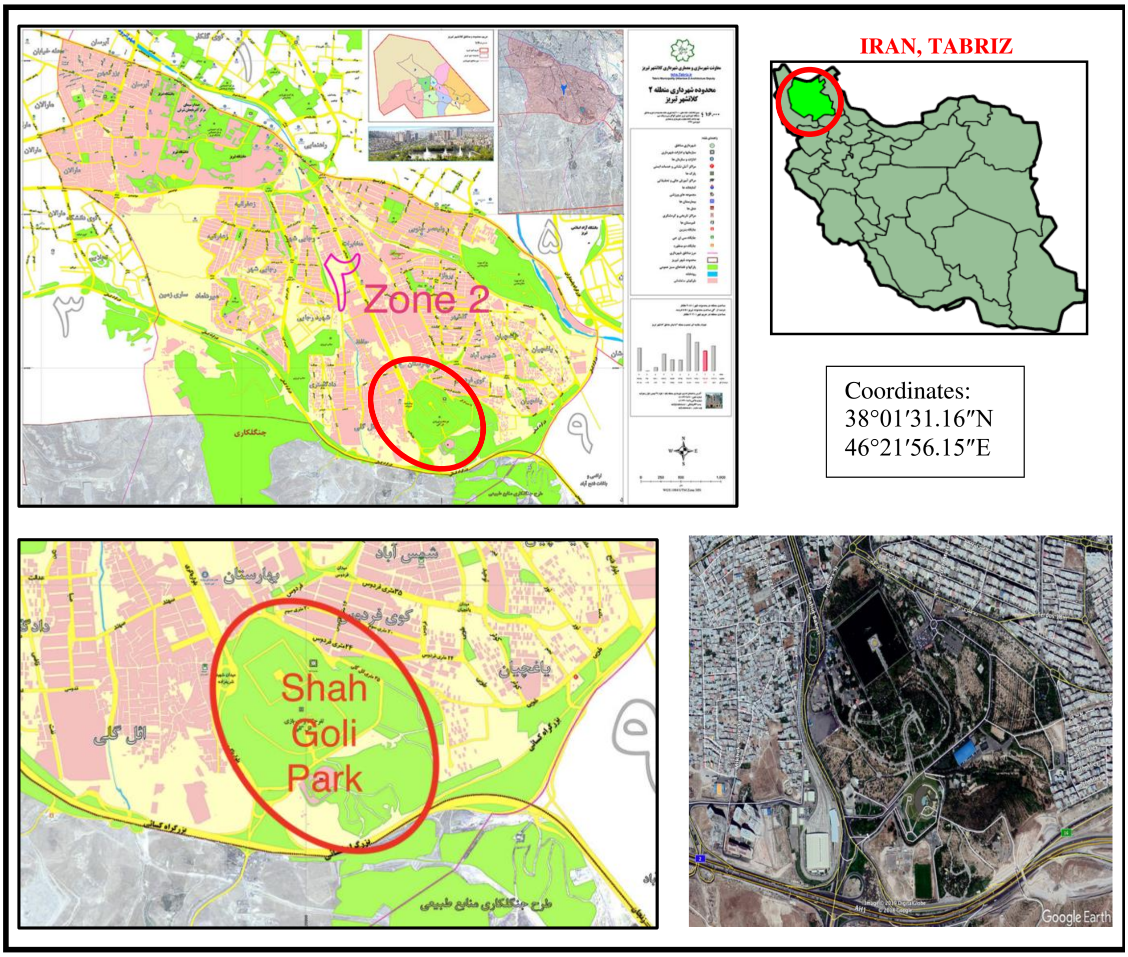

Figure 1. *Location of Shah Goli Park (Tabriz City Regional Planning, 2018)*

History of urban park in Iran

Iran's history of public space can be traced from the period when the Persian Empire around 500 years B.C. Throughout the history public spaces were being used for various purposes like a place for national ceremonies, declaring important news, crowning ceremonies, etc. During 14th century A.D., public spaces like "Chahar Bagh" were the most essential parts of the city. In this period, public spaces were used as a place of social communication, as well as national and religious ceremonies. Additionally, Persian gardens were introduced as an area in order for experiencing the nature, as well. During the early 20th century A.D., in Iran, including Tabriz, for recreation and socialization of people, Persian gardens started to be recognized as public urban parks in most of the large cities (Romianfar, 2008). What is more is that these public spaces can be recognized as multipurpose areas, too (Hami et al., 2011). Urban parks were not that common in the cities of Iran because of the insufficient amount of water resources; hence, a vast majority of available green spaces in the traditional cities were garden, generally built on a private land. Following the swift and modern urbanization of Persian cities, urban parks became popular choices for accessibility to green space. As per a previous study, people used to prefer urban parks mostly for passive entertainment and individual active activities like hiking (Hami et al., 2011), notwithstanding that,

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some of the parks possess sport facilities like volleyball and tennis courts for the visitors. In general, urban parks in Iran are recognized as places for nature appreciation (Hami et al., 2011; Rostami et al., 2017) and social interactions like family gatherings (Hami et al., 2011). Therefore, landscaping of urban parks require considering up of public demands and needs.

Shah Goli Park

Shah Goli Park is also known as the “Old Park” in Tabriz and was built as a royal park by an unknown king in 1785 (Wilber, 1979). This was later declared as a public urban park in 1930 (Romianfar, 2008). El Goli covers an area of 60.7 Hectares (150 acres) (Ghorbani, 2006) and located in the Southwest part of Tabriz. Construction of the original building ponds is ascribed to the reign of Sultan Yaghoob Aghkoyunlular and consider its development from the Safavid period. Completion of especial esplanade and mansion for courtiers and its prosperity is ascribed to the time of Champion Mirza, the son of Abbas Mirza regent- who was the ruler of Azerbaijan. The Safavid dynasty in the pool Shah Goli was the largest water reservoir in the garden. Aghkoyunlular and Safavid and Qajar periods all within the catchment area of sand was empty and it was drawn on the walls of stone and lime. After the Qajar era this area was transferred to Tabriz municipality be a public walkway. With the construction of five-star hotel Elgolu Pars, this area has the international aspect and the space agencies of Tabriz city has become one of the most beautiful public parks (Rostami, 2017). In 1930, renovation was done to the park by Tabriz Municipality and it became a public park (Romianfar, 2008). During the renovation, an artificial lake was added and it becomes a prominent feature of Shah Goli Park. The artificial lake is 213.23 m (700 ft) long and 213.23 m (700 ft) wide covering 54,675 m^2 of area. In the eastern side, some terraces have been built and a row of poplars planted along the walls. The park was further expanded in 1989 and 1996 with new features added (*Fig. 2*) (Romianfar, 2008).

Name of park	**Thematic class of park**	**Position of park from satellite**
Shah Goli Park	Historical national park	
Entrance of park	**Use of plant material**	**International Pars Hotel**
		

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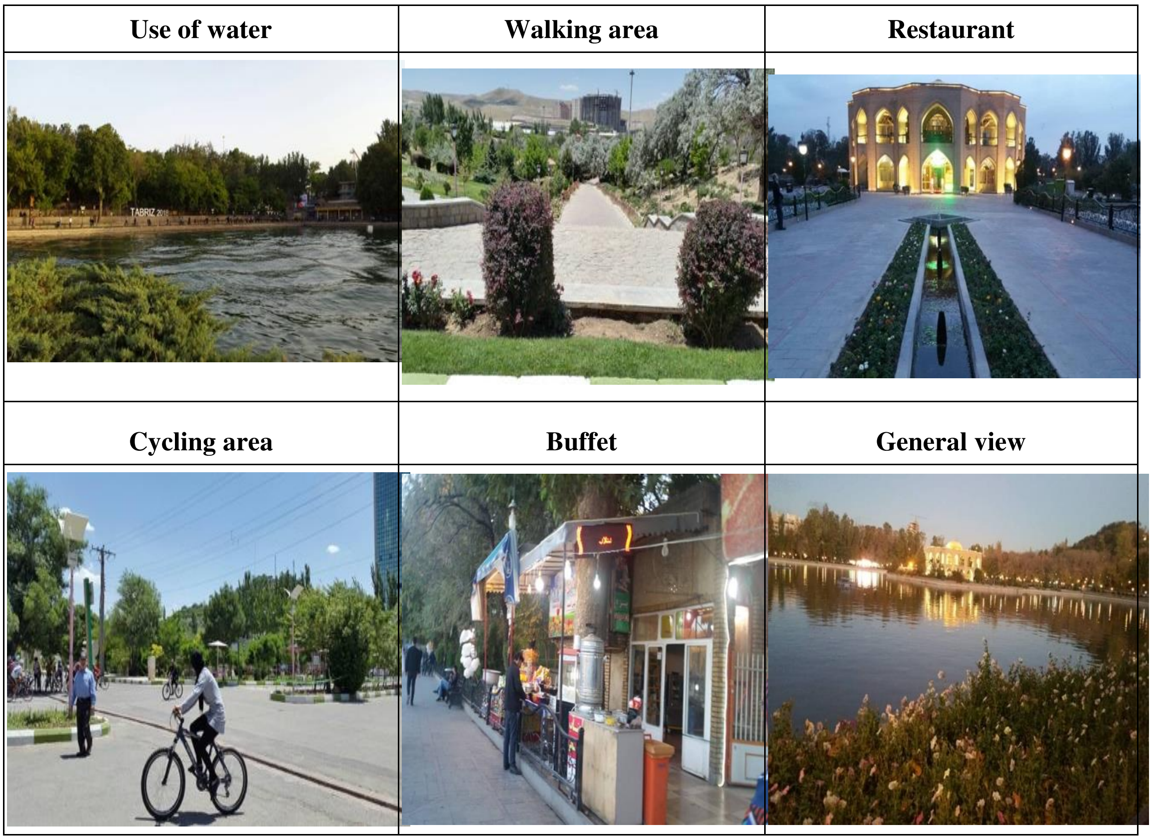

Figure 2. *View of Shah Goli Park (Original, 2018)*

Results

Demographic profile of the participants

A total of 296 participants took part in the study. *Table 4* shows the participants' demographic information. The majority of participants were male (55.1%, n = 163), 18-30 years old (47.3%, n = 140), married (58.1%, n = 172), and had university level education (52.02%, n = 154) (*Figs. 3–6*).

Table 4. *Demographic description of the survey*

Variables	Number	Percent
Gender	296	100
Male	163	55.1
Female	130	43.9
Age		
18-30	140	47.3
31-40	87	29.4
41-50	36	12.2
Above 50	30	10.1
Marital status		
Single	121	40.9
Married	172	58.1
Education		
Guidance school	25	8.4
Under diploma	29	9.79
Diploma	88	29.7
University	154	52.02

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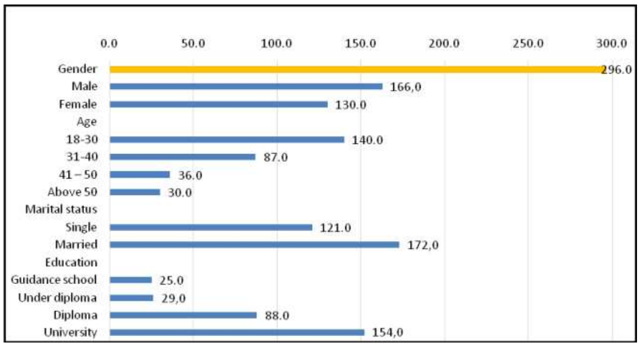

Figure 3. *Description of survey by gender, sex, age, marital status and education*

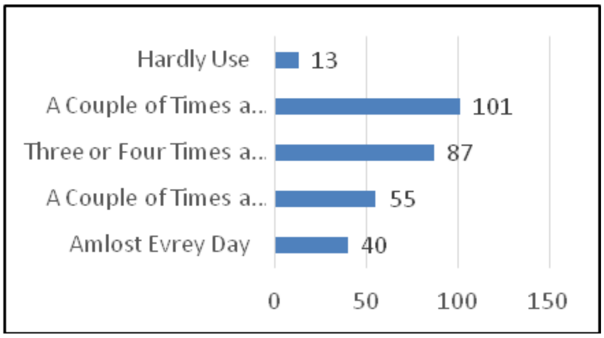

Figure 4. *Usage frequency of Shah Goli Park*

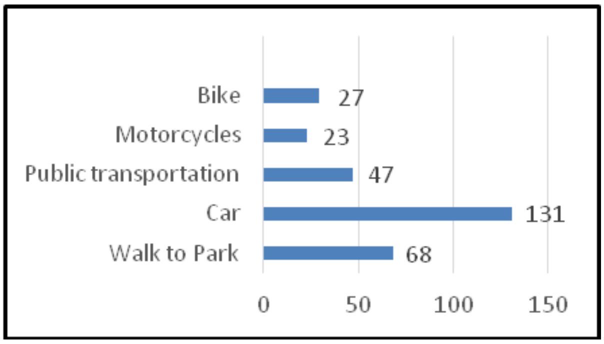

Figure 5. *Type of access to Shah Goli Park*

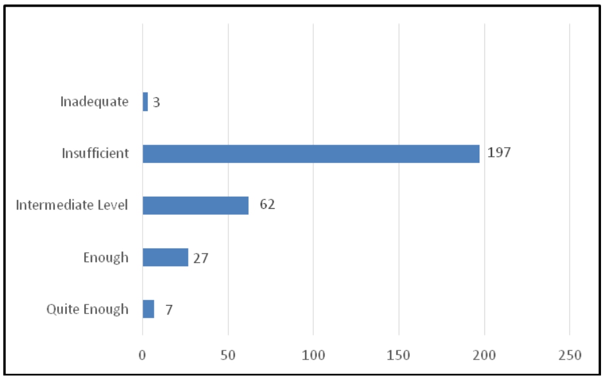

Figure 6. *According to users, Shah Goli Park's field competence*

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Preferences for park usability

What is shown in the collected data is that "enjoy the landscape view" (mean = 4.72, sd = .55) has the highest preference rate among the other activities for parks, followed by 'to walk in the park' and 'to exercise ' (mean = 4.53, sd = 0.66), 'To watch water flow in the park' (mean = 4.46, sd= .87), 'To socialize with friends' (mean = 4.33, sd = .83), 'To sit under the trees shade' (mean = 4.37, sd= .78), 'To socialize with friends' and 'To have dinner with family or friends in the park' (mean = 4.33, sd= .83), 'To picnic' (mean = 4.27, sd= .93), 'To be with family' (mean = 4.04, sd = 1.11), 'To play with children in the park' (mean = 3.93, sd = .90), 'To socialize with people' (mean = 3.66, sd= .1.18) (*Table 5; Fig. 7*). These results show the areas that the participants preferred for highly social and environmental activities in the park. It seems clear that people are not provided with adequate space for exercising and hiking in a crowded city like Tabriz, arising the need for them to go parks, along with open public spaces in order for hiking and similar activities

Table 5. *Mean analysis for usability variables*

Variables	Mean	Std. dev.
To walk in the park	4.53	0.66
To picnic	4.27	0.93
To watch water flow in park	4.46	0.87
To socialize with friends	4.33	0.83
To enjoy the landscape view	4.72	0.55
To rest and sit under tree shade	4.37	0.78
To be with family	4.04	1.11
To socialize with other people	3.66	1.18
To exercise	4.53	0.66
To play with children in the park	3.93	0.90
To have dinner with family or friends in the park	4.33	0.83

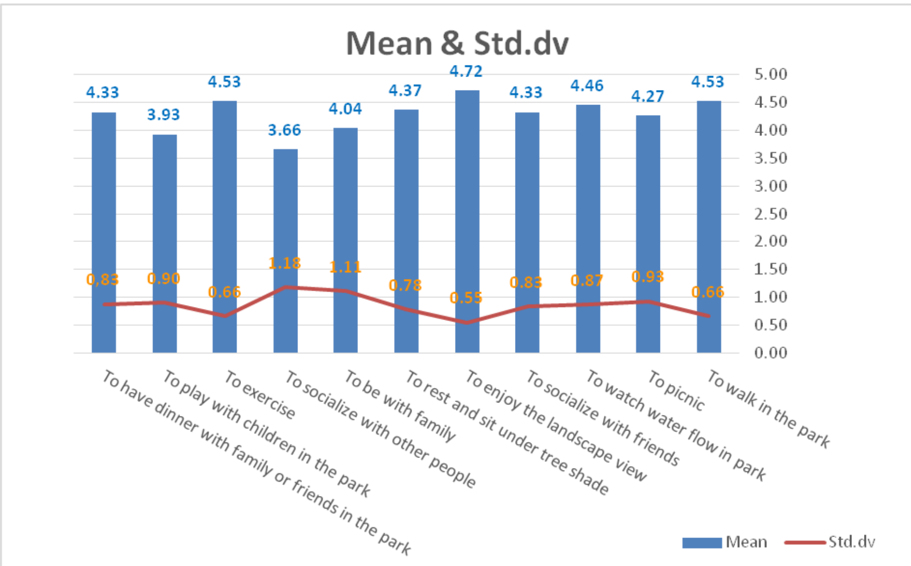

Figure 7. *Mean analysis for usability variables*

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Park usability underlying dimensions

In order to identify the underlying dimensions within the scope of park availability, a factor analysis was carried out on the collected data. Three dimensions were obtained following the analysis on the respective items (*Table 6*).

Activities like nature-related activities may seem appealing to more people for visiting the parks. What is interesting is that there is a relation between plants' contribution to activities done in urban park. A factor analysis on activity set forth that Social Activities (mean = 4.19, sd = 0.69), and five Environmental Activities (mean = 4.64, sd = 0.67) acquired the highest rate, respectively (*Table 6*).

Table 6. *Underlying dimensions of park usability*

Social activities	**Factors**	**Mean**	**Std. dev.**
		4.19	**0.69**
To be with family	.746		
To play with children in the park	.623		
To socialize with friends	.723		
To socialize with other people	.608		
To exercise	.544		
To have dinner with family or friends in the park	.595		
Environmental activities			
		4.64	**0.67**
To have a picnic	.701		
To walk in the park	.607		
To watch water flow in park	.730		
To rest and sit under tree shade	.488		
To enjoy the landscape view	.614		

Discussion

The role of historical parks as the provider of social services, along with its significance for city's sustainability has been determined in this research. For exploring the motives and perceptions of the visitors of Shah Goli Park, certain results of the questionnaire have been presented. Some of the concluded remarks are as follows:

To begin with, the study states that urban planning, along with the contributions it provides in tackling a number of detrimental environmental problems, which have been recently recognized as the characteristic of crowded city centers and urban environments, may be recognized as a premise in terms of creating sustainable urban life. Urban green spaces have the ability to gather people together, while increasing the public congregation and interaction along with increasing accessibility to public services and employment, which, by the way, may be the essential aims of sustainable urban policy.

Depending on the number of users, usage frequency, involved functional facilities and social carriage capacity of people, ecological carriage capacity of the area, the park's surface area may vary. These factors are directly related to the social and environmental structure of people. In this study, the survey was performed on 14 subjects in the park, with a total participant number that is equal to 296. A

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supplementary X2 significance test was carried out with over 296 questionnaires received from the visitors of park, of which results can be seen in *Table 6*. The aim of the study was to determine the individual features of users (gender, marital status, age, education) and differences among these features. From the results obtained, the individual features of 296 participants were as follows: for gender status, 55.1% of subjects was male and 43.9% was female; for marital status, 40.9% was married and 58.4% was single; for age, 47.3% was between 18-30, 29.4% was between 31 and 40, 12.2% was between 41 and 50 and 10.1% was above 50; for education level 8.4% was guidance school; 8.4% was under diploma, 29.7% was diploma and 52% was university graduate.

Conclusion

The results obtained set forth that the participants prefer to perform environmental activities like enjoying the landscape view, watching the water flow in the park and walking around the park. People seemed interested in direct involvement with the natural elements like plant materials and water, while carrying out the respective environmental activities. It is understood that green spaces and water features lead users to feel relieved, thus ensuring serenity in parks. The results indicate that the activities that are performed most frequently in urban parks are as follows: having a picnic, walking, sitting, admiring nature, and watching plant material scenes. So, it can be said for people that they wish to connect with green areas for entertainment and natural involvement in urban parks, attracting more people to visit parks. The study underlined the significance of plant material and water feathers arrangement and configuration within the scope of improving the social life of people. Additionally, activities that are performed by people in urban parks are required to be designed and planned in connection with the natural element's spatial configuration.

Considering the positive effects of open-green areas on human psychology, it can be clearly seen that recreative activities by people in green areas are of vital importance. The leading determinant in this matter is accessibility. People usually prefer going to parks and green areas that are close to where they live, but some people may choose to go distant places in line with the quality of time they spend. Increasing the ratio of urban green areas per capita will not only proportionally increase the options for town-dwellers, but they will also be provided with the opportunity to access areas that offer different activities. Existence of green areas affects both the physical health thanks to the, for example, plants that clear the air, and psychological health of people. In this context the life quality in urban areas increases with green areas, which ensures improvement in the sense of belonging and well-being.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31473163

SELECTIVE ADSORPTION OF U(VI) BY USING U(VI)-IMPRINTED POLY-HYDROXYETHYL METHACRYLATE-METHACRYLOYL-L-HISTIDINE (P-[HEMA-($MAH)_3$]) CRYOGEL POLYMER

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(Received 3rd Jan 2019; accepted 8th Feb 2019)

Abstract. In this study, selective adsorption of U(VI) in aqueous solutions in the presence of various lanthanide ions was conducted by using U(VI)-imprinted cryogel polymer. For this purpose, the pHEMA-$(MAH)_3$-U(VI) cryogel polymer was prepared by free radical polymerization method. U(VI) was desorbed with 5.0 mol.L^{-1} HNO_3 and thus U(VI)-imprints were created on p-HEMA-$(MAH)_3$ cryogel polymer. To determine the optimum conditions, in the process of selective adsorption of U(VI) ion to U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer, some parameters such as pH, flow rate, initial U(VI) concentration were investigated. Under the optimum conditions, the maximum adsorption capacity was obtained as 74.80 mg.g^{-1}. Selectivity studies were also carried out in the presence of Nd(III), La(III) and Y(III) ions using U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer. The obtained adsorption order under competitive conditions was U(VI) > La(III) >Y(III) >Nd(III).
Keywords: *uranium, adsorption, ion imprinting, lanthanides, nuclear energy*

Abbreviations: HEMA: 2-Hydroxyethyl methacrylate; MAH: N-methacryloyl-(L)-histidine methyl ester; TEMED: N,N,N,N-tetramethylethylenediamine; APS: Ammonium persulfate; MBAAm: N,N-methylenebisacrylamide; ICP-MS: Inductively Coupled Plasma-Mass Spectroscopy; SEM: Scanning Electron Microscopy; FT-IR: Fourier Transform Infrared; IIP: Ion Imprinting Polymer; NIP: Non Imprinting Polymer

Introduction

Uranium is an important material in nuclear science and an important actinide in radioactive waste water and environmental samples (Wang et al., 2017a). Uranium is a natural element that quite effective in the nuclear industry and particularly as a fuel for electricity generation by nuclear power plants (Iliaa et al., 2017). Studies on uranium-containing rare earth minerals are causing uranium-containing radioactive pollutants (Li et al., 2018; Wang et al., 2017b; Lu et al., 2016; Wang et al., 2015). Therefore, the solvent extraction and separation of Uranium from rare earth minerals is quite important for the environmental protection (Zhu et al., 2015).

Uranium is the most important fuel element used in nuclear reactors. Therefore, the recovery of Uranium is of great importance for the sustainable development of nuclear industry (Gu, 2007). The content of uranium in the earth's crust is limited (Abdollahy et al., 2011). Anticipating the shortage of nuclear fuels in near future, the selective recovery of Uranium becomes necessary for the sustainable development of nuclear energy (Chmielewski, 2008).

The removal of uranium from the body has been studied by a number of investigators. Mostly, the low concentrations of uranium encountered and the presence of high levels of interfering matrix constituents prevent its direct determination. Because of this, various separation and preconcentration techniques are employed for the determination of uranium. Although liquid–liquid extraction has been widely used

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DOI: http://dx.doi.org/10.15666/aeer/1702_31653178

(Horwitz et al., 1992), it is time consuming. Extraction chromatography (Dolak, et al., 2011), solid-phase extraction (Yener et al., 2017; Kaminski et al., 2000), supercritical fluid extraction (Haerizade et al., 2018), ion exchange (Dolak et al., 2010) and adsorbents (İnam et al., 2001; Sana et al., 2015) have been extensively used for the separation and preconcentration of uranium ions. The process of using adsorbents is an effective method for heavy metals by using metal chelating resins prepared with containing aminoacid monomer ligands (Dolak et al., 2015; Keçili et al., 2018; Dolak, 2018), and for recovering uranium because of the high selectivity for uranium, the ease of handling, and environmental safety. The solid-phase extraction methods using molecular imprinted polymers are the most used methods for the separation and preconcentration of rare earth elements (Harkins and Schweitzer, 1991; Panahi et al., 2012; Wulff, 1995).

Molecular imprinting is a method for making selective binding sites in synthetic polymers by using molecular template. Metal cations can be used as templates for imprinting crosslinked polymers. After the removal of template (the cation), the remaining polymer is more selective. The selectivity of the polymer depends on various factors, like the charge on the cation, the size of the cation, the specificity of the interaction of the ligand, the coordination geometry, and the number of the cations. Transition metals can also be removed by using the molecular imprinting method (Pakdehi, 2016; Dhal and Arnold, 1992; Chen et al., 2017).

Molecular imprinting is a new technique has attracted the attention of researchers for effective recognition of chemical and biological molecules including aminoacids, proteins, enzymes, DNA, drugs and metal ions (Didaskalou et al., 2017; Székely et al., 2012a, b; Dolak et al., 2018). This technique allows selective and sensitive recognition of chosen target molecule by leaving artificial imprinted cavities in polymer matrix that provides high affinity to target molecule. To synthesize molecularly imprinted polymer, the template molecule and functional monomers which can arrange around template are complexed interactively before polymerization. Then the rigid polymer matrix is obtained by polymerization of formed pre-complex and cross-linker reagent. After removal of template molecule from the polymer with suitable desorption agent, the cavities remaining in the polymer that are complementary in shape, size and chemical functionality to the template. Consequently, the resultant polymer able to recognizes and rebinds selectively the template or other molecules that are chemically related to the template (Saylan et al., 2017). This technique is used in many applications such as selectivity recognition and separation (Sellerggren, 2001; Wei and Mizaikoff, 2007; Vedadghavami et al., 2018; Lasáková and Jandera, 2009), drug delivery systems (Alvarez-Lorenzo and Concheiro, 2004), catalysis (Vidyasankar and Arnold, 1995), sensor technology (Monier and Abdel-Latif, 2017). In addition, ion imprinted polymers (IIPs) have been used for the selective removal of metal ions from different matrices (Moussa et al., 2016; Moorthy et al., 2013; Monier et al., 2016; Mitreva et al., 2017; Msaadi et al., 2017; Roushani et al., 2015; Fayazi et al., 2016; Candan et al., 2009; Gao et al., 2015).

In this study, the selective adsorption of U(VI) in aqueous solutions and soil certified reference material in the presence of other lanthanide ions such as Nd(III), La(III) and Y(III) was performed by using U(VI)-imprinted pHEMA-$(MAH)_3$ cryogel polymer purposed. For this purpose, U(VI) was complexed with N-methacryloyl-L-histidine methyl ester (MAH) and the prepared $(MAH)_3$-U(VI) complex monomer was polymerized with 2- hydroxyethyl methacrylate (HEMA) cryogel to prepare pHEMA-

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DOI: http://dx.doi.org/10.15666/aeer/1702_31653178

$(MAH)_3$-U(VI) cryogel polymer by free radical polymerization method. U(VI) was desorbed with 5.0 mol.L^{-1} HNO_3 and thus were created U(VI) imprinted on to p-HEMA-$(MAH)_3$ cryogel polymer. In the process of selective adsorption of U(VI) ion to U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer, several factors such as medium pH, flow rate, initial U(VI) concentration were investigated to determine optimum conditions. Selectivity studies were also carried out in the presence of Nd(III), La(III) and Y(III) ions using U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer. The obtained adsorption order under competitive conditions was U(VI) > La(III) > Y(III) > Nd(III).

Materials and methods

Chemicals and reagents

The chemicals used in the study and their properties are given in *Table 1*.

Table 1. *Used chemicals and their properties*

Chemicals	For what	Manufacturer
Methacryloyl cloride	For monomer synthesis	Sigma Aldrich-Steinheim, Germany (purum, dist., ≥97.0% (GC), contains ~0.02% 2,6-di-tert-butyl-4-methylphenol as stabilizer)
L-histidine	For monomer synthesis	Sigma Aldrich-Steinheim, Germany (ReagentPlus®, ≥99%)
2-Hydroxyethyl methacrylate (HEMA)	For polymer synthesis	Sigma Aldrich-Steinheim, Germany (≥99%, contains ≤50 ppm monomethyl ether hydroquinone as inhibitor)
N,N,N,N-tetramethyl ethylene diamine (TEMED)	For polymer synthesis	Sigma Aldrich-Steinheim, Germany (BioReagent, suitable for electrophoresis, ~99%)
N,N-methylenebisacrylamide (MBAAm)	For polymer synthesis	Sigma Aldrich-Steinheim, Germany (99%)
Ammonium persulfate (APS)	For polymer synthesis	Sigma Aldrich-Steinheim, Germany (reagent grade, 98%)
Uranyl nitrate	For experimental optimization studies	Sigma Aldrich-Steinheim, Germany (extra pure, ≥99%)
Lanthanium(III) nitrate hexahydrate	For selectivity study	Sigma Aldrich-Steinheim, Germany (extra pure, ≥99%)
Neodymium(III) nitrate hexahydrate	For selectivity study	Sigma Aldrich-Steinheim, Germany (extra pure, ≥99%)
Yttrium(III) nitrate hexahydrate	For selectivity Study	Sigma Aldrich-Steinheim, Germany (extra pure, ≥99%)
All organic solvents	For synthesis ımprinted polymer	Sigma Aldrich-Steinheim, Germany

Instrumentation

The analysis of the U(VI) and the other lanthanide ions was performed using a Agilent 7700 Series inductively coupled plasma-mass spectroscopy (ICP-MS). System

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with the following parameters: RF Power = 1600 W, sampling depth = 5.3 mm, analyzer pressure = 7.92×10^{-5} Pa, helium flow in the collision cell = 4.98 $mL.min^{-1}$ and plasma temperature = 9883 K. the measurements were done with three replicates (95% confidence level). A Perkin Elmer model Spectrum 400 FT-IR spectrometer was used for the Fourier transform infrared (FT-IR) measurements. Scanning electron microscopy (SEM) analyses were carried out by using a FEI Quanta FEG 250 SEM system.

Preparation of U(VI)-imprinted and non-imprinted p-HEMA-$(MAH)_3$ cryogel polymer

Synthesis of N-methacryloyl-L-histidine methyl ester (MAH)

The preparation and characterization of N-methacryloyl-(L)-histidine methyl ester (MAH) was reported elsewhere (Bereli et al., 2011). The following procedure was applied for the synthesis of MAH monomer: 5.2 g of L-histidine methylester and 0.22 g of hydroquinone were dissolved in 150 mL of CH_2Cl_2 solution. Solution was cooled down to 0 °C. 12.83 g triethylamine was added to the solution. 4.0 mL of methacryloyl chloride was poured slowly into this solution under nitrogen atmosphere and then this solution was stirred magnetically at room temperature for 1 h. At the end of this chemical reaction period, unreacted methacryloyl chloride was extracted with 10% NaOH. Aqueous phase was evaporated in a rotary evaporator. MAH was crystallized in 20 mL (1:1) ethanol and ethyl acetate. The reaction efficiency was determined as 84% in the stoichiometric calculations based on the amount of reagents added to the reaction medium and the amount of MAH monomer obtained as a result of the process. In addition, it was determined that the purity of MAH monomer synthesized as a result of the characterization processes was greater than 98%.

Preparation of $(MAH)_3$-U(VI) complex monomer

For preparation $(MAH)_3$-U(VI) complex monomer, MAH (0.669 mg, 3.0 mmol) was dissolved in deionized water. After slow addition of $UO_2(NO_3)_2$ (0.394 mg, 1.0 mmol) to this solution, the solution was stirred for 24 h at room temperature. The obtained complex monomer was then filtered and extensive washed with EtOH and deionized H_2O. Then, it was dried at 50 °C for 24 h. For the yield of the synthesis of the synthesized MAH-Th(VI) complex monomer, it was initially determined by ICP-MS analysis of the remaining Th(VI) in the medium which was added to the medium and the complex formation yield was determined to be greater than 95%.

Synthesis of p-HEMA-$(MAH)_3$-U(VI) (IIP) and p-HEMA-$(MAH)_3$ (NIP) cryogel polymer

p-HEMA-$(MAH)_3$-U(VI) and p-HEMA-$(MAH)_3$ cryogel polymers was prepared according to a previously reported method (Baysal et al., 2018). For this purpose, 0.25 g MBAAm was dissolved in deionized H_2O, and then 2.0 mL HEMA and 2.0 mL $(MAH)_3$-U(VI) complex monomer were mixed with this solution. Initiator APS (25 mg)/TEMED (25 µl) was added and the final mixture was placed into a syringe closed with parafilm and allowed to polymerize at -18 °C for 24 h. The frozen solution was allowed to thaw at room temperature. Finally, the prepared imprinted cryogel polymer was washed with 100 mL (1:1) EtOH and deionized H_2O to remove impurities, which was then stored at +4 °C. Non-imprinted polymer were prepared in the same way but in the absence of the template.

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DOI: http://dx.doi.org/10.15666/aeer/1702_31653178

Removal of U(VI) from p-HEMA-$(MAH)_3$-U(VI) cryogel polymer

To obtain the 3-D cavities for desorption of U(VI), the template U(VI) was successfully desorbed from the p-HEMA-$(MAH)_3$-U(VI) cryogel polymer (*Fig. 1*). For this purpose, the cryogel polymer was desorbed with 10 mL 5.0 mol.L^{-1} HNO_3 as the desorbtion solvent for 1 h by using a peristaltic pump. This washing step was repeated until no U(VI) was determined in the desorption solvent.

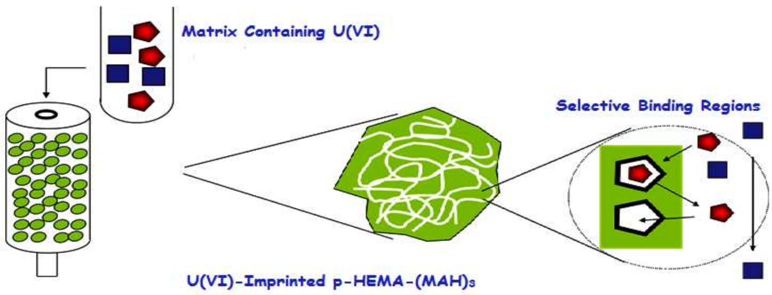

Figure 1. *The prepared U(VI)-imprinted column system and selective separation scheme of U(VI)*

Figure 1 shows schematically that only U(VI) is selectively bound when the mixture of La(III), Nd(III), Y(III) and U(VI) is passed through a prepared U(VI) imprinted p-HEMA-$(MAH)_3$ cryogel column.

Characterization studies

MAH monomer and prepared $(MAH)_3$-U(VI) complex monomer were characterized by FT-IR spectroscopy, whereas U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer were characterized by FT-IR spectroscopy and SEM technique.

To obtain FT-IR spectrum U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer, KBr was mixed with the dried polymer particles and pressed into a pellet form, and the spectra were then recorded.

For the SEM analysis of U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer were covered on the surface of platinum and coated with gold (thickness of 20 nm). Then, SEM analyses were carried out.

To calculate the swelling ratio of p-HEMA-$(MAH)_3$ cryogel polymer, the cryogel was dried and weighed until constant weight (m_{dried}). Then, it was placed in a 30 mL vial containing distilled water and kept at 25 °C for 2 h. The cryogel was removed from water, wiped by a filter paper and weighed again (m_{wet}). The swelling ratio was calculated according to *Equation 1:*

$$S = m_{wet} - m_{dried} / m_{dried} \quad \text{(Eq.1)}$$

For the measurement of macroporosity percentage (M%) of cryogels, the mass of water-saturated cryogels (m_{wet}) was weighed. The cryogel was squeezed to remove free water which is found in the pores ($m_{squeezed}$), and the mass of cryogel without water was weighted. M% was calculated according to *Equation 2:*

$$M\% = m_{wet} - m_{squeezed} / m_{wet} \quad \text{(Eq.2)}$$

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Adsorption studies of U(VI) ion to U(VI)-imprinted (IIP) and non-imprinted (NIP) cryogel polymer

Continuous column system was used to adsorption U(VI) to U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer (IIP) and non-imprinted p-HEMA-$(MAH)_3$ cryogel polymer (NIP). For this purpose, firstly, columns containing IIP and NIP was washed with deionized H_2O and equilibrated with 0.1 mol.L^{-1} phosphate buffer at pH. 7.0. Then, aqueous solution of U(VI) was passed through the columns containing IIP and NIP at 1 mL.min^{-1} flow rate for 1 h. The amounts of U(VI) was determined by ICP-MS. Then, 5.0 mol.L^{-1} HNO_3 was used to desorption of U(VI) bound to the IIP and NIP. Several factors such as pH, flow rate and initial U(VI) concentration were also investigated to obtain the optimum conditions for the adsorption of U(VI). 10 ppm U(VI) solutions in different pH values (pH 3 to 10) was passed through the columns containing IIP and NIP at 1 mL.min^{-1} flow rate for 1 h in order to test pH influence on U(VI) adsorption to the IIP and NIP. Then, the samples came out from the column were analyzed by ICP-MS. The flow rates between 1.0 mL.min^{-1} and 5.0 mL.min^{-1} were applied for the investigation of the effects of these parameters on the adsorption of U(VI). The initial U(VI) concentration was varied between 10 ppm and 2000 ppm to determine maximum adsorption capacity.

Selectivity study

The selectivity of the prepared U(VI)-imprinted (IIP) and Non-imprinted NIP cryogel polymers toward U(VI) were investigated in the presence of U(VI)-Nd(III), U(VI)-La(III) and U(VI)-Y(III) ion pairs. For this purpose 25 mL of 10 ppm lanthanide solutions in 10 mM phosphate buffer, pH 7.0 were passed from columns containing IIP and NIP at a flow rate of 1 mL.min^{-1} at room temperature. Analysis of the lanthanide ions in the column output samples was performed by ICP-MS.

The distribution coefficient of U(VI) ion between the columns containing IIP and NIP and aqueous solutions was calculated using *Equation 3*:

$$K_d = (C_i\text{-}C_f/C_f) \times (V/m) \qquad \text{(Eq.3)}$$

where K_d is the distribution coefficient, C_i is initial U(VI) concentration and C_f is final U(VI) concentration, V represents the solution volume (mL) and m is the polymer mass (g).

The selectivity coefficient (k) and relative selectivity coefficient (k^1) for U(VI) in the presence of other competing lanthanide ions can be calculated applying *Equations 4* and *5:*

$$k = K_{(U(VI))} / K_{(\text{interfering ion})} \qquad \text{(Eq.4)}$$

$$k^1 = K_{(\text{imprinted})} / K_{(\text{nonimprinted})} \qquad \text{(Eq.5)}$$

where $K_{(U(VI))}$ is the distribution ratio of U(VI) ion and $K_{(\text{interfering ion})}$ is the distribution ratio of potentially interfering ions.

Reusability of U(VI)-imprinted cryogel polymer (IIP)

For the reusability studies, adsorption and desorption studies were repeated 10 times using same IIP. After each step, column was washed with 10 mL 5 mol.L^{-1} HNO_3 and deionized water.

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Adsorption studies of U(VI) ion from soil certified reference material

Soil certified reference material was selected as the real sample for the selective adsorption of U(VI). For this purpose, 0.1 g soil certified reference material powdered was leached using concentrated HNO_3 and H_2SO_4 by microwave irradiation. Then, solution pH was adjusted to 7.0 using phosphate buffer and volume of the final solution was distilled to 100 mL by deionized water. The prepared solution was passed through the columns containing IIP and NIP the under the optimum conditions. Analysis of the ions in the column output samples was performed by ICP-MS.

Results and discussion

Characterization of MAH monomer and $(MAH)_3U(VI)$ complex monomer

Prepared $(MAH)_3$-U(VI) complex monomer were characterized by FT-IR spectroscopy, which proved that monomer and complex monomer were synthesized. The obtained FT-IR spectrum of the MAH monomer and $(MAH)_3U(VI)$ complex monomer is given in *Figure 2*.

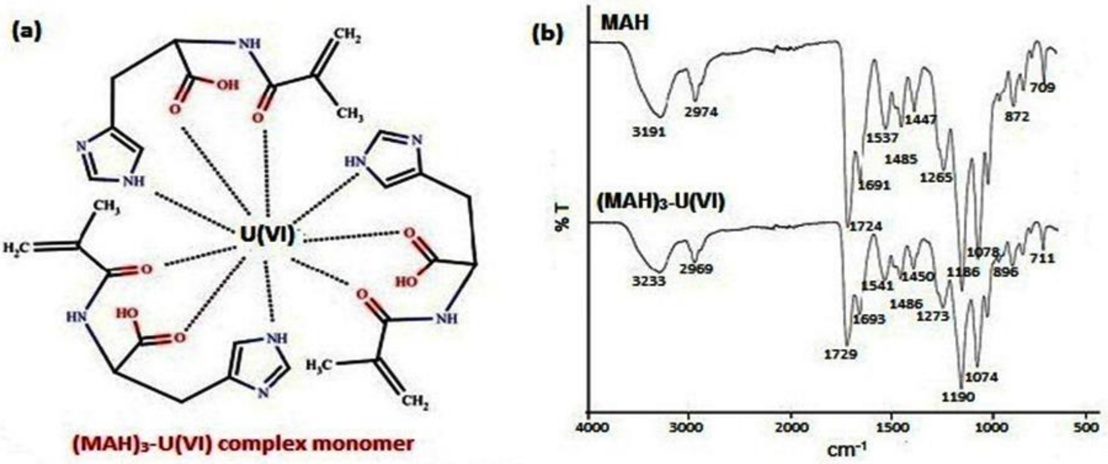

***Figure 2. a** Proposed structure for complex formed; **b** FTIR spectrum for the MAH monomer and $(MAH)_3U(VI)$ complex monomer*

Characterization of U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer (IIP)

p-HEMA and Th(IV)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer (IIP) were characterized by FT-IR and SEM. *Figure 3* shows the FT-IR Spectra p-HEMA and U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer. As can be seen, p-HEMA and U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer exhibited FT-IR patterns with small differences which confirms the similar polymer backbone.

The pore structure and pore size of U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer were visualized with SEM images as seen in *Figure 4*. As shown in *Figure 3,* the U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer has interconnected pores and porous structure. Pore size was found about 50 µm.

The equilibrium swelling degree and macroporosity of the U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel were 6.12 g H_2O/g cryogel and 81.04%, respectively.

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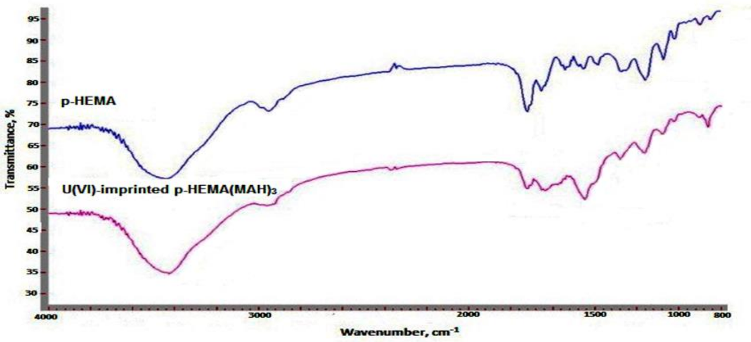

Figure 3. *FT-IR spectrum p-HEMA cryogel and U(VI)-imprinted p-HEMA-$(MAH)_3$ cryogel polymer*

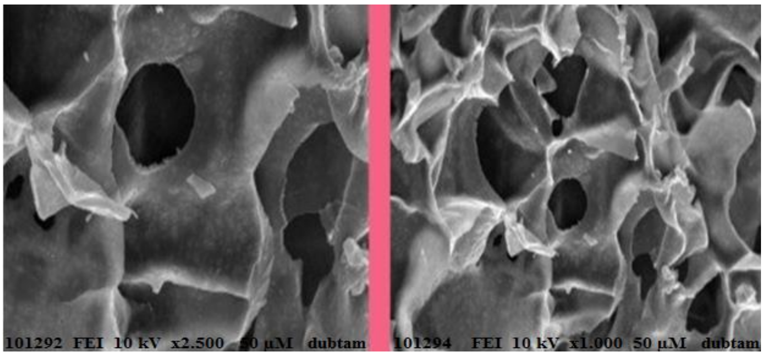

Figure 4. *SEM images U(VI)-imprinted p-HEMA-$(MAH)_2$ cryogel polymer (IIP)*

Adsorption studies of U(VI) on IIP and NIP

pH effect on U(VI) adsorption

The change in amount of U(VI) adsorption to the IIP and NIP as a function of pH was investigated, as seen in *Figure 5*. The maximum U(VI) binding to the IIP and NIP pH 7.0. This could be explained by electron transfer based covalent cross-linking between U(VI) and L-histidine of the functional monomer at pH 7.0. *Figure 5* clearly shows effect of pH on U(VI) binding to the IIP and NIP. As seen in the figure, the values higher and lower than pH 7.0 lead to low adsorption of U(VI) to the IIP and NIP, which can be explained by the repulsive electrostatic interactions between bound U(VI) ion and MAH monomer. The adsorption efficiency may decrease because of the size of conformation and the lateral electrostatic interactions between adjacent U(VI) ion on the IIP and NIP. The experiments were repeated 3 times and the statistical values were given as 95% confidence level relative standard deviation (RSD).

Flow rate effect on U(VI) adsorption

The flow rate of the U(VI) solution pumped through the cryogel is one of the crucial parameter for the control of binding process (Fayazi et al., 2016). The flow rate effect on the adsorption of U(VI) was explored by changing the flow rate from 1.0 to 5.0 $mL.min^{-1}$. 10 ppm U(VI) solution was used for this purpose. Owing to the back pressure produced by the column, the flow rates higher than 5.0 $mL.min^{-1}$ could not be

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investigated. The experiments were repeated 3 times and the statistical values were given as 95% confidence level relative standard deviation (RSD). As shown in *Figure 6,* increasing flow rate resulted in a decrease in the adsorption of U(IV) from 96.56 ± 1.52% to 70.84 ± 1.26% adsorption capacity.

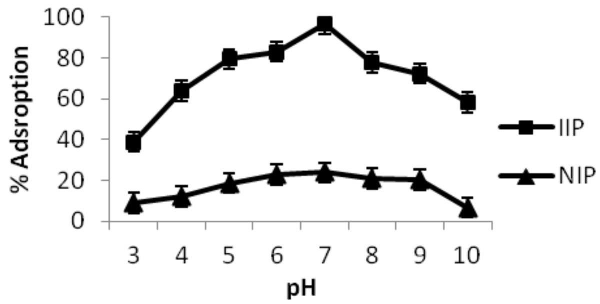

Figure 5. *Effect of pH on U(VI) adsorption (experimental conditions: initial U(VI) concentration: 10 ppm; temperature: 25 °C; flow rate: 1 mL.min*$^{-1}$*; time: 1 h)*

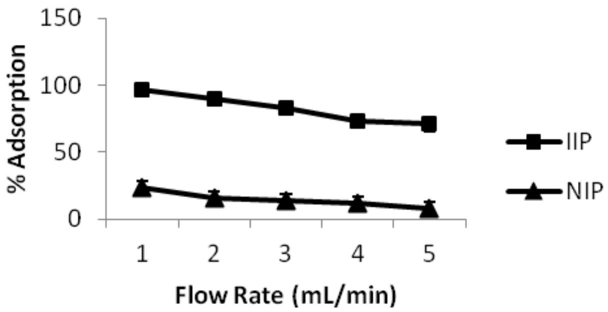

Figure 6. *Effect of flow rate on U(VI) adsorption (experimental conditions: pH:5; initial U(VI) concentration: 10 ppm; temperature: 25 °C; time: 1 h)*

The determination maximum adsorption capacity of IIP and NIP

Initial U(VI) concentration dependence of the bound amount of the U(VI) on to IIP and NIP is depicted in *Figure 7*. As can be seen *Figure 7,* U(VI) adsorption increased when initial U(VI) concentration is increased, and an equilibrium was obtained at a U(VI) concentration of 2000 ppm. The maximum adsorption capacity was obtained as 74.80 ± 1.33 mg.g^{-1} for IIP, while that of NIP was 14.76 ± 1.09 mg.g^{-1}. The experiments were repeated 3 times and the statistical values were given as 95% confidence level relative standard deviation (RSD). it was found that maximum adsorption yield obtained was good result when compared to other studies (Iliaa et al., 2017; Li et al., 2018; Zhu et al., 2015).

Regeneration and reusability of the IIP

One of the crucial advantages for an affinity material for the recognition and separation processes is its reusability (Kupai et al., 2017). To test the reusability of the prepared IIP, U(VI) adsorption and desorption cycle was repeated 10 times using the same cryogel (*Fig. 8*). The elution of U(VI) from the IIP was performed by using 10 mL 5.0 mol.L^{-1} HNO_3 as the desorption solution and complete removal of U(VI) was

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DOI: http://dx.doi.org/10.15666/aeer/1702_31653178

achieved after the desorption step. It was found that the adsorption behavior of the IIP towards U(VI) did not changed significantly after ten adsorption and desorption cycles. Thus, one can easily say that the IIP are stable and the IIP can be used many times without significantly loss of their adsorption capacity.

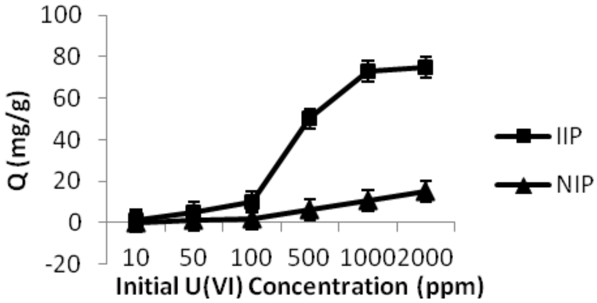

Figure 7. *Effect of initial U(VI) concentration on Th(IV) adsorption (experimental conditions: pH:5; temperature: 25 °C; flow rate: 1 mL.min^{-1}; time: 1 h)*

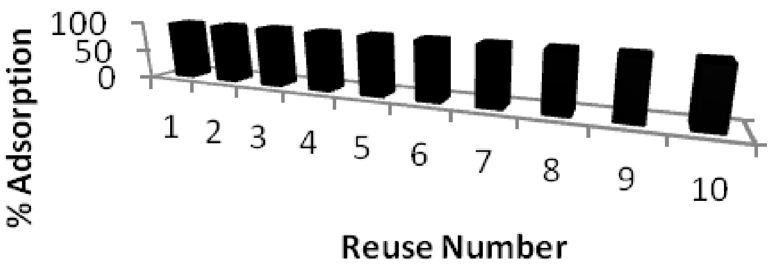

Figure 8. *Reusability of IIP (experimental conditions: pH:5; initial U(VI) concentration: 10 ppm; temperature: 25 °C; flow rate: 1 mL.min^{-1}; time: 1 h)*

Selectivity studies

Competitive adsorption of U(VI)-Nd(III), U(VI)-La(III) and U(VI)-Y(III) were also explored in a column system. The obtained results are given in *Table 2*. U(VI) imprinted cryogel polymer (IIP) exhibited higher selectivity toward U(VI) ions over Nd(III), La(III) and Y(III) ions. K_d values for the IIP were compared with NIP. The obtained results confirmed that the relative selectivity coefficients of the IIP for the U(VI)/Nd(III), U(VI)/La(III) and U(VI)/Y(III) were 129, 60 and 79 times higher than the corresponding NIP, respectively. As a result, it was found that the prepared U(VI)-imprinted cryogel polymer (IIP) exhibited a selectivity to U(VI) ion in the presence of other lanthanides.

Selective adsorption of U(VI) from soil certified reference material

The outcomes of the adsorption of U(VI) from certified reference material are given in *Figure 9.* The results showed that the IIP displayed 93.11 ± 1.47% adsorption toward U(VI) while NIP showed 18.74 ± 0.91% adsorption. The experiments were repeated 3 times and the statistical values were given as 95% confidence level relative standard deviation (RSD).

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Table 2. *K_d, k and k' values of Nd(III), La(III) and Y(III) with respect to U(VI) (experimental conditions: pH:5; temperature: 25 °C; flow rate: 1 mL.min^{-1}; time: 1 h)*

Cryogel column	**U(VI) (ppm)**	**Nd(III) (ppm)**	**K_d (U(IV))**	**K_d (Nd(III))**	***k***	***k'***
Non-imprinted	10	10	761.2	1972.1	0.38	-
U(IV)-imprinted	10	10	98090	2760.1	35.5	129
Cryogel column	**U(VI) (ppm)**	**La(III) (ppm)**	**K_d (U(IV))**	**K_d (La(III))**	***k***	***k'***
Non-imprinted	10	10	1827.9	2633.5	0.70	-
U(IV)-imprinted	10	10	108155	2086.0	51.3	60
Cryogel column	**U(VI) (ppm)**	**Y(III) (ppm)**	**K_d (U(IV))**	**K_d (Y(III))**	***k***	***k'***
Non-imprinted	10	10	927.8	2331.5	0.40	-
U(IV)-imprinted	10	10	73258	2041.0	36.0	79

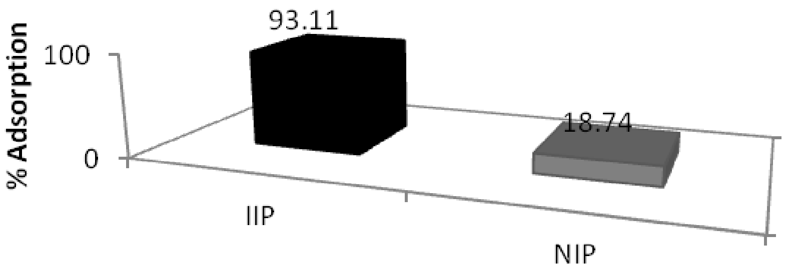

Figure 9. *Selective adsorption of U(VI) from certified reference material (experimental conditions: $C_{U(VI)}$ = 0.93 ppm (in the SRM), pH = 5.0, flow rate = 1 mL.min^{-1}, T = 25 °C)*

Conclusions

We have shown that U(VI)-imprinted cryogel polymer (IIP) that contains Poly-Hydroxyethyl Methacrylate-Methacryloyl-L-Histidine is selective and has high adsorption capacity for U(VI) ion. A High adsorption rate was observed at the beginning of the adsorption process and saturation values are reached within 60 min. The maximum U(VI) adsorption capacity of the cryogel polymer was 74.80 mg.g^{-1} for U(VI)-imprinted cryogel polymer, while that of non-imprinted cryogel polymer (NIP) was 14.76 mg.g^{-1}. The adsorption amount of U(VI) was maximum at pH 7.0. Competitive adsorption studies showed that, U(VI)-imprinted p-HEMA(MAH)$_3$ are only selective to U(VI) ion, even in the presence of other lanthanide ions such as, Nd(III), La(III) and Y(III) ions. Distribution (Kd), selectivity (k), and relative selectivity (k') coefficients were also calculated. The value of k' was found, 129, 60, and 79 for Nd(III), La(III), and Y(III), respectively. These k' values are high values if they are compared with reported research values. The obtained adsorption order under competitive conditions was U(VI) > La(III) >Y(III) >Nd(III). As a result, in our study to selectively remove the uranium used as fuel in nuclear reactors, it was determined that the prepared uranium-imprinted polymer exhibited effective uranium bonding activity and thus selectively separated from the matrix environment where uranium was found. After this stage, it is advisable to use sustainable membranes to purify the separated uranium and make it ready for use in the reactors (Fodi et al., 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31653178

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PHYTOTESTING OF THE SOILS OF URBAN PEDOCOMPLEXES IN RESIDENTIAL AREAS OF PERM, RUSSIA

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(Received 12th Nov 2018; accepted 12th Feb 2019)

Abstract. The response of watercress *Lepidium sativum* L. to the basic properties and toxicity of the soils has been established. When assessing the ecological state of the soil cover in residential areas of the city of Perm, the concept of the urban pedocomplexes as combination of soil and man-made surface formations on the same soil-forming rocks within a certain functional zone was applied. The problem of choosing a test control was solved by using vermiculite with Knop solution as a root substrate. Phytotesting showed mainly satisfactory conditions of the upper layers of the soils in urban pedocomplexes. At the same time, in the area of relatively old buildings, a trend towards the emergence of soil toxicity was revealed.
Keywords: *ecological state, urban soils, watercress, toxicity, test-control*

Introduction

The soil cover of urban landscapes has a complicated mosaic pattern; it consists of transformed and degraded soils and technogenic surface formations (TSFs) which are distinctly different from natural soils and often times are instantiated by lower biological activity and toxicity level. The process of the urban soil cover formations, without reference to the location, involves the following general factors: destruction of natural soils and formation of organo-mineral and mineral layers instead, accumulation of urban artifacts and rock formations in the soil profile, discontinuity of soil formation due to building activities, accumulation of heavy metals, oil products and salts (Dobrovolskiy, 1997; Marcotullio, 2011; Bardina et al., 2013; Ivanov and Kudeyarov, 2015; Yang and Zhang, 2015).

Perm is a large industrial city of the Russian Federation (its area is 780 km^2, population – over 1 million people). It was founded in 1724 as a settlement at a copper smelter. Originally the current center of the city was mainly represented by wooden two- and three-storey buildings. The central part of the city was formed in the period between 1780 and 1860. There were a few stone buildings - mainly administrative buildings, churches, cathedrals, some of which have survived to the present day. In between 1930’s-1970’s new residential areas appeared in the place of former villages and pine forests; also selective reconstruction of the old part of the city was pursued as well. Currently, the city is located on both banks of the Kama River. The essential industrial and residential buildings are located on the left bank, as well as the public center of the city (Nechaev, 2000).

In the residential part of Perm artificial groups of plants predominate (parks, lawns, plantings in yards). The air quality of the urban environment depends on the state of the soil and vegetation. Therefore, optimization of the environment is not possible without the regulation of the soil cover functions and properties.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31793197

The territory of the city of Perm, which is located in the valley of the Kama River, has a natural soil-lithological heterogeneity. Some specific factors were involved in urban soil formation: the widespread use of carbonate gravel in building and road works; the use of de-icing salts (sodium chloride and, to a lesser extent, potassium) on the roads, dumping lowland peat on the surface of organo-mineral and mineral grounds; relatively short duration of urban soil formation (several decades). Due to the high horizontal and vertical heterogeneity of the current soil cover, it grants a possibility for the concept of urban pedocomplexes – a combination of soil and man-made surface formations on the same soil-forming rocks within a certain functional zone (Shestakov et al., 2013). The concept of urban pedocomplexes has greatly facilitated the mapping and assessment of the soil structure of the Perm Region cities (Eremchenko et al., 2016).

Toxicity and low biological activity of urban soils could be induced by both factors – unfavorable properties (compaction, alkalinity, etc.) and accumulation of pollutants (heavy metals, salts, petroleum, etc.) (Dobrovolskiy, 1997; Poyat et al., 2007; Byrne, 2008; Sizov, 2008; Pickett and Cadenasso, 2009; Lisovitskaya and Terekhova, 2010; Marcotullio, 2011; Ivanov and Kudeyarov, 2015; Yang and Zhang, 2015). Currently, biotesting methods are widely used in soil quality control. These methods are mandative in the environmental practice in the USA, France, Germany, Sweden, Japan (Keddy et al., 1995; Juvonen et al., 2000; Maxam et al., 2000; Rivett et al., 2011; Van der Vliet et al., 2012; Romero-Freire et al., 2015). Method of soil biotesting evaluates the reactions of animals, microorganisms and plants. However, the priority is often given to higher plants that creates photosynthesizing cover, which is the basis of trophic interactions in biocenosis. The sensitivity of plants to soil and chemical effects is reflected in growth, morphological and biochemical parameters. Phytotesting is the basis of a method for assessing soil toxicity and their resistance to pollution (Gong et al., 2001; Voronina, 2009; Mayachkina and Chugunova, 2009; Kolesnikov et al., 2010; Lisovitskaya and Terekhova, 2010; Timofeev et al., 2010; Terekhova, 2011; Bardina et al., 2013, 2014; Nikolaeva and Terekhova, 2017; Gareeva, 2018). Many authors showed the effectiveness of the use of cultivated plants' small seeds, in particular of the watercress *Lepidium sativum* L. This species showed its sensitivity in response to pollutant analysis, both by individual pollutants (heavy metals, hydrocarbons, radioactive substances), and their integrated effect (Shunelko and Fedorova, 2000; Czerniawska-Kusza et al., 2006; Sujetovienė and Griauslytė, 2008; Lisovitskaya and Terekhova, 2010).

The goal of our research is to assess the ecological state of the surface soil layers in urban pedocomplexes of residential areas of Perm using the phytotesting method.

Materials and Methods

Laboratory experiment design

At the first stage of our research, we worked on a problem of examining the response characteristics of *Lepidium sativum* L. as a testing culture to the basic properties of regional soils and their contamination with some heavy metals. We used upper horizons of Chernozems, Retisols (Humic) and Albic Retisols. Soils were contaminated with lead nitrate at the rate of 1000 mg Pb per 1 kg of soil, cadmium sulfate at the rate of 500 mg Cd per 1 kg of soil. Lead was introduced in an amount corresponding to the high level of pollution noted in the urban soils (Eremchenko and Moskvina, 2005). The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31793197

toxicity of Cd is several times higher than that of other heavy metals (Kabata-Pendias, 2011), so its dose was halved relating to Pb.

The study of the soil cover was carried out in the residential zone of the multistorey building on the left-bank part of the city, which occupies about 60 km^2. As an object of study, we took the surface soil layers within the urban pedocomplexes (UPC) on eluvial-deluvial loams with outcrops of indigenous carbonate rocks (UPC-1), on ancient alluvial sands (UPC-2), on low-power deluvium, underlain by sand and sandy sediments (UPC-3), on alluvial sediments (UPC-4). From 15 to 28 soil samples from a depth of 0-15 cm were taken within each UPC (*Fig. 1*).

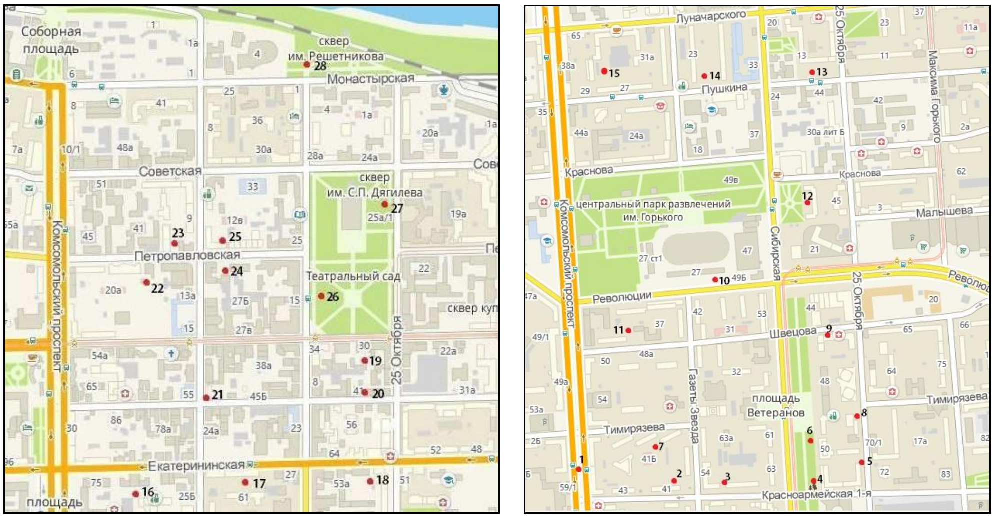

Figure 1. *Sampling points within the UPC-1*

In the samples of natural and urban soils there was determined:

- The content of organic carbon according to Tyurin.
- pH_{water}, pH_{KCl} –by potentiometric method.
- Hydrolytic acidity was determined by the Kappen method.
- Absorption capacity was calculated by adding the sum of the bases and hydrolytic acidity.
- Absorption capacity in carbonate samples - by the Melich method.
- Mobile phosphates and potassium - according to Kirsanov.
- The mobility of heavy metals, expressed in terms of their activity (-lg [Cd] and -lg [Pb]), by the ion-selective method using a pH-meter-ionomer.

Watercress was grown on natural and urban soils within 10 days. The total weight of plants (threefold), the height and weight of one plant in 30-fold repetition were measured. Of a test control there were plants grown on vermiculite with a Knop nutrient solution (1 g/l $Ca(NO_3)_2$, 0.25 g/l KH_2PO_4, 0.25 g/l $MgSO_4$, 0.125 g/l KCl, 0.0125 g/l $FeCl_3$), which was watering once after the seeds had been planted.

In the biotesting of urban soils, choosing the control turns to be very difficult. There are no analogues of these formation in nature. Their toxicity and low bioactivity can be caused by both a multitude of pollutants and common adverse properties. As a test control, we suggested plants grown on vermiculite, a natural mineral from the group of hydromica. Vermiculite of small fractions (up to 1 mm) is produced in the Chelyabinsk region, Russia, and is recommended for growing seedlings, germinating seeds and

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):3179-3197.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31793197

rooting plants. The method we have developed has a patent of the Russian Federation (Eremchenko and Mitrakova, 2017), is positively recommended in the practice of various soil grounds biotesting.

For statistical processing of the obtained data, regression and correlation analyses were used at a 95% probability level. Comparison of the samples was carried out by the dispersive non-parametric method (Kruskal-Wallis test). Significant differences between the compared average values were considered with a confidence level of 95% and higher ($P < 0.05$). The significance of differences with the test control was evaluated statistically by Student's criterion ($P < 0.05$). The figures and tables show the arithmetic mean of biological replicates and standard errors.

Results

The response of the test culture on the basic properties of regional soils

During the phytotesting experiment of regional soils, the watercress responded to the humus content, pH, sufficiency of potassium and phosphorus available forms. The height and weight of the testing culture was closely correlated with the main indicators of soil fertility (*Figs. 2-6*).

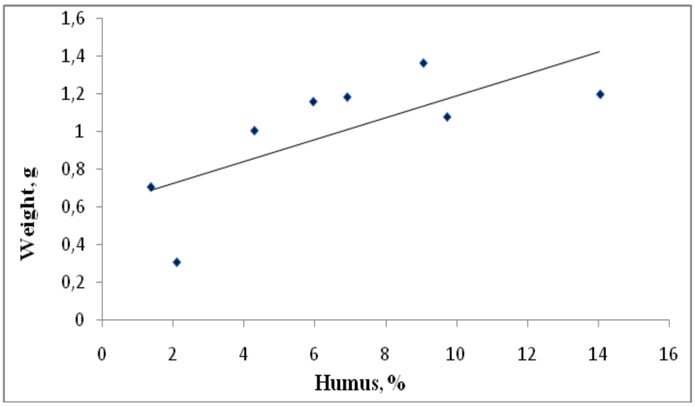

Figure 2. *Dependence of the total weight of watercress (g) on the content of humus (%) in soils:* $y = 0.676 + 0.055x$; $R = 0.64$; $F = 4.92$; $P = 0.021$ *(here and after: R – correlation coefficient, F – Fisher's criterion, P – the level of significance of the null hypothesis)*

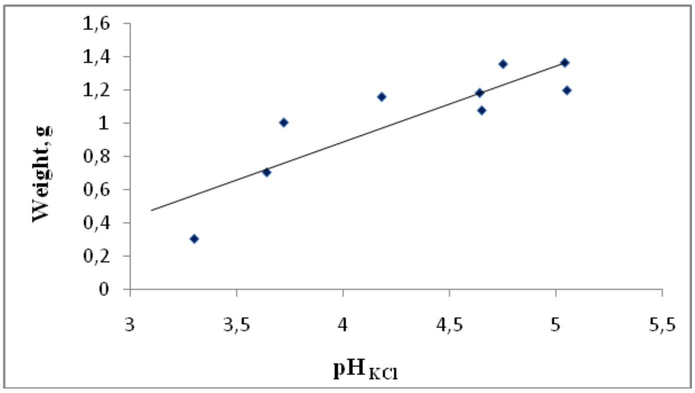

Figure 3. *Dependence of the total weight of watercress (g) on* pH_{KCl} *of the soil:* $y = -0.98 + 0.46x$; $R = 0.84$; $F = 17.4$; $P = 0.002$

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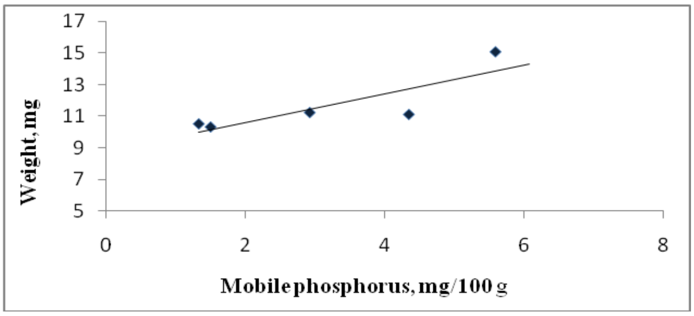

Figure 4. *Dependence of the plant weight (mg) on the content of mobile phosphorus (mg/100 g soil) in the dark gray soil: y = 8.8+ 0.9x; R= 0.84; F= 7.3; P= 0.004*

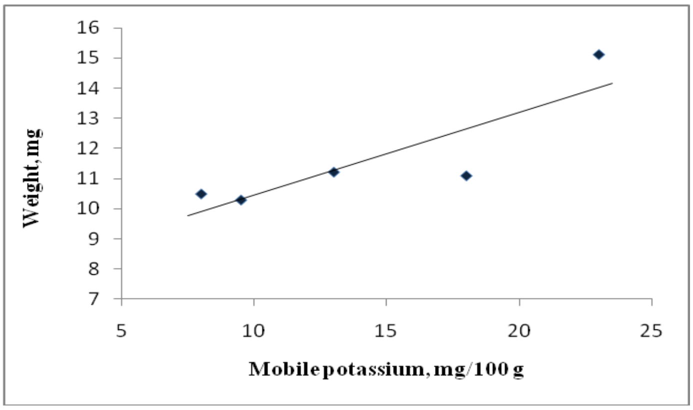

Figure 5. *Dependence of the plant weight (mg) on the content of mobile potassium (mg/100 g of soil) in dark gray soil: y = 7.71 + 0.27 x; R = 0.86; F = 8.9; P = 0.0024*

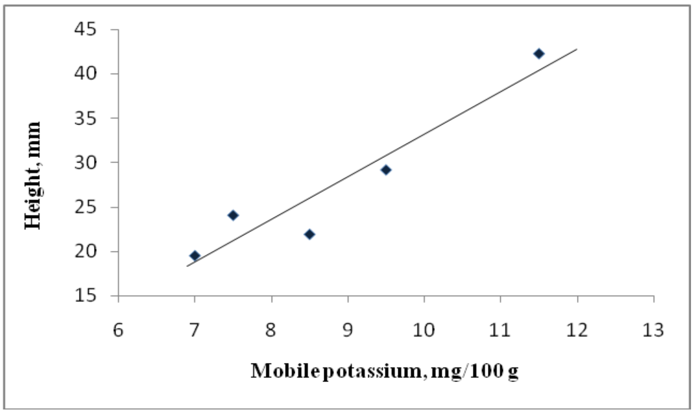

Figure 6. *Dependence of the plant height (mm) on the content of mobile potassium (mg/100 g) in dark gray soil: y = -14.7 + 4.8 x; R = 0.95; F = 28.0; P = 0.0001*

In Perm Krai, which is considered to be an industrialized region with high transport load, indigenous soils accumulate Pb, Cd, Zn, Cu, Cr and other heavy metals (Voronchikhina and Zaporov, 1998; Eremchenko and Moskvina, 2005; Vasilyev and Chashchin, 2011).

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In our experiments on the pollution of natural soils, it was established that the height and weight of the watercress depended on the contamination by cadmium and lead. Some dependencies between height, mass of the testing culture and the mobility of metals in dark gray soils are presented in the *Figures 7-10.*

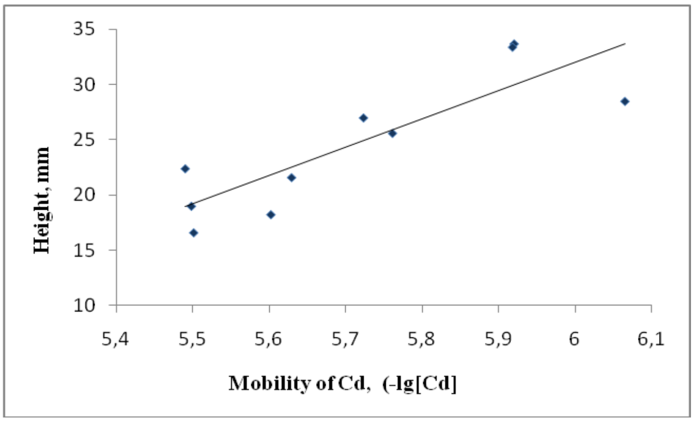

Figure 7. *Dependence of the plant height on cadmium mobility (-lg [Cd]), in dark gray soils: y = -116.8+ 24.8x; R = 0.84, F = 19.8; P = 0.005*

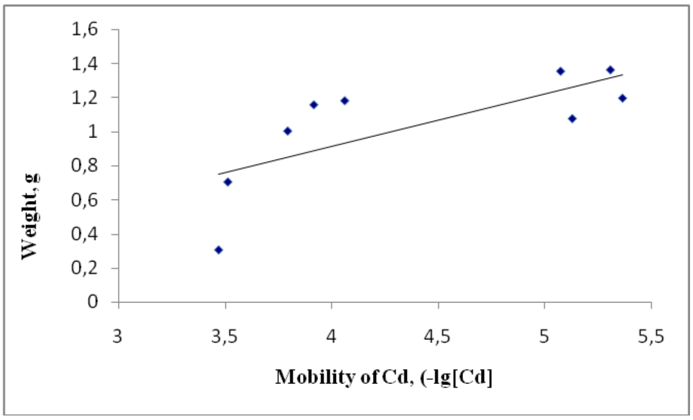

Figure 8. *Dependence of the total weight of watercress (g) on the mobility of cadmium (-lg [Cd]) in soils: y = -2.297+ 0.638 x; R = 0.98; F = 104.8; P = 0.003*

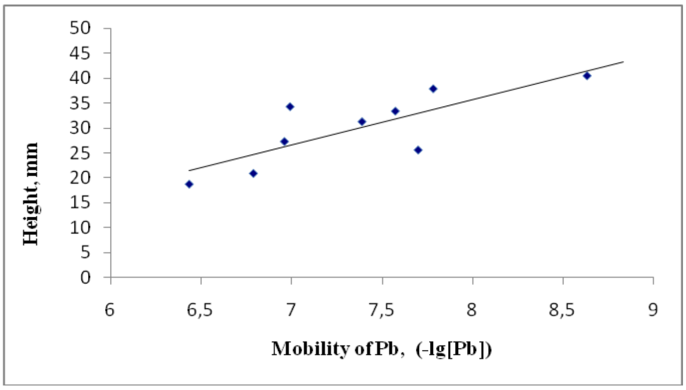

Figure 9. *Dependence of the plant height (mm) on the mobility of lead (-lg [Pb]) in dark gray soils: y = -36.7+ 9.064x; R = 0.80, F = 12.1; P = 0.0002*

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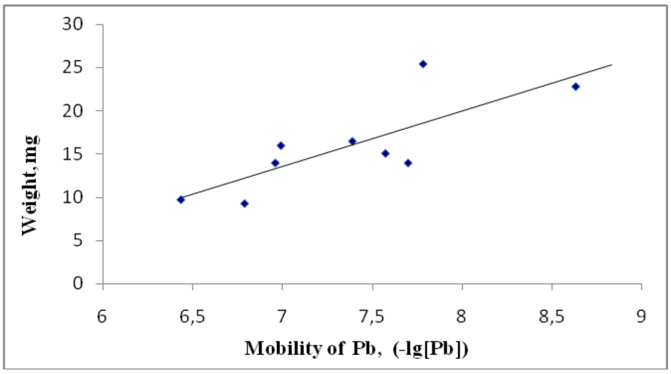

Figure 10. *Dependence of the plant weight (mg) on the mobility of lead (-lg [Pb]) in dark gray soils: y = -31.61+ 6.4x; R = 0.80, F = 11.9; P =0.003*

In the biotesting of urban soils, we considered plants grown on vermiculite with Knop's solution as a test-control. We compared height and weight of plants with watercress grown on the natural soils of our region.

Chernozems are the most fertile soil, so the height of plants that grew on chernozem was lowered by 30% compared to the test control, on dark gray soil (Retisols (Humic)) - by 50-60%, sod-podzolic soil (Albic Retisols) - 60-70%. The decrease in the mass of the plant was 33% on black soil, and 60-70% on gray soils and sod-podzolic soil relative to the test control. Thus, the properties of black soil were the most favorable for the plants, however, the height and weight of the plants were still lower than that of vermiculite. Apparently, the nutrients from the Knop solution turned out to be more accessible.

Based on the results of the experiment, we proposed the state of anthropogenic (man-made) soil or ground to be satisfactory with a decrease in the development of watercress relative to the test control by 10-30%; unsatisfactory - with a decrease of 30-50%; and at a reduction rate of more than 50%, it is environmentally hazardous (Eremchenko and Mitrakova, 2017).

Soil properties of residential areas of Perm

Urban soil is synlythogenic soil, as soil formation proceeds simultaneously with the accumulation of mineral and organic material on the surface; as a result, a profile of different thickness and degree of layering is being formed. The main diagnostic horizon of urban soils is the urbic horizon. In WRB (2006), the urbic horizon was defined as a qualifier for Technosols by the presence of a layer containing 20% of artifacts, including 35% of construction waste. In the Russian Federation, urban soils with a horizon urbic are classified as urbostratozem types (Prokof'eva et al., 2014).

Urbostratozems were prevailed in the UPC of residential areas of the city of Perm (*Fig. 11*). They are mainly formed during the cultivation by dumping (and repeated) lowland peat to the surface. Some properties of urbic are given in *Table 1*. Urbostratozems are not rich in humus, they have a slightly alkaline pH and an average cation exchange capacity.

In domesticated (peat-eutrophied) urbostratozems (*Fig. 12*), on the average, the amount of humus is 2 times higher, the pH is close to neutral, and the absorptive capacity is markedly increased. In the urbostratozem, as a rule, there was a small amount of carbonates.

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Figure 11. *Urbostratozem*

Table 1. *Properties of urbic horizon (0–15 cm) in urbostatozems of residential areas of Perm*

Parameters	Valid N	Mean	-95% confidence limits of mean	+95% confidence limits of mean	Minimum	Maximum	Standard Deviation	Standard error of mean
Urbostratozem								
C org, %	41	2.20	1.90	2.50	0.65	4.70	0.95	0.15
pH $_{water}$	41	7.82	7.70	7.94	6.74	8.66	0.38	0.06
pH $_{KCl}$	41	7.04	6.84	7.25	4.16	7.63	0.63	0.10
Cation exchange capacity, meq/100g	41	23.71	20.88	26.55	10.20	46.00	8.98	1.40
CO_2 of carbonates, %	41	0.45	0.24	0.66	0.00	1.42	0.47	0.10
Peat-eutrophied urbostratozem								
C org, %	22	5.81	4.81	6.80	3.10	11.30	2.24	0.48
pH $_{water}$	22	7.48	7.33	7.63	6.97	8.07	0.33	0.07
pH $_{KCl}$	22	6.97	6.83	7.11	6.32	7.77	0.32	0.07
Cation exchange capacity, meq/100 g	22	38.60	34.76	42.43	15.60	50.30	8.66	1.85
CO_2 of carbonates, %	22	0.50	0.29	0.70	0.00	1.42	0.47	0.10

Figure 12. *Domesticated (peat-eutrophied) urbostratozem*

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Quasi-soils, in which mineral soils are covered with a layer of low-moor peat with a thickness of about 10 cm, are formed on well-maintained plots in relatively new residential areas. In quasi-soils, the “fresh” organogenic layer is characterized by the structure and properties of the peat (*Fig. 13*).

Figure 13. *Peat quasi-soil*

Over time, this layer is enriched with mineral matter, peat is humified, thus forming a compost-humus quasi-soil (*Fig. 14*). Quasi-soils, especially peaty ones, contain a lot of organic carbon, they are often characterized by acidity and high cation exchange capacity (*Table 2*).

Figure 14. *Compost-humus quasi-soil*

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Table 2. *Properties of the surface layer of quasi-soils (0-10 cm)*

Parameters	Valid N	Mean	Minimum	Maximum
Compost-humus quasi-soil				
C org, %	7	6.23	3.57	12.52
pH $_{water}$	7	7.30	6.28	7.83
pH $_{KCl}$	7	6.71	5.54	7.35
Cation exchange capacity, meq/100 g	7	33.59	14.95	56.20
CO_2 of carbonates, %	7	1.44	0.00	3.12
Peat quasi-soil				
C org, %	7	18.90	14.44	27.30
pH $_{water}$	7	6.24	4.64	7.74
pH $_{KCl}$	7	5.48	4.03	6.86
Cation exchange capacity, meq/100 g	7	76.21	46.70	88.50

The investigated UPCs were represented by Urbostratozems and Quasi-soils, which caused a high heterogeneity of the surface soil layers. Variability of soils properties is demonstrated through the example of UPC-1 on eluvial-deluvial loams (*Fig. 15-19*).

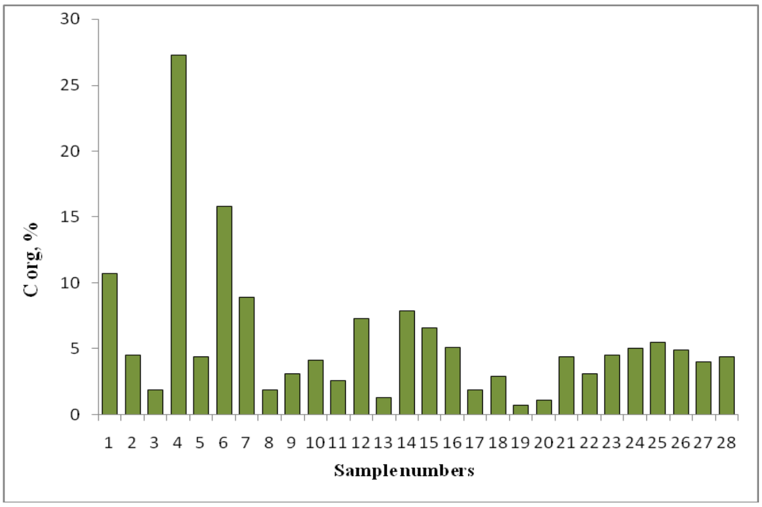

Figure 15. *The content of organic carbon in the upper soil layers of the UPC-1*

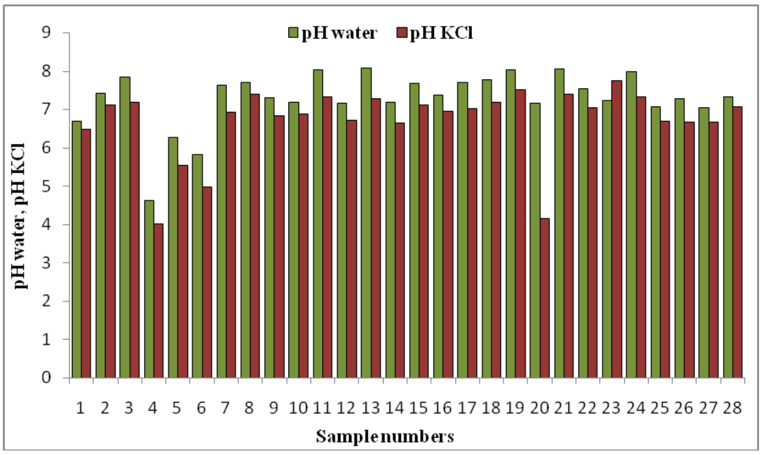

Figure 16. *pH value in the upper soil layers of the UPC-1*

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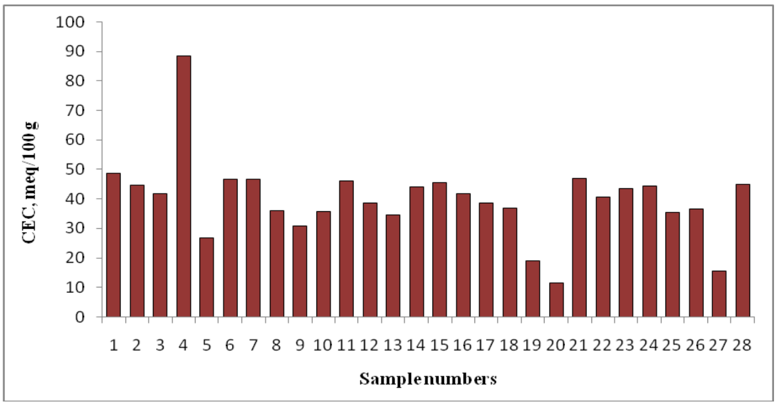

Figure 17. *Cation exchange capacity (CEC) in the upper soil layers of the UPC-1*

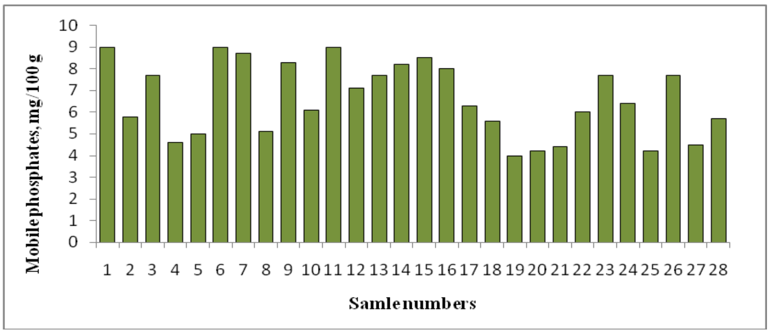

Figure 18. *The content of mobile phosphates in the upper soil layers of the UPC-1*

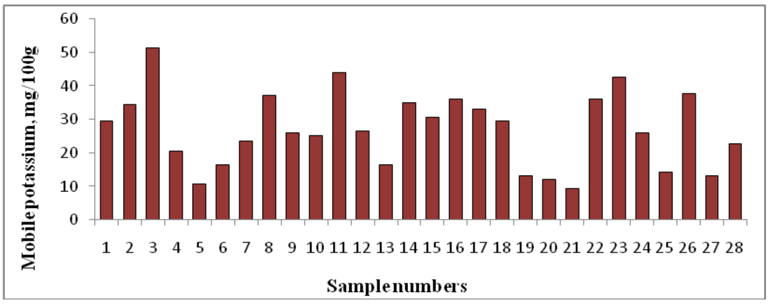

Figure 19. *The content of mobile potassium in the upper soil layers of the UPC-1*

In the upper layers of urbostratozems and quasi-soils, the mobility of heavy metals (*Fig. 20*), as a rule, did not exceed the threshold of previously established toxicity for the testing culture, the highest activity was shown by Cd.

At the same time, the state of the surface soil layers was influenced by the lithological differences of the UPC and the age of urban development. On the average, the increased content of organic carbon in the soils of the UPC-1 (*Fig. 21*) is due to the longer cultivation in this relatively old part of the city. The smallest soil humus content in the relatively young area (UPC-2) is due to weak cultivation, and initially low organic matter content in the sandy soils of the Kama River terraces. In addition, the sandy mineral base of urban soil formation does not contribute to the accumulation of humus.

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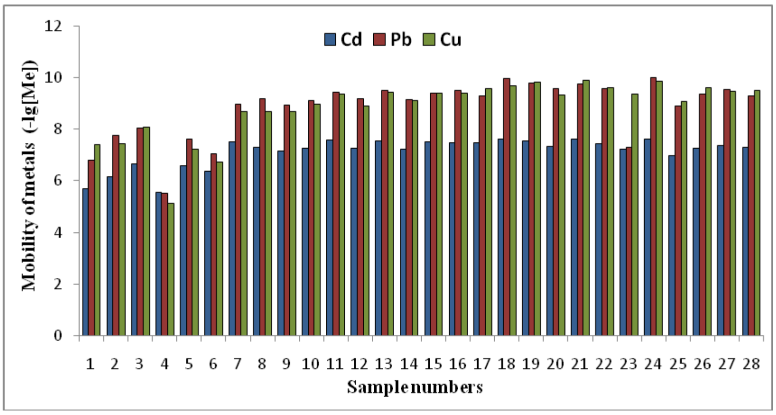

Figure 20. *Mobility of metals (-lg [Me]) in the upper soil layers of the UPC-1*

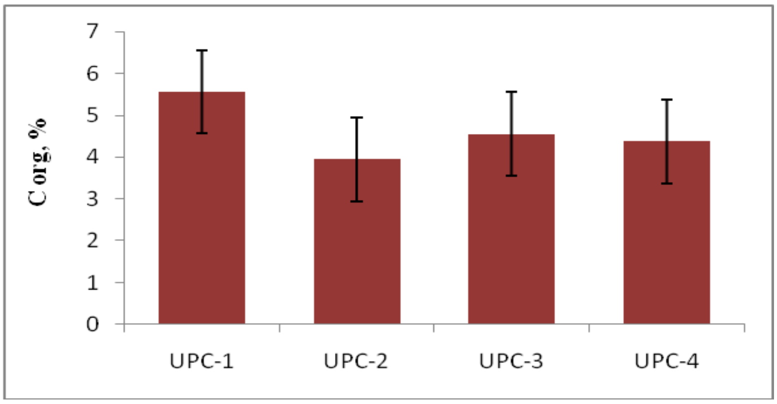

Figure 21. *The average content of organic carbon in the upper soil layers of the UPC*

The surface soil layers in UPC-1 were, on the average, characterized by lower alkalinity due to the introduction of sour peat (*Fig. 22*).

Due to the accumulation of organic matter and predominantly heavy loamy granulometric composition, the surface soil layers in UPC-1 were characterized by the highest absorption capacity (*Fig. 23*). The smallest absorption capacity was in sandy and sandy-loam soils in UPC-2.

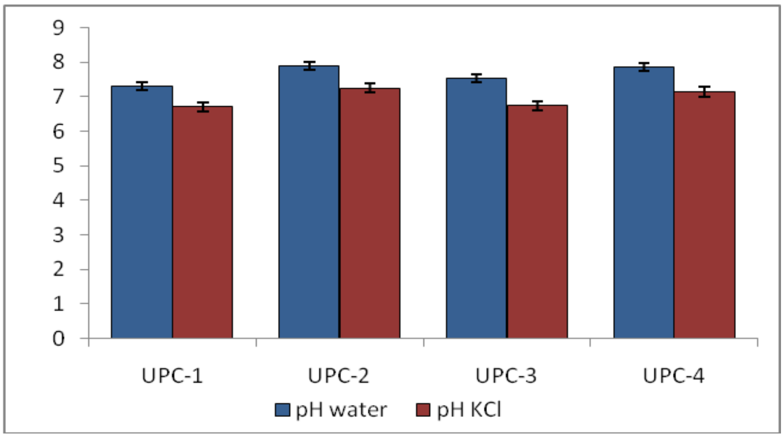

Figure 22. *The average pH in the upper soil horizons of the UPC*

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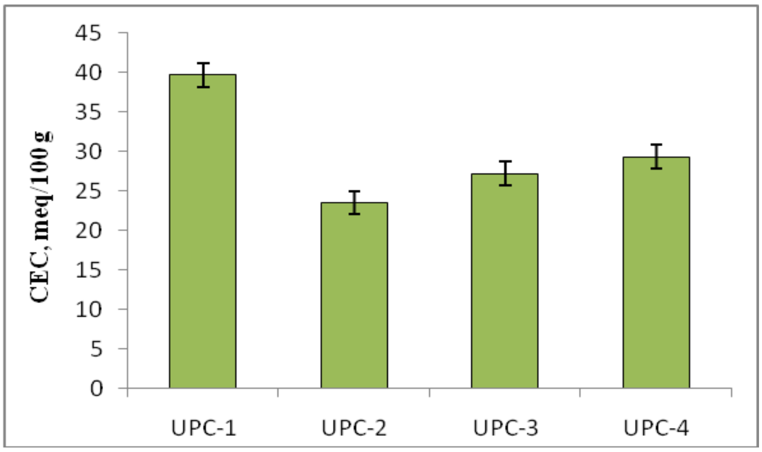

Figure 23. *The average cation exchange capacity in the upper soil horizons of the UPC*

Soils on ancient alluvial sands (UPC-2) and on alluvial sediments (UPC-4) are less supplied with mobile potassium (*Fig. 24*), probably because of the lighter granulometric composition of the rocks.

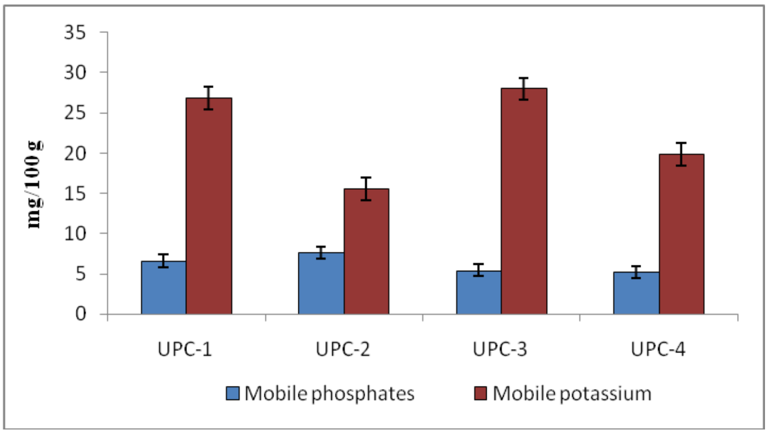

Figure 24. *The average content of mobile phosphates and potassium in the upper soil layers of the UPC*

Phytotesting of the soils of urban pedocomplexes in residential areas of Perm

Plants grown on samples from different soils and TSF, differed significantly in morphometric parameters. Watercress on soil samples from UPC-1 had a slight excess in height, or an allowable decrease (less than 30%) in height and weight relative to the test control, thus the soils had satisfactory ecological condition (*Fig. 25*). However, in 11% of soil samples watercress height was reduced by 31–36% and in 33% of samples weight was reduced by 31–55%. Thus, about the third part of the samples taken within the given residential areas showed the unsatisfactory condition of the surface layers of urbostratozems and quasi-soils.

Comparison of the height and weight of watercress grown on samples from different UPC showed, on the average, a less favorable ecological condition of the surface soil layers in the UPC-2, probably due to sandy and sandy-loam granulometric composition, poverty of organic matter and low content of mobile potassium (*Fig. 26*). Relatively

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better condition was found in plants on the soils of the UPC-3; that occurs due to the average granulometric composition, the cultivation of the soil in the household plots, and generally reduced anthropogenic load in the area of low-rise buildings.

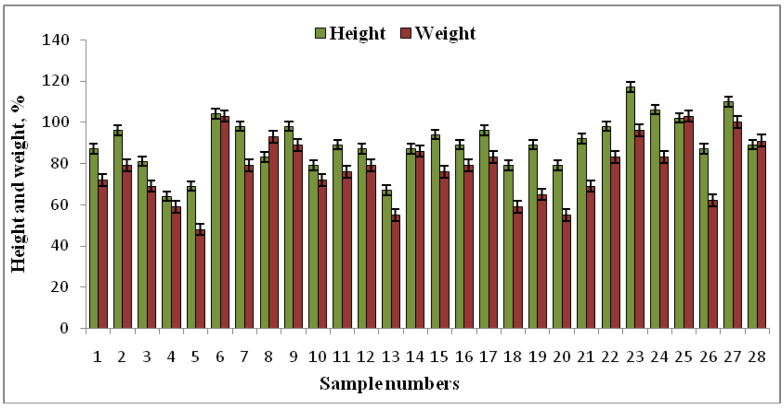

Figure 25. *The height and weight of the testing culture, grown on samples from the soil layers of the UPC-1, % of the test control*

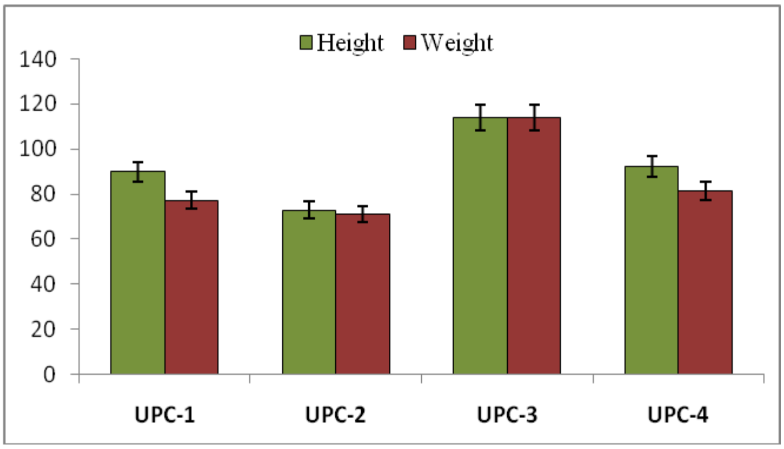

Figure 26. *Height and weight of the testing culture (% of test control) grown on samples from the upper soil layers of the UPC*

Using the correlation analysis, there was established the dependence of the watercress status on soil properties (*Table 3*).

Table 3. *Correlation between the state of the testing culture and the properties of the surface layers of urbostratozems and quasi-soils*

Parameters	Height	Weight
Organic carbon content	**0.36***	**0.37***
Cation exchange capacity	**0.42***	**0.41***
pH water	**-0.42***	**-0.46***
pH KCl	**-0.44***	**-0.47***
Mobile phosphorus	-0.11	-0.08
Mobile potassium	**0.44***	**0.45***
-lg[Pb]	-0.06	-0.20
-lg[Cd]	**-0.32***	**-0.39***

Note. * -significant correlation coefficients at 95% probability level are highlighted in semibold

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The height and weight of plants were positively effected by the content of organic matter, the capacity of cation exchange, the content of mobile potassium in the surface layers of the soil. The test culture showed a negative reaction to the alkalinity of the soil. A positive response of plants to cadmium mobility was revealed (alongside its low activity), which is possibly due to the indirect effect of a decrease in soil alkalinity, in which the availability of the metal is slightly increased.

Discussion

Despite the general feasibility of using biotesting methods in studying the state of the soil, it is not easy to determine the response of organisms. It is believed that when assessing soil toxicity, it is necessary to involve several organisms from different trophic groups (Keddy et al., 1995; Debus and Hund, 1997; Bierkens et al., 1998; Stephenson et al., 2000; Lisovitskaya and Terekhova, 2010; Rivett et al., 2011; Terekhova, 2011; Pleshakova and Belyakov, 2014; Bardina et al., 2014; Romero-Freire et al., 2015). However, the cultivation of animals, plants, microorganisms requires professional skills. In addition, the reaction of remote groups (producers, decomposers, consumers) to the state of the soil is not straightforward; as a result, the authors often recognize that there is no universal method of biotesting that is suitable for all cases.

In our experiment to assess the ecological state of the soil, we used one type of higher plants - watercress, which showed a response to the genetic properties of the soil, to the mobility of lead and cadmium in them. Certainly, there is a large variety of pollutants in the tested urban soils, which are not determined by the reaction of this testing culture. Thereby, the test control was grown on vermiculite with Knop's solution, the ability of which to create conditions for watercress was compared with the ability of natural soils, including black soil - a standard of regional fertility. The application of the test control allowed to bypass the rule of the only difference that is required to be observed during the phytotesting of the test (polluted) and control soil (Eisentraeger et al., 2004; Lisovitskaya and Terekhova, 2010).

Natural-anthropogenic soil formation in residential areas of the city of Perm has led to the formation of a very heterogeneous soil cover, the properties of the surface soil layers vary in a wide range. Urbostratozems with the urbic horizon prevailed in the soil cover of residential areas, in relatively old areas they were formed alongside certain reclamation - by repeatedly dumping peat from the lowlands. There is a general tendency towards accumulation of carbonates in urban soils (Yang and Zhang, 2015), but on the territory of Perm the enrichment of the soil with dispersed carbonates is due to the widespread use of carbonate rubble and gravel. Due to the presence of carbonate salts, the soils have a neutral alkaline pH, which is affected for a relatively short period by the introduction of acidic peat.

The bioavailability of metals depends on the acid-base, redox properties, the quality and quantity of organic substances, the strength of the absorbing complex and other parameters; it decreases with the increase of pH and in the presence of other metals and chelators in the soil. The total accumulation of heavy metals does not always mean the increase of the soil toxicity, since the soil has a certain buffer capacity (Duchovskis et al., 2003; Romero-Freire et al., 2015). Despite the general accumulation of heavy metals in the non-acidic soils of our city (Eremchenko and Moskvina, 2005), their mobility was low. The negative effect of Cd, Pb mobility on the height and weight of the test culture has not been established.

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The concept of "urban complexes" is used in the soil cover mapping of some large cities (New York City Soil Survey Staff, 2005; Sobocka, 2010). Our research has shown that a similar approach is very promising in assessing the ecological state of the surface soil layers. The soils of the UPC in Perm inherited some of the properties of the original soil-forming rocks and soils, primarily related to the particle size distribution, which regulates the water retention and absorption capacity, the availability of chemical elements, the rate of mineralization of organic matter, etc.

Despite the high variation of the soil and TSF properties in urban pedocomplexes, the use of phytotesting shows a generally satisfactory condition of the upper soil layers of residential areas. Soil biotesting in St. Petersburg also showed a satisfactory ecological condition of urban soils, in summer they had no toxicity (Bardina et al., 2013). However, in a relatively old area of the city of Perm, there were revealed the areas where the state of the soil was unsatisfactory. The tendency towards deterioration of the soil ecological condition will develop with modern ways land use and the accumulation of pollutants. According to the results of phytotesting, the most favorable condition was in the soil of a residential area with the distribution of low-rise buildings located near the park area.

Conclusions

This study validates the response of watercress to the leading parameters of soil fertility and the level of soil toxicity. This plant as a testing culture showed mainly a satisfactory condition of the upper layers of soil cover in residential areas of the city of Perm. At the same time, in the areas of relatively old development, a trend towards the emergence of soil toxicity was revealed. In the environmental assessment of soils and the subsequent monitoring of their condition, the idea of urban pedocomplexes occurred to be promising, since the included soils inherit lithological features, on which the specificity of urban soil formation depends. The usage of vermiculite with Knop solution as a test control can be used in the study of various man-made soils and grounds, and we recommend the preliminary comparison of the plants' condition (height and weight) on vermiculite with plants grown on fertile regional soils.

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SEDIMENT OXYGEN CONSUMPTION AND HYDROGEN SULFIDE RELEASE IN HYPOXIC AREAS OF GAMAK BAY, KOREA

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(Received 7th Nov 2018; accepted 4th Feb 2019)

Abstract. This study investigated sediment oxygen consumption rates and geochemical characteristics using chamber experiments and geochemical analyses in the hypoxic area of Gamak Bay, Korea. The organic carbon contents of surface sediments in Gamak Bay were higher in the northern inner bay area, and dropped off toward the southern outer bay. The vertical profiles of calcium carbonate content in piston core sediments indicated that hypoxia has frequently occurred during the past century in the northern inner bay. Sediment chamber experiments were conducted in February, May, August, and November 2010 and 2011 in the hypoxic area of the bay. In the sediment incubation experiments at site C3 in the northern inner bay and site C17 in the southern outer bay, the sediment oxygen consumption rate ranged from 3.98–12.43 mmol m^{-2} d^{-1} and 3.28–8.18 mmol m^{-2} d^{-1}, respectively. When the oxygen was completely depleted, hydrogen sulfide was released at 1.38 and 1.3 mmol m^{-2} d^{-1} at sites C3 and C17, respectively.
Keywords: *hypoxia, ocean acidification, dissolved oxygen, sediment oxygen consumption rate, sediment incubation chamber*

Introduction

Hypoxia occurs seasonally in coastal areas such as estuaries, coastal upwelling areas, and eutrophic waters due to the excessive input of nutrients from rivers. Hypoxic water masses have a dissolved oxygen (DO) concentration $\leq$ 2 mg L^{-1} due to biochemical factors, and are mainly seen throughout the world in highly productive waters and semi-enclosed bays, such as lakes and upwelling areas (Severdrup et al., 1972; Hanazato, 1997; Karim et al., 2002; Wu, 2002; Turner et al., 2005). When there is a large input of organic matter to the ocean bottom layer, and the flow of seawater between the surface and bottom layers is blocked due to stratification, an environment is created in which hypoxia can occur.

Hypoxia is known to occur in the coastal areas of many Asian countries (Wu, 1982; Hong, 1987; Jeng and Han, 1996; Lim et al., 2006). In Korea, hypoxia is reported to occur often in the summer season in connection with red tides (Cho, 1991; Kim, 1990). There is insufficient evidence supporting the occurrence of hypoxic water masses in Cheonsu Bay and Gamak Bay (GB), but these Bays are a focus of concern due to massive organism deaths (Shin, 1995). The depletion of oxygen in a water mass directly affects the respiration and physiological functions of fish and shellfish, as well as the species composition and abundance of benthic organism populations (Hong, 1987; Wu,

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2002; Karim et al., 2003). Bottom layer hypoxic water masses, which occur in coastal areas, not only directly damage benthic organisms, but can have various secondary effects on the coastal ecosystem. It is also known that the upwelling of hypoxic bottom layers can directly lead to organism deaths and cause the water to lose its self-purification functions (Yoon, 1998; Karim et al., 2002).

DO in ocean sediment is consumed by organic matter decomposition, benthic organism respiration, and the oxidation of chemical species. Most DO is consumed by organic matter decomposition, but it is also consumed by nitrification reactions in the nitrogen cycle, the reoxidation of reduced manganese and iron species, and the oxidation of hydrogen sulfide (Lee et al., 2008, 2010). These reactions occur after most of the oxygen has been depleted. If the flow of seawater is disrupted, the reactions can show high abundance and rapidly lead to an anoxic environment. If the anoxic bottom layer environment persists, organic matter decomposition occurs in an anaerobic state and produces ammonia, hydrogen sulfide, and methane. This can limit the distribution and movement of benthic organisms (Yoon, 1998).

The GB is a semi-closed bay in the central part of the Korean peninsula's South Sea. It has an oval shape with a north-south length of 15 km, an east-west length of 9 km, and large and small islands located at its entrance, giving it an almost-closed form. The central area of the GB has a depth of 6–7 m, and the northern inner bay has a depth of 9–10 m. The seabed has a concave, basin-like topography (*Fig. 1*). The bay's sedimentary layer was deposited over the last 6,000 years in accordance with high water levels. The thickness of the sedimentary layer is 5–25 m, being thickest in the central part of the bay and thinner toward the northern inner bay (Kim et al., 2014). The GB is surrounded by islands that strongly affect the conditions therein. Water from the open sea flows in through the bay entrance, and also from another source, Gwangyang Bay, causing different water masses to appear (Lee and Cho, 1990). Approximately 80% of the seawater flows in and out of the bay through its northern entrance, while ~20% flows in and out through a narrow channel. Seawater from both sides meets in the middle of the bay (Lee and Chang, 1982). In summer, stratification occurs readily in water masses in the northern inner bay due to the increased temperature of the surface water and the inflow of freshwater. During flood tide and ebb tide, seawater flow only occurs within the surface layer. It is known that in the northern inner bay, which has a concave seabed, the bottom water is very stagnant (Lee and Cho, 1990).

Hydrogen sulfide is a toxic gas produced when sulfate-reducing bacteria reduce sulfur compounds to hydrogen sulfide in an anaerobic state. Hydrogen sulfide is easily oxidized, which results in the formation of sulfur precipitates. In anoxic environments, hydrogen sulfide is released within the water column and causes mass death of organisms (Milby and Baselt, 1999; Cuevasanta et al., 2017). Hydrogen sulfide is highly toxic and causes great harm to organisms at concentrations of 500 ppm or more; above 1,000 ppm, it causes death (Guidotti, 1994).

The purpose of this study was to investigate the effect of sediment on the hypoxia occurring in GB in the summer of each year. We also investigated the phenomenon that can occur as the duration of hypoxia increases. This study used sediment chamber experiments to measure the oxygen consumed by sediments in regions of the GB where hypoxic water masses occur, to understand how oxygen consumption affects the formation of hypoxic water masses. This study also measured the hydrogen sulfide flux released from sediments during continual hypoxia.

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DOI: http://dx.doi.org/10.15666/aeer/1702_31993214

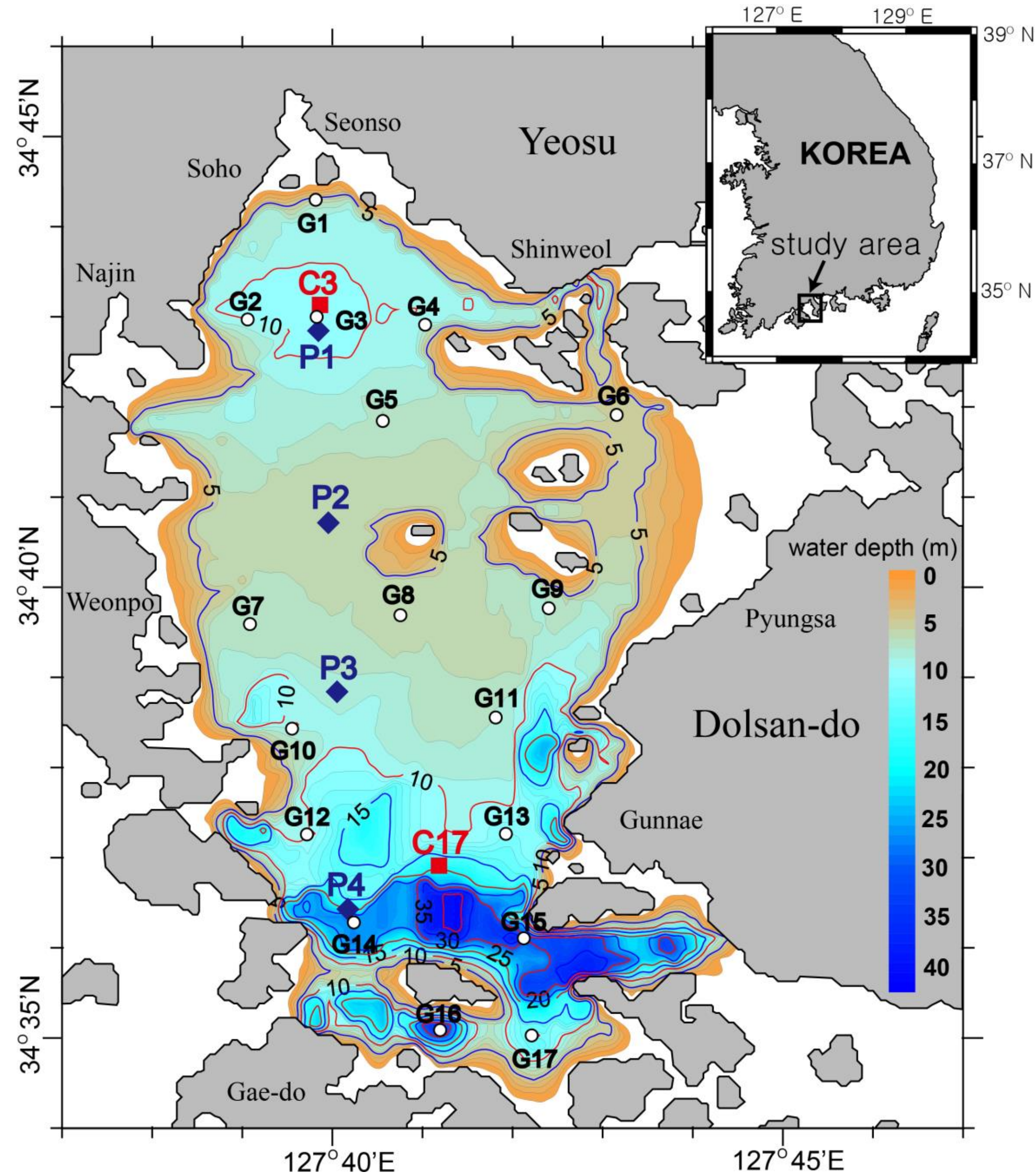

Figure 1. *The locations of piston coring sites (P1–P4), grab sampling sites (G1–G17), and chamber experiment sampling sites (C3 and C17) in Gamak Bay, Korea. The solid lines are contours of water depth; water depth is in meters*

Materials and methods

Surface layer sediments were collected for analysis at 17 sites (G1-G17) using a grab sampler. At 4 sites (P1-P4), a piston corer was used to collect core sediments. At 2 sites (C3 and C17), a box corer was used seasonally to collect sediments for incubation experiment (*Fig. 1*). In the bottom layer of site C3, a hypoxic water mass is formed every summer season. In the bottom layer of site C17, DO concentration is normally distributed. The incubation experiment samples were collected from 2 sites in May,

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August and November, 2010 and February, May, August and November, 2011, respectively.

The surface layer sediment was placed in a sample bag and placed in cold storage. In the laboratory, sediment samples were dried and powdered for geochemical analysis, and particle size analysis was also performed. The core sediment was cut in the laboratory, and subsampling was conducted at each measured depth. After pre-treatment of the surface layer and core sediments, the particle sizes were analyzed. The pre-treatment consisted of a 10% hydrochloric acid (HCl) solution, used to remove carbonates, and a 30% hydrogen peroxide (H_20_2) solution to remove organic matter. The pre-treated sediment samples were cleansed several times with distilled water, and a 4 ϕ sieve was used to divide the sediment into particle sizes > 4 ϕ and < 4 ϕ. The particle sizes of sediments < 4 ϕ were analyzed with a particle size analyzer (Sedigraph 5100), and the proportion of sediment > 4 ϕ was evaluated statistically (Folk, 1954). After the sediment samples were transported to the laboratory and dried, their weight was measured to assess their water contents. The dried sediment samples were powdered, and their total carbon and inorganic carbon contents were measured. The total carbon and nitrogen contents were analyzed using a CHNS elemental analyzer (Carlo-Erba), and the inorganic carbon content was analyzed using a coulometric carbon analyzer (UIC). The total organic carbon (TOC) content was calculated by subtracting the inorganic carbon content from the total carbon content. The calcium carbonate ($CaCO_3$) content was calculated by multiplying the inorganic carbon content by 8.33.

After collecting sediment samples in a chamber, incubation experiments were performed in an environment similar to the field conditions in terms of temperature and light. Using a temperature-adjustable water bath, the chamber temperature was kept as similar as possible to that of the bottom water. A dark room with almost no natural light was created, so that exposure to light was minimal. Bottom water (~ 2 L) collected sites C7 and C13 was carefully poured into the chamber containing the sediment samples to cause minimal disturbance to the sediment, and contact with the air was completely blocked. The chamber was equipped with a Clark-type oxygen sensor, a pH sensor, and a hydrogen sulfide sensor (Unisense), and the hourly changes in all three parameters were recorded. The stirrer was connected to the decelerating motor and stirred at 9 rpm in the overlying water (*Fig. 2*). The sediment incubation was performed until the oxygen was completely depleted or the reduction in oxygen became steady. At the beginning and end of the incubation, overlying water was collected, and the DO concentration was adjusted via Winkler titration. The sediment oxygen consumption rate (SOCR) and hydrogen sulfide flux were estimated by reference to the changes in the concentrations measured by the respective sensors in the chamber, and using *Equation 1:*

$$F = dC/dt \times V/A \qquad \text{(Eq.1)}$$

where F is the net flux of substance via the water-sediment boundary layer (mmol m^{-2} day^{-1}), dC/dt is the slope of the changes in the concentration of substance over time (mmol L^{-1} d^{-1}), V is the chamber volume (L), and A is the area of the chamber's horizontal plane (m^2).

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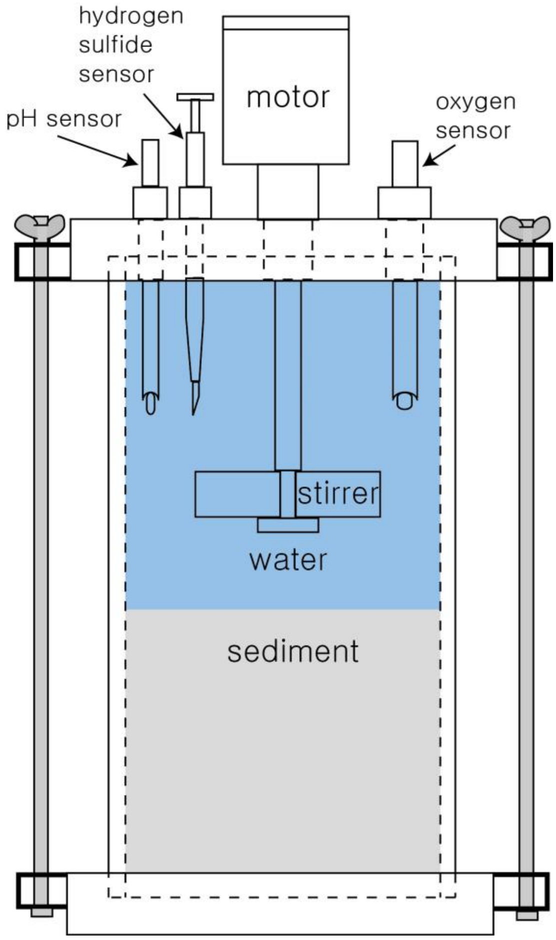

Figure 2. *A schematic diagram of a sediment incubation chamber*

Results

Surface sediment distribution

The surface sediment in the GB is mostly mud, and the amount of sandy sediment increases as one moves toward the southern outer bay. Some gravel sediment is distributed in the deep waterways in the southern outer bay (*Fig. 3a*). The mean particle size of the sediments tends to be fine in the northern inner bay, becoming coarser toward the outer bay. However, the overall bay contains fine sediment with a particle size above 8 ϕ, except at the entrance to the open sea (*Fig. 3b*). The surface layer sediment TOC content is high in the inner bay, at > 2%. In the middle of the bay, the TOC content is < 1% (*Fig. 3c*). The C/N ratio is > 8 in several regions that are adjacent to cities in the northern inner bay. However, the overall bay has a low C/N ratio of < 8 (*Fig. 3d*).

Core sediment vertical distribution

The TOC content was the highest at site P1, at > 1.5% of the sediment surface, with a gradually decreasing trend as the depth increases. However, at sites P2, P3, and P4, there was almost no change in organic carbon content according to depth, and the TOC was relatively low, at < 1% (*Fig. 4*). The $CaCO_3$ content showed an unusual distribution

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at site P1, with > 1% $CaCO_3$ in the surface sediment, but a very low content of < 0.1% below 20 cm in depth. Conversely, at sites P2, P3, and P4, the $CaCO_3$ content showed almost no vertical change and was fixed at ~2%. At a depth of ~200 cm at site P4, the $CaCO_3$ content was relatively high, at 4%.

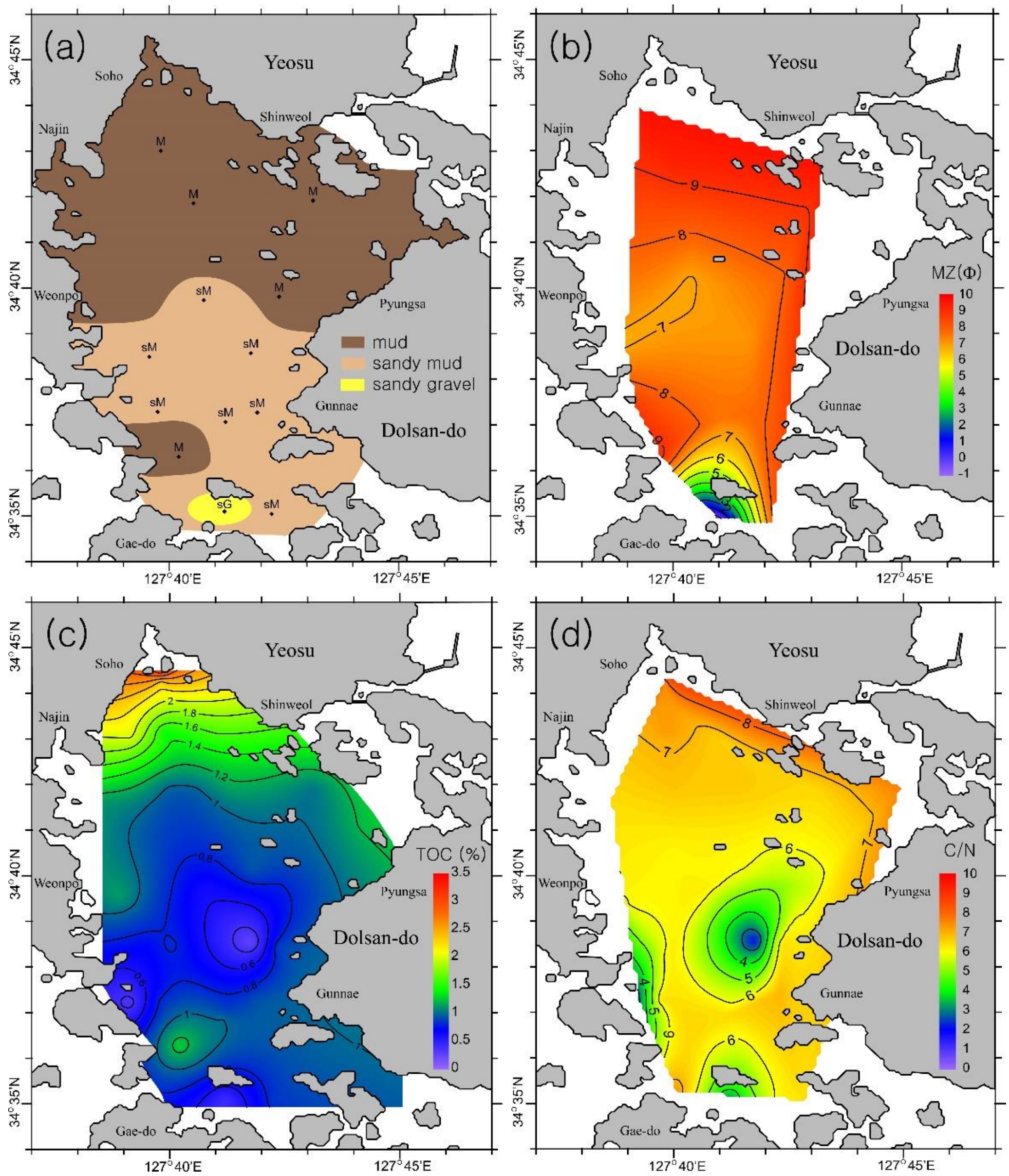

Figure 3. *(a) Sediment type, (b) mean grain size, (c) total organic carbon (TOC) content, and (d) C/N ratio in surface sediments of Gamak Bay*

Sediment oxygen consumption rate (SOCR)

In the sediments collected from sites C3 and C17 in May 2010, the DO concentration began to decrease as soon as the incubation experiment was started in the chamber, and the decrease was more rapid in the sediment from site C17 than in that from site C3 (*Fig. 4*). At site C17, there was a rapid decrease from the start of the incubation until 12 h, followed by a gradual decrease. This differed from site C3, where the decrease

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was gradual from the outset. At site C3, the SOCR was 3.58 mmol m^{-2} d^{-1}; at site C17, it was 9.25 mmol m^{-2} d^{-1}. Regarding the incubation results for May 2011, site C3 showed trends similar to those in 2010, while site C17 showed a smaller reduction in DO at the start of the incubation experiment. However, compared to site C3, the extent of DO reduction was larger; 32 h after the start of the incubation, the DO was completely depleted and the water became anoxic. The SOCR in May 2011 was 3.98 mmol m^{-2} d^{-1} site at C3 and 7.17 mmol m^{-2} d^{-1} at site C17.

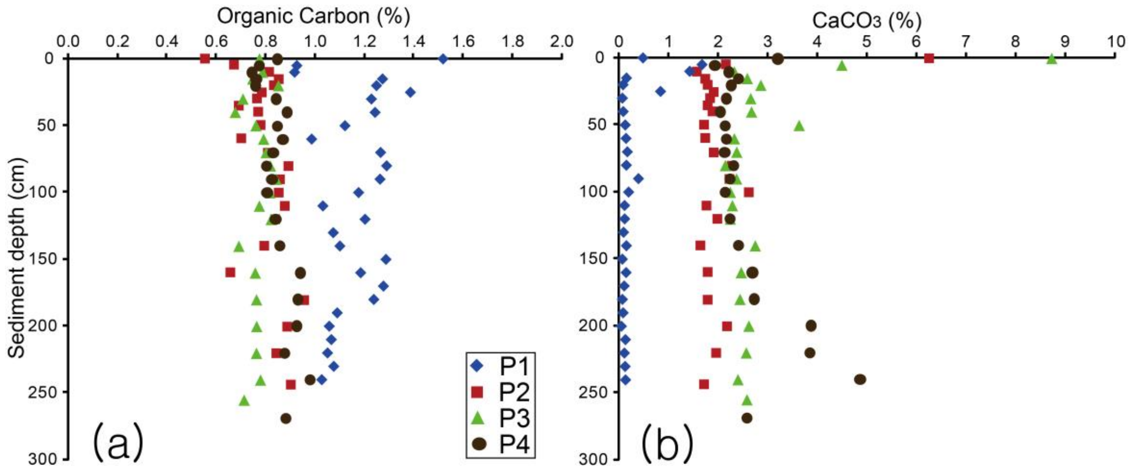

Figure 4. *(a) Vertical profiles of organic carbon contents and (b) calcium carbonate ($CaCO_3$) contents at four piston coring sites*

In the August 2010 sediment incubation experiment, there was no great difference between sites C3 and C17. Site C17 showed a slightly larger reduction in DO, but the reduction trends were almost the same. In contrast, in August 2011, the DO reductions at both sites were more rapid than in 2010. Site C3 became anoxic at 18 h after the start of the culture, and site C17 became anoxic after ~28 h (*Fig. 5*). In August 2010, the SOCRs were 3.94 and 4.31 mmol m^{-2} d^{-1} at sites C3 and C17, respectively; in August 2011, the SOCRs were 12.43 and 8.18 mmol m^{-2} d^{-1}, respectively.

In the November 2010 sediment incubation experiment, the DO decreased more rapidly than in August. The DO concentration in the bottom water was higher in November than in August, and the DO consumption was accelerated. The SOCR at site C17 remained high. Conversely, in 2011, the SOCR at site C17 was markedly lower than at site C3 (*Fig. 6*). The bottom water temperature in the GB in November 2011 remained comparatively high at 18 °C, and the DO consumption was accelerated by the high water temperature. In November 2010, the SOCRs at sites C3 and C17 were 5.35 and 6.21 mmol m^{-2} d^{-1}, respectively; in November 2011, the SOCRs were 6.40 and 3.28 mmol m^{-2} d^{-1}, respectively.

In the February 2011 sediment incubation experiment, sites C3 and C17 showed similar DO reductions. Despite having the highest DO concentration at the start of incubation, the SOCR was not large (*Fig. 6*). In February, the SOCRs at sites C3 and C17 were 4.43 and 4.23 mmol m^{-2} d^{-1}. As the sediment was incubated, the pH decreased in accordance with the reduction in DO concentration. The reduction in pH showed a similar trend to the oxygen consumption: when the DO decreased rapidly, the pH also

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decreased rapidly, and when the decrease in DO was gradual, the decrease in pH was also gradual (*Fig. 7*).

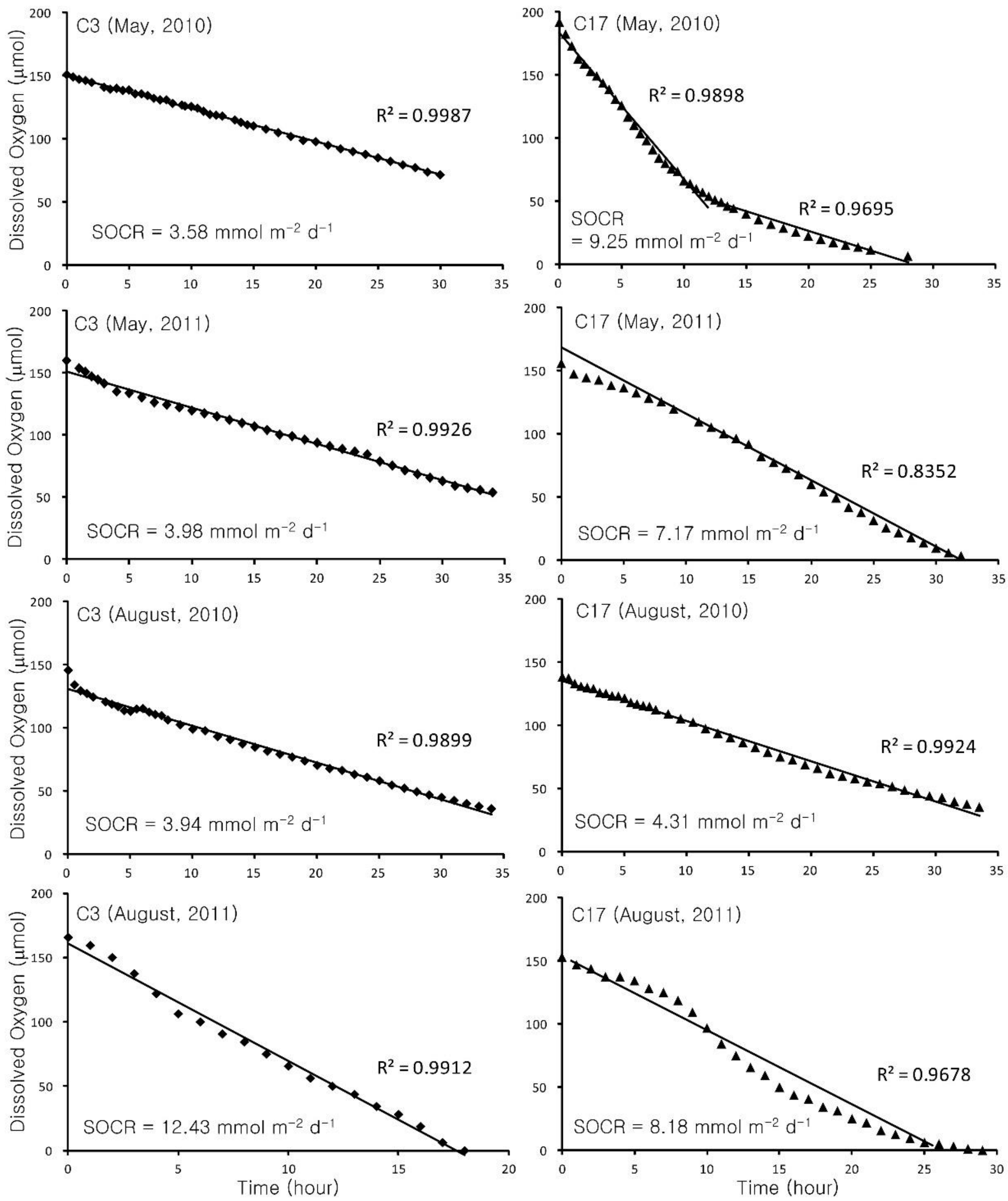

Figure 5. *Time-series of bottom water dissolved oxygen (DO) concentrations in chamber experiments using surface sediments from sites C3 and C17 in Gamak Bay, in May 2010, May 2011, August 2010 and August 2011. The solid lines indicate the results of linear regression at considerable oxygen reduced section*

Hydrogen sulfide release

To examine the substances released from sediments under anoxic conditions, a hydrogen sulfide sensor was installed in a sediment culture chamber in August 2011. During the experiment, the maximum oxygen consumption occurred, and the oxygen

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was depleted not long after the start of the culture, creating an anoxic environment. At site C3, hydrogen sulfide was released only a few hours after the DO was depleted. At site C17, hydrogen sulfide was released 50 h after the oxygen was depleted (*Fig. 8*). The hydrogen sulfide flux at the sediment surface layer was 1.38 mmol m^{-2} d^{-1} at site C3 and 1.31 mmol m^{-2} d^{-1} at site C17.

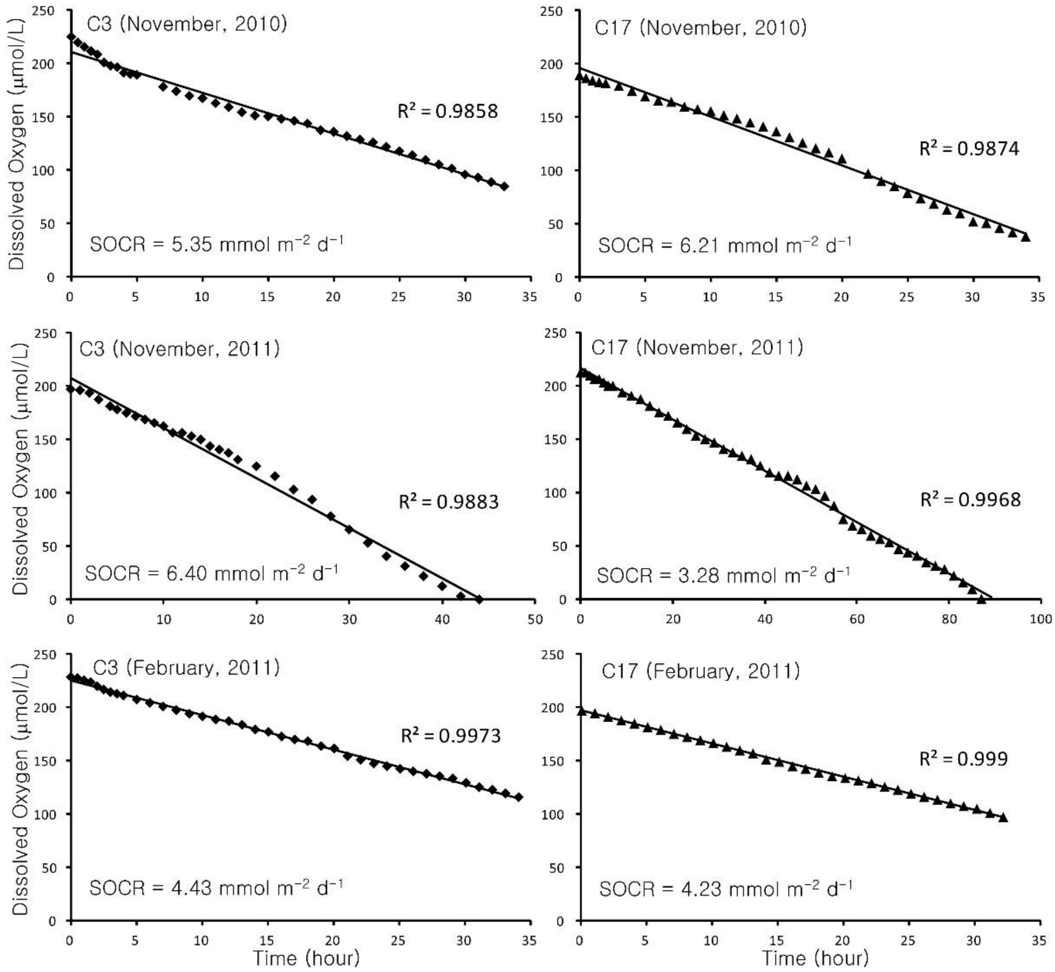

Figure 6. *Time-series of bottom water DO concentrations in chamber experiments using surface sediments from sites C3 and C17 in Gamak Bay, in November 2010, November 2011 and February 2011*

Discussion

Characteristics of sediment distribution

The northern area of the GB is close to a region with a dense urban population, and the ocean currents in the area are very slow. The sediment in the area is artificially inflowing organic matter, which immediately sinks and accumulates. The central area of the GB has a low organic carbon content due to the relatively steady ocean current flow, and deposited organic matter is actively decomposed. As the overall C/N ratio in GB sediment is < 8, the organic matter deposited in the bay primarily originates from the ocean (Prahl et al., 1994). In the inner area of the GB, some organic matter flows in from the land.

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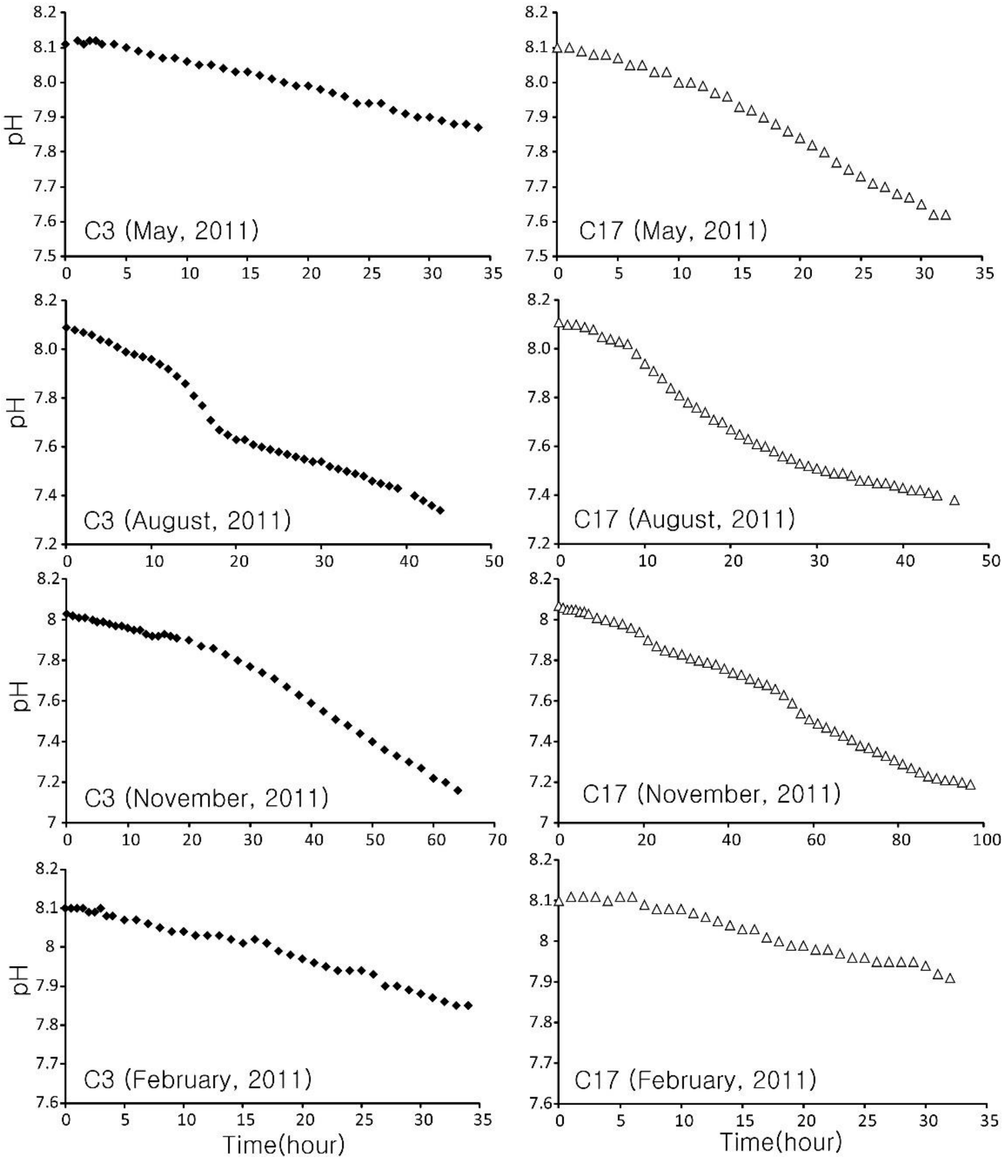

***Figure 7.** Time-series of bottom water pH in chamber experiments using surface sediments from sites C3 and C17 in Gamak Bay, in May, August, November, and February 2011*

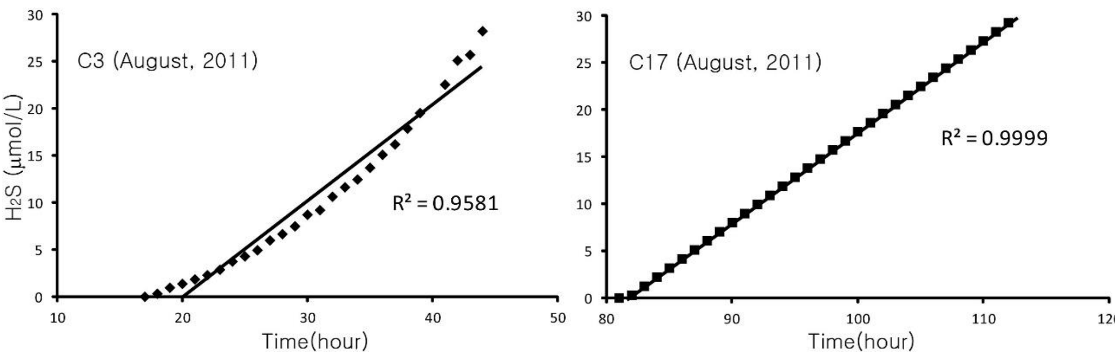

***Figure 8.** Time-series of bottom water hydrogen sulfide concentrations in chamber experiments using surface sediments from sites C3 and C17 in Gamak Bay, in August 2011*

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The surface layer sediments around site P1 had a relatively high organic carbon content distribution compared to the other sites, which corresponds with the core sediment analysis. The organic carbon content was high at the surface, and decreased with depth. This is due to the remineralization process caused by the decomposition of organic matter. However, at other sites, there was almost no change in organic carbon content with sediment depth, due to the relatively low level of organic matter remineralization. Due to the small proportion of organic matter that sinks to the bottom, relatively little remineralization of organic matter occurs.

At site P1, the vertical distribution of $CaCO_3$ exhibited relatively high contents at the sediment surface, but very low contents at a level only slightly deeper. The surface sediments had a relatively low $CaCO_3$ content compared to the other sites, but the contents further decreased with depth due to dissolution within the sediment. The vertical distribution suggests an environment in which $CaCO_3$ easily dissolves. When the pH is low, the $CaCO_3$ dissolution rate tends to increase, and it can be inferred that site P1 had a very low pH for some time; such low pH mainly occurs in hypoxic or anoxic environments. Deoxygenation can result in hypoxia (oxygen depletion detrimental to many organisms), and the combined processes are often referred to as ocean acidification and hypoxia (Klinger et al., 2017). The calcification process of marine organisms has mostly exhibited negative sensitivity to ocean acidification, which would lead to a decrease in larval supply and recruitment of adult population (Hendriks et al., 2010; Waldbusser et al., 2015). Ocean acidification can have a significant impact on oyster production in GB, where a large number of oyster farms are well developed.

According to Shin et al. (2012), the sedimentation rate in the central area of the GB is estimated at 2.5 cm $year^{-1}$, while that in the bay interior is estimated to be slightly lower. Based on this, sediments at the 250 cm depth are estimated to be 100 years old, and hypoxia may have occurred in the vicinity of site P1 for the past 100 years. This hypoxia is likely the result of the topography of the seabed, which is convex and basin-like in shape.

Sediment oxygen consumption and hydrogen sulfide release

The larger SOCR at site C17 compared to site C3 is due to numerous benthic organisms, such as bivalves, living in the sediment, where their respiration rapidly reduces the DO. There are relatively fewer benthic organisms living at site C3, and most of the DO is likely consumed by the chemical decomposition of organic matter and the oxidation of chemical species caused by early diagenesis. Similarly, the SOCR is higher at site C17, but hypoxia does not occur due to the relatively active seawater flow between the open sea and the southern outer bay (Lee and Chang, 1982), which provides a steady supply of DO.

The release of carbon dioxide during organic matter decomposition causes the bottom water carbon dioxide concentration to increase and the pH to decrease. The resulting formation of hypoxic water masses contributes to ocean acidification, and the lower pH can affect organisms directly and indirectly. In particular, the increased solubility of $CaCO_3$ affects both the $CaCO_3$ content in the sediment and the sediment-dwelling organisms, the bodies of which contain $CaCO_3$.

The incubation experiments showed similar SOCRs at sites C3 and C17. However, hypoxic water masses form at site C3, but not at site C17, likely due to the action of bottom layer currents. Stratification exists at site C3, and the flow of bottom water is

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completely blocked, while the inflow and outflow of water from the open sea is relatively active at site C17 (KORDI, 2012). The concave basin-shaped seabed topography at site C3 is likely also a contributing factor.

Anthropogenic processes during the last century (e.g., nutrient and organic matter loading) have caused large increases in the number of areas suffering from hypoxia in the world's coastal oceans (Breitburg et al., 2018; Yoann et al., 2018). Global warming as a causes of oxygen loss in the global ocean (Brewer and Peltzer, 2016). Ocean warming reduces the solubility of oxygen and decreasing solubility is estimated to account for ~15% of current total global oxygen loss (Helm et al., 2011; Schmidtko et al., 2017).

Kim et al. (2010) examined the river pollution load that flows into the GB. The total monthly flux of the rivers was 87,220–191,686 m^3 day^{-1}, with a total nitrogen load of 1,112 kg day^{-1}, a total phosphorus load of 89 kg day^{-1}, and a TOC load of 285 kg day^{-1}. Higher pollution loads were seen in May and July when there was a lot of seasonal rain, as well as at the sewage treatment plant near point C3 and the drainage area near Seonso. It can be inferred that the pollution sources flowing in via rivers directly affect the formation of hypoxic water masses in the northern inner bay. Environmental restoration is greatly affected by the nature of the substances polluting the environment (Gray et al., 2002; Pereira et al., 2004). In the case of organic pollution, the environment recovers relatively slowly from substances with slow, complex decomposition processes, such as sewage, in comparison to the recovery from pollution caused by feed and primary production (Munari, 2003; Smith and Shackley, 2006). In the northern inner bay, excessive organic matter has accumulated due to human activity (Yoon et al., 2008). Therefore, the recovery will likely be very slow, and hypoxia will occur yearly. The SOCR is high when the bottom layer DO concentration is low and the bottom layer temperature is high (*Fig. 9*). Hypoxic water masses mainly occur during summer in the GB, because the SOCR increases due to high temperatures and the oxygen supply is interrupted by the formation of stratification. Hypoxic water masses in the GB onset in May and continue into November. The chamber experiments demonstrated the possibility of formation of an anoxic environment if the hypoxic water mass continues for a long time, depleting the oxygen in the bottom layer. If the bottom layer becomes anoxic, the pH might rapidly decrease, causing the release of nutrients and gaseous components, such as hydrogen sulfide, into the sediment.

Kim et al. (2011) estimated that over 70% of the phosphorous in the GB is labile, and can easily move into the water column due to environmental changes such as sediment disturbances or oxidation/reduction, thus becoming a large source of dissolved phosphorous in the water. Deoxygenation of the sediment increases the possibility of a large release of dissolved phosphorous into the water column. In an anoxic environment, hydrogen sulfide and methane would also be released into the water column.

In the chamber experiments conducted in this study, the hydrogen sulfide concentration in the overlying water was ~30 μmol L^{-1}, a level that is harmful to organisms. Hydrogen sulfide concentrations of 20–30 μmol L^{-1} cause liver damage and fatal gill necrosis in fish (Keimer et al., 1995). Hydrogen sulfide released from anoxic sediments is immediately oxidized by oxygen in the water column (Brooks and Mahnken, 2003). However, even at concentrations < 10 μmol L^{-1}, fish experience physiological stress and stunted growth (Black et al., 1995). Long-term anoxic conditions increase the risk that hydrogen sulfide concentrations will rise to levels fatal

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to bay organisms. To date, there have been no reports of organism deaths due to hydrogen sulfide in the GB. However, there have been cases in which hypoxia occurred in July and persisted until October, and the ecosystem may have been disturbed by hydrogen sulfide release. This phenomenon is expected to occur more often in the near future due to global warming.

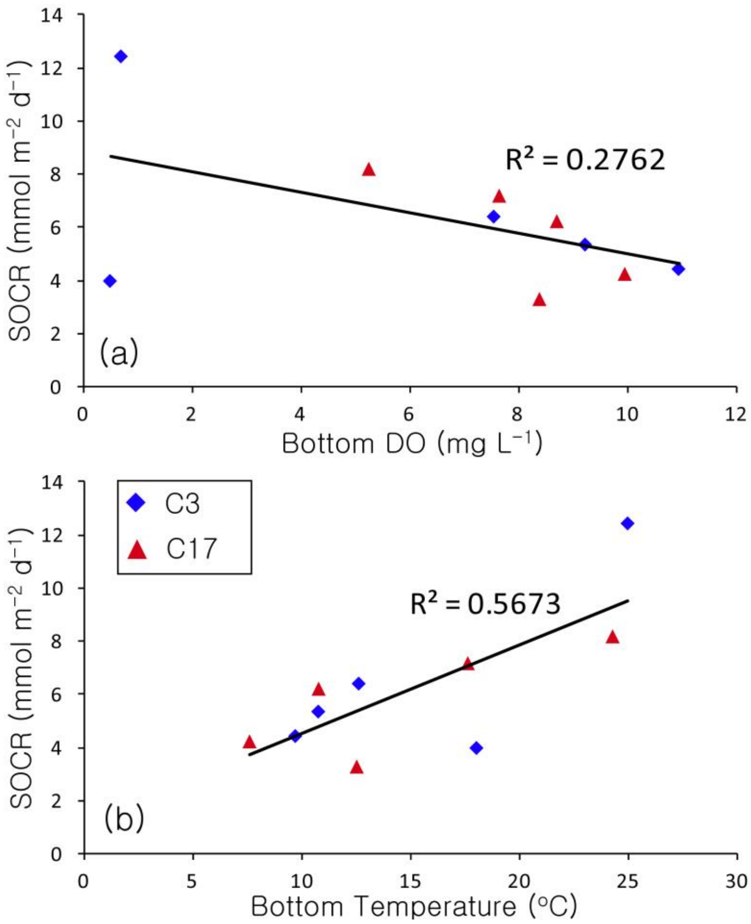

Figure 9. *Sediment oxygen consumption rate (SOCR) versus (a) bottom water DO concentration and (b) bottom water temperature at sites C3 and C17 in Gamak Bay*

Conclusion

In the GB, the surface sediment organic matter content is high in the inner bay, and decreases toward the open sea. Most sediment organic matter in the GB originates from the ocean. However, in the northern inner bay, several areas contain sediment organic matter originating from the nearby land. The hypoxic water masses that frequently occur during summer in the northern inner bay are caused by the high sediment organic matter content and obstruction of seawater flow. The core sediment from the northern inner bay exhibited very low $CaCO_3$ contents down to 260 cm in depth, indicative of a sediment environment with very low pH in which hypoxia can occur. This recurring hypoxic environment has persisted in these waters for hundreds of years.

At site C3, which is in the northern inner bay and frequently experiences hypoxia, the SOCR ranged from 3.98–12.43 mmol m^{-2} d^{-1}, while at site C17, which is near the open water and does not experience hypoxia, the SOCR ranged from 3.28–8.18 mmol

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m^{-2} d^{-1}, which is largely similar. Despite the high SOCR, hypoxia does not occur at the southern part of the GB near the open sea due to the active flow of seawater and the steady supply of oxygen. The SOCR seems to be most closely related to the bottom water temperature. If the temperature is high, the bacteria related to organic matter decomposition are highly active, and when decomposition occurs more actively, more oxygen is consumed.

Hypoxic water masses, which continually occur during summer in the northern inner bay, can cause reductions in pH and affect ocean acidification. If a hypoxic water mass persists in the bottom layer, the water can become anoxic, and hydrogen sulfide and other chemicals may be released from the sediment. The hydrogen sulfide flux was 1.38 and 1.31 mmol m^{-2} d^{-1}at sites C3 and C17, respectively. The chamber experiment results demonstrate the possibility that hydrogen sulfide could be released at a concentration that is harmful to bay organisms.

Acknowledgements. This research was supported by grant from KIOST project (PE99712), titled "Biogeochemical cycling and marine environmental change studies".

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_31993214

BIOTECHNOLOGICAL CONTROL METHODS AGAINST PHYTOPATHOGENIC BACTERIA IN TOMATOES

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(Received 12th Nov 2018; accepted 11th Jan 2019)

Abstract. Due to the lack of control over the spread of pathogens and effective pesticides with antibacterial activity bacterial diseases cause significant economic losses in vegetable production including tomatoes. Therefore, the search for ways of biocontrol of bacterial diseases pathogens in vegetable crops is an extremely urgent problem. The literature review presents a systematic analysis of modern measures of agrotechnical, chemical and biotechnological control of bacterial diseases in tomatoes, and increasing the resistance degree of these crops against phytopathogenic bacteria using traditional methods, cell selection and the involvement of genetic engineering approaches. It is shown that agrotechnical measures are preventive in nature, while using chemicals and antibiotics has side effects, in particular phytotoxicity and the appearance of resistant strains of pathogens. Effective and economically viable is the use of biotechnological preparations and the cultivation of the varieties resistant to pathogens of bacterial diseases in vegetable crops. Selection of varieties resistant to phytopathogenic bacteria is based on the use of wild species as resistance sources. The creation of genetically modified plants containing avr-genes, resistance genes against bacterial phytotoxins, PR-proteins and AMP is promising.
Keywords: *bacterial diseases, biopreparations, antagonists, cells selection, GM-resistant plants*

Introduction

One of the reasons for the limited production of vegetable products is the significant economic losses caused by bacterial diseases (Khaliluev and Shpakovski, 2013). In vegetable crops bacterial etiology describes 40 widespread pathogens (Hvozdiak et al., 2011). An intensive growth in harmfulness has been recently observed in the case of phytopathogenic bacteria, which is the result of: 1) the emergence of new bacteria strains affecting a wide range of vegetable crops; 2) global climate change, contributing to the expansion of areas of bacteria distribution; 3) lack of reliable control over the infection sources and circulation of pathogens; 4) introduction of new varieties and technologies of plant cultivation; 5) excessive fungicide application (Punyna, 2009).

The basis for the developing control methods for bacterial pathogens is timely and accurate diagnosis. After all, bacterial diseases often have external manifestations similar to the symptoms of diseases caused by micromycetes and mycoplasmas (Hvozdiak et al., 2011). Imperfect laboratory diagnosis leads to improper or even harmful use of the means meant to control pathogens.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

Modern bases of protection of vegetable crops from bacterial diseases are based on complex use of methods of control of pathogens (Khaliluev and Shpakovski, 2013). For their effective use, it is necessary to reliably determine the direction of existing methods with respect to the nature and pathways of pathogens and sources of infection. The review presents agrotechnical and chemical methods, the use of biotechnological preparations, antibiotics, the cultivation of resistant and genetically modified plant varieties, wish are part of a complex system for control of tomato bacterial diseases agents.

Agrotechnical measures

Compliance with optimal temperature regimes (avoiding sudden changes between night and day temperatures), air and soil humidity, planting time, timely application of fertilizers and irrigation with water the temperature of which is not higher than 25 °C. In greenhouses, treating the tomato seeds with disinfectants, removal of crop residues and disinfection of equipment are recommended. In the field, as a result of the intensive development of bacterial diseases, crop rotation is used with the return of the culture not earlier than in the season, the removal of weeds and plant residues, minimization of mechanical damage to the culture, the destruction of infected plants or pruning of infected leaves. However, the existing measures are only preventive in nature (Tkalenko, 2012; Huliaieva et al., 2018).

Chemical measures

In production conditions fungicides are used against pathogens of bacterial diseases, as there are no special preparations with antibacterial activity among those registered in Ukraine (Kolomiiets et al., 2017a). In our research we have been investigated antibacterial action of about 50 pesticides with such active ingredients as dimethomorph, mancozeb metalaxyl, azoxystrobin, fenamidone, propamocarb hydrochloride, copper sulfate and oxychloride, mandipropamid, difenoconazole, aluminium phosphide, phosphorous acid, pyraclostrobin and metiram included in the List of pesticides and agrochemicals and allowed for use in Ukraine on tomato crops. It can be argued that the vast majority of pesticides used, does not suppress the development of pathogens of bacterial diseases in tomato plants. Slight antibacterial activity against strains of the pathogens of bacterial cancer, bacterial speak, and black bacterial spot of tomatoes has only aluminium phosphide and phosphorus acid, as well as mancozeb and mancozeb in combination with metalaxyl (Kolomiiets et al., 2017a). From this point of view, it is important to search, test and develop the methods for the use of special substances for the control of phytopathogenic bacteria (Dankevych et al., 2018).

Now, to control bacterial diseases of tomatoes, preference is given to preparations, in particular, sulfate, oxychloride and copper hydroxide (Khadija, 2002). Copper ions denature proteins and thereby destroy enzymes, which are crucial for the bacterial cells functioning (Mohsin et al., 2016). Copper destroys the cells of pathogens on the surface of plant leaves, but once they penetrate the host tissue, they are no longer sensitive to copper processing. Thus, copper sprays act as protective fungicidal / bactericidal methods in the early stages of infection, but are not characterized by prolonged and stable activity (Behlau et al., 2008).

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Known that the use of copper hydroxide and mancozeb inhibits the development of bacterial cancer, which confirms a synergistic effect, as a separate application of mancozeb does not reduce the population and spread of *Clavibacter michiganensis* subsp. *michiganensis* (Hausbeck et al., 2000).

In our research we have found insignificant antibacterial activity of chemical defenses on the basis of copper hydroxide (770 g/kg) and mancozeb (800 g/kg) relative to gram-positive *C. michiganensis* subsp. *michiganensis* (Kolomiiets et al., 2017a).

The use of copper sulfate and 8-hydroxyquinoline resulted in a significant reduction in the symptoms of bacterial cancer of tomatoes was established (Leon et al., 2008). At the same time, a synergistic effect was observed of 8-hydroxyquinoline/ copper sulfate against *C. michiganensis* subsp. *michiganensis*. The combination of available substances in the conditions of reducing the concentration by half provided a more significant and reliable reduction of bacterial symptoms than the use of individual substances (Leon et al., 2008).

In our research we have established a slight antibacterial activity of copper sulfate (345 g/l) relative to *C. michiganensis* subsp. *michiganensis* with zones of growth inhibition of 20 – 50 mm. Relative to tomato bacterial mottle pathogen *P. syringae* pv. *tomato*, antibacterial activity was observed within the growth inhibition zones from 14 to 54 mm, and for the black bacterial spotting pathogen *X. vesicatoria* it did not exceed 18 mm (Kolomiiets et al., 2017a).

The use of bactericides based on copper in combination with fungicides, ethylene-bis-dithionate or MANCOZEB caused an increase in the level of control of even copper-tolerant populations of pathogens. The inductor of plant resistance acibenzolar-S-methyl (CGA-245704 or Actigard) ensured the formation of low indicators of control of the distribution of tomato black bacterial spotting and bacterial mottle (Itako et al., 2014).

It was found that hexanoic acid (Hx) reduces the development of symptoms caused by *P. syringae* pv. *tomato* DC3000 by 50% in treated plants when compared to untreated ones, which indicates the induction of Hx resistance against this pathogen (Scalschi et al., 2014). The effect of Hx is based on inactivation of bacterial virulence genes and slowing their expression or regression (Scalschi et al., 2014).

In the field (Itako et al., 2014) it was evaluated the effectiveness of acibenzolar-S-methyl, fluosines, pyraclostrobine, copper oxychloride, MANCOZEB/ copper oxychloride and pyraclostrobine/metiram for the control of tomato black bacterial spot. In 40 days after transplantation, the plants were inoculated with *X. perforans* (10^7 CFU/ml) in order to assess the degree of development of the disease. Promising results were obtained only under treatment with fluosine, pyraclostrobine, pyraclostrobine/metiram, copper oxychloride and mancozeb/copper oxychloride. The activity of enzymes of polyphenol oxidase and peroxidase was higher than in terms of application of acibenzolar-S-methyl, pyraclostrobine and pyraclostrobine/metiram on tomato leaves, which confirms their participation in the mechanisms of induction of resistance to the pathogen *X. perforans*.

In our research, in a series of studies, the preparation with the active substance pyraclostrobine (50 g/kg) metiram (550 g/kg) was somewhat active against gram-positive *C. michiganensis* subsp. *michiganensis* and did not show antibacterial activity against gram-negative *P. syringae* pv. *tomato* (Kolomiiets et al., 2017a). In our opinion, this is due to the structural features of the cell wall of gram-positive and gram-negative bacteria. This drug caused the maximum increase in the activity of the enzyme

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

peroxidase in the leaves of plants for 12 h, which was 111.5–112.5 u.mg^{-1}·s^{-1} (Kolomiiets et al., 2017a).

However, in published literature chemical means of bacterial pathogen suppression is only partially effective, and under favorable conditions for the development of the disease (high temperature, precipitation) are generally ineffective. Intensive treatment with copper for commercial tomato cultivation for many years caused the appearance of resistant strains of phytopathogenic bacteria (Patyka et al., 2016), accumulation of copper in soils and water with subsequent toxic effects on plants, soil beneficial microflora and invertebrate organisms. As a result, there is an urgent need to develop fundamentally new alternative measures to protect vegetable crops from bacterial diseases.

Biotechnological preparations

The basis of the creation of biotechnological preparations of different functional orientation for biocontrol of phytopathogenic bacteria is the elective ability of microorganisms to exhibit antagonistic activity against phytopathogens and stimulate plant defense mechanisms. The prospects of creation of biological means of protection of vegetable crops on the basis of bacteria of the genus *Bacillus* are shown. It is believed that the high level of antagonism of bacilli against phytopathogenic bacteria is associated with the synthesis of a wide range of exometabolites (Bais et al., 2004; Stein et al., 2004; Butcher et al., 2007; Nagorska et al., 2007; Ongena et al., 2007; Ongena and Jacques, 2008; Roi et al., 2012). Anyway, the literature does not provide enough information about the exact mechanisms of action *B. subtilis* as an agent of biocontrol of pathogens on vegetable crops.

The antibacterial activity of surfactin (lipopeptide antimicrobial agent) was determinedby testing the mutant strain *B. subtilis* M1 with deletion in the surfactants gene, which was ineffective as a biocontrol agent of *P. syringae pv. tomato* DC3000 (Bais et al., 2004). Wild strain *B. subtilis* 6051 in terms of colonization of the roots formed stable and extensive biofilm and secreted surfactin, inhibiting the growth of the pathogen of tomato bacterial mottle, which confirms its bactericidal activity.

The treatment with the suspension *B. subtilis* (Quadra 136 and 137) and *Rhodosporidium diobovatum* (S33) prevents the development of bacterial cancer in greenhouse conditions, which is caused by *C. michiganensis* subsp. *michiganensis* was reported (Utkhede and Koch, 2004). Similarly, in terms of treatment of seeds with strains *B. subtilis*GBO3, *B. amyloliquefaciens* IN937a and *Brevibacillus brevis* IPC11 maximum protection of tomatoes against bacterial cancer was recorded.

In our research experiments, biological preparations Phytohelp, Phytocid and Extrasol on the basis of *B. subtilis* had different antibacterial activity to pathogens. Thus, Phytohelp and Phytocid showed high antibacterial activity against *C. michiganensis* subsp. *michiganensis* and *X. vesicatoria*, and the diameter of the zone of absence of growth ranged from 70 to 80 mm. For strains *C. michiganensis* subsp. *michiganensis* and *X. vesicatoria* in terms of action of the Extrasol preparation, it did not exceed 40 mm. Active to the causative agent of tomato bacterial mottle *P. syringae* pv. *tomato* was Extrasol with a diameter of the zone of growth absence of 20–26 mm. Other preparations did not affect this pathogen (Kolomiiets et al., 2017a).

It was described the antagonistic activity of endophytic bacteria *B. pumilus* and *B. amyloliquefacies* against *P. syringae* pv. *tomato* NS4, transformed by GFP-gene (Green

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DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

Fluorescent Protein), and a wild-type NW strain. After using endophytic bacteria was monitored population decrease of *P. syringae* pv. *tomato* NW and NS4 on phyloplan, and number of bacterial spots on the leaves of tomatoes compared with standard chemical protection of the copper oxychloride. In terms of fluorescence microscopy it was monitored small number of labeled GFP cells *P. syringae* pv. *tomato* NS4, which colonized rich in the carbon organic compounds areas of phytoplan. Untreated with antagonists plant leaves contained a significant amount of labeled GFP cells *P. syringae* pv. *tomato* NS4 (Filho et al., 2013).

It was shown the efficiency of epiphytic bacteria *Paenibacillus macerans* and *B. pumilus* for biocontrol of *X. vesicatoria* (Lanna et al., 2010), that reduce by 70% the number of phytopathogenic bacteria cells in phytoplan. The test for antagonistic activity confirmed that epiphytic bacteria effectively inhibit the growth of phytopathogens.

According to data (Fousia et al., 2015), treatment of seeds with *B. subtilis* QST 713 significantly reduces the development of bacterial diseases and provides an increase in plant height when compared to the control. In addition, quantitative PCR-analysis of expression *PR1a*, *PR1b,* and *Pin2* (encoding enzymes for the biosynthesis of salicylic and jasmonic acids) confirmed the role of *Pin2* in protective activity of *B. subtilis* QST 713, as an ecspression of *Pin2* was significantly higher in the treated with *B. subtilis* QST 713 plants, infected by *P. syringae* pv. *tomato* when compared to the control. An early increase was determined in the activity of antioxidant enzymes of superoxide dismutase, catalase, peroxidase and polyphenol oxidase, and a decrease in the content of malonic aldehyde in terms of inoculation of *B. subtilis* QST 713, which plays a key role in reducing oxidative stress and induces systemic resistance of tomato plants against black bacterial spotting (Chandrasekaran and Chun, 2016).

Effective against pathogens of bacterial diseases were biopreparations based on bacteria of the genus *Streptomyces,* which are characterized by selectivity of action and high activity to phytopathogens in low concentrations, which allows avoiding their excessive accumulation in the fruits of vegetable crops. When compared to chemical products, they penetrate more intensively and are metabolized in plant tissues through the leaf surface, stems and roots, are less toxic, decompose quickly, do not pollute the environment and dominate most fungicides in terms of effectiveness (Ferraz et al., 2015).

To prevent loss of tomato crops in greenhouses, caused by the pathogen of bacterial wilt *Ralstonia solanacearum*, it was proposed the root treatment of plants with bacterial isolates *B. thuringiensis* CR-371 and actinomyces *S. avermectinius* NBRC14893 (Elsharkawy et al., 2015).

The possibility of using antagonists *S. setonii* UFV618 and *B. cereus* UFV592 to reduce the symptoms of black bacterial spotting and induce the synthesis of protective enzymes in the leaves of tomato plants that are infected by *Xanthomonas* has been established. The final degree of development of the disease decreased by 29.44 and 59.26% in treatments with *B. cereus* UFV592 and *S. setonii* UFV618. The activity of antagonists can be explained by the activation of protective peroxidase enzymes, polyphenol oxidase, β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase and lipoxygenase involved in the formation of systematic resistance of plants against bacterial diseases (Ferraz et al., 2015).

The antibacterial activity of biopreparations based on *Streptomyces:* Avercom, Avercom nova, Violar, and Phytovit, synthesizing antibiotic substances active to a wide range of microorganisms and fungi have proved (Elsharkawy et al., 2015). Areas of no

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DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

growth of strains *C. michiganensis* subsp. *michiganensis* in terms of action of piopreparations Avercom, Avercom nova, Violar i Phytovit were 16–50 mm. Biopreparations Phytovit and Violar ware inert the pathogens of tomato bacterial mottle *P. syringae* pv. *tomato* and black bacterial spot *X. vesicatoria* (Biliavska rt al., 2015).

Thus, in vegetable growing, promising is the use of biopreparations, which are based on living cultures and metabolic products of microorganisms. It was confirmed antagonistic activity of bacteria of genera *Bacillus* and *Streptomyces* to phytopathogenic bacteria, and biopreparations based on them are recommended for biocontrol of bacterial pathogens.

Antibiotics as means of protection of vegetable crops against bacterial diseases pathogens

Protection against bacterial diseases includes the treatment of vegetable crops with antibiotics, which have advantages in the fight against phytopathogenic microorganisms in comparison to chemicals. They easily penetrate into the tissues and organs of plants through the roots, stems, leaf surface and are metabolized in seeds, so their action is less dependent on climatic conditions; they have antibacterial effect, are relatively slow inactivated, and are non-toxic to the plant body (Kolomiiets et al., 2016). Especially quickly penetrate into plant tissue antibiotics of neutral and acidic nature (chloramphenicol, penicillin), slower – amphoteric (chlortetracycline, oxytetracycline) and antibiotics-the basics (neomycin, streptomycin) (McManus et al., 2002).

Antibiotics for plants are made in the form of powders that contain from 17 to 20% of the active ingredient, and are dissolved or suspended in water to a concentration of 50 to 300 parts per million, and then are applied as an aerosol to the plant organs susceptible to pathogens. They are relatively expensive, so they are primarily used in vegetable and fruit crops (McManus et al., 2002).

The most promising for vegetable production are streptomycin preparations, which suppress the proliferation of bacteria by binding to ribosomes and inhibiting protein synthesis at the stage of initiation of translation (Schluenzen et al., 2006; Schuwirth et al., 2006). The US environmental protection Agency assigned the lowest toxicity category and lack of carcinogenic and mutagenic activity to streptomycin and oxytetracycline. In New Zealand it was registered streptomycin-containing preparation Keystrepto™ for the control of *P. syringae* pv. *tomato, X. vesicatoria, C. michiganensis* subsp. *Michiganensis,* and *P. syringae* pv. *syringae* on tomatoes (Vanneste, 2011).

It was shown that neomycin from the liquid culture of the fungus *S. fradiae* HTP has antibacterial activity *in vitro* and *in vivo* against phytopathogenic bacteria *R. solanacearum*, *E. carotovora* and *X. vesicatoria*. In terms of concentration 200 mg^{-1} of neomycin the reduction in the degree of development of the disease is ranged from 69.07 to 80.51%, which is more effective than in terms of treating with streptomycin – from 50.00 to 72.56% (Tao et al., 2011).

In Florida (USA) it was estimated the influence of kasugamicine (commercial preparation Kasumin® 2L) on the pathogen of tomato bacterial mottle. During its application in the greenhouse there was a decrease in the degree of development of bacterial mottle by 37.5% when compared to the control (Vallad and Pernezny, 2010; EPA, 2005).

However, the use of antibiotics can cause the proliferation of antibiotic-resistant bacteria and the spread of antibiotic resistance genes in the environment or even in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

humans (McManus et al., 2002). So, the appearance of streptomycin-resistant (Sm^R) pathogens makes it difficult to control bacterial diseases of vegetable crops. For example, in the USA streptomycin is allowed to be used on tomato and pepper plants for the control of *X. campestris* pv. *vesicatoria*, which is rarely used for this purpose, as resistant strains were first discovered in Florida in the early 1960s, which are now widespread. Sm^R include the other phytopathogenic bacteria such as *E. carotovora*, *P. chichorii*, *P. lachrymans*, *P. syringae* pv. *papulans*, *P. syringae* pv. *Syringee,* and *X. dieffenbachiae*. The use of kasugamicine is also contradictory, which, together with streptomycin have similar biological mechanisms of action.

It becomes evident that antibiotics are ineffective in protecting plants against bacterial diseases through their instability, blocking metabolic pathways, phytotoxic side effects, entering the human and animal food chain, high cost and development of resistant bacterial populations. Promising is the use of non-preparative forms of antibiotics, and biotechnological preparations, which are based on strains-producers.

Cultivation of resistant varieties of vegetable crops is one of the promising systems of biocontrol of phytopathogenic bacteria. The problem of complex resistance of genotypes against the most dangerous diseases has not been solved yet (Khaliluev and Shpakovski, 2013). The reasons for this are the genetic complexity of the trait, genome instability and microevolutionary changes in the host-pathogen system, as well as the emergence of highly resistant biotypes of pathogens against the background of the use of increased amounts of pesticides (Khaliluev and Shpakovski, 2013), the gradual increase in the duration of the average temperatures of the growing season, the proportion of monoculture and genetic homogeneity of the varieties that are grown. To provide breeding programs, it is necessary to search for new sources of resistance against bacterial diseases, which will allow optimizing, accelerating and increasing the effectiveness of the breeding process, creating the new varieties and hybrids with high resistance against pathogens (Khaliluev and Shpakovski, 2013).

There is evidence that microscopy of the stems of resistant tomato plants affected by *R. solanacearum*, showed restrictions on the spread of bacteria with thickening of the cell membrane and synthesis of suberin. The available samples are recommended to be used in the program for the selection of tomatoes against the bacterial wilting pathogen (Kim et al., 2016). In our research, the tomato plants of the resistant variety Chaika under the action of virulent strain *P. syringae* pv. *tomato* IZ-28 the cell walls were seeped with suberin and filled with lignin components, which is typical for the reactions of induced immunity. Lignin was intensively deposited on tangent and frontal anticlinal walls according to the potential directions of translocation of phytopathogenic bacteria, which created cell barriers to their spread (Kolomiiets et al., 2017b).

The localization and distribution of *R. solanacearum* in plants of 11 resistant tomato varieties from different genetic sources and susceptible variety Ponderosa was studied (Nakaho et al., 2004). The spread of bacteria in the stems of resistant tomato plants was suppressed by blocking the transition of pathogens from protoxylem or primary xylem to other xylem tissues. It was most noticeable on the Hawaii 7996 breeding line, which may be an alternative genetic source for tomato plant reproduction, resistant to bacterial wilting.

In Bulgaria they were obtained stable against two races T1 and T3 *X. vesicatoria* tomato lines for growing in the field. Lines created by hybridization between wild species *Lycopersicon L. pimpinellifolium* PI 126925, *L. chilense* LA 460, *L. peruvianum* var *humifusum* PI 127829, and *L. hirsutum* f. *glabratum*. PI 134418, were used as

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DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

sources of sustainability. They were tested more than a hundred lines of tomatoes, from which they were selected promising numbers 36, 44, 44/1, 163/1, 165/2, 167, 167/4, 267 and 270, which showed high resistance against the race T3 *X. vesicatoria*. Stable plants from the group I were obtained by hybridization with *L. pimpinellifolium* PI 126925. A significant number of stable lines were selected in the group II with the involvement of *L. chilense* LA 460 and *L. peruvianum* var *humifusum* PI 127829. Group III lines that occurred with *L. hirsutum* f. *glabratum* PI 134418, were less resistant to race T1. At the same time, their natural resistance against the T3 race has not changed. The available nine lines were marked by valuable morphological and agronomic traits, which were selected for the reproduction of resistant varieties of tomatoes and as a starting material in cross-breeding programs (Ivanova et al., 2006).

In Uruguay, where tomatoes are affected by the race T2 *X. vesicatoria*, were identified varieties Hawaii 7981, Loica and Ohio 8245, which can be used as new natural sources of resistance to the pathogen *X. vesicatoria* of the T2 race (Berrueta et al., 2016).

Now in the world market there are no varieties of tomatoes that would be resistant against *C. michiganensis* subsp. *michiganensis*. It was provided the screening of wild tomato species for resistance against *C. michiganensis* subsp. *michiganensis* (Sen et al., 2013). High tolerance was revealed in *S. arcanum* LA 2157, *S. peruvianum* PI 127829 i *S. arcanum* LA385 and average – in *S. habrochaites* LA 407 and *S. lycopersicum* cv. IRAT L3. Partial resistance against different strains of *C. michiganensis* subsp. *michiganensis* was identified in the wild relatives of the cultural tomato *Lycopersicon hirsutum – Lycopersicon entryion* (LA) 407. Resistance in LA407 was determined in population lines obtained by reverse crossing (IBC) BC2S4 in greenhouse and field conditions. Two lines of the IBC population, in particular IBL 2353 and IBL 2361, have been identified as sources that maintain high resistance at the genetic level with theoretical homology of the genome *L. esculentum* 87.5% (Francis et al., 2001).

For a long time the race R0 *P. syringae* pv. *tomato* was successfully controlled by the gene *Pto1*, resistance against which was overcome by race R1, which was discovered in 1982 in Canada. There are currently no commercial varieties of tomatoes that are resistant to the R1 race, although some wild tomato species have such genetically determined resistance. The presence of high level of resistance of isolates to California race R1 in the studied tomato lines was shown (Stamova, 2009). The disease spread index (DSI) of the resistant lines ranged from 1.00 to 1.93 on a five-point scale depending on isolate virulence. At the same time, the DSI of susceptible control varieties Chico III and ONT 7710 ranged from 4.70 to 5.00. The level of resistance of F1 plants was equal to the resistance of the maternal line.

Tomato lines with fruits different from the traditional red color were studied in order to find sources of resistance to races R0 and R1 *P. syringae* pv. *tomato* (Ganeva and Bogatzevska, 2017). Lines L1078 and L1083 with brown-red (black) fruits and L1130 with purple-red fruits were highly resistant against races R0 and R1. It was found that two lines with pink fruits L1088 and L584, which are resistant against the race R1 *P. syringae* pv. *tomato*, can be used in combined and heterosis breeding for breeding varieties of tomatoes resistant to bacterial mottle.

In order to create resistant varieties and hybrids of vegetable crops against bacterial pathogens, the use of cell selection methods is an alternative. Joint cultivation of plants with phytopathogens has become one of the effective means of plant breeding for resistance against bacterial diseases (Kolomiiets et al., 2017a). In cell selection, various

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DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

plant cells and organs are used, as well as types of selective agents, which under optimal conditions can trigger a cascade of reactions to the pathogen similar to the whole plant. A plant or its tissue or organ, surviving under the pressure of selective assortment, is a potential source of resistance/ tolerance (Kolomiiets et al., 2017a).

In order to reduce the time of selection of tomato genotypes resistant against bacteriosis pathogens, we have developed a biomethod, which is based on the use of *in vitro* cultures of plant cells and tissues. It has been used to test the stability of 16 determinant varieties of tomatoes of Ukrainian selection. In our research, it was proven that tomato varieties Chaika, Klondike and Zoreslav are resistant against pathogens of bacterial cancer, mottle, and spotting; Flandriia, Lehin – against bacterial spotting, and Oberih, Atlasnyi, Hospodar and Kimmeriets – against bacterial mottle (Kolomiiets et al., 2017a). The selected promising genotypes can serve as a starting material for the creation of tomato varieties with high resistance against bacterial diseases.

Consequently, genetic resistance is gaining significant practical interest in the integrated biocontrol of bacterial diseases to reduce crop losses of vegetable crops. Genetic diversity, high mutation capacity and overcoming the genetic barriers of the pathogen are challenges for breeders in creating varieties that are resistant to bacterial diseases. An alternative strategy is to use partially resistant sources that involve several non-specific genes.

Genetically-modified (GM) plants are a priority in program for growing bacterial-resistant vegetable crops in many countries. However, efforts to obtain GM-resistant plants have been slowed down by complex resistance genetics and variable pathogen races (Horvath et al., 2012).

In the case of bacterial pathogens, the study of genes and mechanisms of pathogenesis and natural or induced plant resistance and parallel work with antibacterial proteins of different origins have become the fundamental basis for the implementation of molecular approaches, in particular the involvement of GM plants for the cultivation of new resistant forms. They are divided into three main categories: 1) the introduction of bacterial genes for avirulence, 2) inclusion of bacterial resistance genes against bacterial phytotoxins, and 3) expression of antibacterial proteins of plants, insects or bacteriophages as bactericidal or bacteriolytic agents (Horvath et al., 2012).

The first category includes several types of *avr*-genes that control the synthesis of race-specific elicitor (*Table 1*) (Singh et al., 2012). In order to increase plant resistance against phytopathogens, special attention is drawn to the use of genes responsible for pathogen recognition and signal transduction (R-genes) according to the concept of “gene for gene” (Singh et al., 2012), which is widely used in genetic programs of vegetable crops mainly due to the acquisition of plant full resistance to a particular pathogen. However, a significant disadvantage of the system is exceptional racial specificity. Resistance of plants is achieved due to the development of necrosis at the site of the lesion, as a result of which the infection does not get further spread. Necrosis induction requires the presence of signal peptide genes in the pathogen and the corresponding receptor in plants, the interaction of which is the trigger for the induction of hypersensitivity reaction (Khaliluev and Shpakovski, 2013).

To date, most of the known resistance genes have been characterized, in particular coding proteins with nucleotide binding site (NBS) and the region of leucine-rich repeats (LRR), which are the most abundant among cloned R-genes. Due to the established molecular structure and biochemical functions of encoded proteins, R-genes

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DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

of tomato plants are divided into four classes: TNL, CNL, RPL and mixed (Kopfmann et al., 2016; Martins et al., 2016) (*Table 2*).

Table 1. *The bacterial genes for avirulence*

Pathogen	Genes for avirulence
Pseudomonas syringae pv. *tomato*	*avrD (avrPtoA1), avrE (avrPtoE1)* *avrPto (avrPtoC1), avrRpt2 (avrPtoB1)* *avrBs1, avrBs2* *avrBs3, avrBsT* *avrBsP (avrBs3-2)*
Xanthomonas vesicatoria	*avrRxv, avrb7* *avrB101, avrBln* *avrB102, avrBn*

Table 2. *Resistance genes of tomatoes against bacterial pathogens*

Class	Resistance genes	Pathogen
TNL (TIR-NBS-LRR)	*Bs4* *Bs2*	*Xanthomonas campestris*
	Rx3	*Xanthomonas campestris*
CNL (CC-NBS-LRR)	*Prf*	*Pseudomonas syringae*
Mixed	*Pto*	*Pseudomonas syringae*

Gene of *Bs4* NBS-LRR class contains TIR-domain, encoding aminoterminal sequences with homology to the cytoplasmic regions of the receptor protein and the IL-1 receptor of mammals and provides for the formation of tomato resistance against *X. campestris* (Khaliluev and Shpakovski, 2013). Similarly, the resistance gene *Bs2* of pepper specifically recognizes and provides resistance against strains *X. vesicatoria*, which contains the corresponding bacterial avirulent gene *avrBs2*. It is proved that the presence of the gene *Bs2* in sensitive line VF 36 reduces the progression of the disease to very low levels, and VF 36 plants have the lowest percentage of the disease among the tested varieties and the commercial tomato lines. The yield of commercial fruits from GM lines was by 2.5 times higher than that of parents, which ranged from 1.5 to 11.5 times depending on weather conditions and infectious background of the disease (Horvath et al., 2012).

The second class includes genes encoding proteins, in which there is a spiral-twisted domain. Representatives of this class are genes that cause the formation of high resistance of tomato plants against *P. syringae* (*Prf*).

Another example of an R-gene is a gene *Pto,* encoding the intracellular serine/threonine specific protein kinases, which was isolated from wild species of tomato plants *S. pimpinellifolium* L. A gene *Pto* causes resistance of plants against *P. syringae* pv. *tomato* and expresses genes of avirulence *avrPto* and *avrPtoB* (Pedley and Martin, 2003). Transfer of cDNA of the gene Pto into susceptible varieties of tomato Moneymarker and Urfa-2 caused the development of resistance against bacterial spotting of fruits (Khaliluev and Shpakovski, 2013).

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The second category comprises the toxin-antitoxin (TA) system which is localized in plasmids and chromosomes of bacteria and contains dicistronic operons, encoding two small genes, one for the toxic component and the second – for antitoxin (Kopfmann et al., 2016). Most of the toxins are endoribonucleases that operate in free or ribosome associated mRNA, the others are focused on DNA-gyrase (CcdB, ParE), tRNA-synthetase (HipA), EF-Tu (Doc) and peptidoglycan predecessors (Martins et al., 2016).

In the third category, genes of hydrolytic enzymes that degrade the cell walls of phytopathogenic bacteria were used to create GM plants with high resistance against pathogenic bacteria. For this purpose, they are used genes of β-1,3-glucanase and inducible genes of plant chitinase with lysozyme activity (PR-2 and PR-3 families of protective proteins) (Goyal and Manoharachary, 2014).

Expression of heterologous plant genes of PR-proteins and antimicrobial peptides (AMP) is the most used genetic engineering concept to improve the degree of plant resistance against diseases of bacterial nature (*Table 3*).

Table 3. *Expression of antibacterial proteins of plants and antimicrobial peptides*

Family	Gen	Resistance
PR-1	*CABPR1*	*Pseudomonas syringae* pv. *tomato*
PR-2	*GLU*	*Ralstonia solanacearum*
PR-5	*thauII*	*Xanthomonas vesicatoria*
PR-12	*alfAFP*	*Ralstonia solanacearum*
PR-13	*Thi2.1*	*Ralstonia solanacearum*
PR-14	*LjAMP1*	*Ralstonia solanacearum*
Snakins	*SN2*	*Clavibacter michiganensis* subsp. *michiganensis*

According to the modern classification, 17 families of PR-proteins and 9 – of AMP are distinguished by similarity of amino acid sequences, biochemical characteristics, biological activity and cell localization (Khaliluev and Shpakovski, 2013). Protective proteins and peptides are relatively small in size, which are positively charged and contain a significant amount of cysteine residues, which stabilizes their tertiary structure by the formation of disulfide bonds (Rahnamaeian, 2011). It was found that the majority of protective PR-proteins and AMP in micromolar concentrations have high antibacterial activity (Edreva, 2005; Van Loon, 2006).

To increase the resistance of tomato plants against pathogens promising was the use of taumatin proteins, which are isolated in the family PR-5. In research (Korneeva et al., 2008), integration into the genome of a gene *thau II*, encoding a native super sweet protein thaumatin, allowed obtaining theGM lines, which have essentially high resistance against the pathogen of tomato black bacterial spot (Korneeva et al., 2011).

Antimicrobial peptides include cecropins, magainins, sarcotoxin IA and tachyplesin I. From potato tubers they were selected antimicrobial peptides (SN1, SN2) with a unique amino acid sequence, which are assigned to individual families AMP – snakins (Mohan, 2011). Their distinctive feature is the presence in the amino acid sequence of the site, which is characteristic of the hemolytic venom of snakes. Snakin SN2 super-production in GM tomato plants contributed to a significant delay and reduction in symptoms of the development of bacterial cancer when compared to the control (Khaliluev and Shpakovski, 2013; Balaji and Smart, 2012).

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Using the method of agrobacterial transformation of the obtained tomato plants with the gene of a synthetic analogue of magainin II (MSI-99) – AMP, which is isolated from the skin of the African clawed frog (*Xenopus laevis*) (Alan et al., 2004). According to the results of plant testing for resistance against *P. syringae* pv. *tomato* it was found that the degree of symptoms of the disease in some lines was much less than in the control.

Vector construction pBI121-spCB, as part of the T-DNA of which it is localized natural gene of cationic lytic cecropin peptide B, isolated from the silkworm (*Hyalophora cecropia*), and with the signal sequence of the gene of α-amylase of barley used to produce the GM tomato plants resistant against bacterial wilt and bacterial black spotting (Jan et al., 2010).

It can be concluded that there is still no reliable information on the production of GM tomato plants that are resistant to bacterial diseases and suitable for commercial use. The complexity of obtaining such plants is explained by the genetic complexity and versatility of this feature, as well as the rapid loss of plant resistance acquired. It is predicted that the partial overcoming of the existing problems is expected by embedding into the genome of plants of simultaneously several genes of different families, protein products of which have different mechanisms of action (Khaliluev and Shpakovski, 2013).

Summary

It is shown that agrotechnical measures are clearly preventive in nature, while chemical and antibiotic use are low effective, showing side effects, in particular, phytotoxicity and spread of resistant strains of pathogens. It was confirmed antagonistic activity of bacteria of genera *Bacillus* and *Streptomyces* to phytopathogenic bacteria, and biopreparations based on them are recommended for biocontrol of bacterial pathogens. Effective and proved measures are the cultivation of resistant varieties, the selection of which is based on the involvement of wild species as natural sources of resistance, and GM vegetable crops containing avr-genes, genes of resistance against bacterial phytotoxins, PR-proteins and AMP.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32153230

FIRST INVESTIGATION OF THE COMPOSITION AND SPATIAL DISTRIBUTION OF POLYCHAETE FEEDING GUILDS FROM ESSAOUIRA PROTECTED COASTAL AREA (ATLANTIC COAST OF MOROCCO)

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(Received 22nd Nov 2018; accepted 28th Jan 2019)

Abstract. Several researches have been conducted to explain patterns of the abundance, richness and taxonomic diversity of benthic polychaetes; however, such analyses have ignored the functional diversity of polychaete communities, especially feeding guilds in intertidal rocky shores. The present study was carried out to describe and analyse the polychaete feeding guilds on intertidal rocky shores and then examine the effects of environmental factors. Twelve intertidal rocky shores from the coastal protected area of Essaouira (Atlantic coast of Morocco) were sampled during the summer of 2016. A total of 42 polychaete species belonging to 29 genera and 16 families were identified among the 4517 specimens collected. The medium biomass per sampling site was found to be 37.61 ± 15.80 $g.m^{-2}$. The polychaete species were classified into five feeding guilds, and nine feeding modes. The filter feeders were the dominant feeding guild (32%) followed by omnivores (23%), burrowers (20%), carnivorous (15%) and surface deposit-feeders (10%). The FDT (filter feeder, discretely motile, with tentacles) was the most abundant feeding mode, accounting for 24% of abundance (mainly represented by *Sabellaria alveolata*), followed by the ODJ feeding mode (omnivorous, discretely motile, with jaw apparatus) with 22%, and the SDT feeding mode (surface deposit feeder, discretely motile, with tentacles) with 18.9%. The highest trophic importance index and index of trophic diversity values were recorded in the southern region of Essaouira coastline. Based on the canonical correspondence analysis, composition and spatial distribution of polychaete feeding guilds were mainly related to the length of rocky shores and water temperature.
Keywords: *rocky shores, intertidal, environmental factors, functional diversity, feeding modes, filter feeders, trophic importance index*

Introduction

Polychaetes (Annelida) are one of the most important taxa in macrobenthic communities in coastal and marine environments, in terms of diversity and abundance (Fauchald and Jumars, 1979; Grassle and Maciolek, 1992; Hutchings, 1998). With more than 13,000 species described to date, polychaetes species are considered the most numerous among benthic taxa (Minelli, 1993; Jumars et al., 2015; Han et al., 2016).

Indeed, the polychaetes represent about one third of the total macrobenthic species in terms of richness and up to 80% of the total abundance (Fauchald and Jumars, 1979; Belan, 2004; Manokaran et al., 2013; Han et al., 2016). They are also crucial in marine food chains as important prey and predators for many organisms (Serrano Samaniego, 2012), and exhibit a high diversity of feeding modes, reproductive strategies and different levels of tolerance to the negative impacts induced by pollution and natural perturbations (Giangrande et al., 2005; MacDonald et al., 2010; Sivadas et al., 2010; Han et al., 2016).

According to Fauchald and Jumars (1979), feeding guild of any organism is defined as the set of relations among food particle size and composition, the mechanism involved in food intake, and the motility patterns associated with feeding. These authors classified feeding guilds of each polychaete family as herbivore, carnivore, filter feeders, surface deposit feeders, and sub-surface deposit-feeders. Later, Ruppert and Barnes (1994) suggested that omnivores also exist in the marine environment. According to Pinnet (2000), organisms of the same feeding guild would dominate a particular type of substrate. Indeed, the filter-feeders are the dominant feeding guild on hard substrate (Porras et al., 1996; Damianidis and Chintiroglou, 2000), while deposit-feeders are the dominant feeding guild on soft substrates (Paiva, 1994; Muniz and Pires, 1999; Mattos et al., 2012). Deposit feeders usually inhabit low-energy, muddy substrates because such substrates tend to have a high content of organic matter (Muniz and Pires, 1999; Wang, 2004; Méndez, 2013). By contrast, filter-feeders would not find enough suspended food in muddy substrates, but could be abundant on gravel and coarse-sand bottoms, since currents provide a continuous supply of suspended organic detritus and plankton (Wang, 2004).

Several biotic or abiotic factors can affect the composition and distribution patterns of polychaete feeding guilds. These include total organic carbon (Denisenko et al., 2003; Taurusman, 2010; Han et al., 2016), food availability (Dauwe et al., 1998; Rosenberg, 2001), depth and salinity (Rosenberg, 2001; Han et al., 2016). Physical characteristics of substrates and hydrodynamic factors may also modify distributions (Arruda et al., 2003; Sanders, 1958). Thus, feeding guilds can be used as a tool to analyse polychaete assemblage patterns, due to their dependence on the environmental variables, and studies on feeding guilds can permit ecologists to understand the ecological function of each species and predict if an ecosystem was susceptible to invasion by certain species (Fauchald and Jumars, 1979).

In Morocco, polychaetes represent an important group in terms of abundance and diversity on the continental shelf. The first attempt to explain patterns of abundance, richness and diversity of benthic polychaetes goes back to the beginning of 20th century with Charrier (1921) who drew up the first list of polychaetes from the region of Tangier. Subsequently, many studies have been carried out on the polychaetes of Morocco, up to the recent description of *Boccardia polybranchia* as a new species along the rocky shores of Safi (Goumri et al., 2017). However, these analyses did not consider the overall functional structure of polychaete communities. Some studies examined polychaete feeding guilds in sandy beach environments, such as the investigations of macrofauna communities carried out on the Atlantic coast between Tangier and Tarfaya by Bayed (1991), in Bou Regreg estuary by Cherkaoui et al. (2003) and in Merja Zerga by Bazaïri et al. (2003) and Touhami et al. (2017). On the Mediterranean coast, the lagoon of Smir was also studied by Chaouti and Bayed (2011). However, analyses of polychaete feeding guilds on intertidal rocky shores are virtually absent. The aim of this

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

paper was to describe, for the first time, the composition and spatial distribution patterns of polychaete feeding guilds in the intertidal rocky shores of Essaouira (Atlantic coast of Morocco), and their dependence to various environmental variables.

Materials and methods

Study area

The study area is located along the Atlantic coast of Essaouira province, Morocco. It lies between longitudes 9° 20' and 9° 90' West and between latitudes 31° 10' and 31° 50' North (*Fig. 1*). This coastal zone is made up of sandy beaches, rocky shores and dunes (fixed and shifting) with elevations ranging from 0 to 43 m. The lithology is composed of quaternary conglomerate, colluvial, and alluvial deposits and Jurassic limestone (Molua Mwambo et al., 2007). The hydrographic network includes a few rivers. The main ones are oued Ksob, oued Tidzi and oued Aghbalou which are located in the south of Essaouira while oued Tahria is located in the north. The area climate is semi-arid with a long dry period that lasts from April to October. The aridity decreases from east to west. In the western narrow coastal fringe around the town of Essaouira, the influence of the cold currents that flow onshore from the Canaries Island, create a microclimate with a very homogeneous average temperature of about 16.7 °C during the year (Bazairi et al., 2010). There is little difference between the average temperatures of the hottest and the coldest months. The coastal zone of Essaouira is endowed with ecological assets that have earned it a double protection status: Arganeraie Biosphere Reserve and site of biological and ecological interest (SIBE). The major economic activities in the Essaouira province include agriculture, fishing, mining, trade, industries, tourism, artisanal activities and handicraft industry.

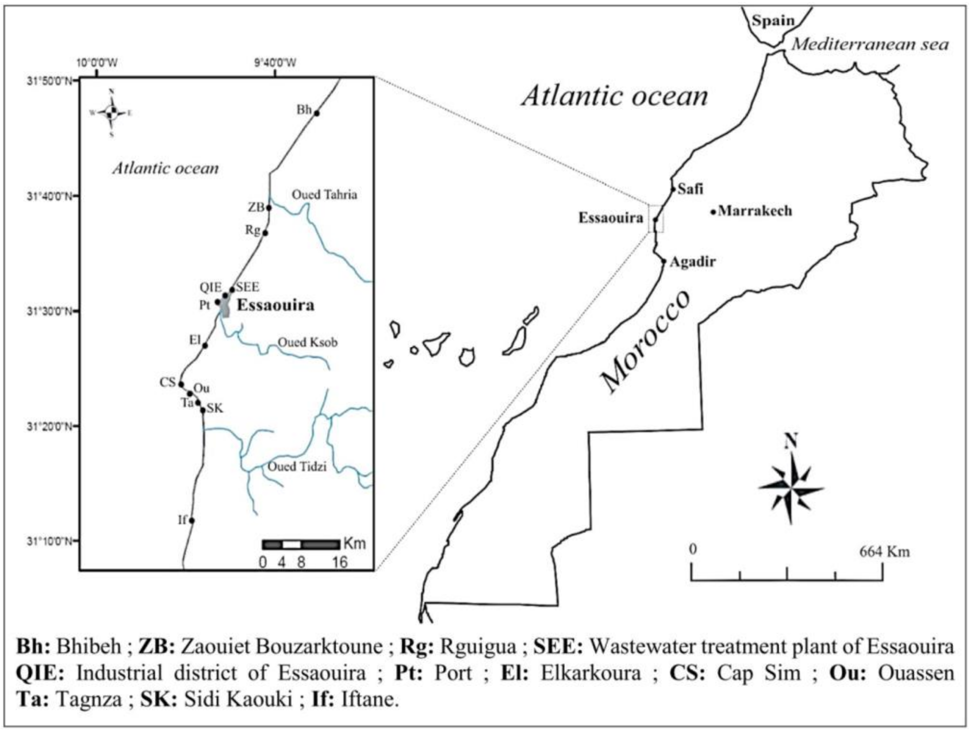

Figure 1. *Geographical position of study area and sampling sites for benthic polychaetes along the coast of Essaouira (9° 20' and 9° 90' W; 31° 10' and 31° 50' N), NW Morocco*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Sampling and laboratory techniques

Twelve sampling sites were investigated in summer 2016 (*Fig. 1; Table 1*). The samples were collected using a quadrat method (Misra, 1968) with four quadrats (each 25 × 25 cm, surface area 0.0625 m^2) in the intertidal zone of each site. Sampling was done by scraping the surface layer (sediments, algae) and digging 2 cm deep in the hard substratum using a hammer. The material was manually collected and then fixed in formaldehyde (8%). Water parameters such as pH, conductivity and temperature were recorded *in situ* using a multi-parameter (type VMR MU 6100 H, Germany), and the salinity was calculated from the conductivity and temperature of sea water (Aminot and Kérouel, 2004). At the same time, the length (m) of each rocky shore was measured from the low water to high water using a measuring tape. Rocks of 20 cm in length and 15 cm in width were collected for each sampling site using a hammer. All these samples were at least triplicated at each site to ensure the representativeness of the samples and measured parameters. In the laboratory, biological samples were rinsed in freshwater and preserved in 70% ethanol and the polychaetes were identified to species when possible with assistance of Dr. Patrick Gillet (Catholic University of the West, France) using various guides, e.g., Fauvel (1923, 1927) and Fauchald (1977), and then counted and weighed. The rock samples were cut and dozens of thin sections were prepared (*Fig. 2*), then grains size from these thin sections were measured using polarizing optical microscope (OLYMPUS BH 2), and the void percentage in each thin section was estimated using “Chart for estimating mineral grain percentage composition of rocks and sediments” (Compton, 1962).

Table 1. *Numbers, names, abbreviation codes and GPS coordinates of the sampling sites*

Site number	Site name	Abbreviation code	Latitude	Longitude
1	Bhibeh	Bh	31° 47' 55" N	9° 34' 59" W
2	Zaouiet Bouzarktoune	ZB	31° 38' 51" N	9° 40' 41" W
3	Rguigua	Rg	31° 36' 46" N	9° 41' 12" W
4	Wastewater treatment plant of Essaouira	SEE	31° 31' 53" N	9° 45' 02" W
5	Industrial district of Essaouira	QIE	31° 31' 21" N	9° 45' 38" W
6	Port	Pt	31° 30' 43" N	9° 46' 27" W
7	Elkarkoura	El	31° 26' 55" N	9° 48' 01" W
8	Cap Sim	CS	31° 24' 02" N	9° 50' 37" W
9	Ouassen	Ou	31° 23' 02" N	9° 49' 12" W
10	Tagnza	Ta	31° 22' 18" N	9° 48' 31" W
11	Sidi Kaouki	SK	31° 21' 24" N	9° 48' 08" W
12	Iftane	If	31° 11' 54" N	9° 49' 20" W

Polychaetes feeding guild assignments

In this study, we assigned feeding guilds to all polychaetes collected according to the definitions of Fauchald and Jumars (1979), MacDonald et al. (2010) and Jumars et al. (2015), which are based on the stomach content analysis of polychaetes and also direct observations of feeding behavior. Feeding guilds of polychaetes are divided into two modes (macrophagous and microphagous) and five or six sub-modes (herbivores, carnivores, filter feeders, surface deposit feeders, burrowers and omnivores), according

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DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

to their major feeding modes. The motility pattern was classified into motile, discretely motile and sessile, and the morphological structures used in feeding were classified into jawed, pumping, tentaculate or other structures, which are usually eversible sac-like pharynges (Fauchald and Jumars, 1979; MacDonald et al., 2010; Jumars et al., 2015). The feeding guilds annotations was performed as follows (*Table 2*): in the three letter codes, the letter in the first position indicates major mode, the second the motility pattern, and the last letter indicates the morphological structure used in feeding; in position 1:B, subsurface deposit feeder (burrower); C, carnivore; F, filter feeder; H, herbivore; O, Omnivore; S, surface deposit feeder; in position 2:D, discretely motile; M, motile; S, sessile; in position 3:J, jawed; T, tentaculate; X, other structures, usually eversible sac-like pharynges.

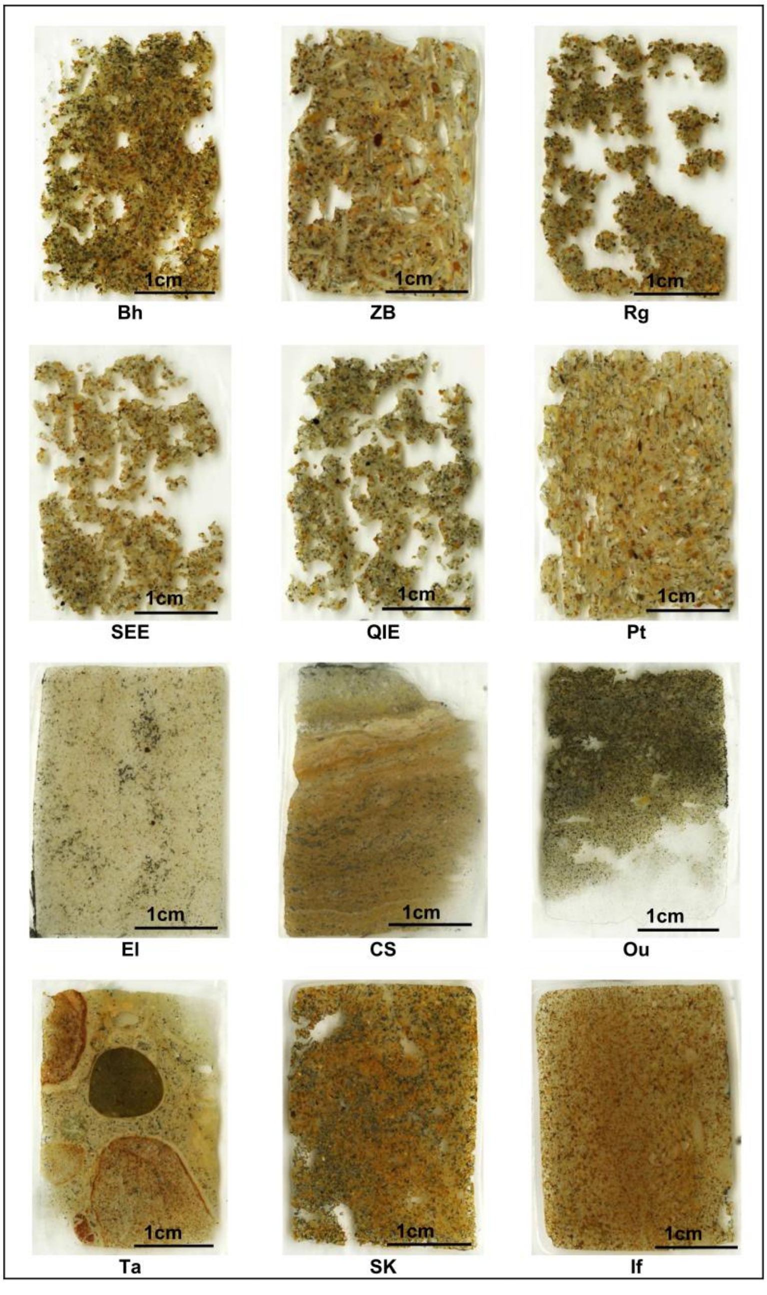

Figure 2. *Photos of thin sections showing grain size of sampled rocks in the study area*

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DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

Table 2. *Feeding guilds annotations of polychaetes after Fauchald and Jumars (1979), MacDonald et al. (2010) and Jumars et al. (2015)*

	Motile	**Discretely motile**	**Sessile**
Macrophagous modes			
Herbivores			
Unarmed pharynx	HMX		
Jawed pharynx	HMJ	HDJ	
Carnivores			
Unarmed pharynx	CMX		
Jawed pharynx	CMJ	CDJ	
Microphagous modes			
Filter feeders			
Tentaculate		FDT	FST
pumping		FDP	FSP
Surface deposit feeders			
Unarmed pharynx	SMX	SDX	
Jawed pharynx	SMJ	SDJ	
Tentaculate	SMT	SDT	SST
Burrowers			
Unarmed pharynx	BMX	BDX	BSX
Jawed pharynx	BMJ		
Tentaculate	BMT		

Statistical analysis

The trophic importance of each group was evaluated according to the trophic importance index (TI) proposed by Paiva (1994) and modified by Muniz and Pires (1999), using the following equation (*Eq. 1*):

$$\mathrm{TI} = \sum_{i=1}^{s} \ln ni\ (+0.1) \quad \text{(Eq.1)}$$

where s is the number of species of a trophic group in the sample, ln is the natural logarithm, n_i is the number of individuals of the *i*th species in the sample, and 0.1 is a constant.

The index of trophic diversity (1-ITD) was calculated as follows (*Eq. 2*):

$$\mathrm{ITD} = \sum \theta^2 \quad \text{(Eq.2)}$$

where θ is the contribution of the density of each trophic group to the total polychaete density. The 1-ITD ranged from 0.90 (the highest trophic diversity) to 0.0 (the lowest diversity; i.e. one trophic guild accounts for 100% of the polychaete density: Heip et al., 1985). The taxonomic similarity between sampling sites was elucidated by a cluster analysis (Bray-Curtis similarity, Complete Link) based on the values of trophic

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importance index (TI). This analysis was carried out using the BioDiversity Pro statistical program (Version 2.0).

One-way ANOVA (analysis of variance) and Tukey's test were used to evaluate statistically significant differences of environmental variables means and Trophic Index means between the sampling sites. CCA (canonical correspondence analysis) was performed to explore the distribution of the polychaete feeding guilds in relation to the rocky shores and environmental factors. One-way ANOVA, Tukey's test and CCA analysis were conducted using the PAST software package (Hammer et al., 2001), Statistical Version 3.15 for Windows software.

Results

Environmental variables

The environmental characterization of the study area was presented in *Table 3*. The longest and shortest rocky beach measured were of 271.67 ± 12.58 m (CS) and 88.33 ± 7.64 m (Pt), respectively. The void percentage in the rock ranged from 1.00 ± 0.04% (El) to 43.00 ± 5.77% (QIE). The mean grain sizes varied from 0.19 ± 0.10 mm to 6.83 ± 6.27 mm on the (CS) and (Ta) rocky shores, respectively. The seawater temperature showed fluctuations between the sampling sites with a minimum value recorded at Pt (17.50 ± 0.50 °C) and a maximum value at SEE (21.00 ± 0.15 °C). The pH value showed a similar trend, ranging from 8.00 ± 0.01 (Ou) to 8.51 ± 0.01 (If). The values of conductivity were typical of seawater, (~50 $ms.cm^{-1}$, Rodier et al., 2009). The salinity was relatively high compared to the average oceanic salinity (35‰) and ranged from 36.26 ± 0.24 ‰ (SEE) to 39.81 ± 0.08 ‰ (Pt). The differences in mean values of these environmental variables were found to be significant at all sites ($p < 0.05$) except for conductivity.

Table 3. *Environmental characterization of the study area, based on variables measured at the 12 rocky shores of Essaouira coastline (mean value ± standard deviation, N = 3)*

Site	Length (m)	Void percentage in the rock (%)	Grain size (mm)	Water temp. (°C)	pH	Conductivity ($ms.cm^{-1}$)	Salinity (‰)
Bh	160.33 ± 7.09^{D*}	20.00 ± 3.92^{B}	0.47 ± 0.29^{B}	18.70 ± 0.60^{C}	8.20 ± 0.07^{CD}	50.90 ± 0.90^{A}	38.65 ± 0.21^{BC}
ZB	150.00 ± 7.07^{DE}	9.00 ± 1.41^{C}	0.67 ± 0.53^{B}	20.90 ± 0.14^{A}	8.28 ± 0.01^{BC}	50.80 ± 0.24^{A}	36.59 ± 0.11^{FG}
Rg	164.33 ± 5.13^{CD}	42.00 ± 10.61^{A}	0.28 ± 0.17^{B}	19.10 ± 0.08^{BC}	8.18 ± 0.03^{CD}	50.90 ± 0.13^{A}	38.28 ± 0.05^{CD}
SEE	109.33 ± 9.02^{FG}	38.00 ± 3.56^{A}	0.38 ± 0.20^{B}	21.00 ± 0.15^{A}	8.15 ± 0.13^{CD}	50.50 ± 0.44^{A}	36.26 ± 0.24^{G}
QIE	131.67 ± 10.41^{EF}	43.00 ± 5.77^{A}	0.60 ± 0.24^{B}	18.38 ± 0.17^{CD}	8.08 ± 0.13^{CD}	50.90 ± 0.52^{A}	38.95 ± 0.29^{BC}
Pt	88.33 ± 7.64^{G}	7.00 ± 0.41^{C}	0.68 ± 0.25^{B}	17.50 ± 0.50^{D}	8.04 ± 0.14^{D}	50.90 ± 0.56^{A}	39.81 ± 0.08^{A}
El	188.33 ± 10.41^{C}	1.00 ± 0.04^{C}	0.23 ± 0.07^{B}	20.00 ± 0.87^{AB}	8.10 ± 0.01^{CD}	50.60 ± 0.26^{A}	37.22 ± 0.65^{EF}
CS	271.67 ± 12.58^{A}	1.50 ± 0.41^{C}	0.19 ± 0.10^{B}	20.70 ± 0.30^{A}	8.43 ± 0.03^{AB}	50.80 ± 0.13^{A}	36.76 ± 0.21^{FG}
Ou	163.33 ± 5.77^{CD}	10.00 ± 3.65^{C}	0.33 ± 0.11^{B}	19.00 ± 0.03^{BC}	8.00 ± 0.01^{D}	50.00 ± 0.01^{A}	37.61 ± 0.03^{DE}
Ta	230.00 ± 15.00^{B}	1.00 ± 0.12^{C}	6.83 ± 6.27^{A}	18.00 ± 0.50^{CD}	8.10 ± 0.03^{CD}	51.00 ± 0.08^{A}	39.41 ± 0.42^{AB}
SK	172.67 ± 7.51^{CD}	7.00 ± 1.15^{C}	0.67 ± 0.16^{B}	18.20 ± 0.27^{CD}	8.19 ± 0.05^{CD}	51.00 ± 0.62^{A}	39.22 ± 0.37^{AB}
If	170.00 ± 10.00^{CD}	3.00 ± 0.41^{C}	0.52 ± 0.10^{B}	18.50 ± 0.10^{CD}	8.51 ± 0.01^{A}	50.90 ± 0.09^{A}	38.84 ± 0.05^{BC}

*The different upper-case letters in the same row show the differences between sites ($p < 0.05$)

Taxonomic and feeding guild composition

In this study, 42 polychaete species belonging to 29 genera and 16 families were identified among the 4517 specimens collected (see *Appendix*). The mean biomass

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(expressed as g wet weight per m^2, with 0.001 g accuracy) was found to be 37.61 ± 15.80 $g.m^{-2}$. The abundance of polychaetes varied from 388 individuals per m^2 ($ind.m^{-2}$) at Elkarkoura site to 3388 $ind.m^{-2}$ at Iftane site. The number of species ranged between 13 and 27 with the highest number (27 species) observed at Iftane site which is 51 km from any source of urban pollution of Essaouira city, and the lowest species number (13 species) at the site SEE. The latter site is within the effluent outflow of the wastewater treatment plant of Essaouira. Families such as Sabellaridae (24% of the individuals), Nereididae (23%), Orbiniidae (9%), Lumbrinereidae (9%) and Terebellidae (8%) were found to be dominant in terms of abundance. *Sabellaria alveolata* ranked as the top species in abundance (24%), followed by *Perinereis cultrifera* (12%), *Platynereis dumerilii* (8%), *Terbella lapidaria* (8%), *Scoletoma impatiens* (8%) and *Scolaricia typica* (8%).

The polychaete assemblage found in the study area comprised five different feeding types, namely; surface deposit feeders, carnivores, filter feeders, subsurface deposit feeders, omnivores, and nine feeding modes: BDX (burrower, discretely motile, other structures), CMX (carnivore, motile, other structures), SDT (surface deposit feeder, discretely motile, with tentacles), CMJ (carnivore, motile, with jaw apparatus), BMJ (burrower, motile, with jaw apparatus), ODJ (omnivore, discretely motile, with jaw apparatus), BMX (burrower, motile, other structures), FDT (filter feeder, discretely motile, with tentacles) and FST (filter feeder, sessile, with tentacles) (*Table 4*).

Table 4. *List of polychaete taxa recorded throughout the sampling sites and relevant feeding guilds. In the three letter codes, the letter in the first position indicates major mode, the second the motility pattern, and the last letter indicates the morphological structure used in feeding; in position 1:B, subsurface deposit feeder (burrower); C, carnivore; F, filter feeder; H, herbivore; O, Omnivore; S, surface deposit feeder; in position 2:D, discretely motile; M, motile; S, sessile; in position 3:J, jawed; T, tentaculate; X, other structures, usually eversible sac-like pharynges*

Species	Feeding guilds	Feeding modes	Species	Feeding guilds	Feeding modes
Capitellidae sp	B	BDX	Orbiniidae sp	B	BMX
Bhawania goodei	C	CMX	*Eulalia viridis*	C	CMX
Cirratulidae sp	S	SDT	*Mysta picta*	C	CMX
Cirriformia tentaculata	S	SDT	*Pyhllodoce maculta*	C	CMX
Lysidice ninetta	C	CMJ	*Lepidonotus clava*	C	CMJ
Marphysa sp	C	CMJ	Polynoidae sp	C	CMJ
Nematonereis unicornis	C	CMJ	*Sabellaria alveolata*	F	FDT
Scoletoma funchalensis	B	BMJ	*Dasychone lucullana*	F	FST
Scoletoma impatiens	B	BMJ	*Jasmineira elegans*	F	FST
Lumbrinereis sp	B	BMJ	*Spirobranchus triqueter*	F	FST
Johnstonia clymenoides	B	BDX	*Spirorbis sp*	F	FST
Petaloproctus terricola	B	BDX	*Serpula vermicularis*	F	FST
Maldanidae sp	B	BDX	Serpulidae sp	F	FST
Perinereis cultrifera	O	ODJ	*Nerinides cantabra*	S	SDT
Perinereis marionii	O	ODJ	*Polydora ciliata*	S	SDT
Platynereis dumerilii	O	ODJ	*Aonides oxycephala*	S	SDT
Nereis irrorata	O	ODJ	*Syllis armillaris*	C	CMJ
Nereis sp	O	ODJ	Syllidae sp 1	C	CMJ
Nereididae sp	O	ODJ	Syllidae sp 2	C	CMJ
Scolaricia typica	B	BMX	*Terebella lapidaria*	S	SDT
Nainereis laevigata	B	BMX	Terebellidae sp	S	SDT

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On the intertidal rocky shores of Essaouira, the filter feeders were the dominant feeding guild (32%) followed by omnivores (23%), burrowers (20%), carnivores (15%) and surface deposit-feeders (10%) (*Fig. 3A*). The FDT was the most abundant feeding mode, accounting for 24% of abundance (mainly represented by *Sabellaria alveolata*), followed by the ODJ feeding mode with 22%, and the SDT feeding mode with 18.9% (*Fig. 3B*), it seems that these 3 groups were present in almost similar proportions. Most feeding modes occurred at all studied sites (12 sampling sites), except for SDT feeding mode absent from the EL site, and the BMX feeding mode absent from the SEE and EL sampling sites.

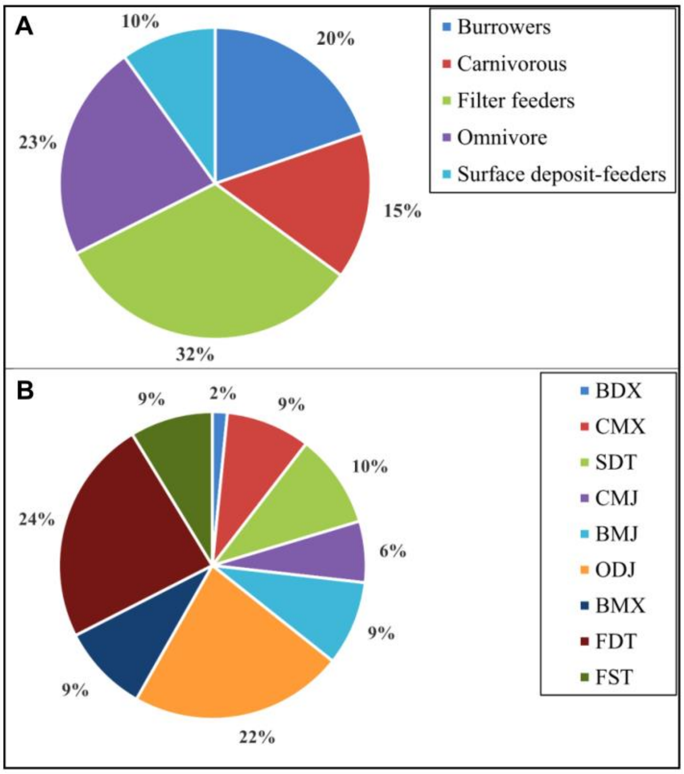

Figure 3. *Relative abundance (%) of the feeding guilds (A) and feeding Modes (B) of polychaetes sampled in the study area. BDX (burrower, discretely motile, other structures); CMX (carnivore, motile, other structures); SDT (surface deposit feeder, discretely motile, with tentacles); CMJ (carnivore, motile, with jaw apparatus); BMJ (burrower, motile, with jaw apparatus); ODJ (omnivore, discretely motile, with jaw apparatus); BMX (burrower, motile, other structures); FDT (filter feeder, discretely motile, with tentacles); FST (filter feeder, sessile, with tentacles)*

Feeding guild characterization

The spatial distribution of feeding guilds at different sampling sites of Essaouira intertidal rocky shores is presented in *Figure 4*. The results of a one way ANOVA shows a highly significant difference (F = 2.46; df = 11; N = 5; Pp < 0.05) of Trophic Index mean between the investigated sites. The burrowers were very common in the studied area, and the most abundant species were *Scoletoma impatiens* and *Scolaricia typica*. The highest TI of this feeding guild was recorded at CS, SK and QIE sampling sites, with TI = 33.97, TI = 32.91 and TI = 32.62 respectively. The lowest TI was

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recorded at ZB (TI = 12.23) and EL sites (TI = 15.58). Carnivores were very abundant in the study area, and the main species of this group were *Bhawania goodei* and *Eulalia viridis*. Their Trophic index was highest at If (TI = 42.45) and lowest at CS (TI = 12.80). Filter feeders were abundant in the whole study area, and represented mainly by *Sabellaria alveolata*. The highest TI of this group was recorded in the southern region of Essaouira, with TI = 34.54, TI = 32.91 and TI = 31.86 at Ta, SK and If, respectively. The lowest TI was recorded north of Essaouira at SEE (TI = 8.86) and ZB sites (TI = 12.80). Omnivores were also common in the study area, but not very abundant as compared to filter feeders. Their main representative species were *Perinereis cultrifera* and *Platynereis dumerilii*. The highest TI of this group was recorded in Essaouira city with TI = 22.40 at Pt. The lowest TI was recorded south of Essaouira at Ou (TI = 7.92). Finally, the surface deposit-feeders, represented mainly by *Terebella lapidaria*, were not very common in the study area because of their absence in the El sampling site. The highest TI of surface deposit-feeders was recorded at the southernmost site at If (TI = 26.57), and the lowest TI was recorded north of Essaouira at ZB (TI = 4.14).

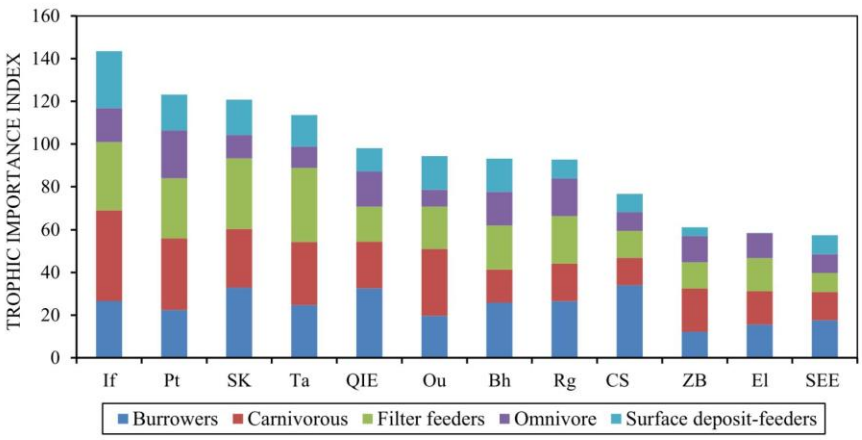

Figure 4. *Trophic importance index values for sampling sites of Essaouira rocky shores*

The index of trophic diversity (1-ITD) values were highest south of Essaouira (Ou, If, Ta, CS, El and SK sampling sites) and lowest in the urban area of Essaouira at QIE (*Fig. 5*).

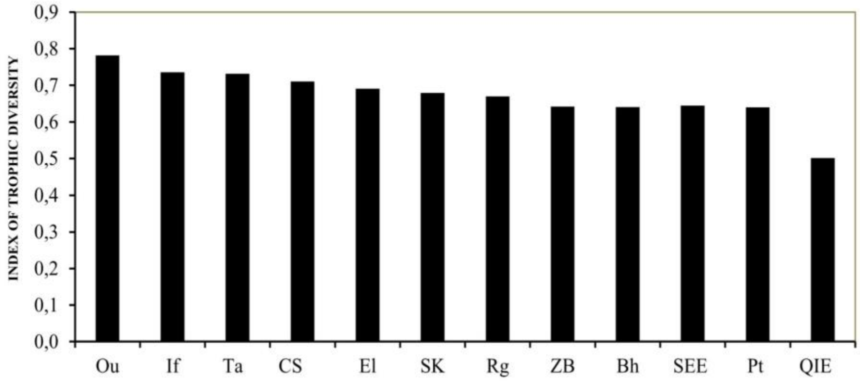

Figure 5. *Index of trophic diversity values for the sampling sites of Essaouira rocky shores*

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The results of the cluster analysis based on the values of trophic importance index (TI) are illustrated in *Figure 6*.

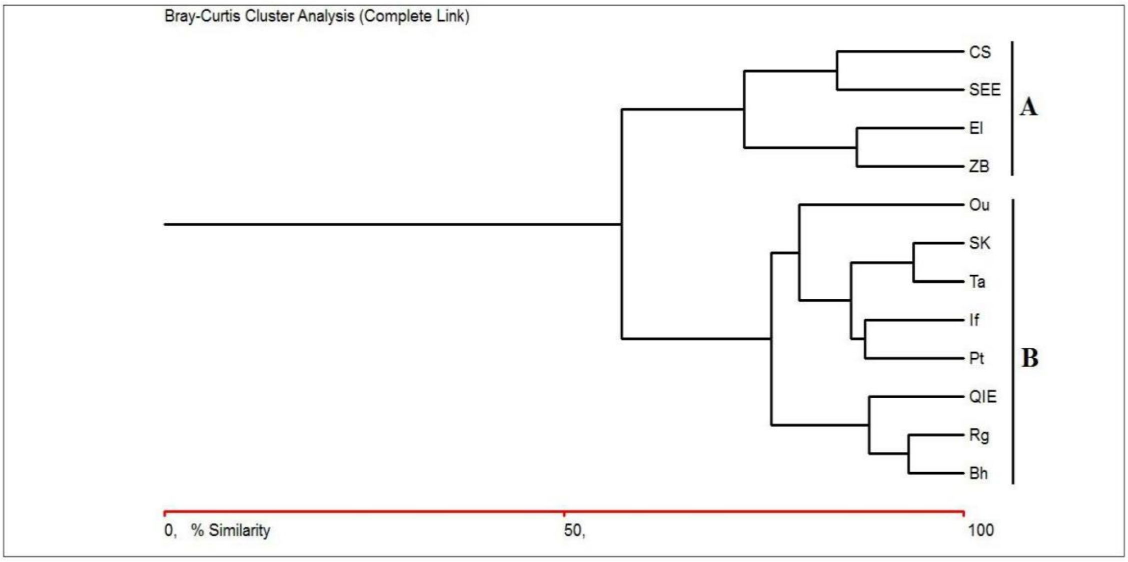

Figure 6. *Distinct groups of sites established by cluster analysis for the 12 sampling sites considered on Essaouira coastline*

According to these analyses, there are two distinct assemblages (A and B) with a level of dissimilarity less than 50% even though both groups included five feeding guilds and nine feeding modes:

- Group A consists of four rocky sites (ZB, El, SEE, and CS) and the dominant feeding guilds, burrower and filter feeder groups, comprised 26.89% and 26.77% of all individuals respectively, while ODJ was the most abundant feeding mode (23.84%). This assemblage was characterized by a medium Trophic Importance index (TI = 63.47 ± 8.96) and moderately high Index of trophic diversity (1-ITD = 0.67 ± 0.034) (*Table 4*).
- Group B includes eight rocky sites (Ou, SK, Ta, If, Pt, QIE, Rg and Bh). The dominant feeding guild was filter feeder with 35.84% of all, while FDT was the most abundant feeding mode (26.48%). This assemblage was characterized by a high Trophic Importance index (TI = 109.93 ± 18.47) and moderately high Index of trophic diversity (1-ITD = 0.67 ± 0.085) (*Table 5*).

Table 5. *Feeding guild assemblage characteristics in the Essaouira coastline*

Parameters	Group A	Group B
Site number	4	8
Total feeding guilds	5	5
Total feeding modes	9	9
Mean (TI)	63.47 ± 8.96	109.93 ± 18.47
Mean (1-ITD)	0.67 ± 0.034	0.67 ± 0.085
Dominant feeding guild	Burrower and Filter feeder	Filter feeder
Dominant feeding mode	ODJ	FDT

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Relationships between feeding guild composition of polychaetes and abiotic factors

The canonical correspondence analysis (CCA) indicated that abiotic factors contributed significantly to explaining the variation in polychaete feeding guilds among the rocky shores studied. The first and second axes accounted for 39.7 and 29.9% of the variance observed in the feeding guild data, respectively. On the CCA plot (*Fig. 7*), communities at Bh, ZB, Pt, El, SK and If are moderately associated with salinity. By contrast, communities at Ta and Ou (but not Rg) positively related to the length of the rocky shores, higher grain-size and higher water temperature but negatively with salinity.

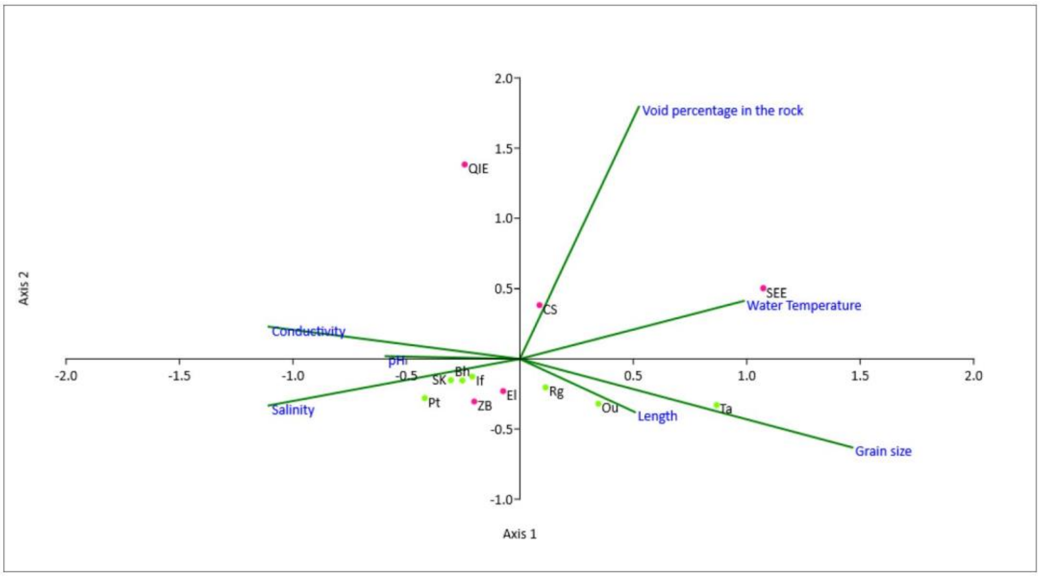

Figure 7. *Canonical correspondence analysis (CCA) ordination of the polychaete feeding guild scores and environmental parameters in the study area (Axis 1: 39.7%; Axis 2: 29.9%)*

Discussion

The present study provides the first characterization of the composition and spatial distribution patterns of polychaete feeding guilds along the rocky shores of Essaouira on the Atlantic coast of Morocco, and their relationship with environmental variables such as temperature, pH, conductivity, salinity, length and void percentage in the rock, and grain size of the rock. In this study, polychaete feeding communities at 12 sites were classified into five feeding guilds (Surface deposit feeders, Carnivores, Filter feeders, Subsurface deposit feeders and Omnivores), and nine feeding modes (BDX, CMX, SDT, CMJ, BMJ, ODJ, BMX, FDT and FST). We have included the omnivore feeding guild in this work since it is difficult to assign some Nereids in a particular feeding guild. Fauchald and Jumars (1979) classified several Nereid species as both carnivores and herbivores. Later, Gaston (1987) characterized them as surface deposit-feeders, Bianchi and Morri (1985) featured them once again as herbivores, and Jumars et al. (2015) specified that Nereids are clearly omnivorous, although digestive physiology suggests more likely specialization. According to Lindsay and Woodin (1995), Hentschel and Larson (2005), there are some species that can change their feeding mode depending on the availability of resources. As there are no consensus in the literature, we preferred classifying the Nereids of Essaouira intertidal rocky shores as omnivores.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

The filter feeders were the dominant feeding guild (32%) followed by omnivores (23%), burrowers (20%), carnivores (15%) and surface deposit-feeders (10%), and the FDT (filter feeder, discretely motile, with tentacles) was the most abundant feeding mode, followed by the ODJ feeding mode (omnivores, discretely motile, with jaw apparatus) and the SDT feeding mode (surface deposit feeder, discretely motile, with tentacles) accounting for 24%, 22% and 18.9% of abundance, respectively. Denny (1988) suggested that the wave action is one of the main factors influencing the establishment and development of intertidal and shallow subtidal benthic communities. This factor represents a constant stress source that forces populations to acquire specific adaptations that allow them to survive in a stressful environment. One of these adaptative aspects is the suspension-feeding mode, which allows organisms to feed on material brought in by water movements (Okamura, 1990; Mann and Lazier, 1991). Thus, it is not surprising that in our case this feeding guild was dominant and present in all study areas, and is probably further enhanced by the influx of nutrient-rich cold waters brought to the surface by upwelling. This phenomenon usually occurs from June to October (Belvèze, 1983; Gentile, 1997; Simone, 2000; Bazairi et al., 2010; Benazzouz, 2014). The main representative species of this guild was *Sabellaria alveolata*, contributing 73.2% to total filter feeder abundance. Similarly, other studies have shown a large number of polychaete feeding guilds in marine environments (*Table 6*). According to these studies, the filter-feeders were the dominant feeding guild on hard substrate while the deposit-feeders were the dominant trophic group on soft substrates.

Table 6. *Polychaete feeding guilds from different marine ecosystems and climate areas*

Number of trophic group (number of guilds)	Number of species (number of family)	Dominant trophic guild	Substrate	Ecosystem	Geographical area	Reference
5 (9)	42 (16)	Filter-feeders	Hard	Intertidal	Essaouira (Atlantic coast of Morocco)	Present study
4 (9)	48 (16)	Filter-feeders	Hard	Mussel bank	Thessaloniki Bay (Greece)	Damianidis and Chintiroglou (2000)
4 (4)	22 (3)	Filter-feeder	Hard	Reef	Valencia Gulf Coast (Spain)	Porras et al. (1996)
5 (16)	160 (44)	Filter-feeders	Muddy to coarse sand	Subtidal	Campeche Bank (southern Gulf of Mexico)	Castanedo et al. (2012)
5 (n.a.)	75 (29)	Surface deposit-feeders	Medium sand to fine silt	Continental shelf	Southeast coast of India	Manokaran et al. (2013)
5 (10)	24 (21)	Deposit-feeders	Fine to coarse sand	Sandy beach	Sepetiba Bay (south-eastern Brazil)	Mattos et al. (2012)
3 (n.a.)	28 (13)	Surface deposit-feeders	Soft bottom	Deep-sea	Malta Escarpment (Western Ionian Sea)	Langeneck et al. (2017)
5 (n.a.)	92 (25)	Deposit-feeders	Sand and mud	Intertidal	Gulf of Gabès (Tunisia)	Mosbahi et al. (2017)
4 (13)	83 (12)	Deposit-feeders and carnivores		Subtidal	Hong Kong (China)	Cheung et al. (2008)
3 (n.a.)	15 (10)	Deposit-feeders	Fine to coarse sand	Mangrove	Quebra Pote, Maranhão (Brazil)	Cutrim et al. (2018)
5 (12)	80 (37)	Burrowers	Fine sand to coarse silt	Coastal bay	Sishili Bay, the northern Yellow Sea (China)	Han et al. (2016)

n.a.: not applicable

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Paiva (1994) indicated that the density approach is more coherent to evaluate the importance of each feeding guild, especially when working with polychaete worms, since it indirectly estimates the biomass and production as these communities are composed of species with analogous life cycles. However, recruitment events and patchy distribution, not necessarily linked to increased food supply, can lead to an overevaluation of the feeding guild. Consequently, the use of the species richness gives more reliable results. During this study, we applied “Trophic group Importance index” (TI) proposed by Paiva (1994) which reduces the importance of density by applying log- transformation of abundances thus preserving the species richness factor. TI index was proved to be practical and very informative. Highest values of TI were recorded on the southern region of Essaouira where the beaches are sheltered and relatively far from human activities.

To accurately define the spatial organization of polychaete feeding guilds of Essaouira’s rocky shores, we used the cluster method which allows a discrimination between the sampling sites. Spatial differences in the structure of polychaete feeding guilds clearly discriminated between two groups: the first was group A (sites ZB, El, SEE, and CS) that characterized by the dominance of burrower and filter feeder feeding guilds, and ODJ feeding mode. The second one was group B which includes eight rocky sites (Ou, SK, Ta, If, Pt, QIE, Rg and Bh), with the dominance of filter feeder feeding guild and FDT feeding mode.

Several authors have observed strong relationships between polychaete feeding guilds and environmental factors, among which granulometry of sediments seems to be the most important (Maurer and Leathem, 1981; Carrasco and Carbajal, 1998; Muniz and Pires, 1999; Pagliosa, 2005; Mattos et al., 2012). Muniz and Pires (1999) and Méndez (2013) concluded that organic matter level in sediments could also strongly influence the feeding guilds of polychaetes, while Carrasco and Carbajal (1998) found no relationship with this variable, but a strong influence of depth. According to Paiva (1994), depth contributes to the sediment stabilization and consequently increases the feeding guild variety. Salinity, dissolved oxygen and temperature were also found to be important factors in determining trophic structure (Maurer and Leathem, 1981; Castanedo et al., 2012). In this study, the CCA results indicate that the length of rocky shores and water temperature are the most influential factors on the composition and spatial distribution of polychaete feeding guilds. Indeed, the rocky shores of Essaouira are long and rich in microhabitats, providing habitats for a large range of species which favours the increase of feeding guild diversity in this area. However, water temperature acts negatively on the diversity and abundance of feeding guilds via its negative influence on species richness and abundance of polychaetes.

Conclusion

This study on the composition and spatial distribution of polychaete feeding guilds of Essaouira intertidal rocky shores led to the identification of five feeding guilds (Surface deposit feeders, Carnivores, Filter feeders, Subsurface deposit feeders and Omnivores), and nine feeding modes (BDX, CMX, SDT, CMJ, BMJ, ODJ, BMX, FDT and FST). Compared to other marine ecosystems and geographical areas, we can conclude that the rocky shore of Essaouira is relatively rich in polychaete feeding guilds. The abiotic factors such as length of rocky shores and water temperature are the major factors affecting the composition and spatial distribution of polychaete feeding guilds in this

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area. The data from this study can be used as a baseline reference for future scientific research programs, and may be a useful tool for authorities in charge of protection and management of this protected coastal area. Nevertheless, a detailed study during different seasons is required, in order to better understand the biotic and abiotic factors determining the structure and organisation of feeding guilds, determine the possible anthropogenic influences on this ecosystem, as well as to predict if this protected coastal area can be invaded by some invasive species.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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APPENDIX

Total abundance of polychaete species identified in Essaouira intertidal rocky shores. Data presented has not been standardised to 1 m^{-2}

Species	Bh	ZB	Rg	SEE	QIE	Pt	El	CS	Ou	Ta	SK	If
Capitellidae sp	0	0	2	0	0	0	0	0	0	0	0	1
Bhawania goodei Webster, 1884	1	4	1	4	7	15	0	1	29	113	14	13
Cirratulidae sp	0	0	0	0	0	1	0	0	0	3	2	0
Cirriformia tentaculata (Montagu, 1808)	0	0	0	0	1	2	0	0	6	2	7	7
Lysidice ninetta (Audouin & Milne-Edwards, 1833)	0	2	1	0	0	1	2	0	27	17	4	5
Marphysa sp	0	0	0	0	0	0	0	0	1	0	0	0
Nematonereis unicornis (Grube, 1840)	0	0	0	0	0	1	0	0	0	0	0	0
Scoletoma funchalensis (Kinberg, 1865)	3	0	1	7	5	0	2	4	5	1	2	7
Scoletoma impatiens (Claparède, 1868)	38	7	12	80	58	16	7	19	13	16	29	69
Lumbrinereis sp	0	0	0	0	0	1	0	0	0	0	0	0
Johnstonia clymenoides Quatrefages, 1866	2	9	1	6	3	4	7	1	11	3	1	0
Petaloproctus terricola Quatrefages, 1866	0	0	0	0	1	0	0	1	10	0	0	0
Maldanidae sp	0	0	1	1	0	0	2	3	0	1	0	0
Perinereis cultrifera (Grube, 1840)	22	13	46	12	20	49	16	69	50	43	49	168
Perinereis marionii (Audouin & Milne-Edwards 1833)	11	2	16	0	26	30	0	0	0	0	0	1
Platynereis dumerilii (Audouin & Milne-Edwards 1834)	9	57	13	0	33	25	48	3	10	5	49	115
Nereis irrorata (Malmgren, 1867)	0	0	0	0	0	0	2	0	0	0	0	0
Nereis sp	0	0	0	3	0	0	0	0	0	0	0	0
Nereididae sp	0	0	2	0	0	1	0	0	0	0	0	0
Scolaricia typica Eisig, 1914	0	1	0	0	226	28	0	21	0	0	50	29
Nainereis laevigata (Grube, 1855)	4	0	1	0	8	0	0	8	0	0	2	29

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DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

Orbiniidae sp	2	0	0	0	0	0	0	2	5	1	1	0
Eulalia viridis (Linnaeus, 1767)	16	2	13	7	16	31	2	9	3	31	25	39
Mysta picta (Quatrefages, 1866)	0	0	0	0	0	0	0	0	0	0	0	1
Pyhllodoce maculta (Linnaeus, 1767)	0	0	0	0	0	0	0	0	0	0	0	4
Lepidonotus clava (Montagu, 1808)	0	2	0	0	0	0	0	0	0	0	23	0
Polynoidae sp	0	0	1	3	2	19	1	1	12	21	36	58
Sabellaria alveolata (Linnaeus, 1767)	172	29	81	3	11	264	3	10	22	44	237	199
Dasychone lucullana (Delle Chiaje, 1828)	1	0	3	0	0	0	1	1	5	7	9	15
Jasmineira elegans Saint-Joseph, 1894	7	3	18	4	1	13	2	11	13	40	38	31
Spirobranchus triqueter Linnaeus, 1758	3	7	43	0	3	7	1	0	11	18	25	18
Spirorbis sp	0	0	5	0	0	4	0	0	3	3	0	2
Serpula vermicularis Linnaeus, 1767	0	0	0	0	0	0	0	0	0	4	1	0
Serpulidae sp	0	0	0	0	0	3	0	0	0	5	3	2
Nerinides cantabra Rioja, 1919	1	0	0	0	0	0	0	0	0	0	0	0
Polydora ciliata (Johnston, 1838)	0	0	0	0	0	0	0	0	1	0	0	1
Aonides oxycephala (Sars, 1862)	0	0	0	0	0	0	0	0	18	0	0	2
Syllis armillaris Müller, 1776	0	0	0	0	0	0	0	0	4	1	0	2
Syllidae sp1	4	0	0	0	7	3	1	0	4	4	0	0
Syllidae sp2	0	1	0	0	0	0	0	0	4	0	0	16
Terebella lapidaria Linnaeus, 1767	34	2	42	74	16	18	0	15	10	137	20	6
Terebellidae sp	1	0	4	4	0	0	0	3	0	0	0	7

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32313249

ENZYMATIC BIOREMEDIATION OF ENDOSULFAN IN SOIL USING LIGNINOLYTIC EXTRACT OF SPENT MUSHROOM COMPOST OF *PLEUROTUS OSTREATUS*

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(Received 28th Jul 2018; accepted 31st Oct 2018)

Abstract. Endosulfan is known for its non target toxicity in various compartments of ecosystem. Its presence in hotspots and agricultural fields is an environmental concern. In this study spent mushroom compost extract of *Pleurotus ostreatus* (SMCE) was evaluated for its potential in bioremediation against Endosulfan in different microcosms. Activities of Laccase and MnP were also monitored to observe their possible role in this process. The SMCE alone (M_1) and SMCE with H_2O_2(M_2) exhibited no significant difference in reduction of α- and β-Endosulfan. This suggested that stimulation of ligninolytic enzymes, Laccase and Manganese peroxidase, after addition of H_2O_2 as observed by monitoring their activity had no effect on bioremediation of this pesticide. In soil microcosms however, there was a significantly higher reduction in M_4(H_2O_2 + SMCE) than M_3(SMCE) suggesting that SCME not only served as source of complex enzymes but also as carbon resulting in enhanced soil indigenous microbial communities and enzymes. Under the effect of H_2O_2, certain enzyme complexes either already present in soil or introduced after SMCE addition worked in symbiotic way and resulted in higher reduction. Endosulfan sulfate was detected in all microcosms expect Endosulfan Sulfate while Endosulfan lactone was only formed in M_3 and M_4.

Keywords: *enzymatic bioremediation, spent mushroom compost, Endosulfan, soil, microcosms*

Introduction

In the past few decades use of agrochemical residues increased in the ecosystem, causing momentous contamination in many terrestrial regions resulting in poisoning of human foods (Carvalho, 2017). Organochlorine pesticides (OCPs), group of such agrochemicals are also known for high persistence and non target toxicity within ecosystem at each level of food chain (Jayaraj et al., 2016). Because of their bio accumulative nature (Zhang et al., 2015) these are among the priority pollutants according to the Stockholm convention (Tsai, 2010). Despite the ban, indiscriminate use of OCPs for farm activities is still in practice (Yadav et al., 2017). Majority of these

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32513267

pesticides are located in overpopulated tropic and sub tropic areas (Wang et al., 2016; Buah-Kwofie and Humphries, 2017) but due to atmospheric circulation, they could be transported to areas where there was no history of their usage and production (Hageman et al., 2015).

Endosulfan, an important OCP, was also banned but its residues are still a bio concern in the ecosystem (Hu et al., 2014; Lal et al., 2010; Barcelo-Quintal et al., 2008; Wang et al., 2016) and because of their widespread nature they are considered nowadays a "global pollutant" and a challenge for land management agencies. The conventional methods for remediation of contaminated soil are costly and not ecofriendly (Ojuederie and Babalola, 2017), an alternative effective approach for detoxification of Endosulfan in the soil is an environmental responsibility. Few such methods include bio-augmentation, intrinsic bioremediation and phytoremediation (Singh and Singh, 2017) was investigated in the past and found the best because of their effectiveness in decontaminations (Uqab et al., 2016) and considered as the major pathway for Endosulfan degradation in soil (Ozdal et al., 2017).

One of the Endosulfan bioremediation approach is exploiting white rot fungi (WRF) for its treatment (Kamei et al., 2011). However, direct application of them in the soil under actual treatment is not a viable option because WRF are not stable in the soil under physiological stress due to extreme environmental conditions (Hatakka, 1994; Crawford and Ramachandra, 1993). The resultant end product of certain WRF is spent mushroom compost (SMC) which is left after production of fruiting bodies. Application of farmyard waste (Fogarty and Tuovinen, 1991) and other solid composts have got attention in the past for biodegradation of various OCPs including Endosulfan (Mukherjee, 2012; Al-Hassan et al., 2004). Application of organic wastes (solid and liquid form) alter the physicochemical behavior of pesticides in the soils (adsorption-desorption, mobility, degradation, etc.) and also has positive effects on soil quality (Marín-Benito et al., 2016). However, relatively long microbial lag phases tend to increase contaminant sorption within soil microspores (Manilal and Alexander, 1991).

In the past SMC of *Pleurotus* was used for treatment of polycyclic aromatic hydrocarbon (Li et al., 2010), biocide/fungicide (Juárez et al., 2011), synthetic and textile dyes (Papinutti and Forchiassin, 2010) and many other pollutants (Cole, 1998; Marín-Benito et al., 2016) because of its ability to tolerate and/or detoxify pesticides of complex nature (Ellouze and Sayadi, 2016) at high concentrations. SMC Extract (SMCE) contains variety of immobilized enzymes including Cellulose-degrading enzymes, xylin-degrading enzymes and lignin degrading enzymes (Nakajima et al., 2018) which were reported to be involved in process of bioremediation (Lim et al., 2013; Ko et al., 2005; Karigar and Rao, 2011). These enzymes are viable under stress condition which make SMC the best candidate to treat the contaminants under field conditions. Application of WRF directly at field level has not had much success due to the difficulties in growth to adequate biomass in soil (Chirnside et al., 2011). For bioremediation of certain pollutants in soil, production of sufficient biomass and acclimatization of that particular fungus is essential (Husaini, 2014). Extracellular ligninolytic enzymes of these WRF fungi do not lose their identity in soil and can be successfully used as bio remedial agent for treatment of herbicides Application of WRF directly at field level has not had much success due to the difficulties in growth to adequate biomass in soil (Chirnside et al., 2011). For bioremediation of certain pollutants in soil, production of sufficient biomass and acclimatization of that particular fungus is essential (Husaini, 2014). Extracellular ligninolytic enzymes of these WRF

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32513267

fungi do not lose their identity in soil and can be successfully used as bio remedial agent for treatment of herbicides (Chirnside et al., 2011).

This showed that there is a clear gap in knowledge regarding role of ligninolytic enzymes in effluent treatment. In the present study we sought to determine the role of ligninolytic enzyme extract obtained from SMC of *Pleurotus ostreatus* in the treatment of Endosulfan. For this purpose, we tried to address this gap by extracting and concentrating two ligninolytic enzymes from SMC of this fungus, Laccase and Manganese peroxidase (MnP) and studied their potential for detoxification of this pesticide under incubation studies of soils using biometric flasks.

Materials and methods

Chemicals/gases

Endosulfan (analytical grade) was purchased from Sigma Aldrich (St. Louis, Missouri) (99.99%). Pestanal® grade organic solvents including n-hexane, acetonitrile and acetic acid were used. The salts used for extraction of Endosulfan from samples e.g., magnesium Sulfate (anhydrous) and sodium acetate trihydrate ($NaCH_3COO.3H_2O$) were also of Analytical grade. Gases used for GC-MS (helium) and for GC-µECD (nitrogen) were 99.999% pure. All the other chemicals used to monitor the activity of enzymes, Carbon dioxide, physicochemical properties of soil and SMC were of laboratory grade. Millipore water was used for measuring physicochemical properties of soil and SMC and extraction of pesticides.

Physicochemical properties of soil

Fine loamy Soil obtained from Organic Farming Orchard, National Agriculture Research Centre, Islamabad, Pakistan (73.127855°E, and latitude 33.666042°N) with no history of OCPs (established by GC-µECD analysis) was used for this study. The physical and chemical parameters e.g., particle size distribution (sand, silt and clay), pH, electrical conductivity (EC), total organic carbon, total nitrogen, Calcium Carbonate content ($CaCO_3$) were measured by using standard procedures (Sparks et al.,1996; Dane et al., 2002) and results are given in *Table 1*. The basic characterization of soil and incubation studies were carried out in Soil Environment laboratory, Land resources research Institute, National Agricultural research center, Islamabad, Pakistan.

Table 1. *Physicochemical properties of soil used in experiment (n = 5)*

Properties (units) of soil	
pH	8.04
EC (µS/cm)	2.49
Organic Matter(%)	0.901
Total $CaCO_3$ (%)	3.15
Texture class (sandy clay loam)	
Clay (%)	25.32
Silt (%)	22.55
Sand (%)	52.53

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DOI: http://dx.doi.org/10.15666/aeer/1702_32513267

Concentrated SMC extract (SMCE)

Culture of *P. ostreatus* (WC-814) were shipped from Mushroom Research Program, Pennsylvania State University.

(i) Preparation of Spawn: Spawn was prepared by placing mycelium of above mentioned fungus onto steam-sterilized wheat grains (Cooled) at 22 °C. When the mycelium was completely grown through all the grain, the grain/mycelium mixture (known as spawn) was used to “seed” mushroom compost. (ii) Preparation of SMC: It was prepared by using 2 kg well chopped wheat straw, ½ kg of wheat bran and small quantity of calcium hydroxide $Ca(OH)_2$ in polyethylene bag. The bags were entirely sterilized by autoclaving at 121 °C and 15 psi for 50 min. On cooling the spawn (10 g) was added in each bag and was allowed to yield fruiting bodies. At the end of generation of fruiting bodies, the product leftover was called as SMC. (iii) Preparation of SMCE: 3 g fresh SMC Fresh (ground and sieve through 10 mm) was taken in an Erlenmeyer flask and then 30 mL sodium tartrate buffer (pH = 5.2) was added. The suspension was shaken at 22 °C and 150 rpm for 2 h as described by Lang et al. (1998) with slight modifications. Then, contents were extracted by squeezing manually using mira cloth and fluids was centrifuged (make and model) at the rate of 10,000 × g for 30 min (Márquez Araque et al., 2007). (iv) Concentration of SMCE: It was concentrated using Vivaspin 20 (10 kDa cutoff) – a disposable ultra-filtration device with twin vertical membrane for unparalleled speed in Centrifuge at the speed of 2800 × g.

Physicochemical properties of SMC

Physicochemical properties of concentrated extracellular SMCE e.g., pH (707 soil/compost pH meters), total organic carbon (TOC) (Tandon, 2005), protein and C: N ratio were determined using standard methods already used by Marín-Benito et al. (2016). Total N in SMC was determined by Kjeldahl method (Helrich, 1995) (*Table 2*).

Table 2. *Physicochemical properties of concentrated spent mushroom compost extract used in experiment (n = 5)*

Sr. No	Properties (units)	
1	pH	7.25
2	Total carbon (%)	16.62
3	Total nitrogen (%)	1.036
4	Total protein (%)	8.19
5	Organic matter (%)	30.66
6	C:N ratio	15:1

Ligninolytic enzymes activity of SMCE

UV-VIS (Perkin-Elmer-Lambda-25) at different wavelengths was used to monitor ligninolytic enzymes activities of concentrated extract with/without additions of hydrogen peroxide (H_2O_2) periodically (*Table 3*). Activities of manganese peroxidase (MnP) were determined by O-dianisidine ($C_{14}H_{16}N_2O_2$) oxidation at 460 nm (molar extinction coefficient = 29,400 M^{-1} cm^{-1}) (Paszczyński et al., 1988). Similarly, Laccase activities were monitored using 2, 2’-azino-di-[3-ethyl-benzo-thiazolin-sulphonate (ABTS) as a substrate while activities of Lignin Peroxidase (LiP) were measured by

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oxidation of Veratryl Alcohol ($C_9H_{12}O_3$) to veratraldehyde ($C_9H_{10}O_3$) at 310 nm (Tien and Kirk, 1988).

Table 3. *Ligninolytic enzyme activities of various microcosms during four-week incubation studies (n = 3)*

Days	Microcosms containing Endosulfan					
	M_1** (SMCE*+H_2O_2)		M_2**(SMCE only)		Control 1 and 2	
	Laccase	MnP	Lac	MnP	Lac	MnP
0	32±2.3	18±1.4	22±1.4	8±1.1	ND	ND
7	25±1.3	12±3.5	17±2.2	5±3.1	ND	ND
14	16±2.4	7±2.4	13±3.1	2±1.5	ND	ND
21	9±2.0	4±1.3	7±2.2	0±0	ND	ND
28	3±1.5	1±2.9	1±1.7	0±0	ND	ND

*SMCE: spent mushroom compost extract, M_1 and M_2 mean microcosm 1 and 2

Experimental set up for biodegradation of OCPs

Using concentrated aqueous SMC extract (SMCE)

Concentrated SPME was used as bio remedial to treat Endosulfan. Erlenmeyer flasks (250 ml) were autoclaved twice and were spiked with Endosulfan at the final concentration of 25 mgL^{-1}. Microcosms were treated in the following manner

a) Microcosm 1(M_1): 10 mL of the collected concentrated SMCE
b) Microcosm 2(M_2): 10 mL of the SMCE and 800 µl of H_2O_2 (0.4 mM)
c) Control 1(C_1): 10 mL of boiled SMCE (to denature all the enzymes present in it). This control was used as associated control of M_1
d) Control 2(C_2): 10 mL of boiled SMCE and 800 µL of H_2O_2 (0.4 mM). This control was used as associated control of M_2.

Application of concentrated SMCE in soil

Biometric flasks (250 mL) (Bellco, Glass Inc., Vineland, NJ) were used for preparation of soil microcosms while SMCE extract was used as bio remedial for Endosulfan decontamination. The main body of biometric flasks consisted of soil (25 g) spiked Endosulfan at final concentration of 25 $mgkg^{-1}$. During the experiment, side arm of flasks was filled with 50 ml solution of 0.4N NaOH [CO_2-free Double distilled water (DDW)] and was used as trap to monitor CO_2. Following five treatments were applied in main body of flasks already contaminated with mixture of Endosulfan isomers to access role of SMCE, derived from *P. ostreatus* in the process of bioremediation:

1. Microcosm 3(M_3): Fresh soil (20 g) was amended with 10 mL of freshly prepared SMCE.
2. Microcosm 4(M_4): 20 g fresh soil + 10 mL SMCE + 800 µL H_2O_2 (0.4 mM).
3. Control 3(C_3): Associated control of M_3: 20 g soil+ denatured SMCE (10 ml).
4. Control 4(C_4): Associated control of M_4: 20 g soil + denatured SMCE +H_2O_2(800µL H_2O_2 (0.4 mM) to monitor role of H_2O_2 activated sterilized SMCE in contaminated soil.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32513267

5. Microcosm 5(M_5) (abiotic control): 20 g double autoclaved soil + denatured SMCE to find role of other soil inhabitant microbes in biodegradation.

During both the experiment flasks were incubated at 22 °C for 28 days. During the experiment, moisture level (60%) was maintained by taping DDW. The flasks were weighed and corrected for evaporative water loss by addition of sterile water prior to sampling at each time series. At the end of experiment, the samples were immediately put in boiling water and subsequently mixing with 10 mL acetonitrile to stop enzymes activity. All the experiment was conducted with three replicate flaks to improve the confidence of results. Required number of microcosms were taken off (sampled as whole) after each seven days till the end of experiment.

Measurement of carbon dioxide (CO_2)

Sodium hydroxide (NaOH) present in trap of biometric flasks was used to monitor CO_2 production using traditional acid titration method (Paul et al., 1999). Acid/base titration was done (at the same time intervals) for measurement of total carbon dioxide evolved. Samples were taken at day 0, 7, 14, 21 and 28 and production of CO_2 was monitored. The total amount of CO_2 evolved from the treatment bio meters was used as measure of rate of mineralization.

Extraction of OCPs from microcosms and analysis

Extraction from SPME microcosm

SMCE microcosms contents were transferred to centrifuge tubes and thoroughly mixed for 1 min using vortex mixer (Velp scientific centre, Bohemia, NY). After addition of 10 mL of acetone: water (1:3, v/v), the content was transferred to separating funnel (Pyrex® Squibb, Corning, New York), acetonitrile (2 mL) added and shaken vigorously for 5 min manually followed by addition of petroleum ether (2.0 mL). Aqueous portion was discarded and organic layer was dried with 1 g Sodium sulfate ($NaSO_4$) (anhydrous) and gauged at 2 mL using rotary evaporator (Buchi-Rotavapour.R-210) (Hernandez-Rodriguez et al., 2006)

Extraction from soil microcosm

Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction method (with cleanup) (Boes et al., 2015) was used for extraction of selected OCP from soil with slight modifications. After hydration of samples (10 g) for 30 min using water (10 mL) and acidified acetonitrile (10 mL) (99:1 v/v) in 50 mL tube, the contents were mixed for 5 min using vortex mixer (Velp scientific centre, Bohemia, NY) and dried and buffered by adding 4.0 g magnesium sulfate (anhydrous)($MgSO_4$), 1.0 g sodium chloride (NaCl), 1.0 g trisodium citrate dehydrate ($C_6H_5Na_3O_7.2H_2O$) and 0.5 g disodium hydrogen citrate sesquihydrate ($C_6H_8Na_2O_8$). Centrifugation was done at 5000 × g for 5 min. After separation of acetonitrile layer, it was concentrated to 5 ml and was later mixed with water (2 mL) and n–hexane (10 mL) and swirled for 1 min. After 2 min, an aliquot of 9 ml of the upper n–hexane layer was collected and reduced again to 1.4 mL in an amber glass vials and stored at -20 °C. Analysis of Endosulfan in extracted samples was done using Gas Chromatography (7890B Agilent) equipped with micro-electron capture detector (G3440B). HP-5(30 m × 0.25 µm I.D × 0.320 mm) capillary column was used.

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Gas chromatography (GC- 7890B Agilent) mass spectrometry (MSD-5977A Agilent) with column DB-5 Ultra inert (30 m × 0.25 mm i.d × 0.25 lm). Summary of conditions is shown in *Table 4*. Matching library used was Retention time lock Pesticides and endocrine disruptor MS Library (RTLPEST3.L). Recovery of Endosulfan (*Table 5*) was in accordance with acceptability criteria set in SANCO's (2017).

Table 4. *Conditions for gas chromatography analysis using electron capture detector and mass spectrometer detector*

Conditions		
	GC-MSD	**GC-µECD**
Column	DB-5 Ultra inert (-60 °C to 325 °C (350 °C) (30 m × 0.25 mm i.d × 0.25 µm)	HP-5 (30 m × 0.25 µm i.d × 0.320 mm)
Injector temperature	250 °C	225 °C
Carrier gas/purity/flow rate	Helium gas/99.999%/2 ml min^{-1}	Nitrogen/99.999%/2 ml min^{-1}
Detector temperature	280 °C	280 °C
Oven temperature ramping	Initial oven temperature 60 °C (0.5 min) was increased at 20 °C min^{-1} to 170 °C (3 min hold). Later on, it was increased at rate of 5 °C min^{-1} to 295 °C (44 min) to allow all the metabolites to elute from column	Initial oven temperature 80 °C (0.5 min) increased at 10 °C min^{-1} to 180 °C (held for 10), then increased at 15 °C min^{-1} to 250 °C and finally held for 10 min

Table 5. *Recovery of Endosulfan isomers by selected extraction method (n = 5)*

Compound	**SMCE**		**Soil spiked with SMCE**	
	Recovery (%)	**RSD (%)**	**Recovery (%)**	**RSD (%)**
α-Endosulfan	91.00	2.19	88.59	3.18
B-Endosulfan	99.22	0.59	96.73	0.78

Kinetic studies and statistical analysis

Simple first-order kinetics (SFO) was used found the most fitted kinetic equation for the set of experiments**.** The reduction in extractable amount of Endosulfan from the artificially spiked soil was calculated using *Equation 1* (Yang et al., 2014)

$$lnC = a + K_1 t \quad \text{(Eq.1)}$$

where *lnC* is natural logarithm of concentration, *t* is time given to compound for degradation and K_1 is first order rate constant.

Half-life (DT_{50}) was calculated by *Equation 2:*

$$DT_{50} = t_{1/2} = \frac{0.693}{k1} \quad \text{(Eq.2)}$$

For DT_{50} and DT_{90} calculations, a computer program R (version 3.0.3) with the kinfit software package was used. The best fitted SFO (simple first-order kinetics) was used. The data was statistically analyzed (analysis of variance) using complete randomized

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design with two factors (treatments and times) at $\alpha = 0.05$ by Statistix 8.1 (Analytical Software, USA) computer program.

Results and discussion

This study was designed to find the potential of ligninolytic enzymes extract obtained from SMC to treat this pesticide in soil. In order to make the enzymatic effect clear, concentration of the enzyme and treatment of the microcosms with or without H_2O_2 was done. SMC has many immobilized enzymes that could serve as bio remedial agent (Valentin et al., 2010). Therefore, some researchers applied SMC directly in soil to treat contaminants (Marín-Benito et al., 2016). Direct application is beneficial because it acts as the substrate to change the bioavailability and immobilization of pesticides in the soil. However, while studying the role of ligninolytic enzymes in bioremediation, direct use of SMC is also a big problem because of adsorption of pesticides onto fungal biomass and compost which hinders true revelations. Therefore, extraction of enzymes from SMC and their application in soil is another option.

Bioremediation of Endosulfan using only SMCE

After application of Endosulfan in flask, microcosms were treated with SMCE and bioremediation of both the isomers of this pesticide was studied. In M_1 where H_2O_2 was added in microcosm to stimulate the effect of enzyme in SMCE, 24.99% of applied α-Endosulfan was reduced at the end of first week of incubation. At the end of 4^{th} week, 58% of applied extractable α-Endosulfan was removed. However, 56.85% of removal was observed in M_2, where no H_2O_2 was added. It showed addition of H_2O_2 did not play any substantial role in reduction of applied α-Endosulfan as there was no significant difference ($\alpha = 0.05$, $p < 0.01$) in both above mentioned microcosms, M_1 and M_2 (*Table 6*).

Table 6. *Bioremediation of Endosulfan under different microcosms to access the role of extracellular enzyme extract, where M_1 and M_2 are Microcosm 1 and 2, while C1 and C2 are their associated controls ($\alpha = 0.05$; $p < 0.01$)*

Days	Relative reduction (%) of applied concentration									
	α-Endosulfan					β-Endosulfan				
	M_1	M_2	C_1	C_2	Means	M_1	M_2	C_1	C_2	Means
0	0.00	0.00	0.00	0.00	0^e	0	0.00	0.00	0.00	0^e
7	24.99	17.73	6.68	3.20	13.15^d	7.64	6.85	2.85	1.25	4.65^d
14	40.22	43.37	8.35	6.61	24.64^c	14.65	10.57	3.64	2.99	7.96^c
21	48.25	50.05	10.93	9.01	29.56^b	18.42	16.76	4.96	3.46	10.9^b
28	58.00	56.85	12.36	10.25	34.37^a	20.87	21.49	5.49	4.59	13.11^a
Means	34.29^a	33.6^a	7.66^b	5.184^b	20.34	12.32^a	11.13^a	3.39^b	2.46^b	7.324

α-Endosulfan: LSD for Days 2.69; LSD for treatments 3.21
β-Endosulfan: LSD for Days 1.22; LSD for treatments 1.46

The calculated rate of dissipation using SFO kinetics was not substantially different in both the microcosms which resulted in DT_{50} equal to 22 days and 21 days for M_1 and M_2 respectively (*Table 7*). C_1 and C_2 which were the associated control of M_1 and M_2,

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showed the reduction of only 12.36% and 10.25% of applied α-Endosulfan, respectively. Ultimate DT_{50} calculated for C_1 was 154 days ($K_1 = 0.0045$) while under C_2 it was 173 days ($K_1 = 0.004$). However, there were a significant difference between each microcosm and their associated controls (*Table 6*). In the past, it was reported that presence of H_2O_2 in ligninolytic system is responsible for faster bioremediation or decolonization (Vyas and Molitoris, 1995; Eichlerová et al., 2006). However, in this case, addition of H_2O_2 did not pose any effect on reduction of M_2 than M_1 where no H_2O_2 was added.

Table 7. *Kinetic of dissipation of Endosulfan in spent mushroom compost extract using simple first order kinetic model*

Experimental microcosm	α-Endosulfan				
	Kinetic equation	**K_1**	**DT_{50}**	**DT_{90}**	**R^2**
M_1	LnC=4.56-0.0318t	0.0318	21.80	72.40	0.99
M_2	LnC=4.58-0.033t	0.033	21.00	69.80	0.97
C_1	LnC=4.59-0.0045t	0.0045	154.03	511.69	0.91
C_2	LnC=4.60-0.004t	0.004	173.29	575.65	0.98
Experimental microcosm	**β-Endosulfan**				
	Kinetic equation	**K_1**	**DT_{50}**	**DT_{90}**	**R^2**
M_1	LnC=4.59-0.0087t	0.0087	79.67209	264.665	0.97
M_2	LnC=4.60-0.0085t	0.0085	81.54673	270.8924	0.99
C_1	LnC=4.60-0.00194t	0.00194	357.2924	1186.9	0.91
C_2	LnC=4.60-0.00167t	0.00167	415.0582	1378.793	0.97

The other isomer of Endosulfan, β-Endosulfan showed the similar reduction pattern (*Table 6*) but the rate of this reduction was about ⅓ of that observed in case of α-Endosulfan. Total removal of this isomer, during incubation period, in M_1 and M_2 was 20.87% and 21.49% respectively showing there was no significant difference ($\alpha = 0.05$, $p < 0.01$) in bioremediation efficacy of SMCE with or without H_2O_2 (*Table 6*). It shows that stimulation of enzymes by adding H_2O_2 as were mentioned in another study (Chirnside et al., 2011) had no role in reduction of β-Endosulfan. DT_{50}, calculated for M_1 and M_2 were 80 days and 82 days respectively. It also showed autoclaving to denature the SMCE was responsible for the slow rate of removal (*Table 7*) because it eliminated biotic factor including enzymes and microbes in associated controls.

SMCE of *P. ostreatus* was monitored for two main ligninolytic enzymes MnP and Laccase enzyme activities in all the microcosms (*Table 3*). Immediately after addition of H_2O_2 on day zero, the activities of both the enzymes, Laccase and MnP, were higher showing stimulatory effect of H_2O_2 on M_1 (*Table 3*). All the activities had downward trend at each sampling time which shows that with the passage of incubation time, the enzymes activities were declined. The activities of MnP and Laccase in M_2 where no H_2O_2 was added were lower than M_1 at each time interval (*Table 3*). M_1 showed significantly more activities of Laccase and MnP than M_2 but there was no significant difference in reduction of both the isomers of Endosulfan. For example, a study conducted by Ulčnik et al. (2013) showed that purified laccase obtained from this fungus and other related ligninolytic enzymes were found to play no role in the process of bioremediation. Another study conducted by Purnomo et al. (2010) also showed that

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ligninolytic enzyme are not involved in the bioremediation of OCPs. Same is proved in our case.

During the course of study, oxidation occurred and only one dechlorination metabolite, Endosulfan sulfate was formed in flasks from day 7 and no other metabolite was detected on GC-MSD.

Bioremediation of Endosulfan using SMCE in Soil

For preparation of soil microcosms, biometric flasks containing soils treated with SMCE were incubated for 28 days at 22 °C. NaOH solution was added in the side arm of biometric flask and served as trap of CO_2. Application of SMCE in fresh soil (M_3) resulted in total reduction of 41.62% of applied concentration of α-Endosulfan at the rate 0.013 days^{-1}. Resultantly, calculated DT_{50} was 51 days (r^2 = 0.98). Addition of H_2O_2 along with SMCE in soil (M_4) resulted in total reduction up to 47.16% while under M_5 (abiotic microcosm), the reduction of 7.42% at the rate K_1 = 0.013 days^{-1} was observed which is less than both M_3 and M_4. Denaturation of all the biotic factors present in soil and SMCE (M_5) resulted in least reduction of applied α-Endosulfan (*Fig. 1*). There was a significant difference in reduction between M_3 and M_4 (α = 0.05, $P < 0.01$). Since there are various other constituents in the soil that could be stimulated by addition of H_2O_2 (not necessarily enzymes from added extract) which resulted in significant difference in amount of reduction of α-Endosulfan in M_4 than M_3. The role of addition of SMCE in M_3 and M_4 in bioremediation was assessed by running associated controls (C_3 and C_4). The reduction of α-Endosulfan under M_3 was significantly different from its associated control (C_3). Same was true for M_4 and associated control.

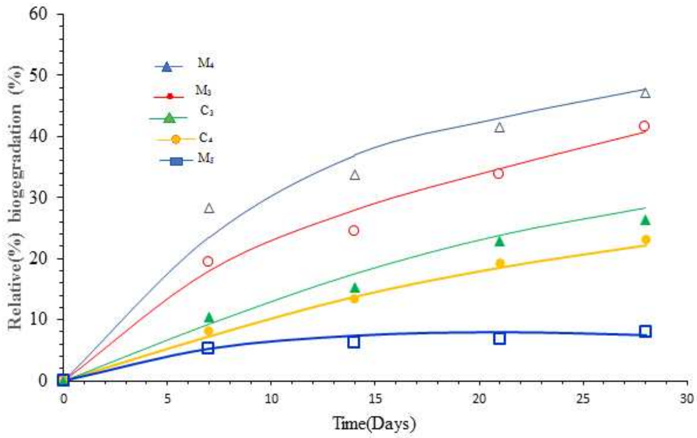

Figure 1. *Relative reduction of α-Endosulfan in soil using spent mushroom compost extract in different microcosms*

Similarly, recorded reduction for β-Endosulfan under M_3 was 32.40% at the rate of 0.016 day^{-1} while its associated control C_3 exhibited reduction equals to 20.05% at the rate of 0.0078 day^{-1} (*Fig. 2; Table 8*). Similar pattern was observed in M_4 and its associated control C_4. Removal of Endosulfan isomers in C_3 and C_4 revealed the role of denatured SMCE as carbon source (Kodjo-Wayo, 2006) (*Fig. 2*). In case of abiotic control (M_5), total of 2.84% of applied β-Endosulfan was reduced. Detailed DT_{50} calculated based on equation 2 and rates of all the treatments are given in *Table 8*.

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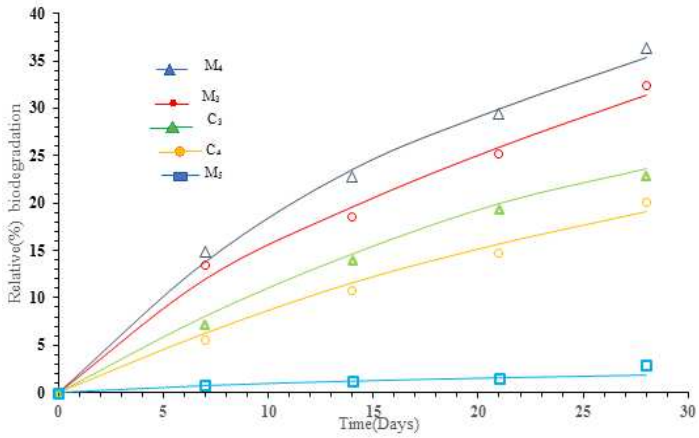

Figure 2. *Relative reduction of β-Endosulfan in soil using spent mushroom compost extract*

Table 8. *Dissipation kinetic of Endosulfan in soil treated with SMCE using SFO model*

Kinetic studies of Endosulfan in soil using SMCE						
Isomers	**Treatments**	**Kinetic equation**	**K_1 (day^{-1})**	**DT_{50}**	**DT_{90}**	**r^2**
α-Endosulfan	M_3	4.58-0.0133t	0.013	51.41	170.77	0.98
	M_4	4.52-0.0211t	0.023	29.65	98.48	0.91
	C_3	4.60-0.009t	0.009	73.38	243.75	0.99
	C_4	4.59-0.011t	0.011	62.45	207.461	0.98
	M_5	4.59-0.002t	0.002	259.71	862.74	0.86
β-Endosulfan	M_3	4.58-0.02t	0.016	43.2	143.49	0.98
	M_4	4.57-0.0186t	0.0186	37.08	123.17	0.97
	C_3	4.60-0.007846t	0.0078	88.33	293.46	0.97
	C_4	4.58-0.0095t	0.0096	72.48	240.79	0.99
	M_5	4.61-0.00094t	0.0009	737.75	2450.76	0.95

Addition of SMCE initiates oxidative pathways and produces free radicals in soil which are removed by soil constituents and cause less reduction of toxic compounds in soil bio meters (Chirnside et al., 2011) than flasks which contained only SMCE (M_1 and M_2). This was evident in this finding where SCME alone reduced more Endosulfan than after addition in soil. The overall reduction of both the isomers was higher in SMCE microcosms without soil (M_1 and M_2) than the microcosms where SMCE was added in soil (M_3 and M_4). Presence of total organic carbon (TOC) in denatured SMCE resulted in decontamination of Endosulfan in soil because of its role in increasing the indigenous population of soil microbes (Guerin, 1999; Özyer et al., 2016). In our case, SMCE was rich in organic carbon (*Table 2*), which further helped to enhance the population of indigenous microbes (Zhang et al., 2013) in the soil. This increase in Endosulfan removal in H_2O_2 amended microcosm of soil M_4 indicated that addition of H_2O_2 in soil amended with SMCE stimulated certain soil enzymes and microbes which were not present in sole SMCE. Addition of SMCE within the soil involves couple of reactions whose nature is very difficult to explore. The less degradation of β-Endosulfan by application of SMCE in soil than α-Endosulfan could be due to inter-conversion of the

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α-isomer to β-Endosulfan (Rice et al., 1997; Schmidt et al., 2001) and also microbial species prefer α-Endosulfan for degradation over β-Endosulfan (Siddique et al., 2003).
The bioremediation pathways for Endosulfan after application of SMCE were built based on the time of appearance of peaks, their position and centre of mass of concentration profiles of the metabolites detected by GC-MS. In all associated controls and treatments of M_1 and M_2, only Endosulfan sulfate was formed (*Fig. 3*). Endosulfan lactone was only formed in M_3 and M_4 soil microcosms. Under M_5, there was no appearance of any metabolites. This is possibly because of absence of process of oxidation because of un availability of microbes or enzymes. Formation of Endosulfan lactone is also in line with the findings of (Kataoka and Takagi, 2013). Other expected bioremediation products could be Endosulfan diol, Endosulfan ether, Endosulfan hydroxyether and Endosulfan monoaldehyde (Kullman and Matsumura, 1996) which were not detected during the course of this study. However, formation of Endosulfan lactone is evident for the formation of other above mentioned intermediate products and the fact that hydrolysis also occurred in the soil treated with SMCE.

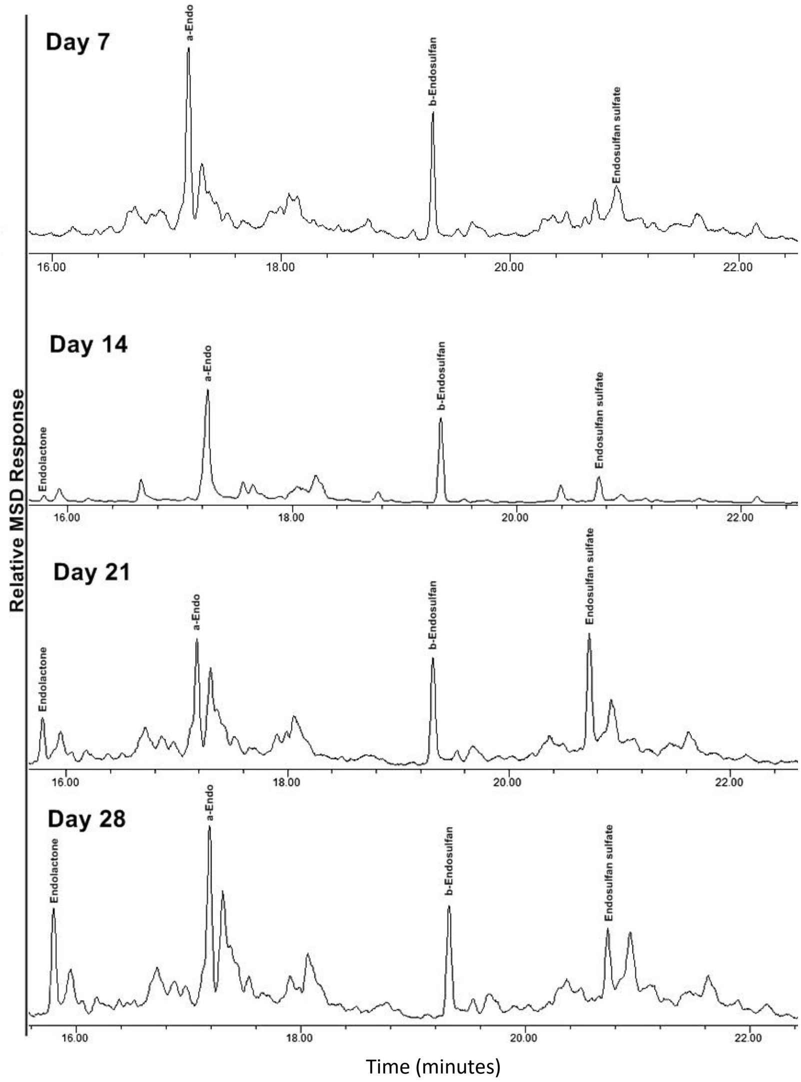

Figure 3. *The metabolic products of Endosulfan detected at GC-MS after incubation of soil with spent mushroom compost extract. Formation of Endosulfan sulfate indicate oxidation while generation of Endosulfan lactone is the result of hydrolytic pathways*

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Emission of carbon dioxide is a bioremediation indicator and contaminant depletion can be estimated from the amount of carbon dioxide emitted from the system (Schoefs et al., 2004). Amount of CO_2 produced in spiked bio-meter flask with M_3 and M_4 was much more than associated controls of containing denatured SMCE (*Fig. 4*). Evolution of CO_2 was increased exponentially as experiment progressed. The evolution of CO_2 from M_5 flask was the slowest of all the microcosms. The highest amount of CO_2 generated was in M_4. It is observed that the emission and measurement of CO_2 was good qualitative indicator of bio mineralization. This similarity also existed for abiotic controls where the CO_2 production stayed very low. It can be assumed that since OCPs are very persistent and complex compounds therefore free radicals did not play any role in their oxidation. Nevertheless, the inactivated extract, used as control, showed significantly less Endosulfan decrease than the activated SCME extract. This suggested that that both wash-off and volatilization had no impact on OCPs loss. This is in accordance with the studies conducted by Juárez et al. (2011). Since it is proved that Laccase and MnP have no role in bioremediation here, there could be another enzyme or enzymatic complex which is responsible for the degradation of Endosulfan. Although role of laccase in biodegradation of many pollutants has been widely studies (Karigar and Rao, 2011) but there is no direct evidence which connect the effect of presence or quantity of this enzyme in biodegradation of Endosulfan.

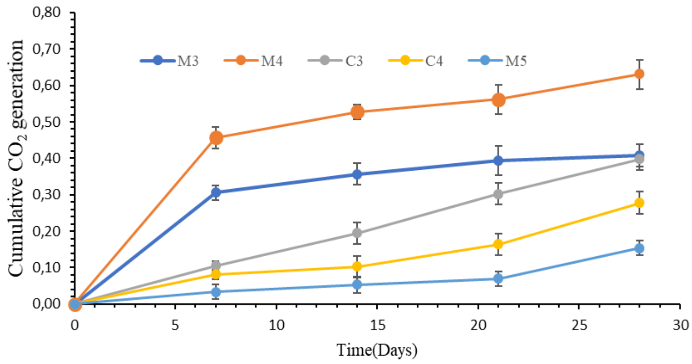

***Figure 4.** Evolution of CO_2 during the incubation studies of the Endosulfan bioremediation by application SMCE treatments in soil, where M_1 is microcosm having fresh soil and SMCE and M_2 is microcosm that contain H_2O_2 along with while C_1 and C_2 are the associated controls of microcosms 1 and 2. M_5 is the abiotic control microcosm*

Conclusion and recommendation

In order to avoid the problem of adsorption, instead of its direct application spent mushroom compost extract was used to evaluate the role of enzyme complex in bioremediation of Endosulfan. Five different microcosms along with associated controls were used to demonstrate the role of SMCE. This study revealed that ligninolytic enzyme stimulation after addition of H_2O_2 does not affect the bioremediation efficacy of SMCE, rather it has certain other extracellular enzymes that could possibly be involved in process of decontamination. This was evident from almost equal reduction of this pesticide in the presence and absence of H_2O_2. In soil, this extract showed some effect of addition of H_2O_2. There was significantly more reduction of both isomers in the soil microcosm containing SMCE spiked with H_2O_2 than the other where H_2O_2 was not spiked. Associated controls showed if SCME was denatured after sterilization, there

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was less reduction in both isomers of Endosulfan. It also indicated the role of stimulation of indigenous soil microbes/enzymes in the presence of H_2O_2 and the role of SMCE as carbon source to enhance the microbial population of soil. All these results provide a basis for the development of new bioremediation strategy for decontamination of Endosulfan in soil. However, there is a strong need of understanding the complex nature of reactions involved in symbiotic interaction between SMCE and soil constituents which were responsible for more reduction of Endosulfan in soil.

Acknowledgements. We are thankful to Staff of Mushroom Research Laboratory, National Agriculture Research Centre, Islamabad, Pakistan for cooperation in mushroom culture, spawn and compost preparation. We are also very thankful to Soil Environment Lab for facilities of SMC and soil physicochemical properties. Many thanks to laboratory staff of Ecotoxicology Research Program especially, National Agriculture Research Centre, Islamabad, Pakistan.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32513267

COMPARATIVE STUDY ON THE EFFECT OF NITRIC OXIDE, CHROMIUM AND EDTA ON OXIDATIVE STRESS RESPONSES IN AGATHI (*SESBANIA GRANDIFLORA* L. PERS)

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(Received 23rd Nov 2018; accepted 28th Jan 2019)

Abstract. Heavy metal contaminations are a major cause of global environmental pollutions. The release of heavy metals into the environment has increased the threat of serious health issues for living organism. Among the known heavy metals, chromium is released into the environment by various anthropogenic activities causing various ill effects on the life forms. Hence in the present study which was carried out in Vellore Institute of Technology, Tamil Nadu, India and the phytoextraction of chromium enhanced with metal chelator ethylenediamine tetraacetic acid was studied in *Sesbania grandiflora* in VIT, India. To asses this, various treatments were prepared with combinations of chromium and chelator. The antioxidant enzyme activities such as Superoxide dismutase, Catalase, Ascorbate peroxidase and Glutathione reductase activity were determined in the presence of metal and chelator. The results showed an increase in all the antioxidant enzymes in the presence of metal and chelator due to the increased metal uptake. To reduce the stress levels created by the metal and chelator, sodium nitroprusside a nitric oxide donor was exogenously sprayed on to the plants and a comparative study was made in the presence and absence of sodium nitroprusside. The results indicated that the presence of sodium nitroprusside had a positive role in combating the stress created by the metal and chelator which was evident by the reduced antioxidant enzyme levels in spite of metal uptake. Hence sodium nitroprusside could be considered for its wider application against metal stress conditions to combat oxidative stress. The plant *Sesbania grandiflora* showed higher withstanding capacity to the heavy metal stress and was effective in extracting the metal out of the contaminated soil.
Keywords: *heavy metal, Sesbania grandiflora, superoxide dismutase, catalase, sodium nitroprusside, chelator, oxidative stress*

Introduction

Heavy metals pose a critical concern to human health due to their increasing usage as industrial inputs (Alloway, 1995; Diels et al., 2002). Chromium (Cr) is an environmental pollutant that ranks seventh in abundance in the earth crust. The major contributors of Cr contamination are the leather tanning, electroplating, and stainless steel industries. India is one of the largest producers of leather, and nearly 80% of the tanneries are engaged in the chrome-tanning process. Chromium being toxic interferes with metabolic processes in plants, which causes several morphological and physiological changes causing reduced growth, impaired photosynthesis and can lead to death of the plant. Chromium exists in two forms Cr (III) and Cr (VI). Among them Cr (VI) exists in the forms of chromate anions (CrO_4^{-2}), dichromate ($Cr_2O_7^{2-}$) and chromium trioxide (CrO_3) which are considered the most toxic forms of chromium, since they possess high oxidizing potential, high solubility, and mobility across the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

membranes in living organisms and in the environment (Azmat and Khanum, 2005). Clean up of contaminated soils needs special attention because these soils support agricultural crops and so the consumption of crop plants grown on these soils may create serious health hazards.

Plants sequester heavy metals in roots and/or shoots and, therefore, significantly contribute to metal removal from the environment through the mechanism of phytoremediation (Jadia and Fulekar, 2009). The uptake and translocation of such heavy metal by plants can be made bio available by the addition of natural or synthetic chelators (Schmidt, 2003; Quartacci et al., 2006) like, ethylenediamine tetraacetic acid (EDTA), ethylenediamine di (o-hyroxyphenylacetic acid) (EDDHA) and hydroxyethylethylene-diaminetriacetic acid (HEDTA). Among them, EDTA is most effective chelating agent which enhances the accumulation of metals, by mobilizing metal cations, water soluble complexes (Lestan et al., 2008) there by modifying the availability of the metals in soils to the above ground parts of plants (Garba et al., 2012b), and increasing the uptake of heavy metals.

One of the mechanisms that make a plant tolerant to heavy metal stress is the presence of strong antioxidant defense system (Metwally et al., 2005; Qureshi et al., 2005; Israr et al., 2006 and Diwan et al., 2008). Heavy metals stress causes molecular damage to plants, either directly or indirectly through reactive oxygen species (ROS) formation. Further, plants growing in chromium-stressed environment suffer due to potential risk from reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2). However, plants possess their own defense system to combat these toxicity problems like cellular antioxidants and antioxidant enzymes that play a vital role in protecting the plant from various physiological damage caused by ROS (Mittler, 2002., Baek and Skinner, 2003) that make a plant species tolerant to heavy metal stress (Metwally et al., 2005; Qureshi et al., 2007; Israr et al., 2006; Diwan et al., 2008). Antioxidant system consists of enzymatic and non-enzymatic compounds. Enzymatic antioxidants include superoxide dismutase, catalase, peroxidases, glutathione reductase and ascorbate reductase. Non-enzymatic antioxidants are ascorbate, glutathione, tocopherol and carotenoids (Mittler, 2002; Candan and Tarhan, 2003).

Nitrite oxide (NO) is a lipophilic molecule that diffuses through membranes. Although first described as a signal molecule in animals, accumulating evidence shows that NO is an important signal molecule involved in plant response to biotic and abiotic stresses (Delledonne et al., 1998; Carlos and Lorenzo, 2001; Uchida et al., 2002).

Based on the above observation, the objective of the present study is to investigate whether exogenous sodium nitroprusside (SNP) plays a positive role in protecting *Sesbania grandiflora* against antioxidative stress created by chromium in the presence of EDTA.

Materials and methods

Soil spiking and experimental setup

The study was done in Vellore Institute of Technology (VIT), Tamil Nadu, India. The experiments were conducted during the months of June-August. Pot experiments were conducted using the soil samples taken from VIT garden. The soil was air-dried and sieved. The study was a comparison made by conducting two experiments (Exp), namely Exp 1 and Exp 2. Five pots each containing 2.7 kg of soil + 0.3 kg of manure

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

was mixed along with five different concentrations of chromium and ethylenediamine tetraacetic acid (EDTA) in case of Exp 1. The study was done to assess the efficiency of chromium accumulation by *Sesbania grandiflora*. This was studied in comparison with Exp 2 containing in addition 10 ml of 250 µM of SNP as foliar spray along with chromium and EDTA. The various treatments that were considered in Exp 1 and Exp 2 are given in the following treatment tables:

Treatment name (Exp 1)	**Concentrations**
C1	20 mg Cr Kg^{-1}
C2	40 mg Cr Kg^{-1}
C3	60 mg Cr Kg^{-1}
C4	80 mg Cr Kg^{-1}
C5	100 mg Cr Kg^{-1}
C1+EDTA	20 mg Cr Kg^{-1} + 0.35 mM EDTA
C2+EDTA	40 mg Cr Kg^{-1} + 0.35 mM EDTA
C3+EDTA	60 mg Cr Kg^{-1} + 0.35 mM EDTA
C4+EDTA	80 mg Cr Kg^{-1} + 0.35 mM EDTA
C5+EDTA	100 mg Cr Kg^{-1} + 0.35 mM EDTA

Treatment Name (Exp2)	**Concentrations**
C1	60 mg Cr Kg^{-1}
C2	100 mg Cr Kg^{-1}
C3	60 mg Cr Kg^{-1} + 0.35 mM EDTA
C4	100 mg Cr Kg^{-1} + 0.35 mM EDTA
C5	60 mg Cr Kg^{-1} + 250 µM SNP
C6	100 mg Cr Kg^{-1} + 250 µM SNP
C7	60 mg Cr Kg^{-1} + 0.35 mM EDTA + 250 µM SNP
C8	100 mg Cr Kg^{-1} + 0.35 mM EDTA + 250 µM SNP
C9	Control + 250 µM SNP
C10	Control

Control plants were free of chromium and EDTA (Exp 1) and contained SNP in case of Exp 2. All the treatments were done in triplicates. The control and the treated soil were left to equilibrate for 3 weeks during which it was occasionally turned and mixed to ensure thorough mixing. The seeds of *Sesbania grandiflora* were sterilized in 3% formaldehyde and sown in plastic trays for one week to attain the seedling stage (*Fig. 1a*). About 6 seedlings were transferred to the spiked soil pots after three weeks of incubation. Totally 33 pots from Exp 1 and Exp 2 were maintained in a randomized block design (*Fig. 1b*). Both the experiments were performed at the same time period. The harvested plants were collected during every morning at the end of 30, 60 and 90 days and were stored at -20 °C for a short duration and all the enzyme assays were performed within a week's time from the harvest.

Enzyme extraction and activity of antioxidant enzymes

500 mg of fresh leaves was weighed and homogenized in 3 ml of extraction buffer containing 0.5 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.5 ml of Triton X-100, 1 g of polyvinyl pyruvate (PVP) and 3.72 mg of EDTA. The homogenate were

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

filtered and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was used for assaying superoxide dismutase (SOD), ascorbate peroxidise (APX) and glutathione reductase (GR) activities.

a **b**

***Figure 1. a** One week old seedling of Sesbania grandiflora; **b** randomized block design of treatment pots*

Superoxide dismutase (SOD) (EC1.15.1.1)

SOD activity was assayed using a modified NBT method according to Beyer and Fridovich (1987). The 2 ml assay reaction mixture contained 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 µM nitro blue tetrazolium (NBT), and 0.025% Triton-X100. 40 µl of diluted (2×) sample and 20 µl of 1 mM riboflavin were added and the reaction was initiated by illuminating the samples under a 15 W fluorescent tube. Duplicate tubes with the same reaction mixture were kept in the dark and used as blank. Absorbance of the samples was measured immediately after the reaction was stopped at 560 nm against blank. The enzyme activity grams^{-1} f.w of a sample was determined from a standard curve obtained by using pure SOD.

Native polyacrylamide gel electrophoresis (PAGE) of crude extract (25 µg) combined with loading buffer (containing neither SDS nor P-mercaptoethanol) was performed in a mini-gel system (Medox) at 150-200 V. SOD activity in native polyacrylamide gels was determined by the nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). SOD activity was visible after several minutes as an achromatic band against a blue background.

Catalase (CAT) (EC 1.11.1.6)

The Catalase assay was performed by the method of (Luck, 1974). The enzyme extract (0.02 ml) was added to the reaction mixture containing 3 ml of hydrogen peroxide (H_2O_2) and 0.067 M phosphate buffer (pH 7.0) and the optical density change was measured at 240 nm, the time taken for decrease in the absorbance from 0.45 to 0.4 is noted as ΔT. The activity of the enzyme is expressed in the terms of µmole of H_2O_2 consumed/min/ mg protein. The activity was determined by calculating the concentration of H_2O_2 using the extinction coefficient 0.036 per µmole per ml.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

Ascorbate peroxidase (APX) (EC1.11.1.11)

The APX activity was determined by the method followed by Nakano and Asada (1987). The 1 ml reaction mixture contained 0.1 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.3 mM H_2O_2 and 50 µl of enzyme extract. The oxidation of ascorbic acid was measured by the decrease in absorbance at 290 nm for 3 min using a spectrophotometer. The enzyme activity was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein)$^{-1}$. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate per min at 25 °C.

Glutathione reductase (GR) (EC 1.6.4.2)

The activity of GR was measured by monitoring the glutathione dependent oxidation of (Nicotinamide adenine dinucleotide phosphate) NADPH described by Rao et al. (1996). 1 ml reaction mixture contained 0.1 ml of 100 mM potassium phosphate buffer (pH 7.5), 0.1 ml of 1 mM EDTA, 0.02 ml of 0.2 mM NADPH, 0.05 ml of 0.5 mM oxidized glutathione and 50 µL of enzyme extract. The reaction was allowed to run for 3 min and the absorbance was measured at 340 nm using a spectrophotometer. The enzyme activity was calculated using extinction coefficient 6.2 mM^{-1} cm^{-1} and expressed in enzyme units (mg protein^{-1}). One unit of enzyme is the amount necessary to decompose 1 µmol of NADPH per min at 25 °C.

Determination of ascorbate oxidase activity (AAO) (EC 1.10.3.3)

Assay of ascorbic acid oxidase activity was carried out according to Oberbacher and Vines (1963). 0.1 ml of the enzyme extract was added to 3 ml of the substrate solution (8.8 mg of ascorbic acid in 300 ml phosphate buffer, pH 5.6). The absorbance change was measured at 265 nm for every 30 s for a period of 5 min. One enzyme unit is equivalent to an absorbance change of 3.58 per minutes.

Non-enzymatic antioxidants glutathione estimation

The level of glutathione in the treatments with and without EDTA containing chromium was determined according to Tukendorf and Rauser (1990), using Ellman's reagent. About 500 mg of the plant material was homogenized with mortar and pestle in 5 ml of cold extraction buffer containing, 0.1 M Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 3 mM Sodium-EDTA. The homogenate was centrifuged for 30 min at 15 000 g at 4 °C. The reaction mixture contained 1 ml of the supernatant, 2 ml of 0.4 M Tris-HCl buffer (pH 8.9), 50 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) DTNB. All the samples were incubated for 1 h at 37 °C. The absorbance was measured at 412 nm after 2 min in a spectrophotometer ($\sum 412 = 13.6$ mM^{-1} cm^{-1}).

Statistical analysis

The data observed in the experiment were statistically analyzed for the mean, standard deviation (SD), standard error (S.E.) and least significant difference (LSD) to determine the critical level of significance ($P \leq 0.05$) of the differences between means of treatments.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

Results and discussion

Effects of Cr, EDTA and SNP on superoxide dismutase activity

The results of SOD activity are presented in *Figure 2a* showing the effect of different concentration of chromium with and without EDTA on superoxide dismutase activity (Units mg^{-1}protein) in *Sesbania grandiflora* and in *Figure 2b* showing the effect of chromium and EDTA in the presence of SNP on superoxide dismutase activity (Units mg^{-1}protein) in *Sesbania grandiflora* respectively. The results after 30 days time period from *Figure 2a* showed an increase in the SOD activity with increase in the concentration of Cr. When compared with control plants the C5 treatment plants showed 80.7% increase with respect to the SOD activity. Further in the presence of chelator EDTA, the SOD activity was observed to be increased in all the treatments with EDTA when compared with control. Significant difference was noted for the treatment with highest concentration of Cr at both the levels $P \leq 0.05$ and $P \leq 0.01$. During 60 days of growth significant difference at both $P \leq 0.05$ and $P \leq 0.01$ were observed for the treatments C4, C5 and C5+EDTA, whereas treatments C3+EDTA and C4+EDTA showed significant difference only at one level $P \leq 0.05$. The highest SOD activity was represented by the treatment C5+EDTA at 60 days of growth with 116.01 units/mg protein. Whereas in absence of EDTA the C5 plants showed 22.48 % decrease in the SOD activity, this justifies the role of EDTA in enhanced metal uptake and increased oxidative stress, thereby increasing the SOD antioxidant enzymes. At 90 days there was a significant difference for C3+EDTA, C4+EDTA and C5+EDTA at both $P \leq 0.05$ and $P \leq 0.01$, whereas in the absence of EDTA, C3 and C4 showed difference only at $P \leq 0.05$, whereas C5 showed significant difference at both the significant levels. Hence the significant difference between the treatments Cr without and with EDTA shows that, in the presence of EDTA the concentration of Cr is higher and it also increased with the time period. The elevation of SOD activity in general is a clear indication to detoxify the O^{-2} and other ROS in downstream pathways under metal stress. But in the presence of EDTA, the levels of SOD are still higher.

SOD activity increased with 150 µg/g Cr in the presence of EDTA in *Cucumis utilissimus* (Sinam et al., 2011). The increase in SOD activity after 12 hr of application of 50 µmol/L of Cr (VI) was also reported in *Vigna radiata* cv. CO_4 (Shankar et al., 2005). In maize the SOD activity increases with 300 µM of Cr in Sartaj variety (Maiti et al., 2012).

The result represented in *Figure 2b* depicts the effects of exogenous nitric oxide in the presence of chromium and EDTA. From the figure it was clear that, when the concentration of chromium is increased from 60 mg/Kg Cr to 100 mg/Kg Cr, the SOD activity also increases corresponding to the heavy metal increase enhanced by EDTA, which is vivid from the treatments C1-C4. This phenomenon of increase in SOD activity corresponding to the metal increase has been reported by different authors. Due to oxidative stress created by the metal the plant has adapted a defensive mechanism by increased production of anti-oxidative enzymes, among the known antioxidative enzymes, SOD is considered to be the first line of defense for plants anti oxidative defense system. In the presence of SNP, this condition is reversed in the present study. From this it is concluded that, C3 and C4 plants when compared with that of C7 and C8 plants, the presence of SNP in latter in spite of metal and EDTA concentration it decreased the SOD activity in those plants (i.e. C7 and C8) by 34.4% and 32.90% respectively at the end of 30 days growth period. Significant difference was depicted in

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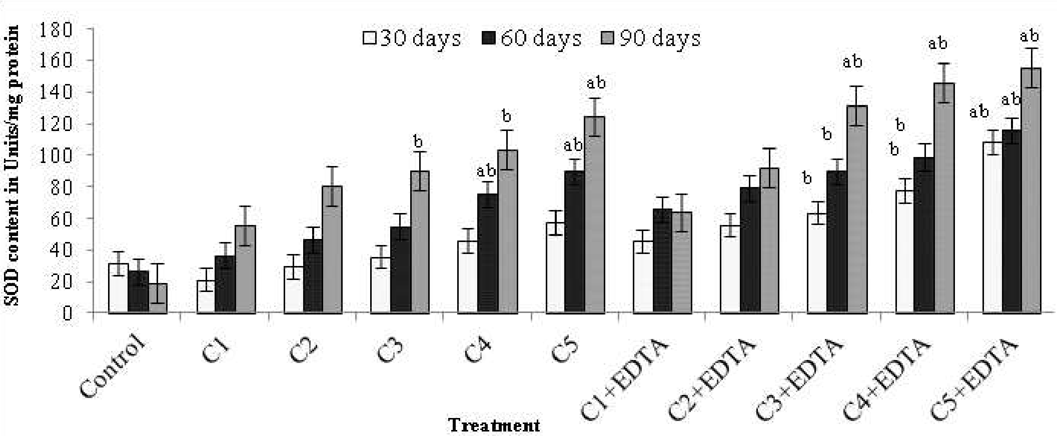
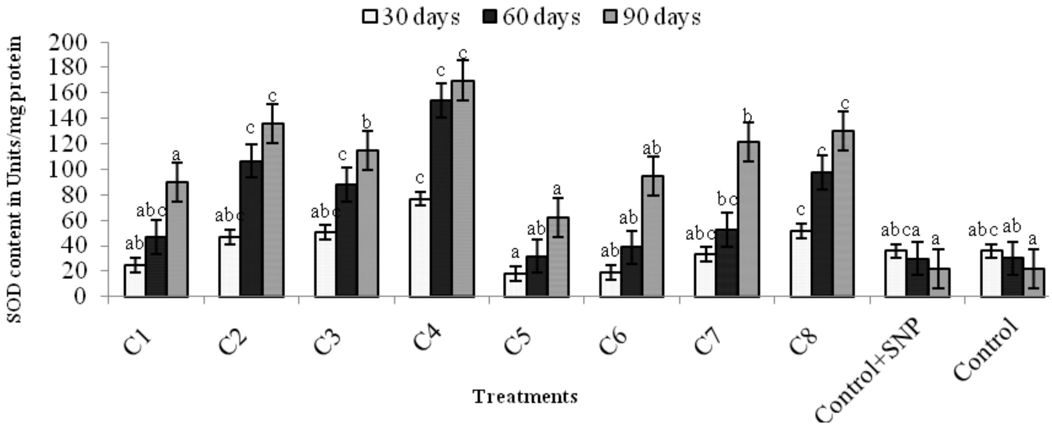
DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

activity of SOD in C3 and C7 for varying time periods. This shows that in the presence of SNP the SOD activity have been reduced. Based on the literature (Ferreira et al., 2010) the decreased SOD activity during treatment with SNP clearly states that nitric oxide (NO) action has created lower substrate availability and created a reversed effect. This fact was detected in the present study with the plants supplied with 250 µM of SNP.

Similar results were reported in ryegrass and in *Arabidopsis* under Cu stress (Dong et al., 2014; Cobbett, 2000). Reports in sunflower and rice leaves under Cd stress by Laspina et al. (2005) and Hung and Kao (2003) have detected that in the presence of SNP, the SOD levels decreases and this low SOD activity was due to decrease in lipoperoxide levels. It increased the photosynthetic ability of the plant and confers the plant protection against oxidative stress. Similar to the above results (Caro and Puntarulo, 1998) observed the lowered levels of substrates for SOD due to NO presence in soybean embryo. Reports also suggested that nitric oxide play role even as an antioxidant thereby directly quenching the super oxide anion and regulates the oxidative damage as seen in *Triticum aestivum* roots grown under Pb induced toxicity (Kaur et al., 2015). Thus based on the above reports by various authors it could be correlated in the present work, that NO gradually scavenged the superoxide radical when SNP is supplied exogenously in the treatments C5 and C6. Even in the presence of combined effect of Cr and EDTA in the treatments C7 and C8, presence of SNP had a positive role in attenuating the oxidative stress generated by the metal and chelator.



a



b

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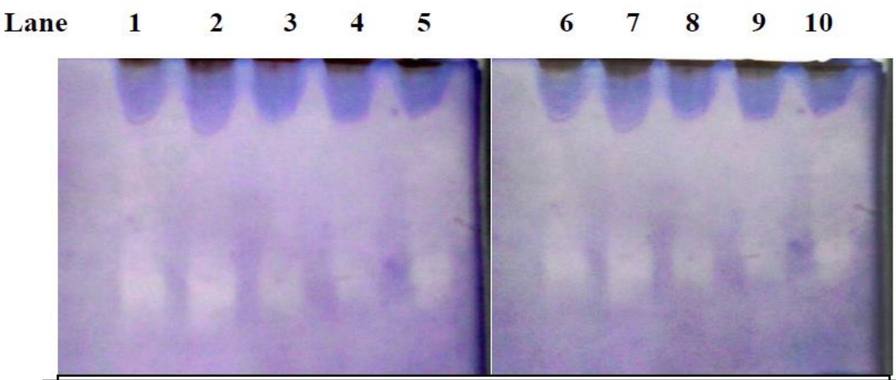
DOI: http://dx.doi.org/10.15666/aeer/1702_32693291



Lane 1: 60mg Cr; Lane 2: 100mg Cr; Lane 3: 60mg Cr+0.35mM EDTA; Lane 4: 100mg Cr+0.35mM EDTA; Lane 5: 60mg Cr+250µM SNP; Lane 6: 100mg Cr+250µM SNP; Lane 7: 60 mg Cr+ 250µM SNP+0.35mM EDTA; Lane 8: 100mg Cr+250µM SNP+0.35mM EDTA; Lane 9: Control+250µM SNP; Lane 10: Control

c

Figure 2. a *Effect of different concentration of chromium with and without EDTA on Superoxide dismutase activity (units mg^{-1} protein) in Sesbania grandiflora. (Significance at $P \leq 0.05$ = b, significance at $P \leq 0.01$ and $P \leq 0.05$ = ab.)* ***b*** *Effect of chromium and EDTA in the presence of SNP on superoxide dismutase activity (units mg^{-1} protein) in Sesbania grandiflora. (Lower case letters that are different denote the treatments which are significantly different. Same letter indicates the treatments which are not significantly different.)* ***c*** *Superoxide dismutase (SOD) isoenzymes activities in native gels in leaves of Sesbania grandiflora under various combinations of chromium, EDTA and SNP*

SOD enzyme was distinguished by native Polyacrylamide gel electrophoresis (PAGE) experiment to determine the isoenzymes of SOD in the presence of SNP-treatment. The finding in *Figure 2c* shows the superoxide dismutase (SOD) isoenzymes activities in native gels in leaves of *Sesbania grandiflora* under various combinations of chromium, EDTA and SNP, this shows the presence of one manganese superoxide dismutase (Mn-SOD) in both the control and chromium treated plants. When plants were exposed to higher concentration of chromium with 100 mg Cr Kg^{-1}, it was observed that MnSOD was apparently affected in SNP treated plants which is clear from the bands which were clearly less intense (lane 8) than the other treatments (lane 4) in the absence of SNP.

Effects of Cr, EDTA and SNP on catalase activity

Catalase, a tetrameric heme-containing enzyme, that degrade H_2O_2 rapidly and more sensitive to excess Cr, where it readily binds to thiol groups and inactivates the thiol-containing enzyme (Luna et al., 1994). Similar to SOD activity, the catalase activity in the study represented in *Figure 3a* shows the effect of different concentration of chromium with and without EDTA on catalase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. The activity was found to be increased with higher concentrations of Cr in the presence of EDTA. When compared with the control, the highest catalase activity was noted for higher concentration of Cr in presence of EDTA (C5+EDTA) which was observed to be 55.5% increased than non-EDTA plants (C5) at 60 days growth period. The CAT activity also increased with the time period from 60 days compared to 90 days due to the persistent effect of chromium action. Hence under this metal stress reactive

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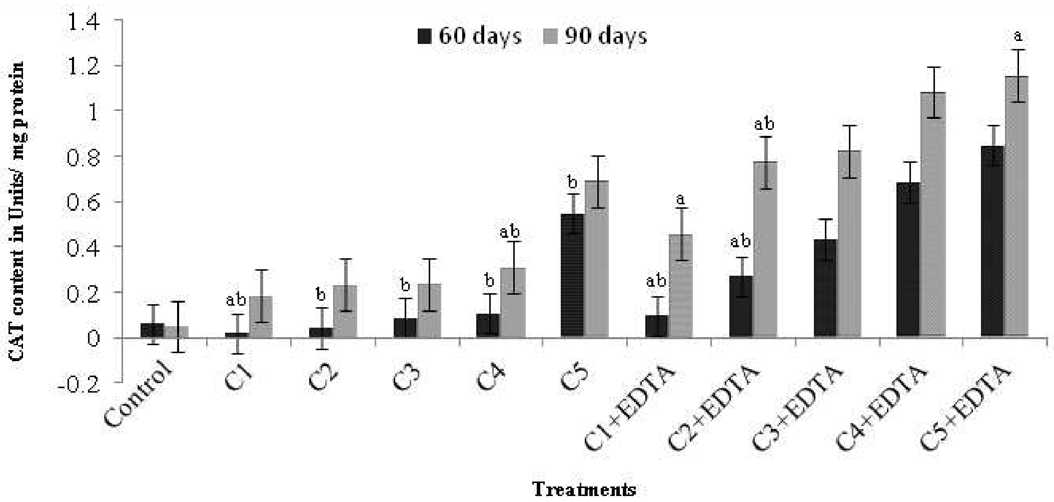
oxygen species (ROS) production is elevated leading to oxidative damage. Statistically it was noted that at 60 days significant change was exhibited at $P \leq 0.05$ and $P \leq 0.01$ for the treatments C1, C1+EDTA and C2+EDTA. Similarly at 90 days C4 and C2+EDTA showed significant difference ($P \leq 0.05$ and $P \leq 0.01$). Statistically also EDTA proves its higher metal enhancement activity, since from *Figure 3* it is clear that, at 60 and 90 days of the growth the treatment C2+EDTA alone showed significant difference at the p values $P \leq 0.05$ and $P \leq 0.01$, stating that the presence of EDTA with higher concentration of Cr had influence on the increased activity. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since SOD show customary action on the superoxide anion converting it to H_2O_2 which is another reactive intermediate and later H_2O_2 gets converted it to water and oxygen (Mates, 2000). Catalase enzyme increases with increasing concentration of Cr in *Ocimum tenuiflorum* and *Sesbania sesban* (Rai et al., 2004; Bakiyaraj et al., 2014).



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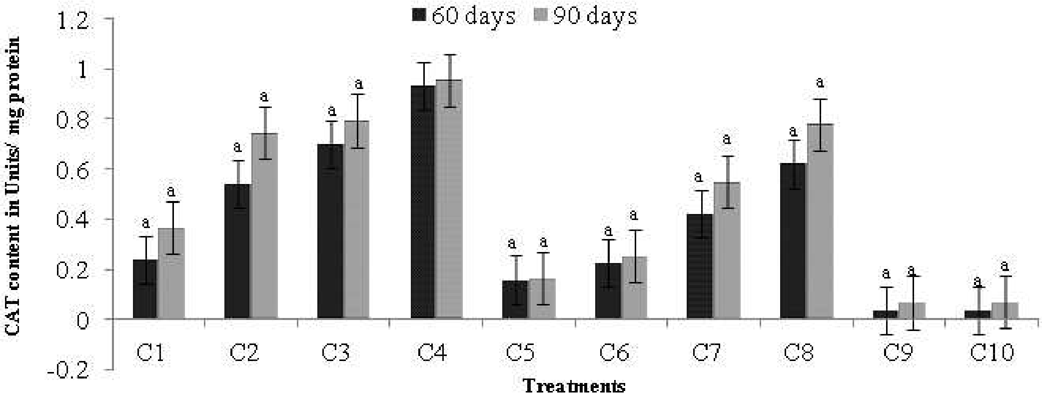

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***Figure 3. a** Effect of different concentration of chromium with and without EDTA on catalase activity (units mg^{-1} protein) in Sesbania grandiflora. (Significance at $P \leq 0.05$ = b, significance at $P \leq 0.01$ and $P \leq 0.05$ = ab.) **b** Effect of chromium and EDTA in the presence of SNP on catalase activity (units mg^{-1} protein) in Sesbania grandiflora. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

The results of catalase activity in the presence of chromium and EDTA, and exogenous application of SNP are represented in *Figure 3b* showing the effect of chromium and EDTA in the presence of SNP on catalase activity expressed in (Units mg^{-1} protein) in *Sesbania grandiflora.* From the figure it is clear that, the catalase activity increased with increase in chromium and EDTA levels, which is apparent from the treatments C2 and C4. But the treatments C5-C8 with SNP the activity of catalase decreased, with corresponding increase in the concentration of chromium.

According to Ferreira et al. (2010), the cause of the decrease in CAT activity was due to reduced substrates for CAT activity as observed in SNP-pretreated soybean plants. This fact was paralleled with the present study that the decreased H_2O_2 production as a consequence of the NO effect, approve protection against ROS in SNP administered C7 and C8 plants. Our study is in concordance with the above statement that, in the presence of SNP the catalase activity is reduced in C5 and C6. This is also applicable to the treatments C7 and C8, where in spite of chromium and EDTA the catalase activity was reduced in these plants. Thus when a comparison was made between C3 and C4 plants with their counter parts C7 and C8 plants, presence of SNP in latter, reduced the CAT activity by 31.6% in case of 60 mg Cr, SNP and EDTA and 18.94% in case of 100 mg Cr, SNP and EDTA at 90 days growth period. Hence compared with the control the antioxidant activity was decreased in SNP treated plants which clearly explains the potential of NO, which confers resistance to heavy metal stress created oxidative damage in *Sesbania grandiflora.* And moreover the reduction of catalase activity and its decreased levels to normalcy was experienced after addition of SNP indicating the cell response to NO action and thus decreasing the ROS generation inside the cell. Similar results were reported by Dalurzo et al. (1996) in Pea leaves and (Cobbett, 2000) in *Arabidopsis* showing the decline in CAT activity.

Effects of Cr, EDTA and SNP on ascorbate peroxidase (APX) activity

The APX activity for Exp.1 is presented in *Figure 4a* showing the effect of different concentration of chromium with and without EDTA on ascorbate peroxidase activity (Units mg^{-1}protein) in *Sesbania grandiflora.* From the figure, it is clear that the APX activity increased in the treatments of chromium in the presence of EDTA than experienced by the treatment in the absence of EDTA. The levels of APX increased in 30 days of plant growth, and when compared with the control, the value of 4.6 units/mg protein observed in C5+EDTA plants, is 10.96% higher than C5 plants with 0.51 units/mg protein of APX. A significant difference in the treatments was observed for the higher concentration at both the levels $P \leq 0.05$ and $P \leq 0.01$ in the presence of EDTA (C4+EDTA and C5+EDTA). After 30 days, the APX activity increased with increase in the chromium concentration. This increase in the enzyme activity could be correlated with action of ascorbate peroxidase that was more efficient in destroying H_2O_2 under Cr stress in the presence of chelator. Statistically at 60 days, there was a significant difference noted for all the treatments at both $P \leq 0.05$ and $P \leq 0.01$, except for C2+EDTA and C5+EDTA which showed significant difference only at one level $P \leq 0.05$. Among the EDTA treated plants C1+EDTA was the only treatment that showed significant difference at $P \leq 0.05$, whereas all the treatments in the absence of EDTA significantly differed ($P \leq 0.05$ and $P \leq 0.01$). When compared with the control, the progressive level of APX showed an increase in the activity of APX in the treatment C5+EDTA at 90 days with 8.6 units/mg protein of the enzyme. The study is in agreement with the reports of (Karuppanapandian et al., 2006a) that, the antioxidant

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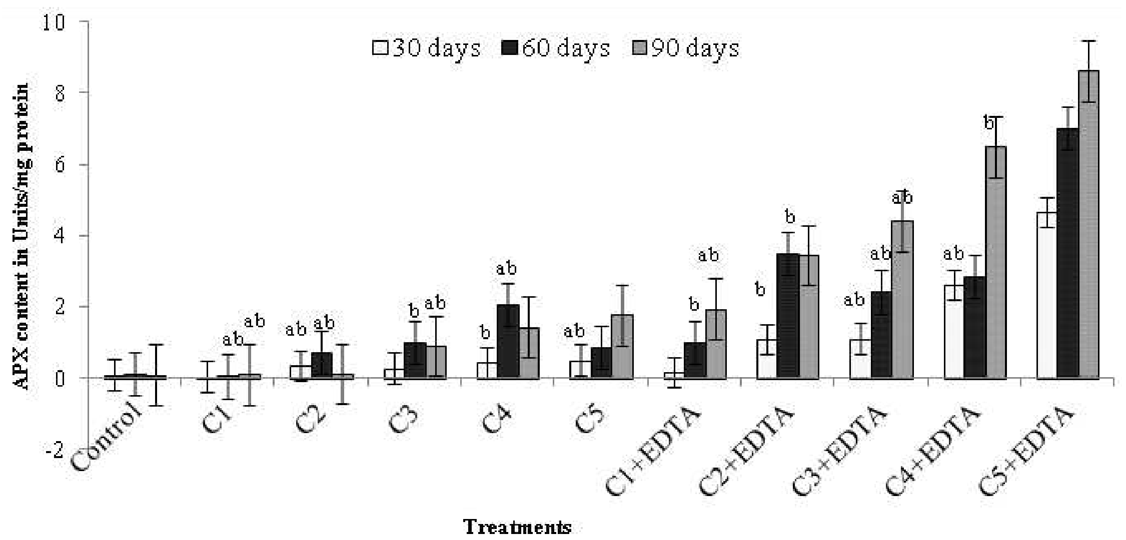
enzyme increase was due the antioxidative capacity stimulated by Cr, which caused the conversion of H_2O_2 to H_2O and O_2. The high levels of APX produced, due to the higher concentrations of Cr in the presence of EDTA, uses ascorbic acid as a reductant in the first step of the ascorbate-glutathione cycle (Mittova et al., 2003 and Smirnoff, 2000). Similar results were reported by Rai et al. (2006) in sugarcane plants exposed to hexavalent chromium. Cr and Al induced H_2O_2 accumulations in wheat and green gram seedlings were also found to significantly elevate the APX activity (Sharma and Sharma, 1996). APX plays an important role in scavenging H_2O_2 which is a systemic signal for the induction of APX (Morita et al., 1999).



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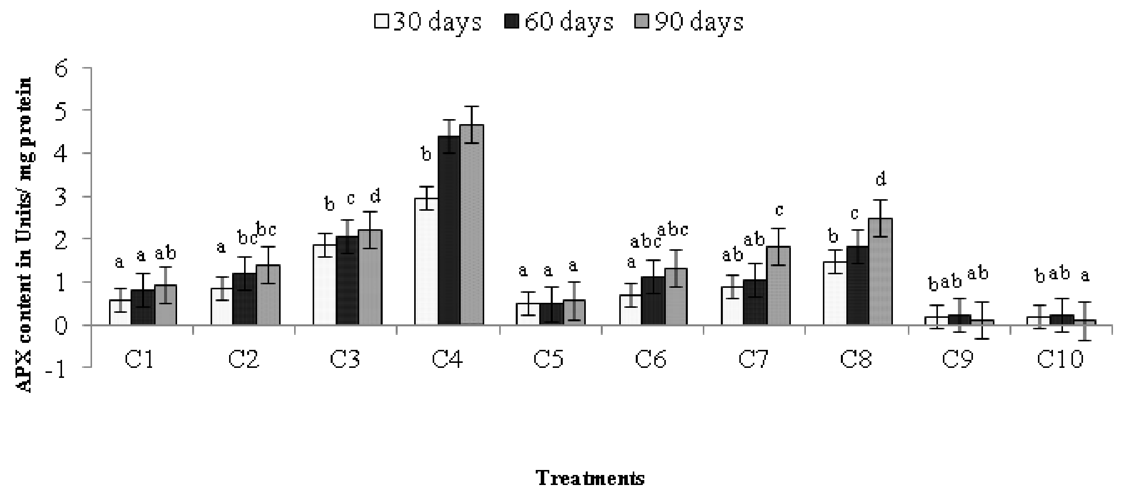

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Figure 4. a *Effect of different concentration of chromium with and without EDTA on ascorbate peroxidase activity (units mg^{-1} protein) in Sesbania grandiflora. (Significance at $P \leq 0.05$ = b, significance at $P \leq 0.01$ and $P \leq 0.05$ = ab.)* ***b*** *Effect of chromium and EDTA in the presence of SNP on ascorbate peroxidase activity (units mg^{-1} protein) in Sesbania grandiflora. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)*

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The effects of APX activity in the presence of chromium and EDTA, with exogenous application of 250 µM SNP are represented in *Figure 4b* showing the effect of chromium and EDTA in the presence of SNP on ascorbate peroxidase activity (Units mg^{-1} protein) in *Sesbania grandiflora.* From Exp.1, it was concluded that, the APX activity was found increased at higher chromium levels with EDTA, due to higher metal accumulation which was assisted in presence of EDTA. From Exp.1 also it is evident that, in order to overcome the oxidative stress, the APX activity increased in EDTA treated plants as a plant adaptive defence mechanism, which is evident from the treatments C3 and C4 (60 and 100 mg Cr kg^{-1} with 0.35 mM EDTA). The increased APX activity was observed for higher concentrations of chromium with increase in time period (C5, C5+EDTA), whereas C2, C4 and C2+EDTA plants exhibited lesser variation in APX activity during 90 days. Similarly the plants supplied with 250 µM SNP, showed decreased APX activity. This is obvious when comparison is made between C3 and C4 and C7 and C8 plants, that presence of SNP has decreased the APX activity by 17.56% (between C3 and C7) and 47% (between C4 and C8). Significant difference was depicted in C8 during 60 and 90 days compared to C4. This decrease in APX activity from the present study could be correlated with Hiner et al. (2000) and Talukdar (2012b) that, low availability of reduced ascorbate, its co-factor, or inhibition of its isoforms by excess H_2O_2 or both, caused reduced APX activity in *Lathyrus sativus* by the latter. Hence in the present study the lower ascorbate levels created by the nitric oxide have reduced the stress levels and hence a reduced APX activity was observed. Similar results were reported in wheat roots (Singh et al., 2008).

Effects of Cr, EDTA and SNP on glutathione reductase (GR) activity

The results for GR activity after 30, 60 and 90 days are presented in *Figure 5a* showing the effect of different concentration of chromium with and without EDTA on glutathione reductase activity (Units mg^{-1} protein) in *Sesbania grandiflora.* The figure denoted an increasing GR activity corresponding to the increasing concentration of the chromium and increased further in the presence of EDTA. The treatment C5 was recorded with 0.38 units/mg protein of GR content which corresponds to a decreased percentage of 58.24%, when compared with that of C5+EDTA plants with 0.91 units/mg protein. Statistically there was significant difference for the treatments C5, C3+EDTA and C5+EDTA after 30 days at $P \leq 0.05$ and $P \leq 0.01$. During 60 days of growth C4, C5 and C3+EDTA showed significant difference. At 90 days there was statistical difference for $P \leq 0.05$ and $P \leq 0.01$ in all the treatments with and without EDTA, except for C1. Thus the determination of GR content from Exp.1 concludes that, the GR levels increased with the concentration of the treatments and it is duration dependent. Similar results were also reported in Indian mustard (Diwan et al., 2009) stating that the activity of GR under Cr treatment was concentration as well as duration dependent.

GR is a flavoenzyme, oxidoreductase, which catalyze the conversion of oxidized glutathione to reduced glutathione, which is found in both prokaryotes and eukaryotes. It is a prospective enzyme for the (Ascorbate glutathione) ASH-GSH cycle and plays a vital role in defense system against ROS by sustaining the reduced status of (Glutathione) GSH (Romero-Puertas et al., 2006; Gill and Tuteja, 2010).

GR content increased under Cr stress in *Vigna radiata* roots (Shankar et al., 2004). In another study (Nehnevajova et al., 2012), the activity of GR was strongly increased in young seedlings exposed to heavy metals and the enzyme activities were particularly

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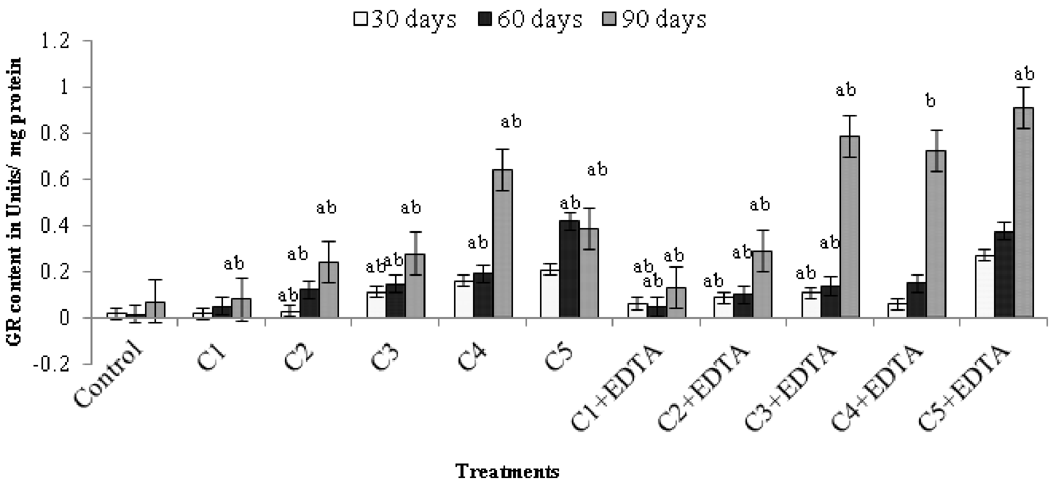
pronounced in mutant lines of sunflower. Increased GR activity was noted (Thounaojan et al., 2012) in Cu treated *Oryza sativa* over control and, the roots showed greater activity than shoot. According to Yadav et al. (2010) chromium stress induced the production of the GR and Cr detoxification in *Jatropha curcus* plant.



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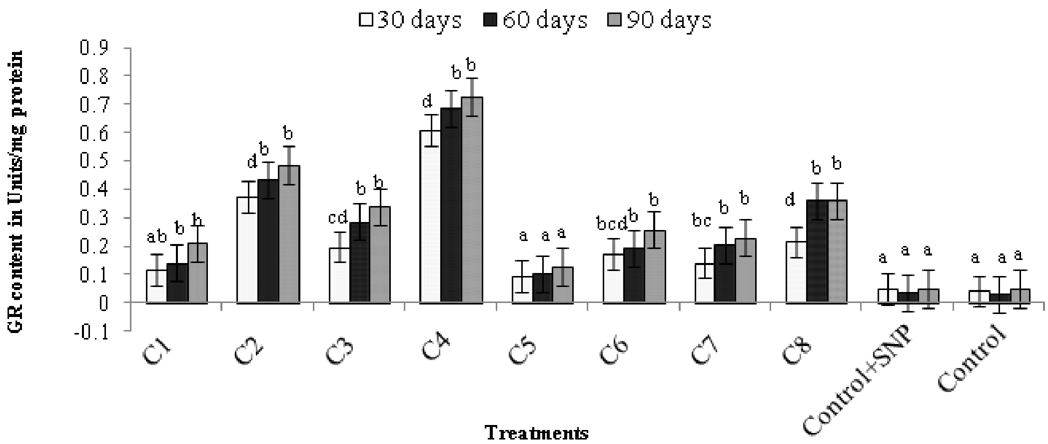

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Figure 5. ***a*** *Effect of different concentration of chromium with and without EDTA on glutathione reductase activity (units mg^{-1} protein) in Sesbania grandiflora. (Significance at P ≤ 0.05 = b, significance at P ≤ 0.01 and P ≤ 0.05 = ab.)* ***b*** *Effect of chromium and EDTA in the presence of SNP on glutathione reductase activity (units mg^{-1} protein) in Sesbania grandiflora. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)*

According to Diwan et al. (2012) APX and GR maintain the H_2O_2 level. The increased level of H_2O_2 when exported to cytosol, create an adverse effect due to formation of hydroxyl radicals through the metal-catalyzed Haber–Weiss reaction. In the present study the increased activities of APX and GR in higher concentrations in Cr treated plants in the presence of EDTA shows that they were functioning concurrently to remove H_2O_2.

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Glutathione reductase catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and anti oxidative processes in plants. GR catalyses the NADPH dependent reaction of disulphide bond of GSSG (oxidized glutathione). It is thus important for maintaining the GSH (reduced glutathione) pool (Reddy and Raghavendra, 2006). GR and GSH play crucial role in determining the tolerance of a plant under various stress (Chalapathi Rao and Reddy, 2008).

The GR activity with SNP treated plants are represented in *Figure 5b* showing the effect of chromium and EDTA in the presence of SNP on glutathione reductase activity (Units mg^{-1} protein) in *Sesbania grandiflora.* It depicts that there is an elevation of the scavenging enzyme in the treatments C3 and C4 in order to counter act Cr-induced stress in the presence of chelator compared to its absence in C1 and C2. According to Shigeoka et al. (2002) APX and GR are the two main enzymes of the ascorbate-glutathione cycle, which are localized mainly in chloroplasts and play an important role in combating oxidative stress. This is proved in the present study that in plants treated with Cr and EDTA, there is a considerable increase in the GR content.

Further comparing with other treatments, it is slightly reversed upon addition of SNP. The treatment plants C5 and C6 supplied with 250 µM of SNP in the presence of 60 and 100 mg Cr kg^{-1}, the oxidative stress created by the metal is reduced. In treatments C7 and C8 despite the presence of EDTA and Cr, addition of SNP reduced the stress by showing reduction in the enzyme activity which reduced the stress by 33.3% (C3 and C7) and 50% (C4 and C8) respectively. In other words, SNP as a NO donor has an ameliorating effect on Cr and EDTA induced stress. Supporting facts by Singh et al. (2008) reported similar reduction in GR activity upon SNP treatment against Cd toxicity in wheat roots.

Effects of Cr, EDTA and SNP on ascorbic acid oxidase activity

The activities of Ascorbic acid oxidase determined in the leaves of *Sesbania grandiflora* are presented in *Figure 6a* showing the effect of different concentration of chromium with and without EDTA on ascorbic acid oxidase activity (units mg^{-1} protein) in *Sesbania grandiflora.* From the results it is clear that when the concentration of the chromium increases from 20-100 mg, there is an increase in the activity of the antioxidant enzyme ascorbic acid oxidase. Moreover when compared with the control, the activity of the enzyme increased by 89.4%, 115.7%, 105.2%, 363.1% and 410.5% in the presence of EDTA in C1+EDTA to C5+EDTA plants respectively corresponding to chromium concentration noted after 60 days of growth. When compared with C5 plants, C5+EDTA showed 94% increase in ascorbic acid oxidase activity. Statistical significant difference was noted in all the treatments at $P \leq 0.05$. After 30 days of growth, treatments C3+EDTA and C4+EDTA showed significant difference at both the levels $P \leq 0.05$ and $P \leq 0.01$. At 60 days of growth C2, C3, and C4 showed significant difference at $P \leq 0.05$ and $P \leq 0.01$. Similarly after 90 days, significant difference was exhibited by the treatments C1, C2 and C3. Thus the present study shows that EDTA had an influence on the ascorbic acid oxidase enzyme concentration determination in the presence of increasing concentrations of Cr.

Ascorbate is a universal soluble antioxidant that is involved in various physiological mechanisms of plant, which exists in photosynthetic organisms. The ascorbate is considered to be the most important substrate for decomposition of hydrogen perodxide H_2O_2 detoxification (Singh et al., 2005). High AO activity is associated with the cell wall ascorbate and cell wall localized ascorbate oxidase (AO) that function to control

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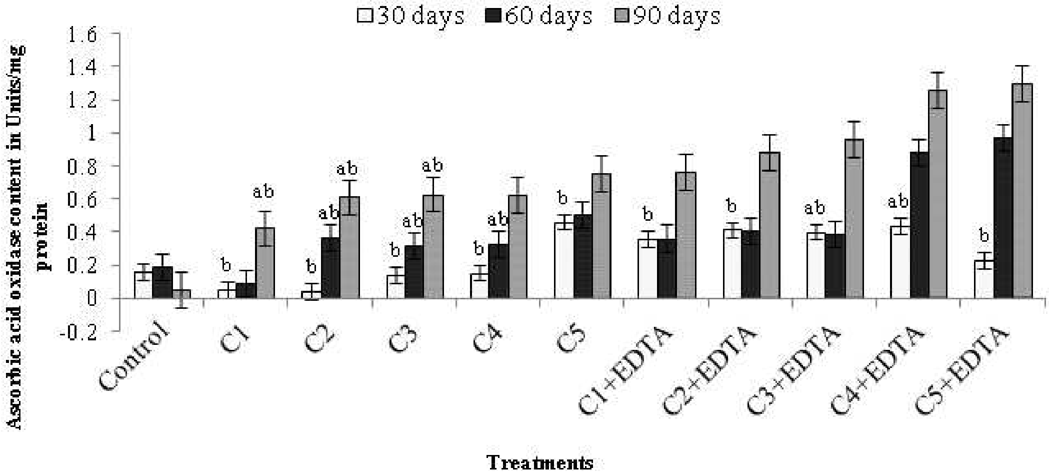
growth and expanding cells and as a model that links wall ascorbate and ascorbate oxidase to cell wall extensibility (Smirnoff, 1996).



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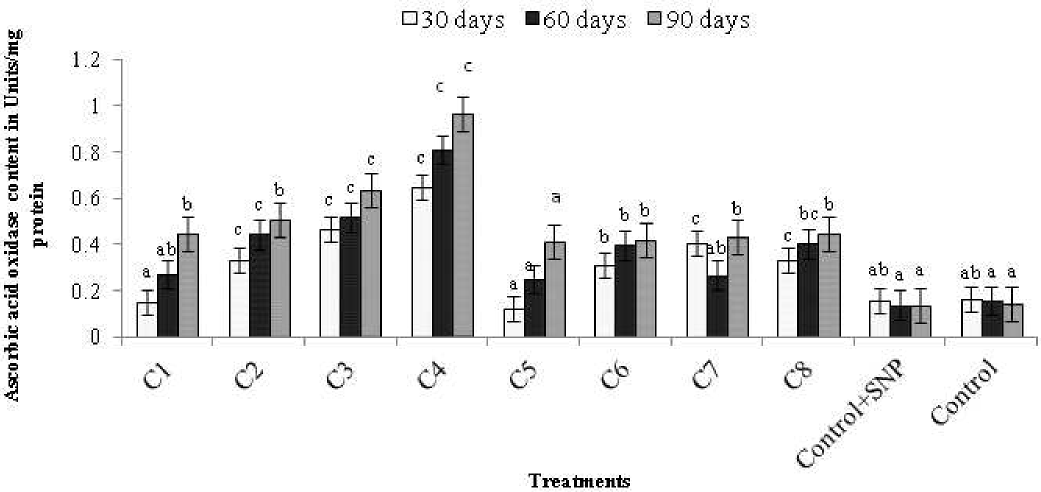

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***Figure 6. a** Effect of different concentration of chromium with and without EDTA on ascorbic acid oxidase activity (units mg^{-1} protein) in Sesbania grandiflora. (Significance at $P \leq 0.05$ = b, significance at $P \leq 0.01$ and $P \leq 0.05$ = ab.) **b** Effect of chromium and EDTA in the presence of SNP on ascorbic acid oxidase activity (units mg^{-1} protein) in Sesbania grandiflora**.** (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)*

Ascorbate is considered to be the primary as well as a secondary antioxidant, which binding to the metals, affects their movement across biological membranes, or act as a reductant. Reports suggested that, the ascorbate content in *Vigna aconitifolia* roots increased significantly under cadmium stressed conditions (Vijendra et al., 2016). Similarly the activity of ascorbate oxidase enzyme was determined for a period of three months for the plants treated with 60 and 100 mg of Cr with 0.35 mM EDTA and 250 µM SNP. The result represented in *Figure 6b* explains the effect of chromium and EDTA in the presence of SNP on ascorbic acid oxidase activity (Units mg^{-1} protein) in

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Sesbania grandiflora. The figure articulates that, the enzyme activity increased from 30 to 90 days in treatments (C1-C4) in the presence of chromium and EDTA as a defensive mechanism for the plant to withstand the oxidative stress formed due to the reactive oxygen species (ROS) liberated due to high metal concentrations. But in the treatments (C5-C8) the result showed that, the presence of nitric oxide in the plants under the treatments showed decline in the enzyme levels, where the C8 plants showed a decreased percentage of 54.1% when compared with C4 plants in the absence of SNP, stating the fact that nitric oxide can counteract Cr and EDTA induced oxidative stress.

Such effects were confirmed by the other antioxidant enzymes studied and such studies reported that NO can detoxify or scavenge ROS directly or it can also react with $O_2^{\bullet-}$ and generate peroxynitrite ($ONOO^-$) (Martinez et al., 2000; Neill et al., 2003). Thus SNP protects plants against Cr+ EDTA induced oxidative stress. The SNP effect in the present study represents the antioxidant property of NO for suppression of high levels of Cr-triggered ROS.

Effects of Cr, EDTA and SNP on glutathione activity

Glutathione (GSH), a tri-peptide is most abundant low molecular weight thiol in all mitochondria-bearing eukaryotes including plants. In plants, GSH is involved in defence against ROS (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), sequestration of heavy metals (Cobbett and Goldsbrough, 2002) and is one of the major source of non-protein thiols in most plant cells (Freeman et al., 2004). The glutathione content in the chromium treated plants with and without EDTA in the present study represented in *Figure 7a* shows the effect of different concentration of chromium with and without EDTA on glutathione content (µg. FW^{-1}) in *Sesbania grandiflora.* When compared with the control the glutathione content increased with the increase in the concentration of Cr in the presence of EDTA. The highest glutathione content was recorded for the treatment with 100 mg of Cr + 0.35 mM EDTA with 280.63µg.

With increase in time period from 30 to 90 days the glutathione content was found to be increased by 92.9% in C5+EDTA plant. All the treatments showed significant difference at both levels $P \leq 0.05$ and $P \leq 0.01$. But at 60 days of growth all the treatments showed significant difference at both levels $P \leq 0.05$ and $P \leq 0.01$ except C5 and C5+EDTA. This validates that, higher concentrations of Cr have influence on glutathione concentration in plant under heavy metal stress and this effect being more pronounced in the presence of EDTA. Thus at higher concentration of Cr/chelator the level of glutathione increased with time.

Furthermore, elevated GSH concentration is correlated with the ability of plants to withstand metal-induced oxidative stress. Enhanced antioxidant activity in the leaves and chloroplast of *Phragmites australis* was mainly associated with a large pool of GSH resulting in protection of many photosynthetic enzymes against the thiophilic bursting of Cd (Pietrini et al., 2003). Similarly, increased concentration of GSH has been reported in *Pisum sativum*, *Sedum alfredii* and *Vigna mungo* (Metwally et al., 2005; Sun et al., 2007; Molina et al., 2008).

Various non enzymatic antioxidants play an important role in plant defense mechanism under heavy metal stress. In *Oryza sativa* there was no observed significant change in the ascorbate (AsA) content in developing seedlings under Cr (VI) (Panda, 2007); however, the reduced glutathione (GSH) content increased by 30% and 52% at 50 mM and 100 mM of Cr (VI), respectively, after 48 h of exposure. Elevated expression of genes encoding these enzymes under Cr (VI) toxicity suggests defensive

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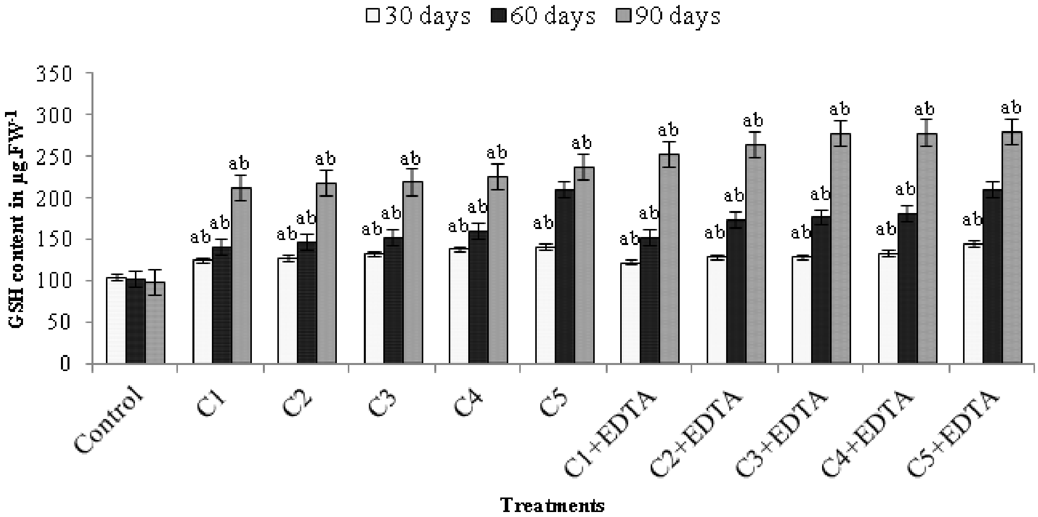
role of glutathione under heavy metal stress according to Zulfiqar et al. (2011), these observations show the involvement of glutathione metabolism in the alleviation of Cr (VI) toxicity by providing protection against oxidative stress.



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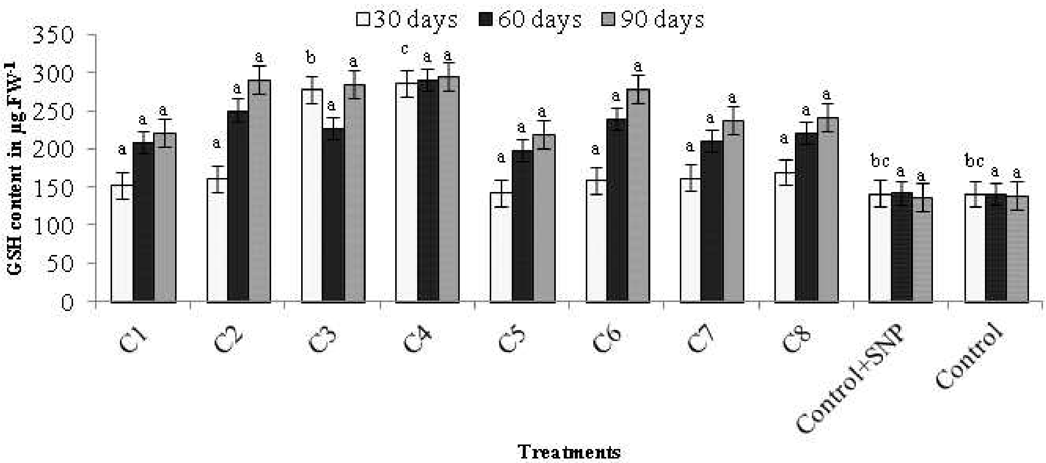

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Figure 7. a *Effect of different concentration of chromium with and without EDTA on glutathione content (µg.FW^{-1}) in Sesbania grandiflora. (Significance at P ≤ 0.05 = b, significance at P ≤ 0.01 and P ≤ 0.05 = ab.)* ***b*** *Effect of chromium and EDTA in the presence of SNP on glutathione content (µg. FW^{-1}) in Sesbania grandiflora. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)*

A marked increase in GSH levels in *Zea mays*, *Brassica spp*. and *L. esculentum* upon exposure to 5–10 mg l^{-1} Cr (VI) (Sanita di Toppi et al., 2002). A positive correlation between GSH and Cr levels has also been observed in *Salvia natans* (Dhir et al., 2009) and *Brassica juncea* (Diwan et al., 2010).

GSH is one of the crucial metabolites in plants which are considered as most important intracellular defense against ROS induced oxidative damage. GSH is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory

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effects of ROS induced oxidative stress. It is a potential scavenger of $^1O_2$, H_2O_2 and most dangerous ROS like OH^-.

The observations made on the glutathione content with SNP treated plants represented in *Figure 7b* show the effect of chromium and EDTA in the presence of SNP on glutathione content (µg. FW^{-1}) in *Sesbania grandiflora* depicts that increased glutathione content with chromium and EDTA treated plants (C1-C4) was reduced in the presence of SNP. From the figure, it shows that treatments, C5 to C8 exhibited reduced glutathione content when compared with control and other treatment plants.

A comparison between the treatments in presence and absence of SNP showed that, presence of SNP in C7 and C8 plants showed a decreased percentage of glutathione content with 16.5% and 17.9%, with that of their respective counterparts C3 and C4 plant.

The reports indicate that the heat-treated *Triticum aestivum* plants showed a reduced GSSG content compared to heat treated plants, due to the SNP supplement (Hasanuzzaman et al., 2012). Literatures also suggest that during stress conditions the glutathione levels inside the cell increases as a consequence to confer resistant against oxidative stress created by heavy metal (Hasanuzzaman et al., 2017). This was paralleled with the present study that the GSH content was found to increase with higher concentrations of chromium in the presence of EDTA. But Exp.2 concluded that, though there was increase in GSH content in chromium and EDTA treated plants, the SNP supplemented plants showed a decreased GSH content than the normal.

This could be justified that the oxidative stress generated by metal and chelator was mitigated by the action of SNP, there by manifesting the key role of nitric oxide in acting as a direct scavenger of ROS and thus reduced the oxidative stress in *Sesbania grandiflora* subjected to metal stress induced by chelator. The reduced GSH levels in the present study could be attributed to the fact that, the reduced stress levels has limited the accumulation of GSH inside the plant due to NO activity.

Conclusion

The present study concludes that exogenous nitric oxide supply ameliorates the Cr-toxicity in the presence of chelator EDTA. Nitric oxide also helped in ameliorating oxidative stress by decreasing the antioxidant levels in response to increasing metal and chelator concentration. Hence SNP when supplemented to heavy metal accumulating plants will reduce the stress levels in plants. Further studies are required to elucidate the changes in molecular levels, to identify the isoenzymes or proteins that are involved in mediating the ameliorating action of NO.

Acknowledgements. The authors would like to thank the VIT management for the constant support provided during the entire period of study.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32693291

THE EFFECT OF EDTA-SE WITH DIFFERENT CONCENTRATIONS ON PHOTOSYNTHESIS OF FRAGRANT RICE (*ORYZA SATIVA* L.)

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(Received 19th Nov 2018; accepted 25th Jan 2019)

Abstract. Selenium (Se), as an essential element, has its effects on the growth and development of plants. Present study aimed to assess Se mediated chlorophyll contents, net photosynthesis, biomass accumulation and activity of carbon metabolism enzymes. Three different levels of Ethylenediamine tetra acetic acid - chelating Se (EDTA-Se) at 10(E1), 30(E2) and 50(E3) μmol L^{-1} were overhead applied at initial heading stage of two aromatic rice cultivars i.e., *Xiangyaxiangzhan* and *Meixiangzhan2*, the treatment without Se were served as control (CK). Results revealed that exogenous Se applications enhanced the chlorophyll content as well as net photosynthetic rate (Pn), meanwhile, grain weight at maturity and the biomass accumulation during the filling had been remarkably improved. Furthermore, growth, yield and quality components were also improved by Se application. Hence, Se proved better for both rice cultivars whereas the E2 was the best treatment with the highest yield, biomass accumulation and net photosynthesis rate.
Keywords: *selenium, rice, net photosynthetic rate, biomass accumulation, chlorophyll content*

Introduction

Rice production is the cornerstone of Chinese food safety system. Recently, as the human populations swell across the world, the demand for rice has been increasing, while available cultivated land for it has been decreasing. The study of Krishnan et al. (2007) showed that it was possible there would be 10 billion people on the earth. In the coming decades, it would lead to increasing the demand for rice. Aromatic rice is a delicacy in rice and has always been the focus of the people because of its predominant tasty, captivating fragrance and other unique features. Despite aromatic rice has been fetching an extremely high price, interestingly, the demand for it keeps growing steadily (Mo et al., 2016). However, just like other rice cultivars, the yield of fragrant rice is affected by many uncertain factors such as climate condition and other environmental factor. For example, Mo et al. (2015) demonstrated that shading the aromatic rice plant during the grain filling stage could significantly reduce its yield. Further study on lead (Pb) stress caused a huge loss in yield of fragrant rice (Ashraf et al., 2018). Meanwhile, a series of researches indicated that certain artificial regulation and application could improve the quality and yield of aromatic rice. Ren et al. (2017) found that fertilizer treatment and severe drought conditions applying separately not only improved the yield of aromatic rice significantly, but also enhanced the 2-acetyl-1-pyrroline (2-AP), the key element of the fragrance of aromatic rice, content in the plant (Yajima et al., 1979). The investigation of Pan et al. (2017) also found that mechanized deep placement of nitrogen fertilizer elevated aromatic rice yield dramatically.

Multiple microelements are available to promote fragrant rice performance. Li et al. (2016) demonstrated that Manganese (Mn) could regulate yield information of aromatic rice and enhanced 2-AP content in grains by improving the synthesis of proline and pyrroline-5-carboxylic acid (P5C) (2-AP's precursors) as well as promoting activity of proline dehydrogenase (ProDH). The report of Mo et al. (2017) revealed that Silicon (Si) application had ability to improve the growth, yield, quality and 2-AP contents of fragrant rice because of the net photosynthetic (Pn) rates, proline contents and activity of ProDH being enhanced.

Selenium (Se), as a significant element, plays an essential role for humans, animals and plants. An analysis (Gladyshev et al., 1999) of Se revealed that poor immune function, high risk of mortality, and cognitive decline had been associated with deficiency of Se while taking Se supplementation had improved the virus inhibitory effect and reduced the risk of catching an autoimmune thyroid disease. With the advance of research, Se was proven to be able to against toxic elements such as arsenic, antimony, mercury and copper (Srivastava et al., 2009; Gotsis, 1982). Moreover, the research of Wang et al. (2012) generated a selenium-enriched rice with high production and enough bioavailability by using selenite fertilization.

Photosynthesis, as an important physiological process in rice, affected rice growth and yield significantly (Zhong et al., 2019; Cao et al., 2018). Normally, the proportion of cop biomass which is directed towards the harvested part could decide the potential of rice yield. To date, there are few reports about fragrant rice photosynthesis and the effect of Se application on fragrant rice. Moreover, Ethylenediamine tetra acetic acid - chelating Se (EDTA-Se) is a new exiting form of selenium element. Hence, it has much room for investigation and based on the hypothesis that exogenous Se application in initial heading stage could enhance the rice photosynthetic characteristics during the filling stage, this study was conducted in Guangdong province (major rice producing province in South China) in order to study the effect of EDTA-Se on rice photosynthesis and dry matter accumulation during the filling stage.

Materials and methods

Plant materials and growing condition

Two aromatic rice cultivars, *Xiangyaxiangzhan* and *Meixiangzhan2*, which are widely cultivated at South China, having 111-114 days of growth period were planted in late season at Experimental Research Farm, College of Agriculture, South China Agricultural University, Guangzhou, (23°09'N, 113°22'E and 11 m from mean sea level) China (*Fig. 1*) during the July and November in 2017. This region has subtropical-monsoonal type of climate with mean annual air temperatures of 26.6 °C, mean annual maximum and minimum air temperatures of 14°C in November and 37 °C in August, respectively. Before sowing, the seeds were soaked in water for 24 h, germinated in manual climatic box for the next 24 h,

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DOI: http://dx.doi.org/10.15666/aeer/1702_32933303

dried in the shade. Then the germinated seeds were sown in PVC trays for nursery raising. 20-day-old seedlings were transplanted to the field at the planting distance (30 cm × 12 cm). The experimental soil was sandy loam with of 25.65% organic matter content, 1.360% total N, 0.956% total P, and 17.460% total K.

Figure 1. *The location of the experimental site*

Treatments descriptions

Experimental treatments were as described:
CK: Overhead sprinkle with double distilled water at initial heading stage of rice.
E1: Overhead sprinkle with EDTA- Se liquor (10 μmol L^{-1} pure selenite concentration) at initial heading stage.
E2: Overhead sprinkle with EDTA- Se liquor (30 μmol L^{-1} pure selenite concentration) at initial heading stage.
E3: Overhead sprinkle with EDTA- Se liquor (50 μmol L^{-1} pure selenite concentration) at initial heading stage.

Plant sampling and determination of biomass accumulation

At heading stage and maturity stage, the rice plants were harvested from six unit sampling areas (1 m^2) in each plot. The leaves, stem-sheaths and grains were separated from the plants and dried under the condition of 80 °C respectively in order to get the estimation of dry matter. The thirty fresh flag leaves were separated and collected from the rice plants at the 15th day after heading stage, washed with double distilled water and stored under the condition of -80 °C for the sake of chlorophyll analysis.

Biomass accumulation during filling stage (BADF) was calculated as: BADF = Total dry weight at heading stage per hill – total dry weight at maturity per hill.

Determination of photosynthesis

Portable photosynthesis system (LI-6400, LI-COR, USA) was used to determine net photosynthetic rate at 09:00–10:30 a.m. according to the standard method (Pan et al., 2015). The photosynthesis was measured on 15th day after the heading stage.

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Estimation of chlorophyll contents

The contents of total chlorophyll (total Chl), chlorophyll a (Chl a) and chlorophyll b (Chl b) were determined by the methods of Anjum (2016). Grinding leaf sample (about 0.1 g) was placed in 15 ml centrifuge tube along with 95% absolute ethyl alcohol (10 ml) and then kept at dark condition until the color of sample transformed turn into white. Then with the help of UV-visible spectrophotometer, Chl a, Chl b and total Chl contents were estimated at 645 nm, 652 nm and 663 nm.

Determination of yield and yield related traits

At maturity stage, the rice grains were harvested from seven unit sampling area (1 m^2) in each plot and then threshed by machine. The harvested grains were sun-dried and weighted in order to determinate the grain yield. Twenty hills of rice from different locations in each plot were sampled for estimate the average effective panicles number per hill. Then, three hills representative plants were taken for estimation of the yield related traits.

The economic coefficient (EC) was calculated as: EC = grain weight / whole plant weight.

Statistical analysis

Data were analyzed using statistical software ‘Statistix 8.1’ (Analytical Software, Tallahassee, FL, USA) while differences amongst means were separated by using least significant difference (LSD) test at 5% probability level. Graphical representation was conducted via Sigma Plot 14.0 (Systat Software Inc., California, USA).

Results

Chlorophyll contents

Exogenous Se applications affected chlorophyll contents at filling stage significantly (*Fig. 2*). For *Xiangyaxiangzhan*, compared with CK, all Se treatments markedly increased the contents of both total chlorophyll and chlorophyll a (except E3) while chlorophyll b content of E2 was 1.32-fold higher than CK. For *Meixiangzhan2*, the highest total chlorophyll, chlorophyll a and chlorophyll b were all recorded in E2 while the chlorophyll contents under E1, E2 and E3 treatments were all higher than CK respectively.

Net photosynthetic rate

As shown in *Figure 3,* net photosynthesis varied with different Se applications in both rice cultivars. The E1 treatment had highest value of net photosynthetic rate while there was no distinct difference between E1 and E2. The trend of net photosynthetic rate was recorded as: E1 > E2 > E3 > CK for both *Xiangyaxiangzhan* and *Meixiangzhan2*.

Dry matter accumulation

Different Se applications affected the dry matter accumulation during the filling stage of fragrant rice (*Table 1*). For *Xiangyaxiangzhan*, E2 treatment significantly increased the weight of stem and leaf as well as total aboveground weight when the

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specimen had been taken in both heading stage and maturity. Only E1 and E2 treatments improved the biomass accumulation significantly. For *Meixiangzhan2*, E2 remarkably improved grain weight at maturity and the biomass accumulation during the filling stage. All Se applications enhanced the total aboveground weight at maturity.

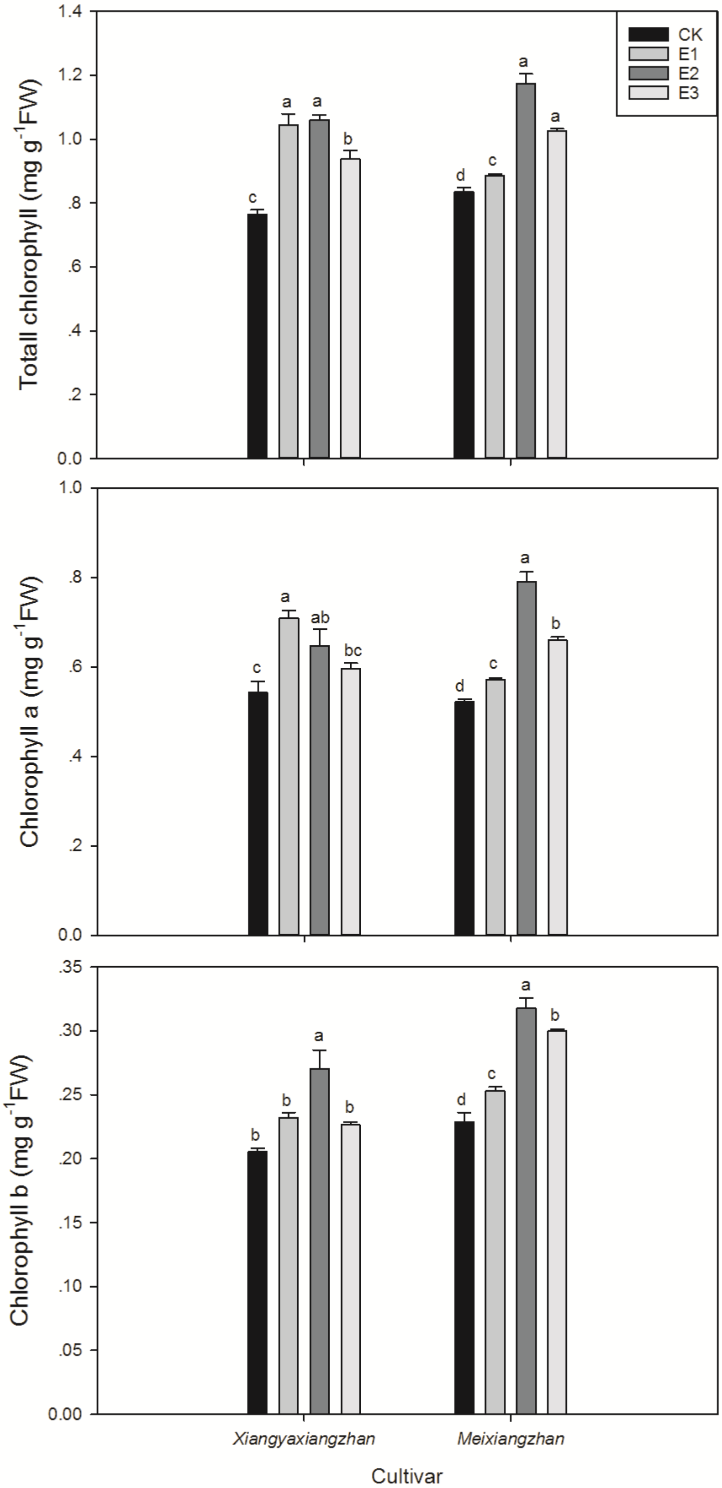

Figure 2. *Effect of EDTA-Se on chlorophyll content. Means sharing a common letter do not differ significantly at (P < 0.05) according to least significant difference (LSD) test for both the seasons. The same as below*

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DOI: http://dx.doi.org/10.15666/aeer/1702_32933303

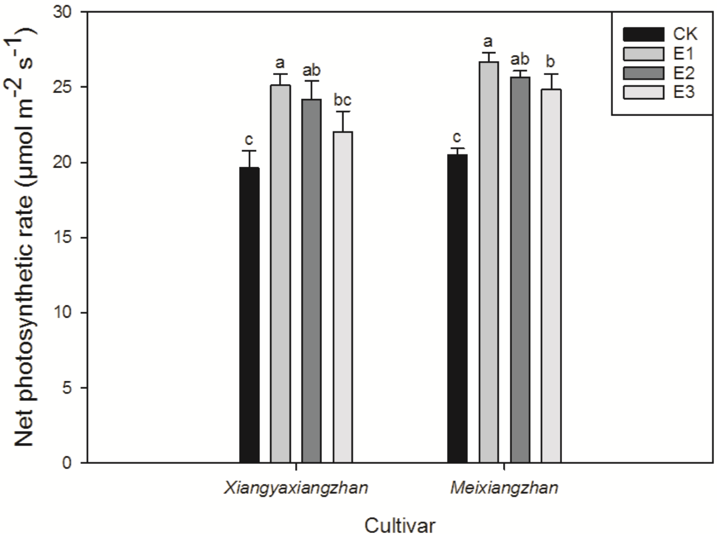

Figure 3. *Effect of EDTA-Se on net photosynthetic rate*

Table 1. *Effect of EDTA-Se on dry matter accumulation during the filling stage*

Treatments	**Heading stage**				**Maturity**				**BDAF ($g\ m^{-2}$)**
	Stem weight ($g\ m^{-2}$)	**Leaf weight ($g\ m^{-2}$)**	**Grain weight ($g\ m^{-2}$)**	**Total aboveground weight ($g\ m^{-2}$)**	**Stem weight ($g\ m^{-2}$)**	**Leaf weight ($g\ m^{-2}$)**	**Grain weight ($g\ m^{-2}$)**	**Total aboveground biomass ($g\ m^{-2}$)**	
Xiangyaxiangzhan									
CK	610.67c	223.49b	110.03a	944.00b	453.0a	117.16b	464.55b	1035.12b	91.05bc
E1	624.67b	240.40ab	114.18a	979.23ab	459.67a	125.37ab	507.53ab	1092.52ab	113.53b
E2	639.67a	253.33a	109.05a	1002.31a	468.67a	134.09a	572.57a	1175.20a	173.23a
E3	621.67bc	231.00ab	111.67a	964.30ab	455.00a	124.16ab	468.98b	1048.30b	83.98c
Meixiangzhan2									
CK	602.58b	218.06c	81.33a	901.91b	420.06a	150.39c	601.53b	1171.90c	269.95b
E1	617.00ab	238.33ab	85.00a	940.33a	430.01a	160.33b	632.89b	1223.21b	282.89ab
E2	637.05a	255.48a	84.67a	977.00a	435.20a	171.67a	680.43a	1287.10a	310.10a
E3	628.12a	235.17bc	79.67a	943.08a	429.19a	156.60bc	630.15b	1215.80b	272.82b

Yield and its traits

Overall, foliar applications of EDTA-Se at initial heading stage can increase grain yield of *Xiangyaxiangzhan* and *Meixiangzhan2* (*Table 2*). For *Xiangyaxiangzhan*, E1, E2 and E3 had significant higher yield than CK, which were 4.20, 4.33, 4.21 and 4.00 t ha^{-1}, respectively. For *Meixiangzhan2*, there was no remarkable difference among CK, E1, E2 and E3 in panicle number, grain per panicle and filling percentage while both E1 and E3 increasing grain weight significantly and the highest yield was also recorded in E2.

Correlation analysis

As shown in *Table 3*, yield of fragrant rice had significant correlation with grain per panicle, biomass accumulation during filling stage and net photosynthetic rate. There exited a positive correlation between 1000-grain weight and biomass accumulation at

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DOI: http://dx.doi.org/10.15666/aeer/1702_32933303

filling stage. Furthermore, net photosynthetic rate had positive correlation with grain per panicle, biomass accumulation during the filling stage and total chlorophyll contents.

Table 2. *Effect of EDTA-Se on fragrant rice yield and it traits*

Treatments	Panicle number (m^{-1})	Grain per panicle	Grain filling percentage (%)	1000-grain weight (g)	Yield (kg m^{-2})
Xiangyaxiangzhan					
CK	236.81±9.06a	112.39±5.72b	81.72±4.32a	18.54±0.19a	0.40±0.01c
E1	257.46±12.78a	113.97±4.62b	83.72±2.16a	18.28±0.32a	0.42±0.01b
E2	230.27±14.47a	148.27±15.21a	81.32±3.82a	18.94±0.34a	0.43±0.02a
E3	259.36±9.88a	124.71±12.41ab	79.73±4.74a	17.46±0.28a	0.42±0.01b
Meixiangzhan2					
CK	214.36±18.48a	178.81±4.44a	72.65±1.47a	18.04±0.60b	0.51±0.01b
E1	218.06±12.10a	177.12±5.06a	78.76±0.64a	19.11±0.55a	0.52±0.01b
E2	218.43±10.87a	177.36±13.73a	80.93±4.64a	18.65±0.39ab	0.55±0.01a
E3	229.89±16.06a	177.79±6.09a	80.50±2.27a	19.27±0.16a	0.52±0.01b

Table 3. *Relationship among yield, yield compounds, biomass accumulation, photosynthesis and chlorophyll contents*

Parameters	Yield	1000-grain weight	Panicle number	Grain filling percentage (%)	Grain per panicle	Biomass accumulation during filling stage	Total chlorophyll content
1000-grain weight	0.1438						
Panicle number	-0.2360	0.3089*					
Grain filling percentage (%)	-0.0654	-0.2402	-0.1826				
Grain per panicle	0.7787**	-0.1666	-0.7047**	-0.0937			
Biomass accumulation during filling stage	0.8111**	0.3900**	-0.2750	-0.2566	0.6138**		
Total chlorophyll content	0.2441	-0.1594	-0.2847*	0.5343**	0.2908*	0.0330	
Net photosynthetic rate	0.4075**	0.1801	-0.1806	0.2475	0.3418*	0.3043*	0.6146*

Significant correlations at *P < 0.05 and **P < 0.01

Discussion

Photosynthesis plays an important role in rice growth and yield information (Zhong et al., 2017). Mitchell and Sheehy (2010) had already explained the relationship between photosynthesis and rice yield, this discovery also proposed the view of supercharging rice photosynthesis to increase yield. Our study evidenced the contributions of photosynthesis to fragrant rice yield by showing the significant positive correlation between yield and net photosynthesis rate at filling stage.

Photosynthesis is a complicated phenomenon in plants and is affected by a variety of factors. A previous study already discussed the relationship between the leaf photosynthetic capacity and leaf nitrogen while reviewing the factors which are limiting the photosynthesis under different growth environments (Mae, 1997). Study of Krishna et al. (2016) showed that cytokinin has ability to delay dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. There were also certain researches about the effects of metallic and non-metallic elements on the crop photosynthesis. The investigation of SultanaIkeda and Itoh (1999) indicated that NaCl salinity decreased the photosynthesis at the reproductive phase by closing the stomatal

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and reducing transpiration rate, leaf water content and biochemical constituents. For example, Chen et al. (2011) demonstrated that Silicon fertilizer could improve plant water status, photosynthesis and mineral nutrient absorption and alleviate the damage to rice plants caused by drought stress. In our study, we discovered that the net photosynthetic rate of fragrant rice at filling stage was enhanced significantly due to exogenous Se application. It might because the Se treatments increased the chlorophyll contents where absorbing and converting light energy in photosynthesis process. It was also strongly corroborated by the investigation of Wu et al. (2000) which revealed that the electron transfer rate of chloroplast increased with low Se concentration while high concentration of selenium leads to electron transfer rate decreased in chloroplast.

Furthermore, the production of rice photosynthesis is carbohydrate and the biomass accumulation of rice plants mostly depends on the net photosynthetic rate in whole growth period. The sucrose is not only the main form of the production of photosynthesis, but also the major transport form for photosynthetic carbon assimilates. The transport rate directly affected the development of library organs and was one of the important factors limiting the further increase of crop yield (Slewinski et al., 2009). Normally, the accumulation of sucrose in leaves is controlled by synthase and decomposition enzyme related to the activity of key enzymes of sucrose metabolism including SPS, SS and sucrose invertase. SPS catalyzes sucrose synthesis reaction whilst SS had the duality of synthesizing and decomposing sucrose (Baxter et al., 2003; Tobias et al., 2008; Miswar et al., 2008). For example, SS mainly catalyzes sucrose synthesis in leaves; however, in grains it catalyzes sucrose degradation. Present study observed that the dry matter accumulation of aromatic rice during the filling stage was improved because of Se treatments. In this study we considered that was the result of the enhancement of net photosynthetic rate.

Normally, the carbohydrate assimilation is the primary source of rice growth while reserving a complementary source for grain filling in rice (Dingkuhn and Gal, 1996). In this study, we observed that yield had remarkable positive correlation with dry matter weight accumulation during the filling stage. This result agreed with the investigation of Dingkuhn et al. (1990) in which study they demonstrated that about 40 to 45% of panicle dry matter come from measured canopy photosynthesis while 55 to 60% might be the translocation. Another study (Dingkuhn et al., 1990) also revealed that canopy photosynthesis provided 58% and 74% for panicle weight at maturity in direct-seeded and transplanted rice, respectively. Present study evidenced that EDTA-Se treatments improved dry matter accumulation during filling stage. Hence, we discovered that Se could be used to increase it because it could improve photosynthesis and finally increase yield.

Conclusion

As a plant growth regulator, 10–50 μmol L^{-1} EDTA-Se concentration could increase the rice photosynthesis at filling stage and thus improved the dry matter accumulation and grain yield. Furthermore, the EDTA-Se applications enhanced the chlorophyll content and activities of SPS and SS and it might be the reason of Se treatments improving rice photosynthesis. For revealing the mechanism of how EDTA-Se affects rice physiology, much work should be done at molecular and physiological level.

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DOI: http://dx.doi.org/10.15666/aeer/1702_32933303

Acknowledgements. This study was supported by National Natural Science Foundation of China (31271646), Student's Platform for Innovation and Entrepreneurship Training Program (201810564029), National Key R&D Program of China (2016YFD0700301), the World Bank Loan Agricultural Pollution Control Project in Guangdong (0724-1510A08N3684), the Technology System of Modern Agricultural Industry in Guangdong (2017LM1098) and Special Funds for Scientific and Technological Innovation and Cultivation of University Students Guangdong (pdjh2019a0075). The authors declare no conflicts of interest.

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APPENDIX

Photos of the experiment

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_32933303

EFFECTS OF DIFFERENT IRRIGATION LEVELS AND PROLINE TREATMENTS ON ORGANIC ACIDS AND SECONDARY METABOLITES OF PEPPER (*Capsicum annuum* L. cv. Yalova Yağlık 28)

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(Received 30th Nov 2018; accepted 4th Feb 2019)

Abstract. Pepper is quite sensitive to water stress, thus changes in irrigation levels may have significant effects on yield, organic acids and phenolics of peppers. Improper irrigation levels may generate a water stress and resultant stress then create serious oxidative damages on cell membranes. Such damages can be prevented with proline-like antioxidative substances. This study was conducted in Çanakkale – Turkey to investigate the effects of different irrigation levels and proline treatments on yields, quality parameters and organic acid composition of pepper *(Capsicum annuum* L. cv. Yalova Yağlık 28*)* in the years of 2013 and 2014. Experiments were conducted in randomized blocks split plots experimental design with 3 replications. Three different irrigation levels were arranged as to apply certain percentages of total evaporation from Class-A pan (Kcp_{50}, Kcp_{100}, Kcp_{150}). Proline treatments (12 mM) were applied at 3 different growth stages (three equal portions on 20, 30 and 40th days, single treatment on 30th day and single treatment on 40th day of growth). In both years of the experiments, decreasing citric acid contents and increasing ascorbic acid contents (in the first year of experiments, Kcp_{50}=84.54, Kcp_{100}=175.48, Kcp_{150}=198.92 mg/100g) were observed with increasing irrigation levels. Different irrigation levels and proline treatments had significant effects on yield (g/m^2), total phenolics (mg/100g), soluble solids content (SSC, %) and internal proline content (µmol/g) of pepper fruits. Adversely affected parameters as yield (g/m^2) and ascorbic acid content due to limited irrigation positively affected with some proline treatments.
Keywords: *Industrial pepper, water stress, ascorbic acid, citric acid, total soluble solids*

Introduction

Besides fresh consumption, capia peppers are also produced for industrial purposes (pepper paste, canned food). They are also called oil-pepper. Since pepper is quite sensitive to water stress, irrigation plays a significant role in pepper production. Pepper roots are quite sensitive to excess water; therefore, sufficient moisture should continuously be available at root zone (Campos et al., 2014; Penella et al., 2014; Vural et al., 2000).

As a cellular adaptation mechanism to water deficits, plants try to preserve proper water potential and cell turgor through accumulation of dissolved osmolites including proline (Bray, 1997). Proline is accumulated as a response to different stress conditions. It is also mentioned as an osmolite and free-radical (reactive oxygen species) scavenger (Reddy et al., 2004; Trovato, 2008).

In previous studies on different irrigation levels (Erken, 2012; Korkmaz et al., 2015), significant increases were reported in proline contents with decreasing irrigation levels. Reactive oxygen species serve as signaling molecules under normal conditions, but they create oxidative damages on stress-exerted cells (Sharma et al., 2012). Proline then

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

emerges as a non-enzymatic antioxidant able to prevent such oxidative damage (Gill and Tuteja, 2010).

Under deficit moisture or water stress conditions, reactive oxygen species generate damages especially on cell membranes where lipids are concentrated. For instance, hydroxyl radicals attack polyunsaturated fatty acids sensitive to peroxidation and initiate lipid peroxidation (Gutteridge, 1995). It was indicated in previous studies that external proline treatments prevented stress-induced negative conditions (Silva Sa et al., 2016; Öztekin, 2009).

Pepper is quite a significant vegetable for our country's economy. Health impacts of antioxidant compounds, ascorbic acid, carotenoids and phenolics of the peppers were pointed out in previous studies (Howard et al., 2000; Marin et al., 2004)

Secondary metabolites are structurally and chemically different from the primary metabolites like sugar, fatty acids, amino acids and nucleic acids. They are not directly needed for basic photosynthesis and respiration metabolisms, but are considered as compounds encountered in specialized cells required for plants to stay alive. Phenolics are the most common secondary metabolites encountered in plants and they play significant roles in various physiological processes in plants (Balasundram et al., 2006; Lattanzio et al., 2006; Nascimento and Fett-Neto, 2010). Several stress factors including high light intensity, low temperature, pathogen infections, mineral deficiency, UV radiation and negative moisture regime may increase the production of free radicals and the other oxidative species. Plant phenolics play important roles as defense compounds against such stressors moreover biotic and abiotic stressors were reported to stimulate the production of secondary metabolites (Chanishvili et al., 2007). Arnnok et al. (2012) reported total phenolics in pericarp section of paprika peppers (*Capsicum annuum* L.) as between 0.782-4.52 g GAE kg^{-1} fresh weight.

Peppers are quite rich in ascorbic acid and several health benefits of ascorbic acid were reported in previous studies (Pereira et al. 2009; Li and Schellhorn, 2007; Siddique, 2006; Carr and Frei, 1999). Citric acid is an organic acid and used in canned foods and other food processing industries to reduce pH levels, heat requirements, to provide sourish taste, to prevent color and flavor losses and tarnishing (Dauthy, 1995). Oxalic acid is a free acid and generally encountered as calcium salt (Çalışkan, 2000). Oxalate quantities greatly vary based on plant family and organs, growing conditions, climate and growth periods. When high oxalate-containing vegetables (spinach: 1959 mg/100g) were consumed, oxalate forms insoluble complexes with divalent cations and results in formation of calcium oxalate stone. As compared to those vegetables, peppers have quite low oxalic acid contents.

Ascorbic acid is an important organic acid and is required mostly to prevent scurvy. There are earlier studies indicating decreasing (Subramanian et al., 2006) or increasing (Nahar and Gretzmacher, 2002) ascorbic acid contents with different irrigation levels. Role of proline was also mentioned in ascorbate glutathione cycle including ascorbic acid (Islam et al., 2009; Aggarwal et al., 2011).

Besides the studies indicating insignificant differences in phenolic quantities with water stress, there are also some other studies indicating increasing proline contents, soluble solids content and total phenolics with water stress treatments. Researchers also reported significant correlations of antioxidant activity with total phenols and ascorbic acid content of peppers (Estiarte et al. 1994; Mohamed et al. 2014; Ghasemnezhad et al., 2011).

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Peppers are known to be sensitive to water stress and proline has protective effects against certain stressors. Therefore, this study was conducted to investigate the effects of different irrigation levels and proline treatments on yield parameters, soluble solids content, total phenolics, proline content and organic acids of peppers.

Materials and methods

This study was conducted at the experimental fields of Research and Implementation Center of Çanakkale Onsekiz Mart University Agricultural Faculty in Turkey from 2013 to 2014 (*Figure 1*). Seedlings of Yalova Yağlık 28 pepper cultivar (Asgen Tarım A.Ş.) *(Capsicum annum* L. cv. Yalova Yağlık 28*)* was used as the plant material of the study which is a preferred cultivar by farmers in region especially for it's speciality as an industrial pepper. Yalova yağlık 28 is a mid-season cultivar growing for industy and fresh consumption and avaliable for field or greenhouse cultivation. It has sweet and industrial type fruits which are large and conic. Average fruit weight is 90 g, fruit colour is dark red and fruit flesh thickness is 4.8 mm and these characters strictly infleunced by irrigation.

Figure 1. *Photos from area of experiment, organic acid, total phenolics and internal proline analyzes*

Three different irrigation levels were used in experiments. For different irrigation levels, pan-coefficients (Kcp) were used (Kcp_{50}, Kcp_{100}, Kcp_{150}). For each pan-coefficient, proline treatments (12 mM) were performed at 3 different growth stages.

Cumulative evaporations from Class-A pan placed at experimental site were measured with a depth gauge and irrigations were performed as to have Kcp_{50}= 50%, Kcp_{100}= 100% and Kcp_{150}= 150% of cumulative evaporation at a single irrigation interval (3-day) through constant-pressure drip lines (4 lt/h) (Yıldırım, 1996; Doorenbos and Pruit, 1992). Calculation of irrigation water quantity to be applied is shown at *Table 1*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Table 1. *Calculation of irrigation water quantity to be applied*

N=1000/LS*DS (Eq.1)	**I=Epan*Kcp*P** (Eq.2)	**Ta=1000*dt/q*N** (Eq.3)	**ET=I+P±Δs** (Eq.4)
N: Number of dripper (dripper/da) LS: Lateral spacing (m) DS: Dripper spacing (m)	I: Amount of irrigation water to be applied (mm) Epan: Cumulative evaporation from Class-A pan (mm) Kcp: Plant-Pan Coefficient P: Plant cover percentage (%)	Ta: Irrigation duration (hours) dt: Amount of irrigation water to be applied (I) (mm) q: Dripper discharge (4 lt/h) N: Number of drippers (dripper/da)	ET: Plant water consumption I: Amount of irrigation water to be applied (mm) P: Precipitation (mm) Δs: Change in soil moisture (mm)

Seedlings were divided into three groups as to cover each pan-coefficient. The first group was subjected to proline treatments three times at 10-day intervals with the first treatment applied 20^{th} day of seedling plantation. The second group was subjected to single proline treatment on 30^{th} day of seedling plantation. The third group had also a single proline treatment on 40^{th} day of seedling plantation.

Proline (Merck KGaA 64271 Darmstadt Germany) treatments were applied at the dose of 12 mM through foliar sprays. In the first group, total dose was separated into 3 equal doses and applied at 3 different periods. In the second and third groups, total dose was applied in a single treatment (Silva Sa et al., 2016). Experiments were conducted in randomized blocks split plots experimental design with 3 replications. Each treatment had 5 plant rows with 7 plants in each row (35 plants for each treatment). Experiments were then composed of 1260 (35*12*3) plants and 36 (12*3) plots. Analyses and measurements were performed on 10 plants randomly selected from 15 plants left after removing 20 plants as to consider side effects. Experimental design was separated into 3 blocks and the first replication of each treatment was placed in every block.

Soil samples were taken from 0-30 cm soil profile and a fertilization program was applied based on soil analysis results (*Table 2*).

Table 2. *Soil analysis results*

	2013		**2014**	
Analysis	**Results**	**Assessment**	**Results**	**Assessment**
Saturation (%)	50	Clay-Loam	70	Clay-Loam
pH	7,79	Slightly alkaline	7,76	Slightly alkaline
Lime (%)	11,06	Limy	14,88	Limy
Organic Matter (%)	2,071	Moderate	2,03	Moderate
Salt (%)	0,044	Unsaline	0,93	Unsaline
Phosphorus (P) kg/da-ppm	12,18-48,72	Moderate	12,30-49,2	Moderate
Potassium (K) kg/da-ppm	66,215-264,86	High	64,5-258	High

In the years 2013-2014, Ammonium Nitrate (33% N), Mono Ammonium Phosphate (12% N + 61% P2O5) and Potassium Nitrate (KNO_3) (13% N, 13% N-NO3, 45,5% K2O) fertilizers were applied through drip irrigation (fertigation). Meteorological data for experimental years is shown in *Table 3*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Experimental Treatments

- Kcp_{50}: Without proline treatment
- Kcp_{50}: 4 mM (20th day) + 4 mM (30th day) + 4 mM (40th day)
- Kcp_{50}: 12 mM (30th day)
- Kcp_{50}: 12 mM (40th day)
- Kcp_{100}: Without proline treatment
- Kcp_{100}: 4 mM (20th day) + 4 Mm (30th day) + 4 mM (40th day)
- Kcp_{100}: 12 mM (30th day)
- Kcp_{100}: 12 mM (40th day)
- Kcp_{150}: Without proline treatment
- Kcp_{150}: 4 mM (20th day) + 4 mM (30th day) + 4 mM (40th day)
- Kcp_{150}: 12 mM (30th day)
- Kcp_{150}: 12 mM (40th day)

Table 3. *Meteorological data for experimental years*

Monthly Average Temperature (°C)						
Year/Month	**January**	**February**	**March**	**April**	**May**	**June**
2013	7,9	8,8	10,4	14,0	19,8	23,0
2014	9,3	9,1	10,6	13,9	18,1	22,1
Year/Month	**July**	**August**	**September**	**October**	**November**	**December**
2013	25,7	26,7	21,6	14,8	13,5	6.6
2014	25,5	26,1	21,4	16,1	11,8	10,2
Monthly Average Sunshine Duration (hours)						
Year/Month	**January**	**February**	**March**	**April**	**May**	**June**
2013	95,3	94,8	172,4	237,5	303,9	322,8
2014	73,0	122,9	160,4	174,1	240,9	273,2
Year/Month	**July**	**August**	**September**	**October**	**November**	**December**
2013	376,3	356,6	292,2	245,4	123,3	79,3
2014	244,7	234,8	238,2	155,5	96,0	69,4
Monthly Average Wind Speed (m s^{-1})						
Year/Month	**January**	**February**	**March**	**April**	**May**	**June**
2013	4,3	3,9	2,2	3,1	3,9	3,2
2014	3,7	3,6	3,5	3,4	3,6	2,8
Year/Month	**July**	**August**	**September**	**October**	**November**	**December**
2013	4,0	4,2	2,8	3,2	3,6	3,5
2014	3,2	3,4	3,0	4,0	2,9	3,4
Monthly Average Precipitation (mm=kg m^{-2}) MANUEL						
Year/Month	**January**	**February**	**March**	**April**	**May**	**June**
2013	167,4	141,6	59,0	90,3	5,6	21,7
2014	54,2	1,0	80,4	101,4	27,0	75,4
Year/Month	**July**	**August**	**September**	**October**	**November**	**December**
2013	0,2	-	10,2	92,7	50,8	10,7
2014	33,3	8,0	66,6	44,4	109,2	154,4

Investigated parameters

Yield (g m^{-2})

Fruit weights were measured with a precise balance (± 0.01) (GM2202, Sartorius, Göttingen, Germany) and total yield of each treatment was determined.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Total Soluble Solids Content (%)

Atago PAL 1 model digital hand refractometer (Pal-1, Atago, Tokyo, Japan) was used to determine soluble solids content (%).

Total Phenolics (mg/100g)

Total phenolics of the samples were determined from 5 g fruit puree in accordance with Folin-Ciocalteu method at 765 nm absorbance value of a Shimadzu UV-VIS spectrophotometer (UV-Vis Spectrophotometer, Shimadzu Corporation, Tokyo-Japan) (mg/100 g). For each sample, 5 g fruit juice was supplemented with 5 ml Methanol. The mixture was centrifuged at 4000 rpm for 10 minutes. Samples were then supplemented with 2,5 ml 10% Folin-Ciocalteu and 2 ml 1 M Na_2CO_3 and this mixture was kept in hot-water bath at 45°C for 15 minutes. Samples were removed from the hot-water bath and readings were performed at 765 nm absorbance value of a spectrophotometer against 10% Folin-Ciocalteu and results were expressed in total gallic acid equivalent (GAE) mg/100 g (Zheng and Wang, 2001).

Internal Proline Content (µmol/g)

For proline contents, 0.5 g fresh leaf samples were finely chopped and homogenized in 10 ml 3% 5-Sulphuric acid for 2 minutes. Resultant homogenate was filtered through Whatman No. 2 filter paper and placed into tubes. About 2 ml of filtrate was supplemented with 2 ml ninhydrine and 2 ml glacial acid in a test tube and put into reaction in a water bath at 100 °C for 1 hour. Then the test tubes were taken into ice-bath and reaction was terminated. Samples were then supplemented with 4 ml toluene and reaction mixture was mixed in a tube-mixer for 15-20 seconds. The chromophore phase was aspired with a thin-probe pipette into spectrophotometer tubes. When reached to room temperature, chromophore-containing toluene tubes were then subjected to absorbance readings in Shimadzu UV-VIS spectrophotometer (UV-Vis Spectrophotometer, Shimadzu Corporation, Tokyo-Japan) at 520 nm. Toluene was used as the control (Bates et al., 1973).

HPLC analysis for organic acids (mg/100g)

The simultaneous determination of oxalic, tartaric, malic, lactic, acetic, citric, succinic acids and ascorbic acids using liquid chromatography was carried out according to (Arnetoli et al., 2008). The chromatography analysis was carried out using a HPLC system (Shimadzu, Japan). The equipments of HPLC system consist of LC-20AD SP pump, SIL- 10AP Auto sampler, SCL-10A vp system controller, SPD-20A Prominence UV detector, CTO-20AC sp column oven and LC solution (version: 1.23 sp1) software. An Inertsil ODS-III C18 column (46x150 ID, 5 µm particle size) was used for the chromatographic separation. The mobile phase was carried out with 125 mM KH2PO4 adjusted to pH 2.5 with o-phosphoric acid. The flow rate of mobile phase was performed as 1 ml/min. The wavelengths of the UV detection were performed at 210 nm for oxalic, tartaric, malic, lactic, acetic, citric, succinic acids, and 254 nm for ascorbic acid.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Statistical analysis

Experimental data were subjected to variance analysis with SAS.9.1.3 software. Significant means were compared with LSD test. Biplot analysis was used for interpretation of organic acids data.

Results

Yield (g m^{-2})

Table 4. *Effects of different irrigation levels and proline treatments on fruit yields (g m^{-2}) (P- = Treatments without proline, P1= Proline treatments on 20, 30 and 40th days, P2= Proline treatments on 30th day, P3= Proline treatments on 40th day)*

Yield (g m^{-2})					
			1st year		
Treatments	**P- (1,5,9)**	**P1 (2,6,10)**	**P2 (3,7,11)**	**P3 (4,8,12)**	**Kcp mean**
Kcp1=0.5	1003 d	1110 c	1113 c	1121 c	1087 C
Kcp2=1	1676 b	1680 b	1684 b	1692 b	1683 B
Kcp3=1.5	1862 a	1866 a	1868 a	1875 a	1868 A
P mean	1514 B	1552 A	1555 A	1563 A	
			2nd year		
Kcp1=0.5	1083 d	1199 c	1210 c	1213 c	1176 C
Kcp2=1	1809 b	1818 b	1826 b	1821 b	1818 B
Kcp3=1.5	2044 a	2055 a	2047 a	2059 a	2051 A
P mean	1645 B	1691 A	1694 A	1697 A	
			MEAN		
Kcp1=0.5	1043 d	1154 c	1162 c	1167 c	1131 C
Kcp2=1	1742 b	1750 b	1755 b	1756 b	1751 B
Kcp3=1.5	1953 a	1961 a	1957 a	1967 a	1959 A
P mean	1579 B	1621 A	1625 A	1630 A	
Yield in 2013 (g/m2): P×Kcp <0.01 LSD=66.624; Kcp <0.01 LSD=82.179; P <0.01 LSD=33.221					
Yield in 2014 (g/m2): P×Kcp <0.01 LSD=50.553; Kcp<0.01 LSD=33.049; P<0.01 LSD=30.972					
Mean yield (g/m2): P×Kcp <0.01 LSD=44.649; Kcp <0.01 LSD=49.883; P <0.01 LSD=23.638					

In the first year of the experiments, fruit yield per plant of irrigation treatments were gathered under three groups. Yields increased with increasing irrigation levels (*Table 4*). With regard to effects of proline treatments on fruit yields, treatments were gathered under two groups. Proline treatments yielded similar values, but greater values than the control treatments. With regard to effects of interactions (irrigation x proline) on fruit yields, interactions were gathered under 4 groups. In treatments without proline (1, 5, 9), increasing fruit yields were observed with increasing irrigation levels (Kcp50=397,48, Kcp100=663,67, Kcp150=737,58).

In treatments without proline, similar yield values were observed at the same irrigation levels.

Similar statistical groups were observed in the second year of the experiments and for the mean values of years.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Soluble Solids Content, SSC (%)

In the first year of the experiments, soluble solids content (SSC) of irrigation treatments were gathered under three groups and increasing SSC values were observed with decreasing irrigation levels (*Table 5*). The differences in SSC values of the proline treatments were not found to be significant. With regard to effects of irrigation x proline interactions on SSC values, interactions were gathered under three groups and increasing SSC values were observed with decreasing irrigation levels (Kcp_{50}= 9,4%, Kcp_{100}= 8,1%, Kcp_{150}= 7,8%)..

The differences in SSC values of the treatments with and without proline were not found to be significant at the same irrigation level.

Similar statistical groups were observed in the second year of the experiments and for the mean values of years

Table 5. *Effects of different irrigation levels and proline treatments on soluble solids content (%) (P- = Treatments without proline, P1= Proline treatments on 20, 30 and 40th days, P2= Proline treatments on 30th day, P3= Proline treatments on 40th day, N.S.: Not-significant)*

Soluble Solids Content, SSC (%)					
1st year					
Treatments	**P- (1,5,9)**	**P1 (2,6,10)**	**P2 (3,7,11)**	**P3 (4,8,12)**	**Kcp mean**
Kcp1=0.5	9.4 a	9.5 a	9.4 a	9.4 a	9.4 A
Kcp2=1	8.1 b	8.2 b	8.2 b	8.2 b	8.1 B
Kcp3=1.5	7.8 c	7.8 c	7.8 c	7.8 c	7.8 C
P mean	8.4	8.5	8.4	8.5	
2nd year					
Kcp1=0.5	8.4 a	8.5 a	8.5 a	8.5 a	8.5 A
Kcp2=1	7.6 b	7.6 b	7.6 b	7.6 b	7.6 B
Kcp3=1.5	7.3 c	7.3 c	7.3 c	7.3 c	7.3 C
P mean	7.8	7.8	7.8	7.8	
MEAN					
Kcp1=0.5	8.9 a	9 a	9 a	9 a	9 A
Kcp2=1	7.9 b	7.9 b	7.9 b	7.9 b	7.9 B
Kcp3=1.5	7.6 c	7.5 c	7.6 c	7.6 c	7.6 C
P mean	8.1	8.2	8.1	8.2	
SSC in 2013 (%): P×Kcp <0.01 LSD=0.284; Kcp <0.01 LSD=0.263; P <0.05 LSD=N.S.					
SSC in 2014 (%): P×Kcp <0.01 LSD=0.2136; Kcp <0.01 LSD=0.1366; P <0.05 LSD=N.S.					
Mean SSC (%): P×Kcp <0.01 LSD=0.1777; Kcp <0.01 LSD=0.1981; P <0.05 LSD=N.S.					

Total Phenolics (GAE mg/100g)

In the first year of the experiments, total phenolics of irrigation treatments were gathered under three different groups and decreasing total phenolics were observed with increasing irrigation levels (*Table 6*). Considering the mean values of proline treatments, it was observed that treatments were gathered under two different groups and proline-treated group had greater total phenolics than the untreated group.

With regard to effects of irrigation x proline interactions on total phenolics, interactions were gathered under three different groups and increasing total phenolics were observed with decreasing irrigation levels in treatments without proline (Kcp_{150}=1317,9, Kcp_{100}=1383,1, Kcp_{50}=1447,3 mg GAE/100g). In Kcp_{50} irrigation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

treatments, total phenolics had similar values in proline-treated groups (2, 3, 4) and such values were greater than non-proline-treated group (1). In Kcp_{100} irrigation treatments, proline-treated and untreated groups (5, 6, 7, 8) had similar total phenolics. In Kcp_{150} irrigation treatments, a similar case was observed in proline-treated and untreated groups (9, 10, 11, 12). Similar statistical groups were observed in 2014 and for the mean values of the years.

Table 6. *Effects of different irrigation levels and proline treatments on total phenolics (GAE mg/100g) (P- = Treatments without proline, P1= Proline treatments on 20, 30 and 40th days, P2= Proline treatments on 30th day, P3= Proline treatments on 40th day.)*

Total Phenolics (GAE mg/100g)					
1st year					
Treatments	**P- (1,5,9)**	**P1 (2,6,10)**	**P2 (3,7,11)**	**P3 (4,8,12)**	**Kcp mean**
Kcp1=0.5	1447.3 b	1497 a	1505.7 a	1502.9 a	1488.2 A
Kcp2=1	1383.1 c	1393.3 c	1391.6 c	1392.4 c	1390.1 B
Kcp3=1.5	1317.9 d	1319.8 d	1320.8 d	1316.8 d	1318.8 C
P mean	1382.7 B	1403.4 A	1406 A	1404 A	
2nd year					
Kcp1=0.5	1418.3 b	1482.4 a	1479.7 a	1478.4 a	1464.7 A
Kcp2=1	1346.7 c	1349.8 c	1352.5 c	1347.5 c	1349.1 B
Kcp3=1.5	1292.4 d	1294.1 d	1297 d	1295.3 d	1294.7 C
P mean	1352.4 B	1375.5 A	1376.4 A	1373.7 A	
MEAN					
Kcp1=0.5	1432.8 b	1489.7 a	1492.7 a	1490.7 a	1476.5 A
Kcp2=1	1364.9 c	1371.6 c	1372.1 c	1369.9 c	1369.6 B
Kcp3=1.5	1305.2 d	1307 d	1308.9 d	1306 d	1306.8 C
P mean	1367.6 B	1389.4 A	1391.2 A	1388.9 A	
Total phenolics in 2013 (mg/100g): P×Kcp <0.01 LSD=46.11; Kcp <0.01 LSD=65.229; P <0.01 LSD=20.261					
Total phenolics in 2014 (mg/100g): P×Kcp <0.01 LSD=41.068; Kcp <0.01 LSD=36.805; P <0.05 LSD=17.267					
Mean total phenolics (mg/100g): P×Kcp <0.01 LSD=31.799; Kcp <0.01 LSD=44.478; P <0.01 LSD=14.159					

Internal Proline Content (µmol/g)

In both years and for the average of years, effects of irrigation treatments on internal proline contents were gathered under 3 different groups and increasing internal proline contents were observed with decreasing irrigation levels (*Table 7*). With regard to effects of proline treatments on internal proline contents in the first year of the experiments, treatments were gathered under 4 different groups and proline-treated groups had greater internal proline contents than the untreated groups. In the second year of the experiments, proline treatments gathered under 3 different groups and again proline-treated groups had greater internal proline contents than the untreated groups. In the years 2013 and 2014 and in average of years, the lowest internal proline content in proline treatments was obtained from the treatment with proline treatments on 40^{th} day.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Table 7. *Effects of different irrigation levels and proline treatments on internal proline contents (µmol/g) (P- = Treatments without proline, P1= Proline treatments on 20, 30 and 40th days, P2= Proline treatments on 30th day, P3= Proline treatments on 40th day.)*

Internal Proline Content (µmol/g)					
1st year					
Treatments	**P- (1,5,9)**	**P1 (2,6,10)**	**P2 (3,7,11)**	**P3 (4,8,12)**	**Kcp mean**
Kcp1=0.5	67.37 c	96.81 a	87.81 b	90.87 ab	85.71 A
Kcp2=1	33.84 f	43.31 e	50.69 d	42.55 e	42.6 B
Kcp3=1.5	13.58 h	22.52 g	29.63 f	21.14 g	21.72 C
P mean	38.26 C	54.21 AB	56.04 A	51.52 B	
2nd year					
Kcp1=0.5	58.6 c	88.73 a	84.23 ab	79.61 b	77.79 A
Kcp2=1	28.76 ef	42.15 d	44.11 d	35.8 de	37.7 B
Kcp3=1.5	12.65 h	22.01 fg	23.79 fg	19.64 gh	19.52 C
P mean	33.34 C	50.96 A	50.71 A	45.02 B	
MEAN					
Kcp1=0.5	62.99 c	92.77 a	86.02 b	85.24 b	81.75 A
Kcp2=1	31.3 f	42.73 de	47.4 d	39.18 e	40.15 B
Kcp3=1.5	13.12 h	22.26 g	26.71 fg	20.39 g	20.62 C
P mean	35.8 C	52.59 A	53.37 A	48.27 B	
Internal proline content in 2013 (µmol/g): PR×Kcp <0.01 LSD=6.763; Kcp<0.01 LSD=3.2089; PR<0.01 LSD=4.2707					
Internal proline content in 2014 (µmol/g): PR×Kcp <0.01 LSD=8.7853; Kcp<0.01 LSD=5.1097; PR<0.01 LSD=5.4559					
Mean internal proline content (µmol/g): PR×Kcp <0.01 LSD=6.626; Kcp<0.01 LSD=2.6893; PR<0.01 LSD=4.2207					

In the first year of the experiments, in non-proline-treated groups (1, 5, 9), the greatest internal proline content (67.37 µmol/g) was obtained from Kcp_{50} irrigation level and the lowest value (13.58 µmol/g) was obtained from Kcp_{150} irrigation level (Yooyongwech et al., 2013). Similarly in proline treatments on 20th day (2, 6, 10), internal proline contents decreased with increasing irrigation levels (Kcp_{50}=96.81 µmol/g, Kcp_{150}=22.52 µmol/g). Decreasing proline contents were observed with increasing irrigation levels also on 30th day proline treatments (3, 7, 11) and 40th day proline treatments (4, 8, 12). In Kcp_{50} irrigation level, proline-treated groups (2,3,4) (Kcp_{50}=96.81 µmol/g, Kcp_{50}=87.81 µmol/g, Kcp_{50}=90.87 µmol/g) had greater values than untreated group (1) (Kcp_{50}=67.37 µmol/g). The proline content of the treatment with proline treatments on 20, 30 and 40th days (2) was significantly greater than the proline content of the other proline treatments at Kcp_{50} irrigation level and it was the greatest value at all. In Kcp_{100} irrigation level, proline-treated groups (6,7,8) (Kcp_{100}=43.31 µmol/g, Kcp_{100}=50.69 µmol/g, Kcp_{100}=42.55 µmol/g) had greater proline contents than the untreated group (5) (Kcp_{100} =33.84 µmol/g). Similarly in Kcp_{150} irrigation level, proline-treated groups (10, 11, 12) (Kcp_{150}=22.52 µmol/g, Kcp_{150}=29.63 µmol/g, Kcp_{150}=21.14 µmol/g) had greater proline contents than the untreated group (9) of the same irrigation level (Kcp_{150}=13.58 µmol/g). In Kcp_{100} and Kcp_{150} irrigation levels, 30th day proline treatments (7, 11) (Kcp_{100}=50.69 µmol/g, Kcp_{150}=29.63 µmol/g) had greater values than the other proline treatments of the same levels (6, 10, 8, 12) (Kcp_{100}= 43.31 µmol/g, Kcp_{150}=22.52 µmol/g, Kcp_{100}=42.55 µmol/g, Kcp_{150}=21.14 µmol/g). Similar statistical groups were observed in the second year of the experiments and for the mean values of years.

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):3305-3323.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Organic Acid Compositions

Effects of different irrigation levels and proline treatments on organic acid composition were assessed through Biplot analysis. Two principle components (PC1 – 1st principle component; PC2 – 2nd principle component) were used to generate biplot graph (*Figure 2*). Organic acid composition values under different irrigation levels and proline treatments in the first year of the experiments (mg/100g) is also shown in *Table 8*.

In the first year of the experiments, PC1 and PC2 scores for 3 organic acids were able to explain 92% of 3 irrigation levels and proline treatment interactions (*Table 8*).

In biplot graph, over the citric acid axis, the Kcp50 irrigation level without proline treatment (1) was the furthest treatment to ascorbic acid axis and such a case indicated that this treatment had quite low ascorbic acid content and high citric acid content. Increasing citric acid contents were reported with drought stress (Sağlam et al., 2010).

Table 8. *Organic acid compositions under different irrigation levels and proline treatments in the first year of the experiments (mg/100g)*

Treatments	Ascorbic Acid	Oxalic Acid	Citric Acid
1	84.54	34.62	486.42
2	171.84	26.30	311.90
3	192.99	27.77	441.20
4	130.43	56.32	260.15
5	175.48	37.83	226.11
6	142.45	28.19	477.73
7	183.92	19.33	380.05
8	191.11	15.21	315.10
9	198.92	20.77	212.79
10	161.55	18.84	274.32
11	185.83	23.27	268.62
12	195.19	24.42	285.22

Considering the treatments without proline (9, 5, 1), at Kcp50 irrigation level, treatment (1) was placed on positive side of oxalic acid axis (PC1>0, PC2>0), negative side of ascorbic acid axis (PC1<0) and on citric acid axis (PC1>0, PC2>0), at Kcp100 and Kcp150 irrigation levels, treatments (5,9) were placed on positive side of ascorbic acid axis and negative side of citric acid axis (PC1<0, PC2<0). Such cases indicated that these treatments (5,9) had greater ascorbic acid and smaller citric and oxalic acid contents than the treatment (1) at Kcp50 irrigation level. As compared to treatment (5) without proline at Kcp100 irrigation level, the treatment (9) without proline at Kcp150 irrigation level was close to ascorbic acid axis and far from citric and oxalic acid axes. Such a case indicated that treatment (9) had greater ascorbic acid and lower oxalic and citric acid contents than the treatment (5).

At Kcp50 irrigation level, 40th day proline treatment (4) was the closest treatment to oxalic acid axis. Such a case indicated that this treatment had the greatest oxalic acid content.

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At Kcp50 irrigation level, the proline treatments on 20, 30 and 40th days (2) was placed on graph origin (*Figure 1*). Such a case indicated that this treatment had quite less variations in all three organic acids than the other treatments. The treatment (1) without proline at Kcp50 irrigation level, treatment (4) with proline treatments on 40th day at Kcp50 irrigation level and treatment (6) with proline treatments on 20, 30 and 40th days at Kcp100 irrigation level were placed further from the ascorbic acid axis as compared to the other treatments. Such a case indicated that these treatments (1, 4, 6) had lower ascorbic acid contents than the other treatments.

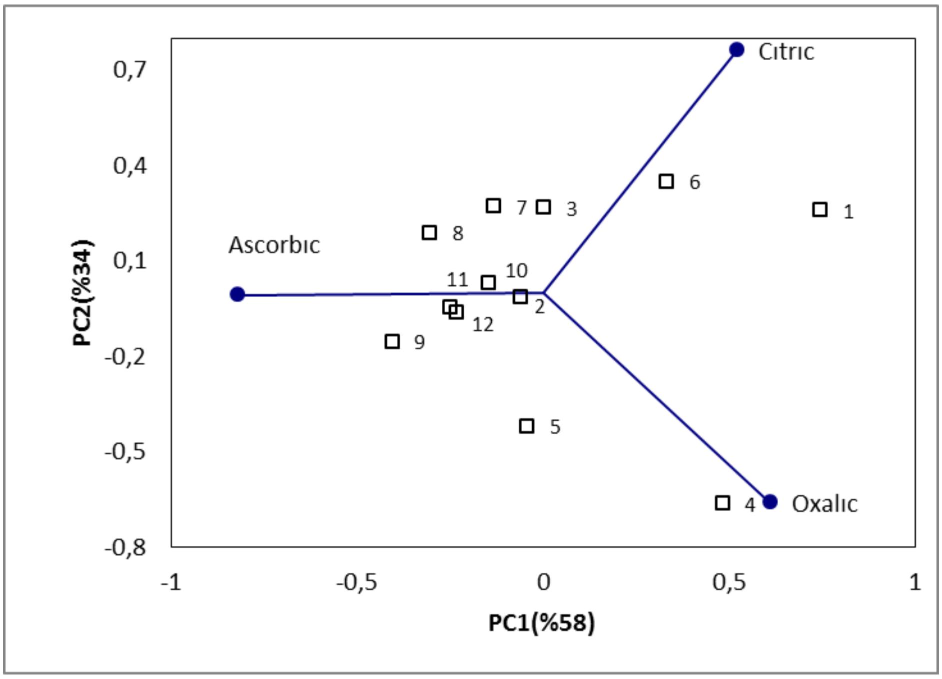

Figure 2. *Biplot for the effects of different irrigation levels and proline treatments on organic acid compositions in the first year of the experiments*

In the second year of the experiments, PC1 and PC2 scores for 3 organic acids were able to explain 94% of 3 irrigation levels and proline treatment interactions (*Figure 3*). Organic acid composition values under different irrigation levels and proline treatments in the second year of the experiments (mg/100g) is also shown in *Table 9*.

Considering the treatments without proline (9,5,1), Kcp_{50} irrigation level (1) was further placed to ascorbic acid axis and closer to citric and oxalic acid axes than the Kcp_{100} and Kcp_{150} irrigation levels (5,9) and Kcp_{150} irrigation level (9) was placed on negative side of citric acid axis (PC1<0, PC2<0). The closest location of Kcp_{150} irrigation level with proline treatments on 40th day (12) to ascorbic acid axis indicated that this treatment had the greatest ascorbic acid content. Again, the closest location of Kcp_{50} irrigation level with proline treatments on 40th day (4) to oxalic acid axis indicated that this treatment had the greatest oxalic acid content.

The Kcp_{100} with proline on 20th day (6), Kcp_{100} with proline on 40th day (8), Kcp_{150} (9), Kcp_{150} with proline on 20th day (10) and Kcp_{150} with proline on 30th day (11) were placed on the negative side of citric acid axis. Such cases indicated that these treatments had lower citric acid contents. The Kcp_{50} with proline on 20th day (2), Kcp_{50} with proline on 30th day (3) and Kcp_{100} with proline on 30th day (7) were placed on positive

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

side of citric acid axis and such cases indicated that these treatments had greater citric acid contents.

Table 9. *Plant organic acid compositions under different irrigation levels and proline treatments in the second year of the experiments (mg/100g)*

Treatments	Ascorbic Acid	Oxalic Acid	Citric Acid
1	142.29	32.09	492.49
2	170.60	28.37	478.87
3	245.42	26.66	512.37
4	119.14	37.24	422.87
5	271.47	25.53	378.87
6	236.91	24.54	325.82
7	251.99	25.88	447.00
8	234.87	25.81	307.53
9	248.67	26.20	301.73
10	232.13	17.86	253.65
11	253.66	24.21	268.41
12	273.92	15.78	316.81

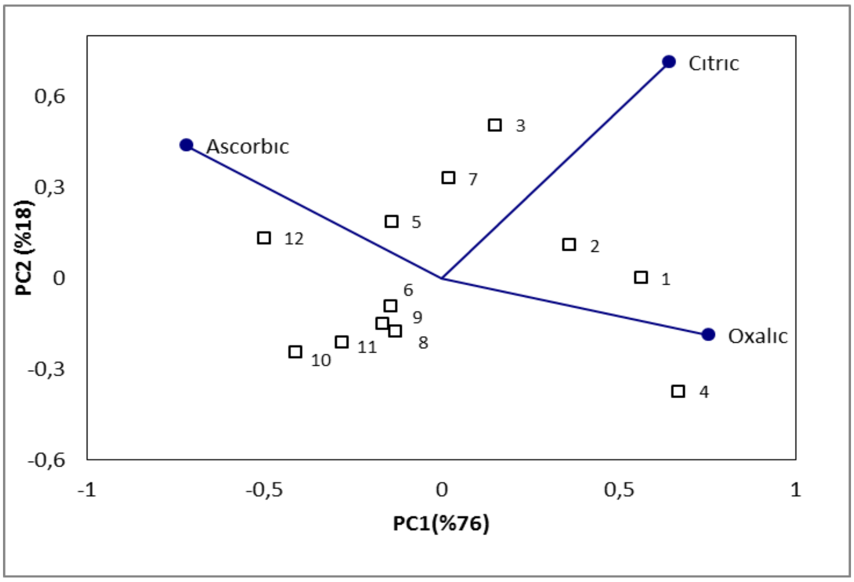

Figure 3. *Biplot for the effects of different irrigation levels and proline treatments on organic acid compositions in the second year of the experiments*

Discussion

Present findings revealed that the increase in yield achieved with increasing irrigation levels from Kcp50 to Kcp100 was greater than the increase achieved with increasing irrigation levels from Kcp100 to Kcp150. Decreasing yields were also reported in pepper, tomato and eggplant with water deficits (Kırnak et al., 2016;

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DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Mitchell et al., 1991; Kırnak et al., 2001). As approached to maximum yield, water use efficiencies decreased and such a case may be related to prevailing climate conditions. Excessive water uses in irrigation is considered as the primary reason of water deficits, thus it was thought that water-yield relations would play significant roles in future water deficits. At Kcp50 irrigation level, an increase was observed in yields with proline treatments, but yields of proline-treated and untreated groups were similar at the other irrigation levels. Such findings indicated that proline treatments were more effective in deficit irrigation conditions.

Farzana (2014) carried out a study with wheat cultivars (BARI Gom-24, BARI Gom-26) and reported increasing yields with proline treatments under water deficit, but reported insignificant differences in yields of BARI Gm-24 cultivar with proline treatments under non-water deficit conditions and increasing values in some parameters of BARI Gom-26 cultivar with proline treatments. Increasing yields were reported in tomatoes with proline treatments under salt stress (Öztekin, 2009).

Soluble solids contents (SSC) increased with decreasing irrigation levels, but did not changed significantly with proline treatments. Generally, high SSC values are desired in industrial-type peppers. Either unchanged or increasing SSC values were reported in peppers and tomatoes with drought stress (Shao et al., 2014; Mitchell et al., 1991; Dağdelen et al., 2004). Dorji et al. (2005) applied deficit irrigation and relative root zone dry out treatments to chili pepper (Ancho St. Luis) and reported 21% increase in SSC values in green and red periods with deficit irrigation. Soluble solids content of tomatoes increased, decreased or remained unchanged with proline treatments under salt stress (Öztekin, 2009). Similar statistical groups were observed in 2014 and for the average of the years. The lowest SSC value (7,3%) obtained from Yalova Yağlık-28 pepper cultivar was greater than the minimum values specified for red peppers (6-6,5%) (Tadesse et al., 2002) and such a case indicated that SSC values of this pepper cultivar will not pose any problems for the producers.

Proline is a non-enzymatic antioxidant (Hossain et al., 2014) and accumulation of proline-like osmatic regulators in cells reduces drought-induced damages in plants (Rontein et al., 2002). Significant correlations were reported between antioxidant capacity and total phenolics of the plants (Li et al., 2008). Anti-radical and antioxidant effects of phenolic acids were also reported (Sroka and Cisowski, 2003) and different free radical-scavenging effects of different phenolics were indicated in previous studies (Aboul-Enein et al., 2007). Nunez Ramirez et al. (2011) investigated the effects of different nitrogen doses (32, 80, 160, 320 kg N ha^{-1}) on Habanero chili pepper (*Capsicum chinense jacq.*) plants and reported increasing TETA values, but unchanged antioxidant activity and total phenols with increasing nitrogen doses. About 86% increase was reported in total phenolics of sugar beet with water stress (Stagnari et al., 2014).

Internal proline contents increased with decreasing irrigation levels. Such a case indicated a correlation between proline treatments and adaptation to stress conditions. Proline treatments at Kcp50 irrigation level contributed plant adaptation to water stress. Besides proline contents, phenolics playing great roles in prevention of various diseases and contributing fruit color and taste parameters also increased with decreasing irrigation levels.

Ascorbic acid contents increased and oxalic acid contents decreased with increasing irrigation levels. Fluctuations were observed in ascorbic acid contents with proline treatments. In both years of the experiments, the Kcp50 irrigation with proline

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DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

treatments on 20, 30, 40th days and on 30th day (2, 3) had greater ascorbic acid contents than Kcp50 irrigation without proline treatments (1). Such a case indicated positive effects of proline treatments on ascorbic acid contents at this irrigation level. Such an increase in ascorbic acid contents with proline treatments was attributed to increasing enzyme activities in ascorbate glutathione cycle (Islam et al., 2009). Decreasing ascorbate contents were reported in tomatoes with drought stress (Ünyayar et al., 2005) and increasing ascorbic acid contents were reported in wheat (Chakraborty and Pradhan, 2012) and tomatoes (Shao et al., 2014) again with drought stress. In a study carried out with soybean, ascorbic acid contents started to increase from the second day of water deficit and started to decrease after the 8th day of water deficit (Angra et al., 2010).

In another study on tomatoes, ascorbic acid contents were similar in normal and slight drought treatments, but greater in moderate and severe drought treatments (Subramanian et al., 2006). However, it was observed in this study that increasing ascorbic acid contents were also related to water deficit conditions, treatment periods and doses. Such cases indicated in general that citric and oxalic acid contents decreased and ascorbic acid contents increased with increasing irrigation levels. Timpa et al. (1986) indicated that 2-3 times increase in citric acid under drought stress pointed out osmatic regulation. Emam et al. (2014) reported increasing oxalic acid contents in paddy with drought stress treatments. Moreover, decreasing ascorbic acid content (Osuagwu & Edeoga 2012, Subramanian et al. 2006) and increasing oxalic acid contents (Emam et al. 2014) were reported with water stress.

In treatments without proline, ascorbic acid content at the lowest irrigation level (Kcp50) was lower than the ascorbic acid contents of the other irrigation levels. Such a case indicated that irrigation quantities below certain levels reduced ascorbic acid quantities, which is a significant quality parameter with various positive impacts on human health. Citric acid contents increased in both years with decreasing irrigation levels. Fluctuations were observed in citric acid contents with proline treatments.

Conclusions and recommendations

In this study, which investigated the effect of proline applications on pepper cultivated with different irrigation levels, the yield was increased with increasing the amount of water. In addition, many quality parameters have also increased. In addition, proline applications had a positive effect on these parameters at the irrigation level with the least amount of water applied, but the effect of proline was found to be decreased at higher irrigation levels. As the irrigation level increased, ascorbic and citric acid values increased and oxalic acid values decreased. It has been determined that proline applications generally have a positive effect on increasing the ascorbic acid value in the subjects where irrigation level is decreased. It can be thought that proline may be effective in improving yield and some other yield and quality parameters, especially in regions with limited irrigation opportunities. High citric acid content is considered as an advantage for the peppers used in canned food industry. Citric acid contents increased with decreasing irrigation levels, thus it was concluded that lower irrigation levels (Kcp50) may be recommended for pepper fruits more available for canned food production. However, more detailed studies on this subject will also help especially the producers producing pepper processed in industry. For this purpose, it will be useful to perform studies in different cultivars.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

Acknowledgements. This study is derived from Tolga Sarıyer's PhD Thesis. Authors wish to thank to Prof. Dr. Mevlut Akçura for performing and interpreting the biplot analyses of organic acids.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33053323

ANALYSIS OF HYDROLOGICAL DROUGHT TRENDS IN THE GAP REGION (SOUTHEASTERN TURKEY) BY MANN-KENDALL TEST AND INNOVATIVE ŞEN METHOD

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(Received 10th Dec 2018; accepted 14th Feb 2019)

Abstract. The analysis of drought trends plays a significant role in the efficient water resource management, especially in arid and semi-arid regions. Assessing the drought trends by drought indices, of which the Standardized Precipitation Index (SPI) is the most popular, is a reasonable way, because the drought indices measure the climatic anomalies through their severity, duration and frequency. In this study, the possible hydrological drought trends in the GAP region (Southeastern Turkey) were investigated by the use of 12- and 24-month SPI series. The SPI series were determined by 48-year-long (1970-2017) monthly precipitation data monitored at nine selected stations (Mardin, Adıyaman, Batman, Diyarbakır, Siirt, Şırnak, Gaziantep, Kilis and Şanlıurfa) in the GAP region. In the analyses, a recent graphical trend detection method entitled Innovative Şen Metod (ISM) and Mann-Kendall trend test (MK) were used. The ISM has non-parametric basis without any restrictive assumption, moreover, its application is rather simple when compared with the other trend identification methods. The ability to identify the trends of low, medium and high values of a series is the innovative side of the ISM. Since the significance test is a crucial factor to identify the possible trends scientifically, the results were statistical tested at the 0.05 significance level in the methods. The ISM and MK test both revealed that, Mardin is the most vulnerable province in the region, due to the significant decreasing trend, which determined by 12-month and 24-month SPI values. Şanlıurfa, Kilis, Siirt, Diyarbakır and Batman are second order drone prone provinces. In accordance with the results of the ISM, there is an increasing trend in normal and wet conditions in Gaziantep, Şırnak and Adıyaman. Specifically, the results highlight that the central of the GAP region has a tendency toward the heavier droughts in the future. It is also concluded that the ISM is a valuable tool with its simplicity as well as the inherent properties to deduce qualitative information.
Keywords: *standardized precipitation index, non-parametric test, 1:1 straight line, graphical technique, sub-trends*

Introduction

In recent decades, managing the water resources has become a major concern due to the impacts of climate change, which is generally characterized by increasing air temperatures and the variations in occurrence frequency and magnitude of the precipitation. Since the changing hydrological system components are not distributed uniformly, the distribution of hazardous events such as floods and droughts also varies around the world. Among these natural disasters, droughts play a more significant role due to its inherent direct effects on socio-economic development and environmental degradation. Therefore, understanding drought phenomena is paramount for the appropriate planning and management of water resources (Yevjevich et al., 1983). However, drought is a complicated phenomenon and is among the least understood natural hazards due to its multiple causing mechanisms or contributing factors operating at different temporal and spatial scales (Kiem et al., 2016).

The general approach to quantify the droughts is using indices. World Meteorological Organization (WMO) and Global Water Partnership (GWP) has given a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33253342

wide and detailed literature and findings of drought indices for researchers in the domains of meteorology, hydrology, agricultural research and application, remote sensing, and water resources management (WMO and GWP, 2016). Using the drought indices, several researchers have analysed drought events in several parts of the world. Among the large, and still increasing number of researches, the most recent ones were Jang (2018), Buttafuoco et al. (2018), Tigkas et al. (2018), and Zhang et al. (2018).

Undoubtedly, the most well-known drought index, the Standardized Precipitation Index (SPI), suggested by McKee et al. (1993), is the most popular among the existing indices for the estimation of drought severity. One of the main advantages of SPI is that it use only precipitation data with easy calculation steps as well as flexible time scales (e.g. 1, 3, 6, 12, 24 months). Hayes et al. (2011) concluded none of the indices has the universal applicability of the SPI, and WMO has recommended the SPI as the main meteorological drought index that countries should use to monitor and follow drought conditions. It has been extensively applied in different countries (Saada and Abu-Romman, 2017; Logan et al., 2010; Manatsa et al., 2010; Patel and Yadav, 2015; Raziei et al., 2009; Zhai and Feng, 2009).

On the other hand, investigations on variations of the drought characteristics are particularly important in identifying and understanding the impacts of climate change on the hydrologic cycle. To this end, the SPI trend analyses, which provide scientific information for better modeling, prediction, and control mechanism of the phenomenon concerned, are the essential efforts (Golian et al., 2015; Zhai et al., 2010; Caloiero, 2018).

There has been a wide range of researches subject to trend analyses on hydro-climate data in the literature (Fathian et al., 2014; Addisu et al., 2015; Chattopadhyay and Edwards, 2016; Bacanli, 2017; Onyutha, 2015; Zhao et al., 2015); however, they principally base on nonparametric tests, as the Mann–Kendall (MK) analysis (Mann, 1945; Kendall, 1975), the Spearman's rho (Yue et al., 2002) and the Sen trend slope calculation methodology (Sen, 1968). As Mohorji et al. (2017) concluded these objective approaches necessitate a set of assumption validity in the historical records (e.g. independent serial correlation structure, probability distribution functions, seasonality) and did not specify if low or high precipitation contributed to the detected trends (Caloiero et al., 2018). However, almost all the hydro-climate records have significant serial correlations at least on short memory basis, and efficient, effective, and optimum management of water resources requires identification of trends not only monotonically over a given time period but also whether the low, medium, and high values have separate trends (Şen, 2012, 2017). For this reason, Şen (2012) proposed the Innovative Şen Metod (ISM), which has found wide application in hydrology researches (Haktanir and Çitakoglu, 2014; Öztopal and Şen, 2017; Sonali and Kumar, 2013; Dabanli et al., 2016; Mishra and Coulibaly, 2014; Timbadiya et al., 2013). The ISM does not require any assumptions, and it base on the comparison of the two ascendingly ordered halves from the original time series.

The Southeastern Anatolia Project (in Turkish the GAP) is one of the most ambitious regional development projects in Turkey encompassing not only hydropower and irrigation infrastructure development, but also all related sectors including industry, transportation, rural and urban infrastructure, environmental protection and social sectors (Kankal et al., 2016). Therefore, an integrated approach that especially focuses on water resources is essential to achieve the planned objectives. In the GAP region, assessment of climate change impacts (e.g. floods and droughts) on water resources

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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plays a crucial role, and climate change models has vital importance on achieving the project targets. To this end, trend analyses, which provide scientific information for better modeling, better prediction and better control mechanism of the phenomenon concerned, are the essential efforts.

This study aims to identify the drought-prone areas in the GAP region through the use of the SPI time series obtained for different timescales and by the Innovative Şen Metod (ISM) that allows the trend identification of the low, medium, and high values. First, the SPI values obtained at various time scales (12 and 24 months) based on 48-year-long (1970-2017) precipitation data at nine selected stations (Mardin, Adıyaman, Batman, Diyarbakır, Siirt, Şırnak, Gaziantep, Kilis and Şanlıurfa) in the GAP region and then the SPI series were investigated by using the ISM and the results were compared with MK test results.

Study area and data

The Southeastern Anatolia Project region, in Turkish GAP region, spreads along 75 10^3 km^2 (9.6% of Turkey's surface area, 780.6 10^3 km^2) and includes nine provinces of Mardin, Adıyaman, Batman, Diyarbakır, Siirt, Şırnak, Gaziantep, Kilis and Şanlıurfa (*Fig. 1*). The Euphrates and Tigris Rivers, located in the GAP, have the largest flow volume of 16.9% and 11.4% of Turkey's average annually runoff (186 10^9 m^3), respectively (Altinbilek and Tortajada, 2012; Kankal et al., 2016). In general, the summer season in the region is very hot and under the influence of dry and warm tropical air mass while the winter is warm and rainy. The mean annual temperature is about 18 °C, due to the high summer temperatures that approach 40 °C in daytime (Yilmaz, 2018).

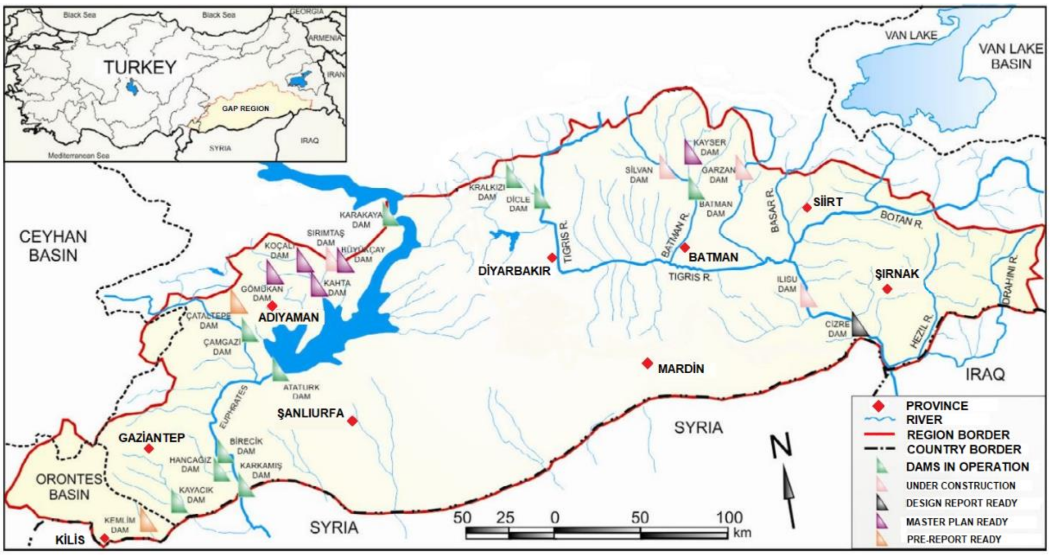

Figure 1. *Water structures and provinces in the GAP region (Kankal et al., 2016)*

The GAP region constitute approximately 10.7% of Turkey's total population (80.8 million, in 2018), and it is estimated that approximately 20% of total irrigable land and 28% of the country's energy potential are in this region (GAP, 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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An irrigable area of 18 10^3 km^2 is planned to be irrigated in the GAP region, and at the first stage, economically irrigable area of 10.6 10^3 km^2 is foreseen to be completed. The progress of the dams and hydroelectric power plants in the GAP region is presented in *Figure 1* according to the project level. In the fertile lands, the agriculture is the main activity, and almost 25% of the economically active population was engaged in agriculture in 2017 (GAP, 2018). That is why the droughts have vital importance on socio-economic development in the region.

For a more detailed description of the GAP, interested readers can refer to the website published by Southeastern Anatolia Project Regional Development Administration (GAP, 2018).

In this study, the monthly precipitation data of the GAP provinces for the period 1970-2017 were obtained from the General Directorate of Meteorological Services of Ministry of Forestry and Water Affairs, Turkey. *Table 1* shows the geographical co-ordinates, elevations and the average annual precipitation of the meteorological stations. The monthly averages of precipitation during the study period (1970-2017) and the seasonal distribution of annual precipitation are presented in *Table 2*. In the GAP region, the annual precipitation ranges from 431 mm to 697 mm, and approximately 80% of the annual total is recorded in winter and spring seasons. Since the summer season is almost dry in the region, the irrigation is a key factor for the agriculture.

Methodology

Standardized precipitation index (SPI)

The SPI quantifies the precipitation deficit and surplus, taking into account the time series (at least 30 years long) of cumulative precipitation for various reference periods (e.g. 6, 12, and 24 months). First, an appropriate probability density distribution is fitted to the frequency distribution of cumulated precipitation and then it is subsequently converted to a standard normal distribution (Tarawneh et al., 2009). In particular, the gamma distribution (two parametric) which generally fits better to the precipitation data is used in the analyses (Ganguli and Reddy, 2014; Zhang et al., 2015). In addition, Guttman (1999) and Angelidis et al. (2012) showed that there are only minor differences between the other distributions (e.g. normal, log-normal) with respect to the results.

Table 1. *The meteorological stations used in the study*

Stations	Station ID	Latitude (N)	Longitude (E)	Elevation (m)
Mardin	17275	37.3103	40.7284	1040
Adıyaman	17265	37.7553	38.2775	672
Batman	17282	37.8636	41.1562	610
Diyarbakır	17280	37.8973	40.2027	674
Siirt	17210	37.9319	41.9354	895
Şırnak	17287	37.5209	42.4523	1350
Gaziantep	17261	37.0585	37.3510	854
Kilis	17262	36.7085	37.1123	640
Şanlıurfa	17270	37.1608	38.7863	550

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 2. *The precipitation data (mm) and seasonal percentiles of annual precipitation (%)*

Months	Mardin	Adıyaman	Batman	Diyarbakır	Siirt	Şırnak	Gaziantep	Kilis	Şanlıurfa
January	101.76	126.18	57.67	62.28	80.50	90.51	92.09	77.59	73.76
February	104.25	99.21	65.40	66.12	94.16	100.27	81.18	69.66	66.80
March	94.99	88.66	73.45	67.20	103.75	110.90	71.79	65.65	62.38
April	78.33	67.98	69.27	65.13	100.07	106.29	56.35	47.05	47.27
May	41.39	37.95	41.57	41.11	60.03	58.95	30.63	25.30	25.30
June	6.30	8.52	8.55	9.45	10.12	5.34	7.49	8.70	4.63
July	2.10	1.55	1.06	0.80	2.56	3.44	5.06	2.15	1.09
August	0.79	1.14	1.14	0.54	1.68	0.31	3.59	4.60	1.43
September	2.99	6.69	4.73	5.56	5.88	7.50	8.27	6.70	4.59
October	32.64	45.58	30.37	33.44	49.27	46.43	39.15	33.91	25.45
November	69.35	73.17	53.65	53.60	81.06	78.71	66.33	58.14	46.58
December	103.37	125.28	63.36	70.06	86.92	88.49	94.61	81.31	71.73
Annual	638	682	470	475	676	697	557	481	431
Winter	48.5	51.4	39.6	41.8	38.7	40.1	48.1	47.5	49.3
Spring	33.6	28.5	39.2	36.5	39.0	39.6	28.5	28.7	31.3
Summer	1.4	1.6	2.3	2.3	2.1	1.3	2.9	3.2	1.7
Autumn	16.4	18.4	18.9	19.5	20.1	19.0	20.4	20.5	17.8

For a given time series of precipitation, the probability density function is defined as (*Eqs. 1-2*):

$$g(x) = \frac{1}{\beta^{\alpha}\tau(\alpha)} x^{\alpha-1} e^{-x/\beta} \quad \text{(Eq.1)}$$

$$\tau(\alpha) = \int_0^{\infty} y^{\alpha-1} e^{-y}\, dy \quad \text{(Eq.2)}$$

where $\alpha > 0$ is a shape parameter, $\beta > 0$ is a scale parameter, $x > 0$ is the amount of precipitation, and $\tau(\alpha)$ is the gamma function. Fitting the distribution to the data requires the estimation of shape (α) and scale (β) parameters, and using the approximation of Thom (1958), these parameters are estimated as (*Eq. 3*):

$$\alpha = \frac{1}{4A}\left(1 + \sqrt{1 + \frac{4A}{3}}\right) \quad \beta = \frac{\bar{x}}{\alpha} \quad A = \ln(\bar{x}) - \frac{\sum \ln(x)}{n} \quad \text{(Eq.3)}$$

where n is the number of observations. Integrating the probability density function with respect to *x* yields the following expression G(x) for the cumulative probability (*Eq. 4*):

$$G(x) = \int_0^{x} g(x)\, dx = \frac{1}{\beta^{\alpha}\tau(\alpha)} \int_0^{x} x^{\alpha-1} e^{-x/\beta}\, dx \quad \text{(Eq.4)}$$

Substituting $t = x/\beta$, *Equation 4* is reduced to (*Eq. 5*):

$$G(x) = \frac{1}{\tau(a)} \int_0^{x} t^{a-1} e^{-1} dt \quad \text{(Eq.5)}$$

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Since the gamma distribution is undefined for x = 0, in order to account for zero values that occur in a sample set, the cumulative probability function for gamma distribution is modified as (*Eq. 6*):

$$H(x) = q + (1 - q)G(x) \quad \text{(Eq.6)}$$

where q is the probability of zero precipitation.

Finally, the cumulative probability distribution is changed into the standard normal distribution to yield the SPI. Following the approximate conversion provided by Abramowitz and Stegun (1965), it results (*Eqs. 7-8*):

$$z = SPI = -\left(t - \frac{c_0 + c_1 t + c_2 t^2}{1 + d_1 t + d_2 t + d_3 t^3}\right) \quad t = \sqrt{ln\left(\frac{1}{(H(x))^2}\right)} \quad \text{for } 0 < H(x) \leq 0.5 \quad \text{(Eq.7)}$$

$$z = SPI = +\left(t - \frac{c_0 + c_1 t + c_2 t^2}{1 + d_1 t + d_2 t + d_3 t^3}\right) \quad t = \sqrt{ln\left(\frac{1}{(1 - H(x))^2}\right)} \quad \text{for } 0.5 < H(x) \leq 1.0 \quad \text{(Eq.8)}$$

where the mathematical constants are: c_0 = 2.515517, c_1 = 0.802853, c_2 = 0.010328, d_1 = 1.432788, d_2 = 0.189269, d_3 = 0.001308.

The average of SPI values is 0, and the standard deviation is 1. For the studied area and studied period, one can identify the wet and dry conditions in accordance with the classification scale of SPI (*Table 3*).

Table 3. *Classifications scale for SPI values (Lloyd-Hughes and Saunders, 2002)*

SPI value	Category	Probability (%)
SPI ≥ 2.00	Extremely wet	2.3
1.50≤SPI≤1.99	Severely wet	4.4
1.00≤SPI≤1.49	Moderately wet	9.2
0.00≤SPI≤0.99	Mildly wet	34.1
-0.99≤SPI≤0.00	Mild drought	34.1
-1.49≤SPI≤-1.00	Moderately drought	9.2
-1.99≤SPI≤-1.50	Severe drought	4.4
SPI≤-2.00	Extreme drought	2.3

The SPI has the ability to reflect the impact of drought on the water resources. However, streamflow, groundwater and reservoir storage reflect the longer-term precipitation anomalies, while soil moisture conditions respond to precipitation anomalies on a relatively short scale (Tsakiris and Vangelis, 2004). In a general classification, it is possible to use the 1 to 3-month SPI for meteorological drought, 3 to 6-month SPI for agricultural drought and 12 to 24-month SPI for hydrological drought analyses and applications (Bonaccorso et al., 2003).

In this study, the SPI values at different time scales (12 and 24 months) were computed by the use of the raw precipitation data, and the software entitled SPI_SL_6.exe which could be download from the link given in the WMO (2012), is used in the calculation process.

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Mann-Kendall trend test

The non-parametric MK test (Mann, 1945; Kendall, 1975) is the most widely applied method to detect the trends in a time series. This test is based on the statistic *S* (*Eq. 9*):

$$S=\sum_{k=1}^{n-1}\sum_{j=k+1}^{n} sgn(X_j-X_k) \quad sgn(\Phi)=\begin{cases}+1 \ if \ \Phi=X_j-X_k>0 \\ 0 \ if \ \Phi=X_j-X_k=0 \\ -1 \ if \ \Phi=X_j-X_k<0\end{cases} \tag{Eq.9)}$$

where *n* is the number of data, *x* is the data point at times *j* and *k* (*j*>*k*) and *sgn(Φ)* is the sign function. In cases where the sample size $n \geq 10$, the mean of *S* and variance are given by (*Eq. 10-11*):

$$E[S]=0 \tag{Eq.10}$$

$$var(S)=\frac{[n(n-1)(2n+5)-\sum_{i=1}^{m} t_i(i-1)(2i+5)]}{18} \tag{Eq.11}$$

where *m* is the number of tied groups and t_i indicates the number of data in the i_{th} tied group. The statistic *S* is approximately normal distributed provided that the following standardized test statistics *Z* as follows (*Eq. 12*):

$$Z=\begin{cases}\frac{S-1}{\sqrt{var(S)}} & S>0 \\ 0 & S=0 \\ \frac{S+1}{\sqrt{var(S)}} & S<0\end{cases} \tag{Eq.12}$$

The positive (negative) value of *Z* shows the upward (downward) trend. In this study, trends were tested with a significance level of $\alpha = 0.05$. The null hypothesis of no trend is rejected if the absolute value of *Z* is greater than 1.96, the critical Z values at 95% two-tailed confidence intervals.

Innovative Şen method (ISM)

In the ISM, proposed by Şen (2012), the time series is divided into two equal parts, which are separately sorted in ascending order. Then, the first and the second half of the time series are located on the *x-axis* and on the *y-axis*, respectively, of a Cartesian coordinate system. If the data are collected on the 1:1 straight line (45°), there is no trend in the time series (*Fig. 2*). If data are located on the upper triangular area of the ideal line, an increasing trend in the time series exists. If data pile up in the lower (upper) triangular area of the 1:1 line, there is a decreasing (increasing) trend in the time series.

It is also possible to have time series in which there are scatter of points on both sides of 1:1 line. These cases correspond to non-monotonic trends where within the same time series there are increasing and decreasing trends at different scales even hidden ones. All possible trend types are given in (*Fig. 2*). Moreover, low, medium and high values of a parameter can be evaluated graphically with this method (Dabanlı et al., 2016; Öztopal and Şen, 2017).

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The statistical significance test of the method has also proposed by Şen (2017), in which a meticulous description could be find. In short, the test is based on the construction of confidence intervals by taking into consideration the difference between two population means. Steps of this method are given by *Equations 13-18.*

In the equations, the notations imply as below, where, $\bar{y}_1$ and $\bar{y}_2$: arithmetic averages of the first and the second halves of the data, ρ: correlation between the first and the second halves of the data, $E(s)$: expectation of the slope (*Eq. 13*), n: number of data, σ: standard deviation of all data, σ_s: slope standard deviation (*Eq. 14*), and s_{cri}: Z critical values in one-way hypothesis at 95% ($\alpha = 0.05$) confidence level ($s_{cri} = \pm 1{,}645$).

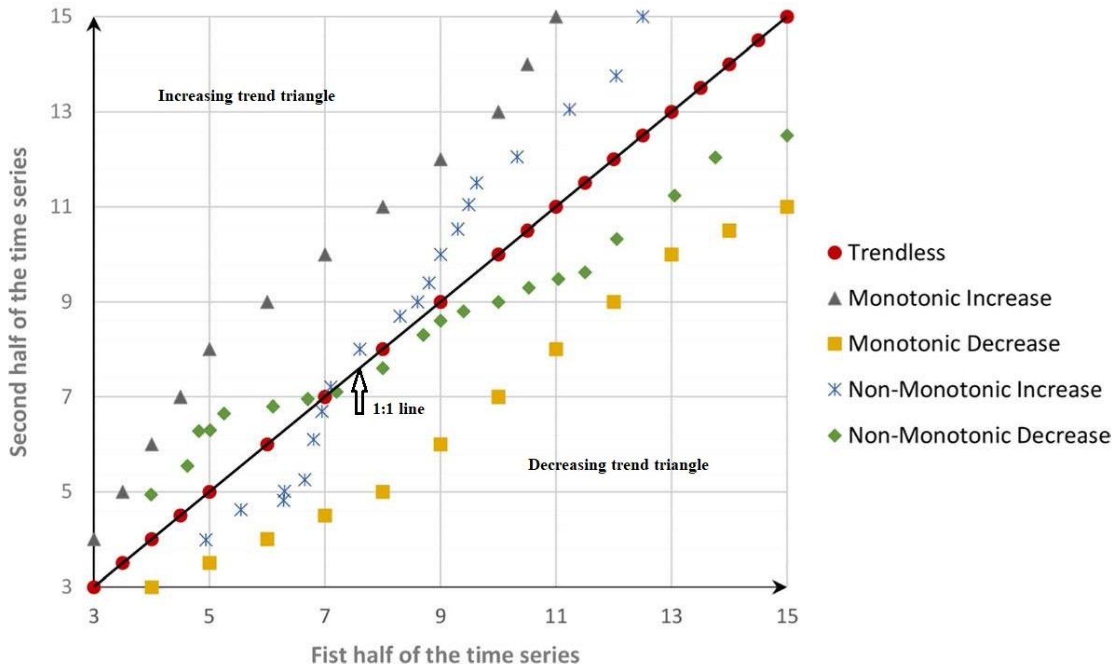

Figure 2. *Illustration of trend types in the ISM (Dabanlı et al., 2016)*

Critical upper and lower limits calculated by *Equation 18* are used to make for hypothesis test. If slope value, s, is outside of the lower and upper confidence limits, thus, the alternative hypothesis, (H_a – *Yes trend*), is approved, otherwise the null hypothesis, (H_0 – *No trend*) is accepted. The type of trend depend in accordance with the slope (s) sign. Slope value (s) can be positive or negative. This means that there is an increasing (+) or a decreasing (-) trend in time series (Şen, 2017; Ay and Özyıldırım, 2017).

$$E(s) = \frac{2}{n}[E(\bar{y}_2) - E(\bar{y}_1)] \quad \text{(Eq.13)}$$

$$\sigma_s^2 = \frac{4}{n^2}[E(\bar{y}_2{}^2) - 2E(\bar{y}_2\bar{y}_1) + E(\bar{y}_1)] \quad \text{(Eq.14)}$$

$$\rho_{\overline{y_2 y_1}} = \frac{E(\bar{y}_2\bar{y}_1) - E(\bar{y}_2)\,E(\bar{y}_1)}{\sigma_{(\bar{y}_2)}\,\sigma_{(\bar{y}_1)}} \quad \text{(Eq.15)}$$

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$$\sigma_s^2 = \frac{8}{n^2}\,\frac{\sigma^2}{n}\left(1 - \rho_{\overline{y_2 y_1}}\right) \qquad \text{(Eq.16)}$$

$$\sigma_s^2 = \frac{2\sqrt{2}}{n\sqrt{n}}\,\sigma\,\left(1 - \rho_{\overline{y_2 y_1}}\right) \qquad \text{(Eq.17)}$$

$$CL_{(1-\alpha)} = 0 \pm s_{cri}\,\sigma_s \qquad \text{(Eq.18)}$$

Results and discussion

In this study, with the aim to detect possible hydrological drought trend in the GAP region, the ISM method was applied to the 12 and 24-month SPI series. All the SPI series were divided into two 24-year sub-series: from 1970 to 1993, and from 1994 to 2017. In order to easily and better identify the possible trend of the dry, normal and wet conditions, two vertical bands have been added in *Figures 3* and *4,* where a red band corresponding to the drought limit (SPI = ≤ -1.0) and a green band corresponding to the wet conditions limit (SPI = ≥ 1.0). The area between the two bands represents the normal conditions. In the figures, 1:1 line indicates neutral (no trend) line and in the case of no trend the centroid point falls on the 1:1, and therefore, $E(s) = 0$. Any deviation from 1:1 line indicates existence of a trend in the given variable (Şen, 2017). The arithmetic averages of the two halves appear as the centroid that falls on the trend line (red dashed line), where the vertical difference between the trend line and 1:1 line is related to the slope (s) of the existing trend in the variable. The vertical distance is equal to the difference between the arithmetic means of the two halves, given as the centroid. It is obvious that in the case of increasing (decreasing) monotonic trend, the trend line falls above (below) the 1:1 line.

Assessment of the 12-month drought trends

The results of the ISM obtained by the use of 12-month SPI values was given in *Figure 3*, and the main findings are summarized below. In *Table 4*, all the necessary calculations of the ISM and the Mann-Kendall significance test results are presented.

In Mardin, there has been a decreasing trend in all SPI values, which lead to heavier droughts and weaker wet periods (*Fig. 3a*). It is also possible to conclude that the droughts follow a monotonic decreasing trend, and the decrement is more evident for the wet periods.

In *Figure 3b*, a slight increasing trend is significant in accordance with the ISM results. In particular, there is an increasing trend in lowest values of SPI and that demonstrates Adıyaman experienced less extreme droughts in 1994-2017 period than the 1970-1993 period. A mild increase in wet conditions is also striking.

Batman, Diyarbakır, Siirt and Şırnak have almost trendless for dry (SPI ≤ -1) and normal conditions; however the index values follow a decreasing trend for wet (SPI ≥ -1) conditions (*Fig. 3c-f)*. Accordingly, the weaker wet periods have been evidenced.

Similar to Adıyaman, in Gaziantep, there is a general increasing trend in normal and wet conditions (*Fig. 3g*). However, here, both the highest and lowest SPI values increase, and that reveals Gaziantep subject to weaker extreme droughts and heavier extremely wet conditions.

In Kilis, decreasing trend in the lowest SPI values correspond heavier drought condition, and there is almost trendless series for normal and wet conditions (*Fig. 3h*).

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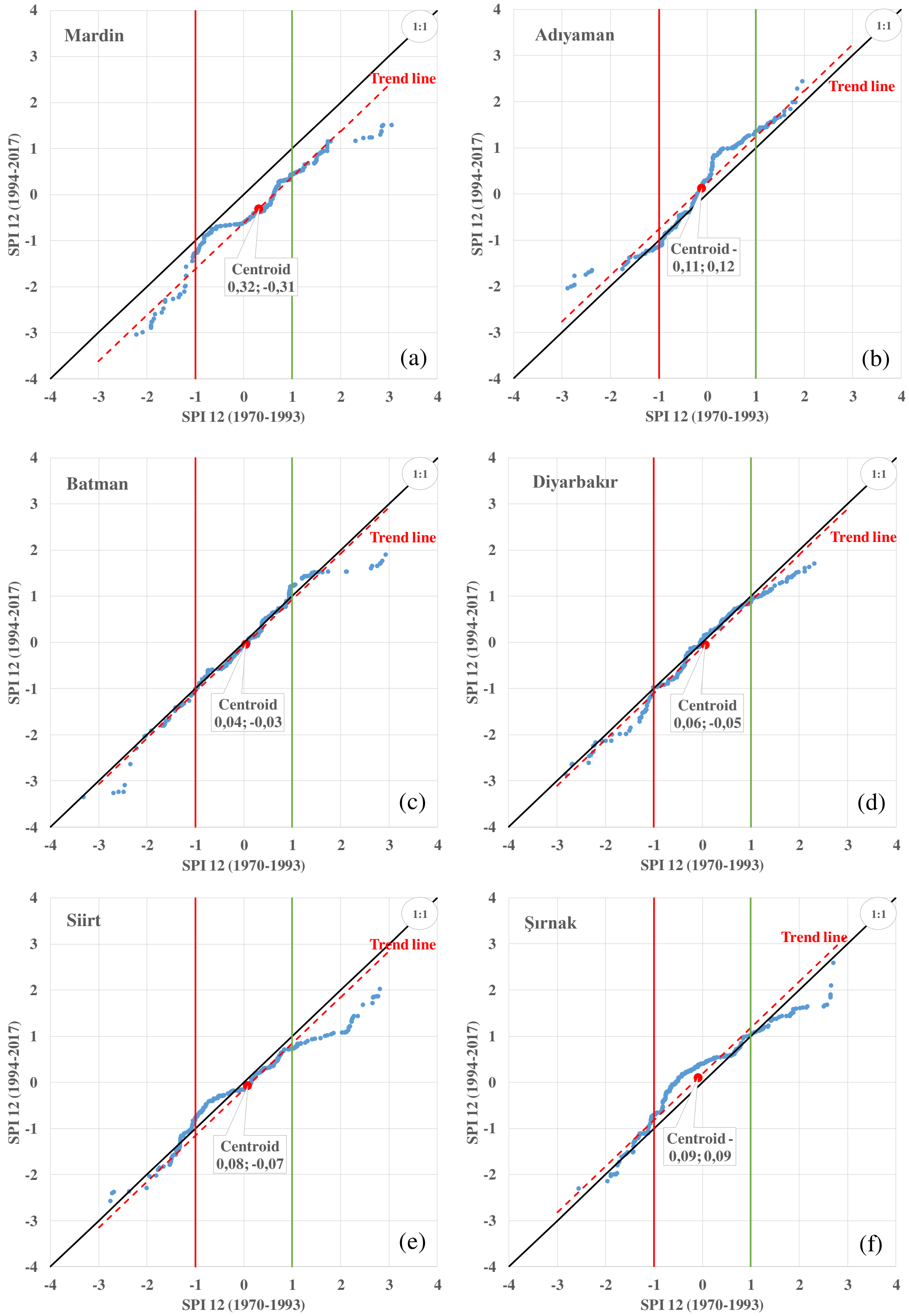
In *Figure 3i,* where the ISM results for Şanlıurfa is illustrated, a sharp increasing trend in the lowest SPI values (weaker extreme droughts) is striking, and the normal and wet periods did not show a clear tendency.



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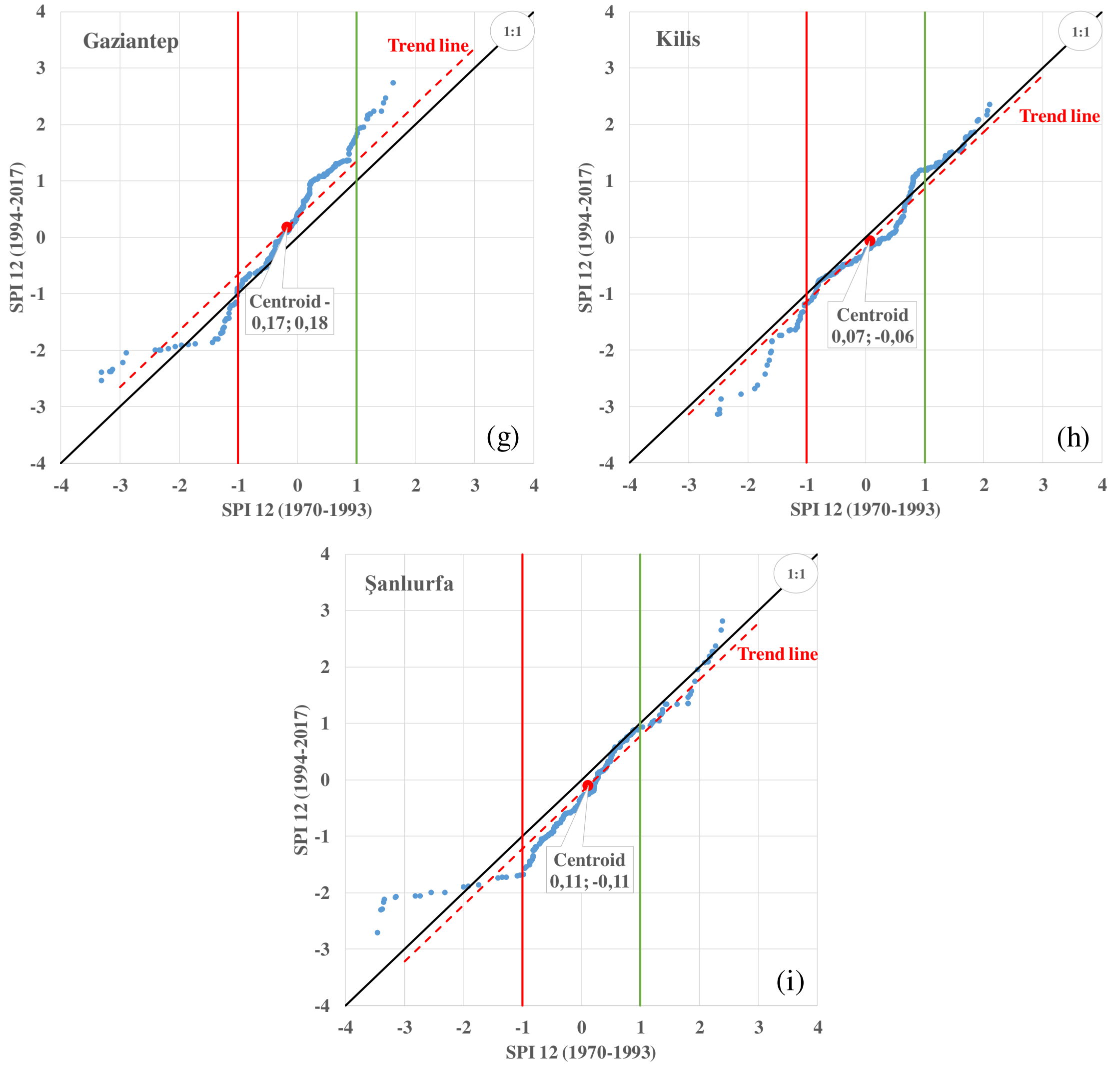

Figure 3. *The scatter diagram of the ISM method for 12-month SPI in the GAP region*

The ISM, a graphical trend analyses approach, allow the above qualitative analyses for dry, normal and wet conditions for the all GAP provinces. The significance analysis for both the ISM and MK test were also performed in accordance with the steps given in the previous sections. Considering the SPI-12 time series as a whole, for five provinces (Mardin, Kilis and Şanlıurfa (decreasing) and Şırnak and Gaziantep (increasing), the same results were obtained both in MK test and in the ISM (*Table 4*).

Assessment of the 24-month drought trends

As regards to the ISM results of 24-month SPI values, almost the same results were obtained. However, it should be noted that there is a clear increase in the trend slope for all provinces (*Fig. 4*). In other words, when the drought trends analyzed with a prolonged time (e.g. 24 months), the lowest SPI values follow a significant decreasing trend with respect to 12-month SPI values, excluding Şırnak and Gaziantep. Another important finding is that there is no significant increase in wet condition through the region, again excluding Gaziantep.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33253342

Table 4. *Results of ISM and MK trend tests for 12-month SPI*

	Mardin	Adıyaman	Batman	Diyarbakır	Siirt	Şırnak	Gaziantep	Kilis	Şanlıurfa
Mean, *y*	0.002677	0.002855	0.002748	0.004592	0.002943	0.001312	0.004060	0.002695	0.003209
Slope, *s*	-0.002221	0.000831	-0.000247	-0.000372	-0.000552	0.000653	0.001239	-0.000478	-0.000792
Intercept, *a*	0.629025	-0.231365	0.072358	0.109379	0.158688	-0.182801	-0.345266	0.137376	0.226649
Standard deviation, σ	1.000675	0.999750	1.000329	0.997984	0.999279	1.001135	0.998790	1.000167	0.999764
Correlation, $\rho_{\overline{y_2 y_1}}$	0.966491	0.970434	0.973991	0.985204	0.975263	0.957829	0.968268	0.983774	0.955506
Slope standard deviation, σ_s	0.000039	0.000036	0.000034	0.000026	0.000033	0.000043	0.000038	0.000027	0.000045
Lower CL (95%)	-0.000064	-0.000060	-0.000056	-0.000042	-0.000055	-0.000071	-0.000062	-0.000044	-0.000073
Upper CL (95%)	0.000064	0.000060	0.000056	0.000042	0.000055	0.000071	0.000062	0.000044	0.000073
Accepted hypothesis (ISM)	H_a	H_a	H_a	H_a	H_a	H_a	H_a	H_a	H_a
Type of trend (ISM)	Decreasing	Increasing	Decreasing	Decreasing	Decreasing	Increasing	Increasing	Decreasing	Decreasing
Sig. level, α (one-way)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Calculated ± Z value	-8.610	1.222	0.460	-0.687	0.730	3.131	1.974	-4.881	-4.434
Z critical value (α = 0.05)	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96
Type of trend (MK)	Decreasing	No trend	No trend	No trend	No trend	Increasing	Increasing	Decreasing	Decreasing

In Mardin, an increasing trend for the extreme droughts is much more pronounced than the SPI-12 trend. It is also possible to say that a decreasing trend under normal and wet conditions (*Fig. 4a*).

For Adıyaman, although there is a decreasing trend in extreme droughts base on SPI-12 evaluations, the ISM results of SPI-24 revealed the increasing trend in normal and wet conditions in addition to almost trendless series in extreme droughts (*Fig. 4b*).

Batman, Diyarbakır and Şanlıurfa have an increasing trend in droughts, especially in the extreme drought cluster (*Fig. 4c-d* and *i*). Moreover, a monotonic decreasing and a non-monotonic increasing trend were determined in normal conditions of Şanlurfa and Batman, respectively. The results revealed that trendless wet conditions for all provinces.

The extreme wet conditions are in a decreasing trend in Siirt (*Fig. 4e*), where the almost trendless series are seen in the normal and dry conditions.

In Şırnak, there is decreasing trend for the extreme droughts, and trendless series in wet conditions. An increasing trend in normal conditions also obtained by the scatter diagram (*Fig. 4f)*.

Figure 4g depicts Gaziantep with an increasing trend both in normal and wet conditions, additionally, it is very clear that there is no trend (increasing or decreasing) in droughts.

In Kilis, a slight increasing trend in wet periods is determined in addition to a mild decrease in normal conditions and trendless series in droughts (*Fig. 4h*).

In *Table 5*, the overall summary of the ISM and MK test results can be found. Similar to SPI-12 results, Mardin, Kilis and Şanlıurfa follow a decreasing trend also in SPI-24 series. Here, a differentation is higher *z* scores of MK test and higher slope values determined by ISM. Adıyaman, Şırnak and Kilis are the provinces with an increasing trend in SPI-24 values, so, they can be seen as the provinces that seem to be the most robust against drought. For Batman, Diyarbakır and Siirt, there is not a consensus between the MK and ISM, since for SPI-24 index values the MK test reveals trendless series while the ISM accept the decreasing trend.

The correlation between the trend slope values and z scores is explanatory for the reliability of the analyses. The correlation coefficient (*r*) is calculated as 0.869 and 0.876 for 12 and 24 month SPI, respectively. That reveals a high correlation between Mann-Kendall test (quantitative, non-parametric and statistical) and Innovative Şen Metod

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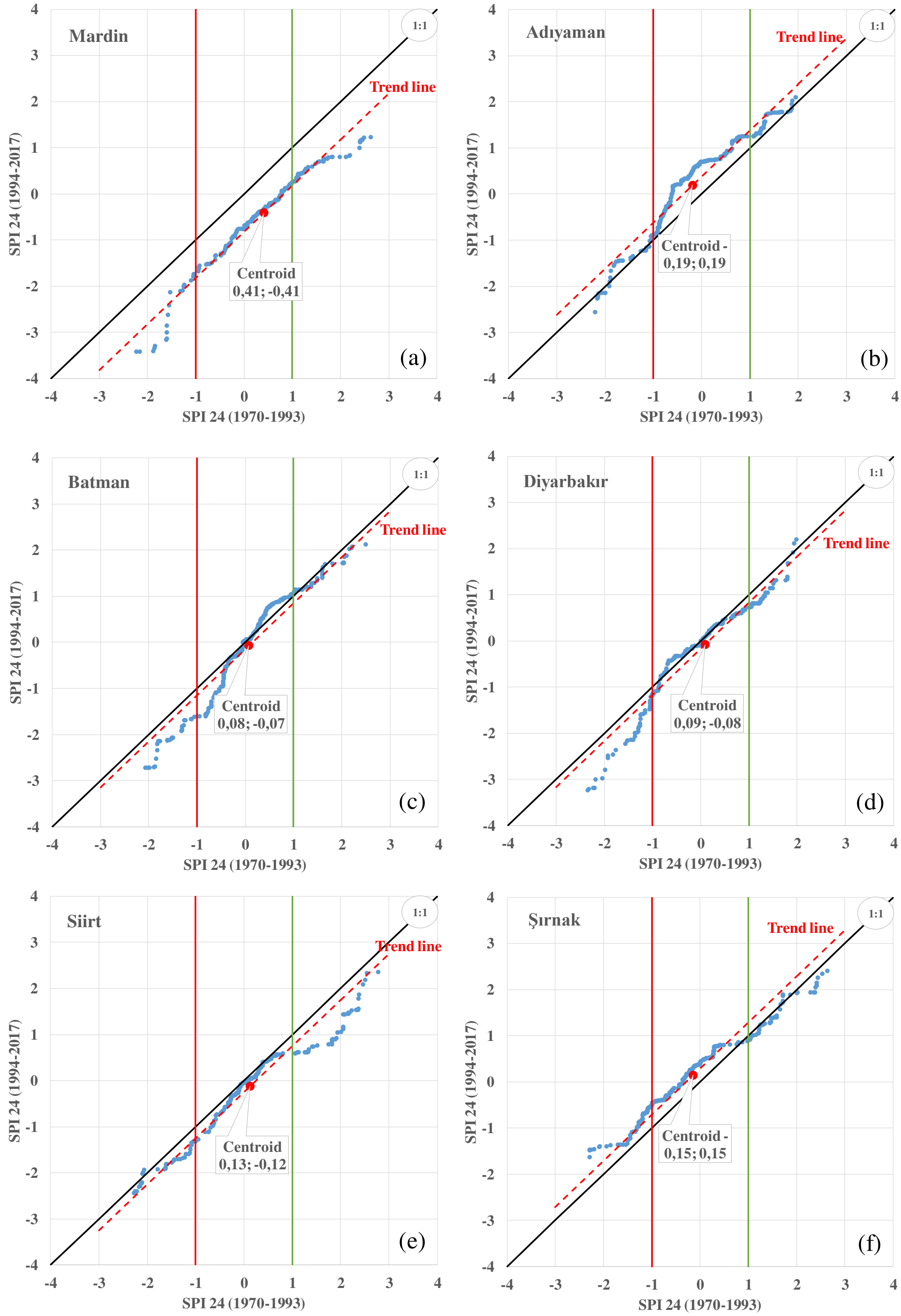
DOI: http://dx.doi.org/10.15666/aeer/1702_33253342

(qualitative and graphical). The increment in correlation is based on the number of overlapping results of the SPI-24 analysis (=6), which is more than the one in the SPI-12 analysis (=5).



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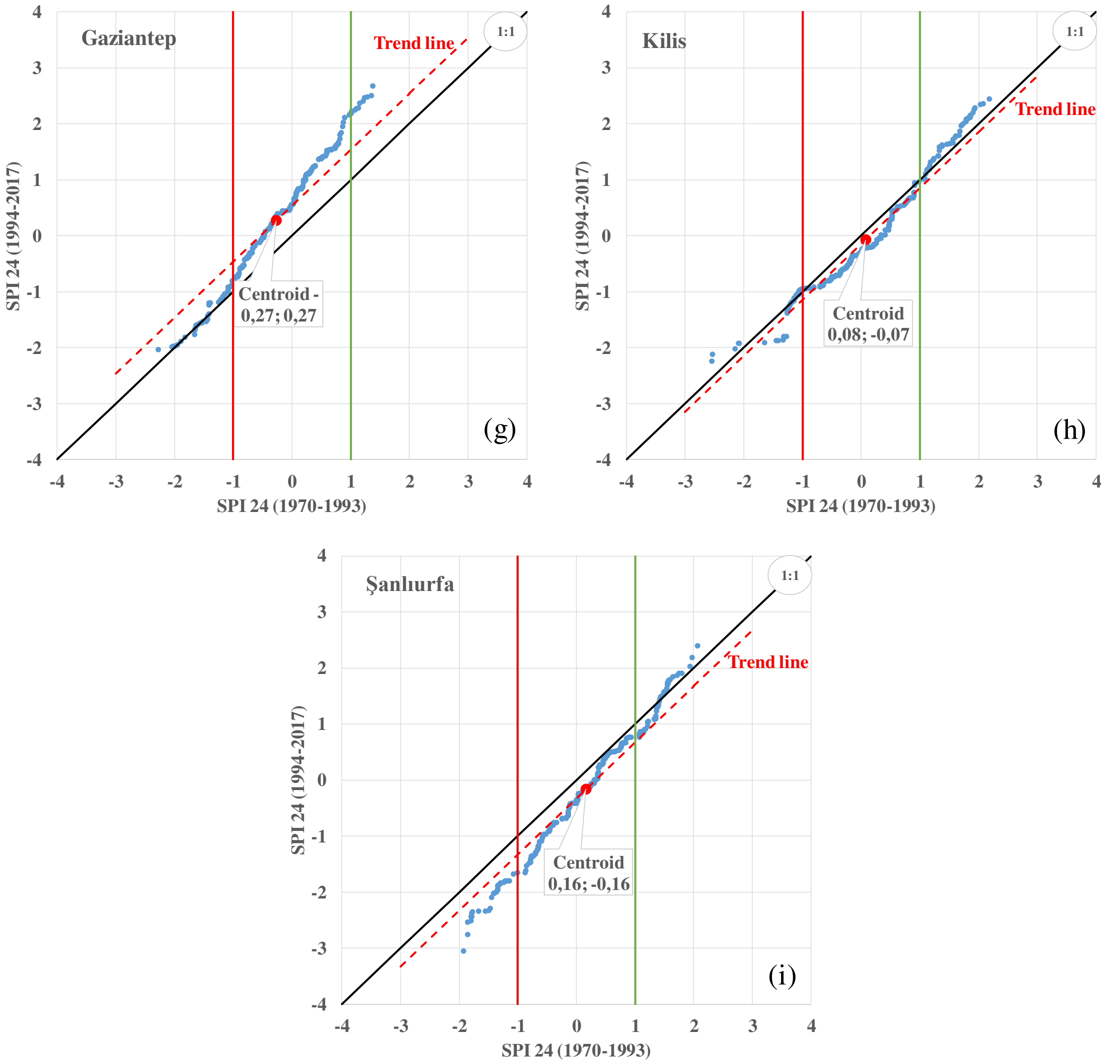

Figure 4. *The scatter diagram of the ISM method for 24-month SPI in the GAP region*

Table 5. *Results of ISM and MK trend tests for 24-month SPI*

Stations	Mardin	Adıyaman	Batman	Diyarbakır	Siirt	Şırnak	Gaziantep	Kilis	Şanlıurfa
Mean, y	0.001667	0.002337	0.002935	0.003243	0.002174	0.000000	0.002482	0.001105	0.001159
Slope, s	-0.002989	0.001391	-0.000537	-0.000625	-0.000915	0.001051	0.001960	-0.000544	-0.001177
Intercept, a	0.826594	-0.381685	0.151051	0.175743	0.254783	-0.290072	-0.538424	0.151286	0.326014
Standard deviation, σ	1.001977	1.000445	0.999506	0.999758	1.000144	1.001955	0.999930	1.001348	1.001712
Correlation, $\rho_{\overline{y_2 y_1}}$	0.978531	0.965260	0.974776	0.969196	0.970922	0.984745	0.993954	0.980526	0.992454
Slope standard deviation, σ_s	0.000032	0.000041	0.000035	0.000038	0.000037	0.000027	0.000017	0.000030	0.000019
Lower CL (95%)	-0.000053	-0.000067	-0.000057	-0.000063	-0.000061	-0.000044	-0.000028	-0.000050	-0.000031
Upper CL (95%)	0.000053	0.000067	0.000057	0.000063	0.000061	0.000044	0.000028	0.000050	0.000031
Accepted hypothesis (ISM)	H_a	H_a	H_a	H_a	H_a	H_a	H_a	H_a	H_a
Type of trend (ISM)	Decreasing	Increasing	Decreasing	Decreasing	Decreasing	Increasing	Increasing	Decreasing	Decreasing
Sig. level, α (one-way)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Calculated ± Z value	-11.112	3.226	0.287	-1.346	0.268	3.817	2.857	-6.236	-5.009
Z critical value (α = 0.05)	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96	± 1.96
Type of trend (MK)	Decreasing	Increasing	No trend	No trend	No trend	Increasing	Increasing	Decreasing	Decreasing

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Conclusions

Trend analysis is one of the most significant elements in any climate change study and provides a foresight for meteorological, hydrological, and climatological variables from past observations to future changes. The well-known trend test, Mann-Kendal (MK) trend test, which is misleading in the presence of data autocorrelation, requires few basic assumptions, which may not be valid in natural hydro-climatological time series. However, the Innovative Şen Method (ISM) does not require assumption, and it is based on the comparison of the two ascendingly ordered halves from the original time series. It also makes it possible to identify significant sub-trends of low, medium and high values. Moreover, due to its simplicity, it may be considered as a first step in the detailed climate change researches, for getting a general idea about the study area.

In this study, the ISM and the MK trend test were applied to the 12- and 24-month standardized precipitation index (SPI) time series, determined for the 1970-2017 period, in order to demonstrate the possible heydrological drought trends in the GAP region (Southeastern Turkey). Since the deficits in the precipitation reflect itself in water resources after a prolonged time, the 12- and 24-month SPI are the main index for demonstration of hydrological drought that refers to shortages of water resources in groundwater, in reservoirs or streams.

The ISM and MK test both revealed that, Mardin is the most vulnerable province in the region, due to the significant decreasing trend determined in 12-month and also in 24- month SPI values. Moreover, the increasing droughts were confirmed by the ISM for all corresponding conditions, referenced as dry, normal and wet. Şanlıurfa, Kilis, Siirt, Diyarbakır and Batman are second order drone prone provinces. In accordance with the results of the ISM, it is noted that there are an increasing trend in normal and wet conditions in Gaziantep, Şırnak and Adıyaman.

This study represent the Innovative Şen Metod with SPI values. However, future studies that focus on the similar methodologies and using the other drought indices that incorporate the evapotranspiration (e.g. Reconnaissance Drought Index) are recommended. When coupled with this study the future efforts would provide more results that are reliable for water resources managers. Moreover, since the various evapotranspiration estimation methods would differ the index values, using a wide range of estimations in the studies would provide possible trends in a more accurate and appropriate manner.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33253342

EFFECT OF FOLIAR SODIUM SELENATE ON LEAF SENESCENCE OF FRAGRANT RICE IN SOUTH CHINA

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(Received 16th Dec 2018; accepted 8th Feb 2019)

Abstract. Selenium (Se) is an essential element to humans, animals and plants, but little is known about roles of Se in yield and antioxidant enzyme activities of rice. In this study, sodium selenate with 10 (T1), 30 (T2) and 50 (T3) μmol L^{-1} concentrations and distilled water (CK) were sprayed onto rice cultivars, or more precisely *Meixiangzhan-2* at rupturing stage during the experiments in South China, 2017. Treatments were arranged in a split-plot design with three replications. The result showed that spraying 10, 30 and 50 μmol L^{-1} sodium selenate at rupturing stage could improve the activities of oxidant enzymes such as peroxidase (POD), superoxide (SOD) and catalase (CAT) and lower the malonaldehyde (MDA) concentrations at filling stage. Furthermore, Se applications could enhance the chlorophyll content at middle and late phase of filling stage and grain yield at maturity. Therefore, Se applications could alleviate the detrimental effects of rice leaf senescence by regulating the activity of enzymatic antioxidants and also increasing chlorophyll content at the filling stage which will be helpful to sustain growth and yield formation in rice production.
Keywords: *aromatic rice, selenate, chlorophyll, antioxidant enzyme, malonaldehyde,*

Introduction

Rice is one of the most important crops, and a staple food all over the world and its production is an essential part of Chinese food security. A previous study showed that the world population may rise to 10 billion people, which means the need for rice will increase dramatically in next few decades (Krishnan et al., 2007). Therefore, it would be a challenging task to ensure the stabilization of rice yield, and exogenous plant growth regulators might come in handy.

A previous study showed that about 90% of the dry matter formed during the growth and development of crops came from photosynthetic assimilates (Petridis et al., 2018). Normally, the photosynthesis of rice will decrease with the leaf senescence at filling stage. The senescence of plant leaves is a complex and highly ordered process accompanied by the degradation of macromolecular materials, which is an important manifestation of plant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

adaptation to the environment in the process of evolution (Zhang et al., 2018; Li et al., 2018; Toscano et al., 2018). Early researches on plant leaf senescence mainly focused on the morphological and anatomical features at the organ level, the changes of physiological and metabolic features of senescence and the index of classification of senescence types (Osborne, 1959, 1962; Shaw and Srivastava, 1964; Jacobs et al., 1962). From early 1960s to late 1980s, plant aging was studied at tissue, cellular, and subcellular levels (Mae, 1990; Davies and Grierson, 1989; Davies et al., 1990; Adams et al., 1990). Since the late 1980s, with the wide application of modern molecular biology technologies such as in vitro translation technology, cDNA difference display, differential subhybridization, antisense RNA technology and functional genome research in the field of aging biology, the molecular mechanism of aging in plant leaves has been gradually revealed (Hafsi et al., 2000; Morris et al., 2000). Many researchers studied the mechanism of plant aging from different perspectives, and put forward some hypotheses about the mechanism of plant aging, including gene regulation theory, light-carbon imbalance theory, nutrition imbalance theory, hormone balance theory and free radical damage theory (May et al., 2017; Diao and Wang, 2018).

Selenium (Se) is an essential element to humans, animals and plant. Se deficiency had been associated with poor immune function, increased risk of mortality, and cognitive decline (Yang et al., 2018; Ishizawa et al., 2018). The research of Lee even revealed that Se could be a cancer-protective agent (Lee, 1996). Plants varied considerably in their physiological response to Se because some plant species growing on seleniferous soils were able to accumulate very high concentrations of Se, but some were Se non-accumulators and Se-sensitive (Terry et al., 2000). Early investigations had evidenced that Se application could against toxic elements such as arsenic, antimony, mercury and copper (Terry et al., 2000). Moreover, the study of Wang (Wang et al., 2012) even generated a selenium-enriched rice with higher yield and bioavailability by using selenite fertilization. Therefore, Se might have potential to be an exogenous regulator which could help to ensure the food security in rice production.

Present study was conducted in Guangdong province (major rice producing province in South China) with the hypothesis that exogenous sodium selenate application in rupturing stage could delay the leaf senescence of rice during the filling stage.

Materials and Methods

Plant materials and growing condition

The fragrant rice cultivar, *Meixiangzhan-2*, having 111-114 days of growth period was in planted in late season of 2017 in Guangzhou, Guangdong, China (23°13’ N, 113°81’ E). The experimental sites had subtropical-monsoon type climate. Before sowing, the seeds were soaked in water for 24 h, germinated in manual climatic box for the next 24 h, shade dried and the germinated seeds were sown in PVC trays for nursery raising. 20-day-old seedlings were transplanted to the field at the planting distance (30 cm × 12 cm). The seeds were sow in July, transplanted in August and harvested in November. The experimental soil was sandy loam with of 20.12% organic matter content, 1.408% total N, 1.068% total P, and 15.767% total K. This region has subtropical-monsoonal type of climate with mean annual air temperatures of 26.6°C, mean annual maximum and minimum air temperatures of 14°C in November and 37°C in August during the experiment, respectively. ‘Special biological organic fertilizer (Dao Feng Xiang)’ manufactured by Guangzhou Huayuan Agricultural Ltd, China comprised of N+ P2O5+K2O ≥74%, active living bacteria ≥20 million g^{-1}, and organic matter ≥10% was applied at 900 kg ha^{-1} with 60% as basal dose and 40% at tillering.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

Treatments and plant sampling

Experimental treatments were as described:

- CK: Overhead sprinkle with double distilled water at rupturing stage of rice.
- T1: Overhead sprinkle with 10μmol L^{-1} sodium selenate at rupturing stage of rice.
- T2: Overhead sprinkle with 30μmol L^{-1} sodium selenate at rupturing stage of rice.
- T3: Overhead sprinkle with 50μmol L^{-1} sodium selenate at rupturing stage of rice.

A special Knapsack Electric sprayer (3WBD-Qianfeng Agricultural machinery, Yangjiang, Guangdong, China) with 0.2–0.5 mPa pressure and 16–18 L capacity fitted with a special windproof atomizing spray nozzle was used for sprinkle. The thirty fresh flag leaves were sampled from the rice at heading stage, 7, 14, and 21 days after heading stage and at maturity (Maturity was 28 days after the heading stage). The leaves were washed with double distilled water and stored at -80°C for physio-biochemical analysis. The measurements were repeated in triplicate and averaged.

Grain Yield

At maturity stage, the rice grains were harvested from six unit sampling area ($1m^2$) in each plot and then threshed by machine. The harvested grains were sundried and weighted in order to determinate the grain yield.

Estimation of malondialdehyde (MDA) and anti-oxidants responses

The MDA content and activities of POD, SOD and CAT were determined according to the methods described by Kong (Kong et al., 2017). MDA reacted with thiobarbituric acid (TBA) and the absorbance was read at the 532 nm, 600 nm, and 450 nm. The content of the reaction solution was calculated as: MDA content (μmol/L) = 6.45(OD532 −OD600) − 0.56OD450, while the final result was expressed as μmol/g FW.

The peroxidase (POD EC1.11.1.7) activity was estiamated after the reaction which the solution was including enzyme extract (50 μl), 1 ml of 0.3% H_2O_2, 0.95 ml of 0.2% guaiacol, and 1 ml of 50 mM l^{-1} sodium phosphate buffer (pH 7.0) while One POD unit of enzyme activity was expressed as the absorbance increase because of guaiacol oxidation by 0.01 (U/g FW). The superoxide (SOD, EC 1.15.1.1) activity was measured by using nitro blue tetrazolium (NBT). 0.05 ml of enzyme extract was added into the reaction mixture which contained 1.75 ml of sodium phosphatebuffer (pH 7.8), 0.3 ml of 130 mM methionine buffer, 0.3 ml of 750 μmol L^{-1} NBT buffer, 0.3 ml of 100 μmol L^{-1} EDTA-Na 2 buffer and 0.3 ml of 20 μmol L^{-1} lactoflavin. After reaction, the absorbance was recorded at 560 nm. One unit of SOD activity is equal to the volume of extract needed to cause 50% inhibition of the color reaction. Catalase (CAT, EC1.11.1.6) activity was estimated by adding an aliquot of enzyme extract (50 μl) to the reaction solution containing 1 ml of 0.3% H_2O_2 and 1.95 ml of sodium phosphate buffer and then the absorbance was read at 240 nm. One CAT unit of enzyme activity was defined as the absorbance decrease by 0.01 (U/g FW).

Determination of Chlorophyll contents

The contents of total chlorophyll, chlorophyll a, chlorophyll b and Carotenoid were determined by the methods of Anjum (Anjum, 2016). Grinding leaf sample (about 0.1 g) was placed in 15 ml centrifuge tube along with 95% absolute ethyl alcohol (10 ml) and then kept at dark condition until the color of sample transformed turn into white. Then, the liquor was read at 665, 652, 649 and 470 nm.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

Statistical analysis

Data were analyzed using statistical software 'Statistix 8.1' (Analytical Software, Tallahassee, FL, USA) while differences amongst means were separated by using least significant difference (LSD) test at 5% probability level. Graphical representation was conducted via Sigma Plot 14.0 (Systat Software Inc., California, USA).

Result

Chlorophyll content

Figure 1 showed the effect of different concentration of sodium selenate on chlorophyll content in flag leaves of rice at different time intervals during the filling stage and heading stage. T2 treatment increased significantly the chlorophyll content in rice flag leaves at whole filling stage. At HS and 7d AH, the highest total chlorophyll content was recorded in T2 while the trend of chlorophyll content at 21d AH and 28d AH was recorded as: T3 > T2 > T1 > CK. Similar conditions were also found in Chlorophyll a, Chlorophyll b and carotenoid content.

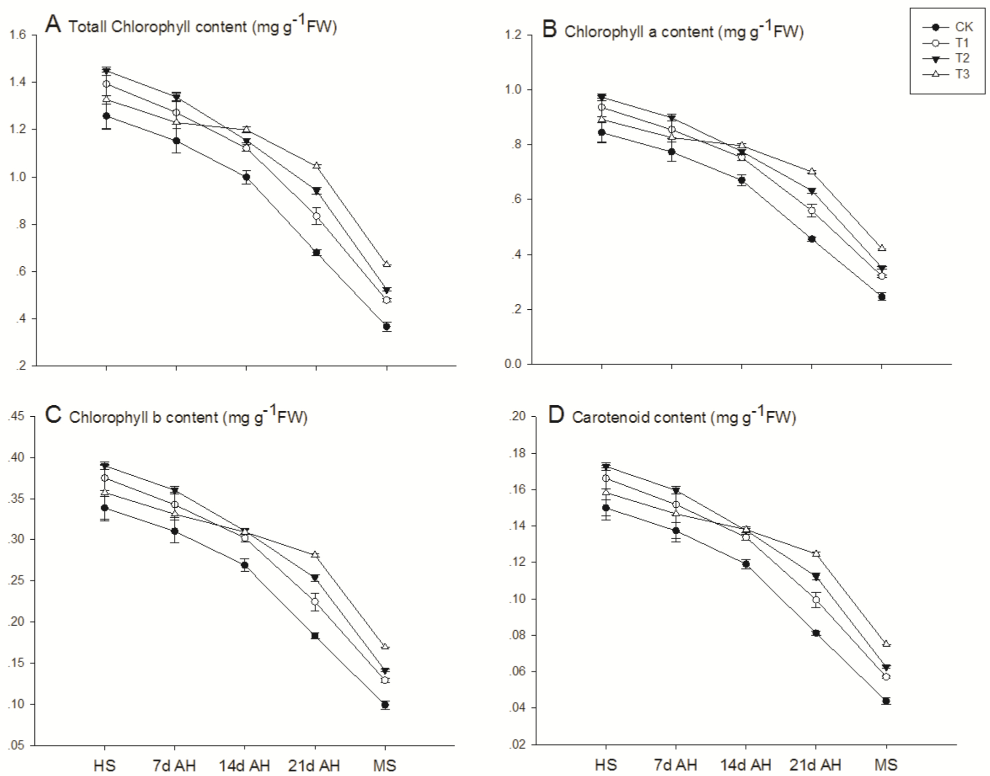

***Figure 1.** Effect of exogenous selenium selenate on chlorophyll content during the filling stage. **A**: Total Chlorophyll conten; **B**: Chlorophyll a content; **C**: Chlorophyll b content; **D**: Carotenoid content; **HS**: heading stage; **7d AH**: 7 days after heading; **14d AH**: 14 days after heading; **21d AH**: 21 days after heading; **MS**: maturity. Capped points represent S.E. of three replicates and significance between treatments at (P≤ 0.05) according to least significant difference (LSD) test. The same as below*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Anti-oxidant enzyme activities and MDA contents

As shown in *Figure 2*, different foliar of selenium selenate affected activities of antioxidant enzymes in rice leaves differently. For SOD activity, both T1 and T2 increased the SOD activities significantly during the early and middle phase of filling stage (HS, 7 and 14d AH). There was no remarkable difference between CK and Se3 at HS and 7d AH whilst the SOD activity of T3 at 14d AH was significantly higher than CK and at 21d AH and maturity, the highest activity was recorded in T3; For POD activity, compared to CK, Se1 treatment increased significantly the POD activity at HS, 7 and 14d AH. At 21d AH and maturity, the POD activity in T2 and T3 were significantly higher than CK while the highest activity was recorded in T3. In addition, there was no significant difference between CK and T1 in POD activity at maturity; For CAT activity, At HS and 7d AH, the highest CAT activity was recorded in T1 and there was no significant difference between CK and T3. However, at 14d AH, the CAT activity of T1, T2 and T3 were significantly higher than CK. At maturity, the highest CAT activity was recorded in T3 while there was no remarkable difference between CK and T1 at maturity; For MDA content, at early phase of filling stage (HS and 7d AH), T1 treatment reduced MDA content significantly compared with CK. At 14 and 21d AH, MDA contents of all Se treatments (T1, T2 and T3) were lower than CK. At maturity, there was no remarkable difference between CK and T1 while T3 remained the lowest level content.

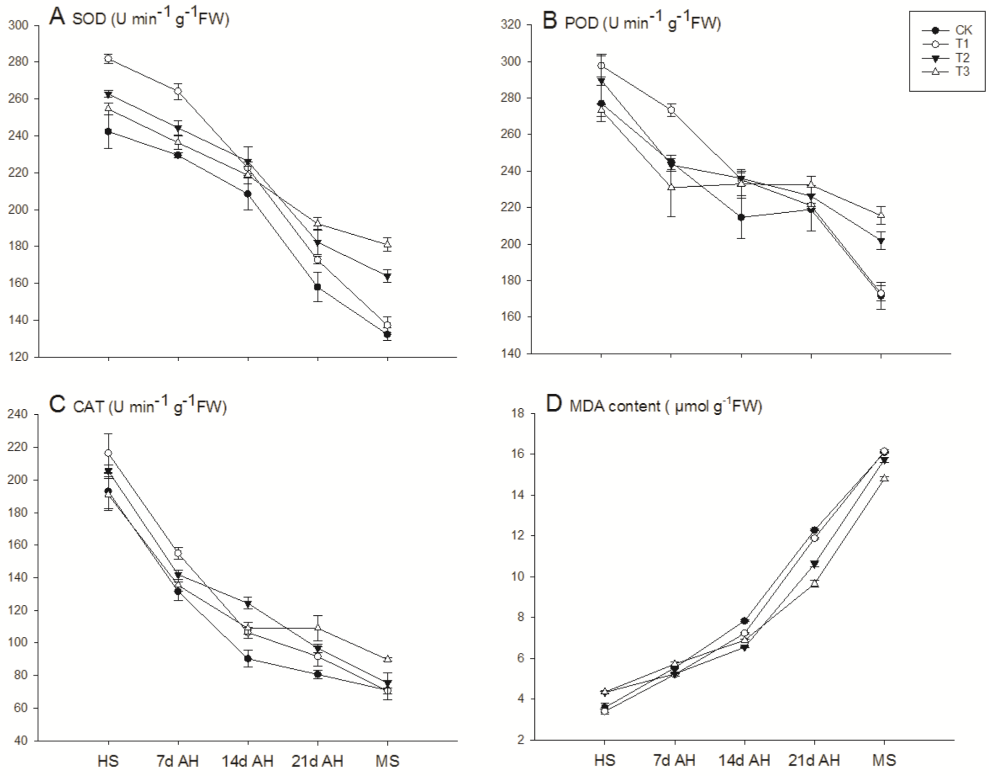

***Figure 2.** Effect of exogenous selenium selenate on antioxidant enzyme activity and MDA content during the filling stage. **A**: SOD activity; **B**: POD activity; **C**: CAT activity; **D**: MDA content*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

Grain yield

Overall, foliar application of selenium selenate at rupturing stage can affected rice grain yield (*Figure 3*). Compared with CK, there was significant difference between CK and T2 while there was no remarkable difference among CK, T1 and T3.

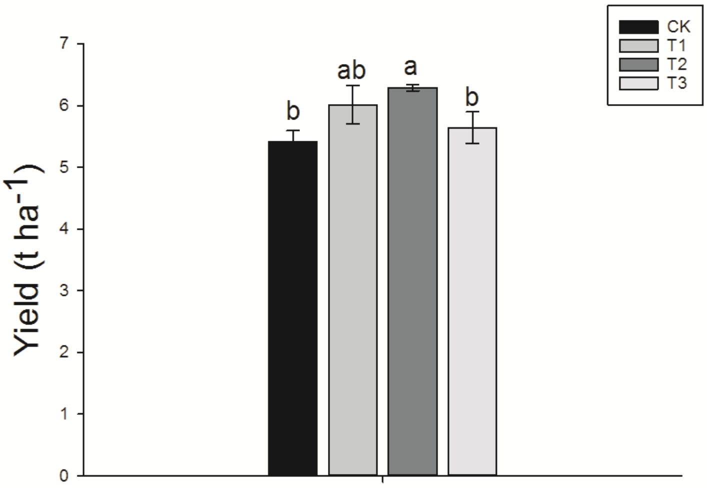

Figure 3. *Effect of exogenous selenium selenate on rice yield. Capped bars represent S.E. of three replicates. Means sharing a common letter don't differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

Discussion

Se plays important roles in plant growth and development (Nawaz et al., 2014). The most important biological function of Se in plant is a consist element of the glutathione peroxidase system (GSH-PX), which was involved in the REDOX reaction in the body, scavenging free radicals, and reducing the body peroxidation damage caused by biofilm (Qiang et al., 2011). The study of Xin et al. (2004) revealed that Se improved the activity of GSH-PX and the oxidizing ability of rice roots while reducing the MDA content under ferrous stress. Yu et al. (2013) demonstrated that Se application significantly enhanced the antioxidant capacity of wheat, corn, soybean and rape while maintain the normal growth of rice. In our study, there were some noticeable effects on rice antioxidant system with foliar application of Se. We observed that Se treatments regulated the antioxidant enzymatic activities at filling stage in terms of POD, CAT and SOD while decreasing the lipid per-oxidation (MDA concentration). This result agrees with the find of Ríos (Ríos et al., 2009) who found that application of selenite at low rate could induce higher increases in activities of enzymes that detoxify H_2O_2, especially glutathione (GSH) peroxidase and SOD. SOD, POD and CAT are key antioxidant enzymes which aid cells to remove the harmful oxygen species. Our result suggested that Se application was able to partially alleviate the detrimental effects of rice leaf senescence by enhancing the activity of antioxidant enzymes which can help in sustaining rice growth at filling stage and yield formation.

The grain yield of rice is one of the most important goals in rice production. There were significant effects on grain yield by spraying exogenous sodium selenate. Boldrin et al. (2012) studied that the applications of both selenite and selenate increased rice

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DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

yield. A previous study (You et al., 1996) also showed that the sodium selenite and selenium-antaining humie acid compound fertilizer increased the yield and selenium content of grain in these crops with low cost and high profit. In present study, foliar sodium selenate at 30μmol L^{-1} increased the grain yield significantly. Our result agreed with the investigation of (Mengxing et al., 2016) who demonstrated that selenium application at appropriate concentration can remarkably increase the seed-setting rate and grain weight and finally enhance the grain yield of aromatic rice.

Furthermore, we observed that the chlorophyll contents under Se treatments were remained at higher level at filling stage. It might because the improvement of activities of antioxidant enzymes delayed the leaf senescence and it also might be the reason why the Se applications improved the grain yield. The study of Cao et al. (2002) revealed that there exited a significant positive correlation between photosynthesis at filling stage and grain yield. The chlorophyll plays an important role in photosynthesis process for absorbing and converting light energy (Wu et al., 2000). Hence, possible that foliar sodium selenate could be a useful application in increasing grain yield and ensuring the stability of rice production.

Conclusions

Spraying sodium selenate with 10, 30 and 50 μmol L^{-1} at rupturing stage could improve the activities of oxidant enzymes such as POD, SOD and CAT and lowered the MDA concentrations at filling stage. Furthermore, Se applications could enhance the chlorophyll content and the grain yield. Whether or not this would be sufficient to apply in rice production should require more detail and long-term investigation in different rice genotypes.

Acknowledgements. This study was supported by National Natural Science Foundation of China (31271646), Student's Platform for Innovation and Entrepreneurship Training Program (201810564029), the Technology System of Modern Agricultural Industry in Guangdong (2017LM1098), the World Bank Loan Agricultural Pollution Control Project in Guangdong (0724-1510A08N3684) and Special Funds for Scientific and Technological Innovation and Cultivation of University Students Guangdong (pdjh2019a0075).

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APPENDIX

Appendix 1. *Photo of the experiment*

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DOI: http://dx.doi.org/10.15666/aeer/1702_33433351

IMPACTS OF LONG-TERM FERTILIZATION ON BACTERIAL COMMUNITY STRUCTURE, SOIL MICROBIAL BIOMASS, AND GRAIN YIELDS IN RED PADDY SOIL IN CHINA

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(Received 16th Dec 2018; accepted 8th Feb 2019)

Abstract. Bacteria play essential roles in soil ecosystems. This study aims to investigate rice rhizosphere bacterial communities, soil nutrition, and grain yield affected by long-term (30 years) fertilization practices. Four fertilizations were applied to experimental red paddy soils: no fertilizer (NF), chemical fertilizer (CF), harvested residue returned to soil (HRR), and 60% organic manure plus chemical fertilizer (OCF). Bacterial communities were then assessed by means of denaturing gradient gel electrophoresis (DGGE) targeting 16S rRNA genes. Results showed that the bacterial community structure of OCF was similar to HRR, which was the most diverse and stable compared to the other treatments. Moreover, CF's effects were similar to NF, which was markedly less diverse. Eight bacterial phyla were identified by sequence analysis of the DGGE bands from rhizosphere inhabitants in the red paddy soils. In addition, soil microbial biomass C and N were significantly higher in HRR and OCF than in CF and NF. Furthermore, double rice grain yield and dry matter in OCF plots were significantly highest ($P < 0.05$). Long-term treatment of organic matter containing fertilizers promoted rhizospheric bacterial diversity and soil quality. The combination of inorganic and organic fertilizers (OCF) improved grain yields more than solely inorganic (CF) or organic (HRR) fertilizers.
Keywords: *bacterial community, rhizosphere soil, denaturing gradient gel electrophoresis (DGGE), long-term fertilization, double rice, dry matter*

Introduction

Soil quality is an important indicator of ecosystem health and soil sustainability. It can be assessed by physical, chemical, and biological approaches (Karlen et al., 2003). Bacterial communities are one of the most sensitive biological indicators of soil quality (Kennedy, 1999). Appropriate community structure, abundant diversity, and high bacterial activity are all significant factors in maintaining the sustainability and productivity of agro-ecosystems. Research on the relationship between bacterial communities and soil quality has gained traction in the past decade, primarily due to environmental issues that arise from soil degradation in various farming systems.

Rice (*Oryza sativa* L.) paddy fields can be defined as an anthropogenic soil type formed under long-term water management with seasonal submergence. In China, red soil is one of the most typical arable soils in the subtropical regions covering approximately 1.13 million km^2, accounting for 11.8% of its land surface, and producing 80% of the rice for 22.5% of the population (Zhang, 2006). However, due to rapid economic and social development, red soils are subject to degradation, characterized by low organic carbon content and low crop productivity (Wang, 1998). In order to maintain the productivity of red soils, different chemical and organic fertilizers have been applied. Studies have shown that crop yields as well as soil physicochemical and biochemical characteristics change

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

with different fertilization treatments (Fierer et al., 2012; Mooshammer et al., 2014; Williams et al., 2013).

Previous studies on the effects of long-term fertilization on microbial community structure and soil quality are however limited. Yet, the few existing studies have revealed that long-term fertilization affects crop yields (Chen et al., 2017), the structure of microbial populations (Gao et al., 2015), the diversity of arbuscular mycorrhizal fungi (Wu et al., 2011), and soil organic matter fractions (Gong et al., 2009). Thus, long-term field trials are important in assessing management, particularly in terms of crop production, soil fertility, and environmental impact (Dobermann et al., 2000).

Therefore, this study has focused on how bacterial community structure, soil quality, and crop yield are affected in double rice-cropping systems by long-term application of inorganic fertilizer (CF), rice straw (HRR), and organic manure plus chemical fertilizer (OCF). This has been done as to provide further information about the complex interactions among soil microbes, soils, and plants. Our highest priority aim has been to evaluate rice rhizospheric bacterial communities in red soils with various fertilization types at different growth stages. Towards this end, denaturing gradient gel electrophoresis (DGGE) and following bands' sequencing were employed to phylogenetically determine the dominant bacterial members.

In sum, we have described the bacterial communities in rice rhizosphere soils that had been subjected to different fertilization regimes since 1986. In addition, the quality of soil and the yield of plants from soils with various treatments were also assessed by evaluating soil biomass, rice grain yields, and dry matter. Our results provide more insight into the effects of fertilization on soil bacteria populations. This in turn helps farmers select best fertilization practices in order to improve soil quality in southern China.

Materials and methods

Experimental site

This study was conducted at the experimental field of the Soil and Fertilizer Institute, Hunan Academy of Agricultural Sciences, Ningxiang County, Hunan Province (110°72'E, 28°52'N), China. The annual average temperature of this region is 14.7-22 °C, and annual rainfall is 1232 mm. The properties of soil before sampling was characterized and listed as follows: soil pH of 5.3, soil containing organic carbon (50.7 g kg^{-1}), total N (2.1 g kg^{-1}), total P (0.73 g kg^{-1}), available N (135.2 mg kg^{-1}), available P (29.8 mg kg^{-1}), and available K (52 mg kg^{-1}).

Experimental design

The rhizospheric soil samples were collected from control and long-term fertilizer treatment soils during April-October 2016. The four treatments were no fertilizer (Gu et al.) as a control, chemical fertilizer (CF), harvested rice straws returned to the soil (HRR), and organic manure plus chemical fertilizer (OCF). The chemical fertilizer was composed of P_2O_5 (75 kg hm^{2-1}), K_2O (100 kg $hm^{2\ -1}$), and urea (200 kg $hm^{2\ -1}$). Rice straws with equal amount of nitrogen as chemical fertilizer were returned to the field in HRR. The manure of OCF was post-fermented and purchased from a pig farm. The average composition of the pig manure was 588 of organic matter (g kg^{-1}), 26.7 of N (g kg^{-1}), 17.6 of P (g kg^{-1}), and 51.6 of K (g kg^{-1}) with a pH of 8.5, the dosage being 1.7 t hm^{-2} (dry weight basis). Twelve plots (4 treatments with 3 replicates) of size 5 × 6 m^2 were

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

randomly distributed, having been used for double-rice and winter ryegrass rotation since 1986.

Grain yields and dry matter

The seeding rate for the rice was 200,000 seedling/hm^2 for both early and late rice. At maturity, three sites of 1 m^2 were chosen randomly from each plot in order to determine the grain yield and dry matter. Plant samples were separated into straw and grain by a manually-operated thresher. The dry weight of roots, parts of above ground, and grain were determined after oven drying at 75 °C to the constant weight. Panicles, spikelets per panicle, grain filling percentages (the filled spikelet number/total spikelet number × 100%), and 1000-grain weights were estimated in order to calculate grain yield.

Soil sampling and analysis

The soils from the rhizosphere were sampled at time points standing for four growth stages: the early rice tilling stage, the early rice mature stage, the late rice tilling stage, and the late rice mature stage; these samplings were taken on May 7, July 7, August 4, and October 12 in 2016. Rhizosphere soil samples were collected as reported by Smalla et al. (2001). One composite sample was taken that consisted of roots of 5 random rice plants. The roots were shaken vigorously in order to separate soil not tightly adhering to its roots. The soils from rhizosphere were then collected, kept on ice, and stored, half of each sample at -70 °C for soil DNA extraction, and the other half at 4 °C for soil microbial biomass.

Soil microbial biomass C (MBC) and N (MBN) were determined through fumigation with ethanol-free $CHCl_3$ and extraction with K_2SO_4 (Vance et al., 1987), the organic C in the extracts was determined using an automated total organic C (TOC) analyzer (Shimadzu, TOC-Vwp, Japan), and the N was detected by the Kjeldahl method. Correction factors of 0.45 (K_{EC}) (Wu et al., 1990) and 0.57 (K_{EN}) (Jenkinson, 1988), were used for the calculated MBC and MBN values, respectively.

DNA extraction, PCR, and DGGE analysis

Three soil replicates were mixed, PCR and DGGE were run 3 times. Total microbial DNA was extracted from approximately 0.5 g of soil by using a Soil DNA Out Kit (TIANDZ, Beijing, China), following the manufacturer's instructions. The variable (V3) region of the 16S rRNA gene was amplified by PCR using the primers of V357F-GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3)'and V517R (5'-ATTACCGCGGCTGCTGG-3'), as described by Muyzer (1993). All amplifications were performed in 50 µl of PCR reactions containing 2.5 ng of template DNA, 1 µl of each primer at 20 µM, 4 µl of 10 mM dNTP, 2.5 U of Taq DNA polymerase, 5 µl of 10×PCR buffer with Mg^{2+} supplied with Taq DNA polymerase (Takara BIO, Tokyo, Japan) and deionized-distilled H_2O. The following parameters were used for PCR amplification: initial denaturation for 3 min at 94 °C, followed by 28 cycles of 30 s at 94 °C, 30s at 59 °C, and 1 min 30 s at 72 °C, with a final extension step at 72 °C for 10 min. The PCR products were verified by electrophoresis in 1.5% agarose gels (containing 0.02% ethidium bromide) within 1×TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3).

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The DGGE analysis of microbial community structure was performed by using the D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). 13 μl of PCR products (approx. 300 ng μl^{-1}) and 7 μl of loading buffer were loaded onto 6-12% (w/v) polyacrylamide gels in 0.5x TAE buffer. The denaturing gradient was established with 30–50% denaturant (100% denaturant corresponding to 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 200 V for 5 h at 61 °C. The gel was stained by SYBRs Green I (Molecular Probes, Eugene, USA) for 30 min, and scanned using the Alpha Imager 2200 Imaging System (Alpha Innotech, USA).

Specific and dominant bands in various treatments were excised from the DGGE gel and eluted overnight in a diffusion buffer at room temperature for re-amplification, as described above. Specific bands were those which were unique between each treatment, whereas dominant bands were those present with a high intensity. The amplified 16S rRNA gene segments were then inserted into pEASY-T3 vectors (TransGen Biotech, Beijing, China) and transformed into *Escherichia coli* DH5α. The recombinants were cultured in LB medium, from which white clones were randomly selected and inoculated in LB/Amp^+, then sequenced by an ABI 3730XL DNA Sequencer (Perkin Elmer, USA) at the Sun Biotech Developing Center (Beijing).

The closest match to each sequence was obtained by using the NCBI basic local alignment search tool (http://www.ncbi.nlm.gov/blast/). The alignment of similar 16S rRNA gene sequences were conducted by Mega 5.

Data analyses and statistics

All results were based on three replications in the field. The data was subjected to analysis of means and standard deviations. The diversity analysis of DGGE patterns was conducted using Quantity One 4.2.3 software (Bio-Rad Laboratories, Hercules, CA, USA) by the unweighted pair-group method with arithmetic averages (UPGMA). Soil microbial community diversity was assessed by the Shannon–Weaver index (H) and richness (S) according to the *Equation 1* (Shannon, 1949), based on the number of bands and their intensities in each lane:

$$H = -\sum_{i=1}^{s}(p_i)(\log 2 p_i) \qquad \text{(Eq.1)}$$

where p_i is the relative intensity of the *i*th band calculated as $p_i = n_i / N$, in which n_i is the intensity of the *i*th band and N is the total intensity of all bands. Richness was recorded as the number of DGGE bands of each sample. The principal component analysis (PCA) of DGGE profiles was performed using the SAS 8.2 statistical program (SAS Institute Inc., Cary, USA). Statistical analyses were performed by one-way ANOVA followed by LSD multiple comparison tests of significance. A probability value (*P*) of <0.05 was considered as statistically significant.

Results

Bacterial community structure and diversity

PCR-DGGE is a molecular fingerprinting method that allows direct comparative overview of the composition and diversity of dominant soil bacteria. DGGE has been

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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used in many studies of environmental microorganisms, and proven a powerful method in investigating bacterial communities in rhizosphere soils (Qiao et al., 2012; Watanabe et al., 2004) since its first application (Muyzer et al., 1993). Soil samples from the 4 different growth stages of the control and 3 different fertilizer treatments were analyzed using PCR-DGGE in order to examine total microbial DNA.

In *Figure 1,* bands 1-25 stand for the common and specific clones of the bacterial species in the control and 3 different treatments. The twenty-five clones included 7 clones from NF, 5 clones from CF, 7 clones from HRR, and 6 clones from the OCF treatment (*Fig. 1*). Bands at the same position of each lane were common bacterial specie in all treatments such as B15, but some bands were only present in one lane or several lanes, such as B1 in lanes 3 and 8, B22 in lanes 3 and 7, B25 in lane 13, etc. Naturally, these bands represent specific bacterial species in different treatments. The more bands that showed up in a given lane, the greater the richness of bacteria identified in the fields.

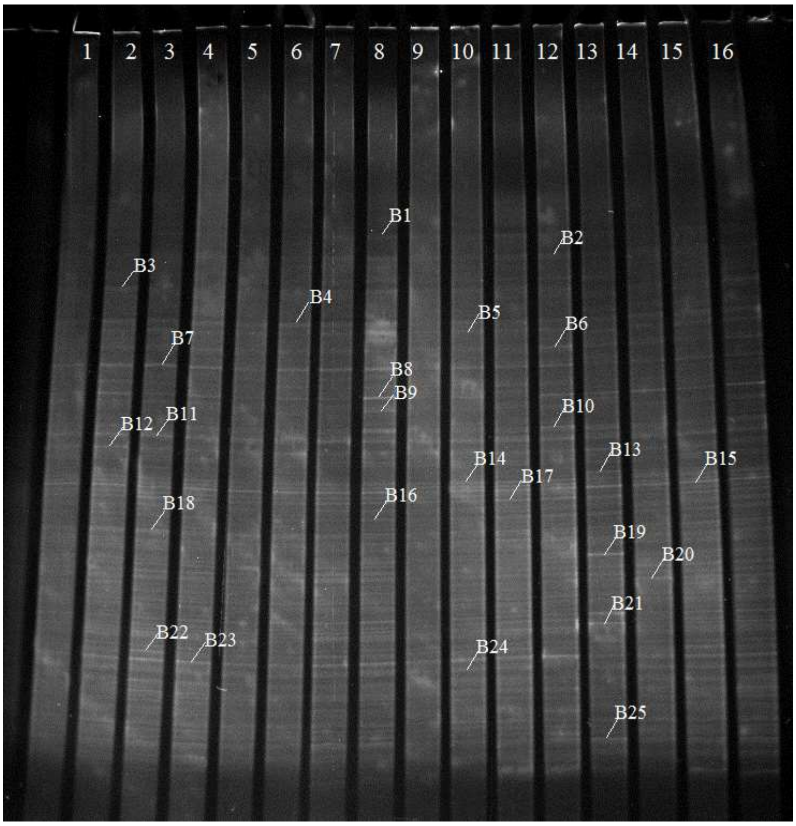

Figure 1. *PCR-DGGE patterns of 16SrRNA gene fragments obtained from soil DNA extracted during four progressive rice growth stages in 2016: May 7, July 7, August 4, and October 12. Lane 1-4: no fertilizer (NF); lanes 5-8: chemical fertilizer (CF); lanes 9-12: harvested residue returned to soil (HRR); lanes 13-16: 60% organic manure plus chemical fertilizer (OCF). Letter B followed by numbers indicates the bands that were extracted from the gel prior to sequencing analysis*

Based on PCR-DGGE banding patterns, the Shannon-Weaver diversity index (H) and corresponding richness (S) were calculated (*Table 1*). Among the three treatments and control, HRR had the highest Shannon-Weaver index at stage 3 (3.88), while the lowest was OCF at stage 1 (3.04).

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Table 1. *Shannon-Weaver diversity index (H) and species richness (S) of the bacterial community derived from the PCR-DGGE profiles*

Fertilization	Date of soil sampling	Lane No.	Richness (S)	Shannon-Weaver (H)
No fertilization (NF)	May 07	1	47.21e	3.70a
	July 07	2	53.15a	3.71a
	Aug 04	3	51.33b	3.67a
	Oct 12	4	49.67c	3.61ab
Chemical fertilization (CF)	May 07	5	47.23d	3.43b
	July 07	6	45.33c	3.44b
	Aug 04	7	49.51b	3.74a
	Oct 12	8	52.87a	3.78a
Harvested residue returned to the soil (HRR)	May 07	9	45.33c	3.67b
	July 07	10	50.84b	3.73b
	Aug 04	11	56.78a	3.88a
	Oct 12	12	49.51b	3.87a
60% Organic manure plus chemical fertilization (OCF)	May 07	13	46.32c	3.04c
	July 07	14	55.33a	3.43b
	Aug 04	15	51.54b	3.75a
	Oct 12	16	46.32c	3.80a

Means in each column by different letters are significantly different at $P < 0.05$ level, n = 3. Lane No. was the number shown in *Figure 1*

Variance analysis between different stages in each group showed that the diversity of the bacterial community had a significant increase ($p < 0.05$) from the early rice stage to the late rice stage in three treatment groups, but not in the control group. Further, except for NF, bacterial community diversity generally increased with a given growth stage in three treatment groups, and increased dramatically from the early rice stage to late rice stage for OCF. This increase was from 3.04 to 3.80, although it should be noted that the index between the two stages of early rice (between early rice tilling and the mature stage) or the last rice stages (between late rice tilling and the mature stage) was not significant in the treatments (*Table 1*). This indicates that the bacterial communities in red paddy soil with various treatments matured from early rice to late rice.

Dendrogram analysis

In order to describe the integrated change of the bacterial community shown by H and S in response to fertilization regimes, PCA and UPGMA cluster analyses were used to compare the different effects of the four groups. PCA based on DGGE banding patterns separated the bacterial communities into two groups: NF and CF in one group, and OCF and HRR in another (*Fig. 2*). The first two principle components of the PCA plot accounted for 20% and 31% of the overall variance, respectively. However, sample 9 in the HRR group (May 2015) was clearly separated from the other two groups and could be considered an outlier. Possible reason for this is that sample 9 was in the early rice tilling stage of HRR, and harvested residue may cause something different to occur in the early days, which would need to be further studied.

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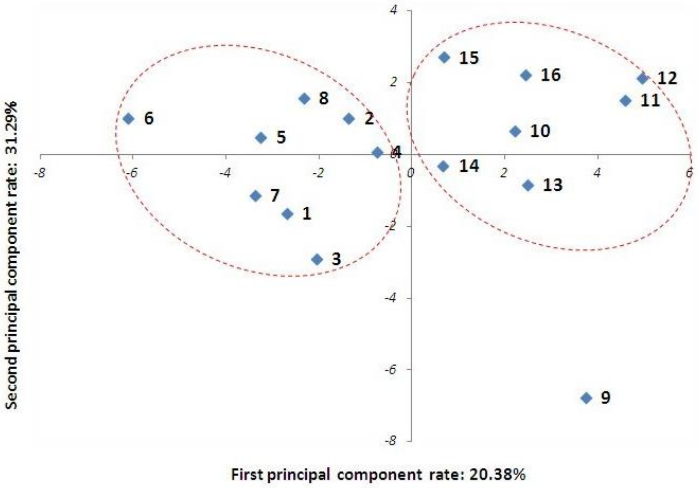

***Figure 2.** Principle component analysis (PCA) of microbial diversity indices of rhizosphere bacterial communities in different fertilization and growth stages based on DGGE profiles of bacterial 16s rRNA. The codes 1–16 are as defined in Table 1*

In general, clustering analysis corroborated the visual interpretation of the DGGE profiles. A dendrogram was obtained using the UPGMA clustering analysis, and the similarity coefficient of Pearson was based on the DGGE profiles of bacterial 16s rRNA gene fragments (170 bp). The clustering analysis revealed that two clusters were separated at 54% similarity, eliminating outlier sample 9. One cluster included samples from HRR and OCF, while another included samples from NF and CF. This suggests that DGGE patterns of CF soil are similar to that of NF soil at all growth stages, while the bacterial community of HRR soil is more similar to that in OCF soil (*Fig. 3*).

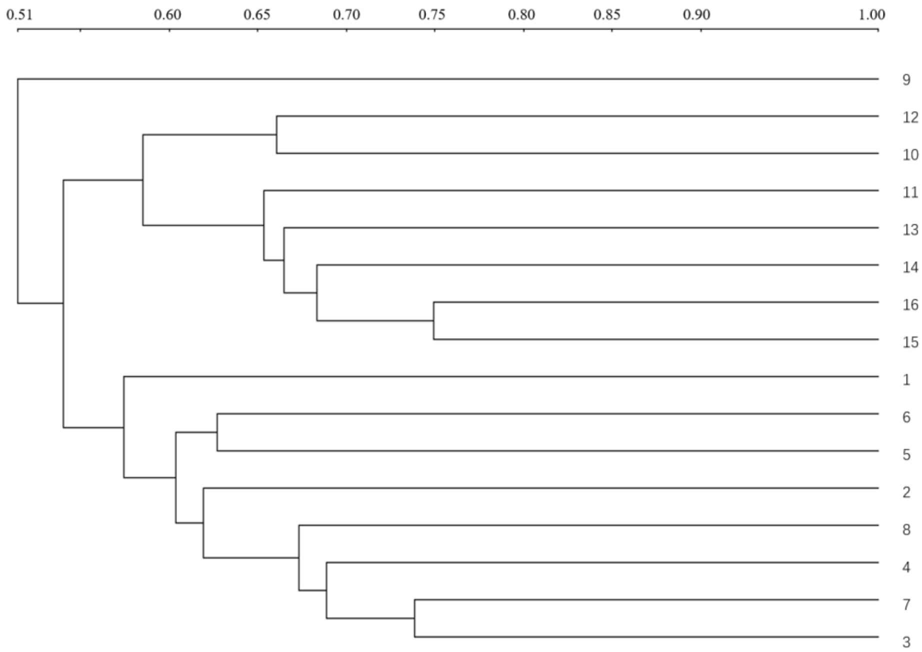

***Figure 3.** UPGMA clustering represents the similarity of 16S rRNA gene fragments from PCR-DGGE profiles generated from soil DNA extracted from different fertilizations and growth stages. The codes 1–16 are as defined in Table 1*

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

At 65% similarity, the soil samples representing the HRR group separated from those of OCF. Four growth stages in the OCF group clustered together, but the growing stages of NF and CF were not separated, which indicates that the difference between the bacterial community structure of NF and CF was not as distinct as that between HRR and OCF.

Sequenced bacterial bands and phylogenetic analysis

Twenty-five specific and intensive bands were excised from the DGGE gel (16S rRNA), then re-amplified, transformed, and sequenced to identify dominant bacterial populations in various soil samples. The 16S rRNA gene sequences (170 bp) of the 25 clones were submitted to the NCBI nucleotide sequence database (*Table A1* in the *Appendix*). Since more than 50% of the retrieved sequences were closely related to 16S rRNA genes of uncultured bacteria, the first hit in Blast-N matching to a culturable strain was also considered. These sequences were classified into eight phyla: *Proteobacteria* (B5, B6, B7, B9, B10, B14, B15, B16, B17, and B18), *Gemmatimonadetes* (B11 and B20), *Chloroflexi* (B22, B23, and B24), *Acidobacteria* (B1, B3, B13, and B19), *Actinobacteria* (B8, B12, and B25), *Firmicutes* (B4), *Bacteroidetes* (B2), and *Planctomycete* (B21) (*Table 2*).

Table 2. *Sequence identification of 16S rRNA gene fragments retrieved from DGGE bands by NCBI blast-N*

16s rDNA fragments	Bacteria with the highest identity	The highest identity (%)	Phylum
B1	*Acidobacteria bacterium* (EF417700.1)	100	*Acidobacteria*
B2	*Uncultured Sphingobacteria bacterium* (EU299921.1)	93	*Bacteroidetes*
B3	*Acidobacteriales* (EU193004.1)	98	*Acidobacteria*
B4	*Uncultured Firmicutes bacterium* (EU753610.1)	100	*Firmicutes*
B5	*Proteobacterium* (EU298752.1)	100	*Proteobacteria*
B6	*Uncultured beta proteobacterium* (AB748625.1)	98	*Proteobacteria*
B7	*Uncultured Rhizobiales bacterium* (FJ477663.1)	100	*Proteobacteria*
B8	*Uncultured Actinomycetales bacterium* (EU449558.1)	95	*Actinobacteria*
B9	*Uncultured Bradyrhizobium sp.* (JX505261.1)	98	*Proteobacteria*
B10	*Uncultured delta proteobacterium* (FJ902393.1)	100	*Proteobacteria*
B11	*Uncultured Gemmatimonadetes bacterium* (EU297425.1)	98	*Gemmatimonadetes*
B12	*Mycobacterium bovis* (AP010918.1)	100	*Actinobacteria*
B13	*Acidobacterium sp.* (GQ287545.1)	92	*Acidobacteria*
B14	*Uncultured delta proteobacterium* (FJ902063.1)	100	*Proteobacteria*
B15	*Uncultured Novosphingobium sp.* (JQ701055.1)	95	*Proteobacteria*
B16	*delta proteobacterium* (GQ406161.1)	91	*Proteobacteria*
B17	*delta proteobacterium* (JQ795294.1)	100	*Proteobacteria*
B18	*Uncultured alpha proteobacterium* (KC449494.1)	100	*Proteobacteria*
B19	*Uncultured Acidobacteria bacterium* (AM935771.1)	98	*Acidobacteria*
B20	*Gemmatimonadetes bacterium* (EF074631.1)	100	*Gemmatimonadetes*
B21	*Planctomycete* (GQ443701.1)	100	*Planctomycete*
B22	*Chloroflexi* (AY149071.1)	95	*Chloroflexi*
B23	*Uncultured Chloroflexi bacterium* (GQ366653.1)	94	*Chloroflexi*
B24	*Uncultured Chloroflexi* (JQ4021491)	91	*Chloroflexi*
B25	*Uncultured Actinobacteridae bacterium* (KC018166.1)	99	*Actinobacteria*

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The relative abundance of dominant bacterial phylum taxa in the control and three treatments are shown in *Figure 4*. All eight bacterial phyla could be detected in the fertilization treatments except for CF, in which *Planctomycete* was not detected in all growing stages. It was clear that the abundance of each bacterial phyla showed explicit differences among the treatments. *Proteobacteria* was the most abundant bacteria under various treatments, with relative abundance ranging from 31 to 48%. More, relative abundance of *Planctomycete* and *Acidobacteria* were greater in the HRR and OCF groups as compared to NF and CF. On the contrary, *Gemmatimonadetes* decreased in HRR and OCF treatments. A hierarchical heatmap of bacterial phyla separated the samples into two groups, with 10, 11, 12, 13, 15 and 16 grouped together, the other samples grouped together. In the first group, all samples were from HRR and OCF, in the other group, most of samples from NF and CF, except 9 and 14 (*Fig. 5*).

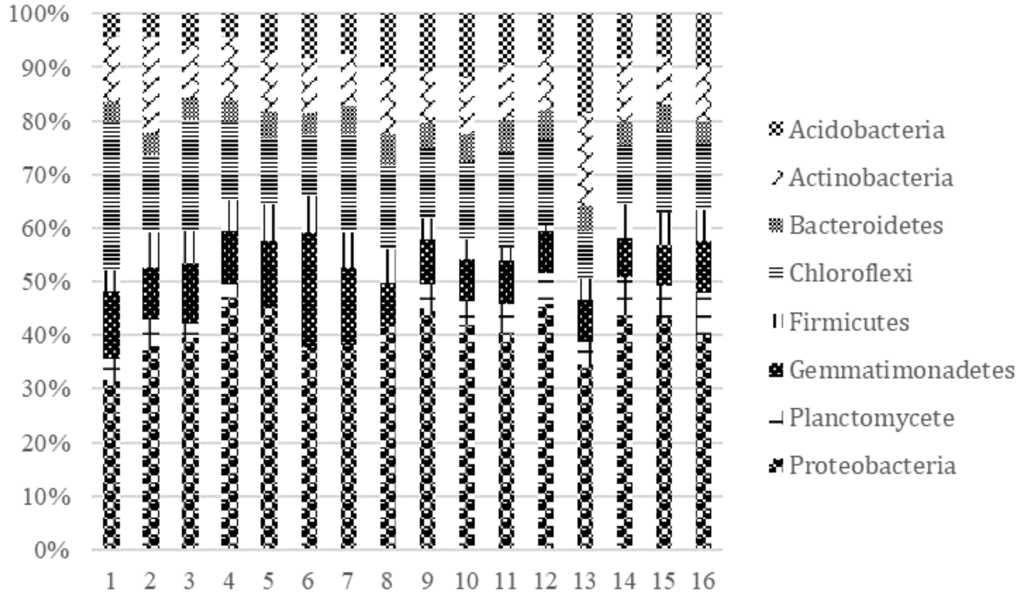

Figure 4. *The relative abundance of all detected phyla in different fertilizations and growth stages treatments. The codes 1–16 are as defined in Table 1*

Soil microbial biomass and crop yields

Plants depend on rhizosphere microbiota to make nutrients accessible. The above data showed that rhizosphere microbiota varied across the fertilizer treatments. In order to examine whether different fertilizers affect both soil quality and crop yields, soil biomass, grain yields, and dry matter were evaluated.

Soil MBN was significantly higher ($P < 0.05$) in HRR and OCF than in CF and NF during the sampling period, whereas MBC was significantly higher ($P < 0.05$) only in May and Oct. Both MBN and MBC did not differ significantly between CF and NF in all stages, but there was a difference between OCF and HRR (*Table 3*). Further, MBN was significantly higher in OCF than in HRR in July and October, while MBC in OCF was significantly higher in May but lower in Oct than HRR. These results indicate that different fertilizer treatments resulted in different values for MBC and MBN, especially fertilizers with organic components such as HRR and OCF, which in turn led to significantly higher biomass in the soils compared to CF or NF.

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

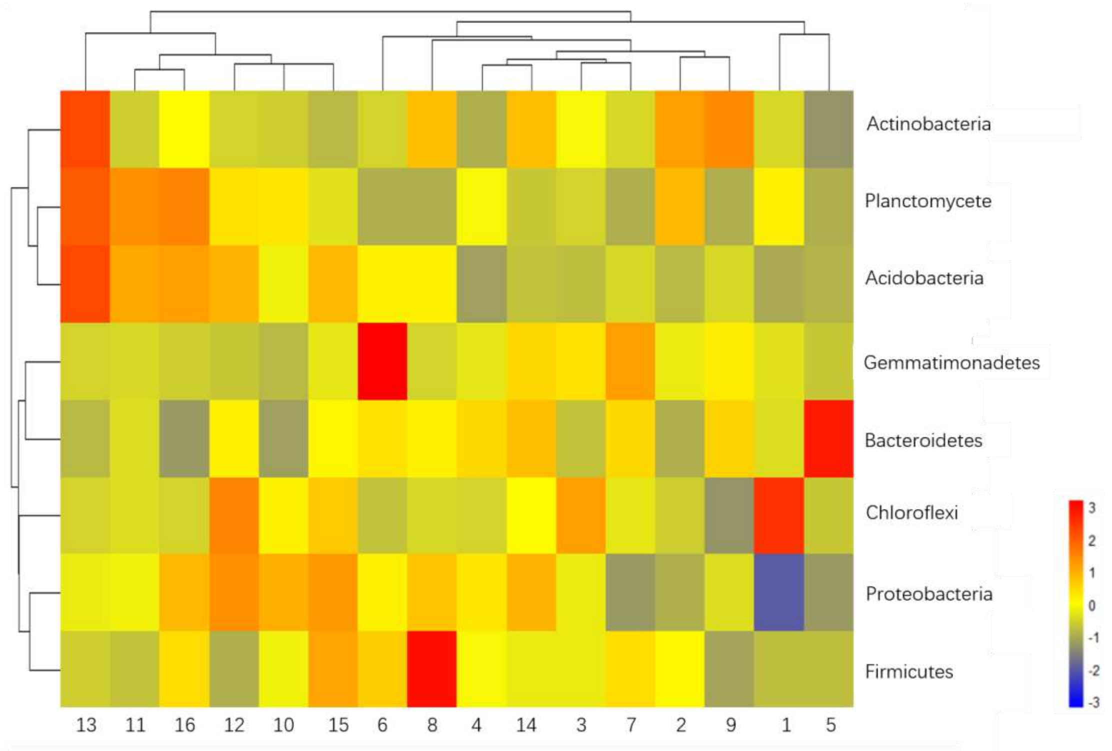

***Figure 5.** Distribution of the phyla and their cluster analysis in different fertilizations and growth stages as visualised by heatmaps (variables clustering on the vertical axis). The relative abundances for microbial phyla are indicated by colour intensity. The codes 1–16 are as defined in Table 1*

***Table 3.** Effect of different fertilizations on soil microbial biomass C (MBC) and soil microbial biomass N (MBN)*

	Treatment	May 07	Jul 07	Aug 04	Oct 12	Average
MBC ($mg.kg^{-1}$)	NF	145.7c	210.6b	266.2a	263.4b	221.5b
	CF	150.2c	209.5b	268.2a	267.1b	223.8b
	HRR	185.4b	238.2a	269.3a	330.0a	255.7a
	OCF	256.4a	215.5ab	267.6a	287.8b	256.8a
MBN ($mg.kg^{-1}$)	NF	7.5b	6.8c	3.3b	9.5c	6.8b
	CF	7.9b	8.0c	3.9b	11.2c	7.8b
	HRR	13.8a	16.4b	6.8a	18.8b	14.0a
	OCF	15.4a	18.9a	6.3a	22.0a	15.6a

Means in each column within MBC and MBN followed by different letters are significantly different at $P < 0.05$ level, n = 3. NF: no fertilizer, CF: chemical fertilizer; HRR: harvested residue returned to soil; OCF: 60% organic manure plus chemical fertilizer

Double rice total grain yield (i.e. the sum of early and late rice grain yield) in all three treatments were significantly higher than that in NF plots. Among the fertilizer treatments, the grain yield of OCF plots was highest ($P < 0.05$), there being no significant difference between CF and HRR. Compared to NF, grain yields of CF, HRR, and OCF were increased by 26.5%, 26.5%, and 38.9%, respectively (*Table 4*). Further,

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

dry matter in OCF plots was highest. Compared to NF, dry root weight of CF, HRR, and OCF were increased by 40.6%, 29.9%, and 50.8%; similarly, the above ground weights were increased by 36.0%, 29.4%, and 44.4%.

Table 4. *Rice grain yield and dry matter of double rice by different fertilizations*

Treatment	Yield (kg.hm^{-2})	Dry matter (kg.hm^{-2})	
		Root	Above ground
NF	9001.2c	837.3c	15543.4d
CF	11391.5b	1177.5ab	21142.0b
HRR	11392.6b	1087.6b	20120.3c
OCF	12503.5a	1262.9a	22448.2a

Means in each column followed by different letters are significantly different at $P < 0.05$ level, n = 3. NF: no fertilizer, CF: chemical fertilizer; HRR: harvested residue returned to soil; OCF: 60% organic manure plus chemical fertilizer

Discussion

In this study, we assessed the structure of microbial communities in response to three types of fertilization on different stages of growth. Broadly, the structure and composition of the bacterial communities were grouped into two clusters: chemical fertilization (CF) and the control treatments in one group, and HRR and OCF in the other (*Fig. 2*).

Previous studies demonstrate that the activity and diversity of soil microorganisms are directly influenced by changes such as soil temperature (Fierer et al., 2005), moisture (Hollister et al., 2010), pH (Rousk et al., 2010), nutrient availability (Cusack et al., 2011), soil type (Xu et al., 2009), and plant type (Berg et al., 2006); following that fertilization is one of the most significant anthropogenic activities that greatly alter soil characteristics. Many studies showed that fertilizers, regardless of type, affect soil bacterial community structure (Enwall et al., 2007; Gu et al., 2009; Marschner et al., 2003). We've concluded that HRR and OCF showed similar influences on soil bacterial communities, but that CF soil had a similar bacterial community structure as NF. Chu et al. previously concluded similar results, in which inorganic fertilization did not affect the DGGE banding pattern (Chu et al., 2007). In contrast, higher bacterial community diversity and structure change were observed in soils with chemical plus organic rice straw treatments in double rice cropping system by using 16S rRNA sequencing (Wu et al., 2011; Yuan et al., 2013), although it should be noted that manure and chemical fertilizers did not change the bacterial community structure significantly in maize or wheat cropping system (Ge et al., 2008; Shen et al., 2010).

In our study, both microbial community diversity and biomass of CF soils were similar to that of NF soils, but were significantly different from that of OCF and HRR soils (*Tables 1* and *3*). A possible reason for this is that organic matter in OCF and HRR provide available substrates for microbial growth and further, that there is a positive correlation between MBC and soil organic matter, as previously reported (Wu et al., 2013).

Compared to inorganic fertilizers, organic fertilizers diversify bacterial communities. Previous studies have found that soil organic matter exerted significant influence on the diversity and structure of soil microorganisms (Cusack et al., 2011; Gu et al., 2009; Hartmann et al., 2015). This is consistent with our results, as HRR and OCF increased

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

the diversity of bacterial communities in soil. Moreover, the diversity of bacterial communities with HRR was highest because straw, the most natural and suitable organic resource of the energy for bacterial activities in soils, was found in greater amounts. Additionally, soil was found to be healthier when straws were returned to fields in HRR (Lou et al., 2011).

Likewise, OCF treatment greatly increased organic substances and adjusted the balance of carbon and nitrogen in soils, both beneficial to soil microbes (Chen et al., 2017). However, OCF showed lower bacterial diversity than HRR, which might be due to its combination with chemical fertilizer. Overall, our results indicated that HRR was the best fertilizer practice, measured as bacterial community diversity against CF and OCF.

In previous studies, microbial diversity and community structure in rice field soils have been reported to change with season and fertilizer management practices (Ahn et al., 2012; Doi et al., 2011). Except for NF, we have found that the growth stages of rice affect bacterial diversity (*Table 1*). Further, the diversity of CF and OCF in two late growth stages were significantly higher than those at the two early stages, while not varying much between early and late stages in HRR. We theorize that CF and OCF having more evident effects than HRR may be that HRR diversified the microbe community quickly then keeping bacterial richness steady at a high level, thus leading to less notable effects with growth stages. Further, diversity at the start of the early stage was low, but significantly increased at the late stage in OCF, which could be explained by OCF being a slow-release fertilizer. Additionally, variation of bacterial communities in various growth stages is also indirectly related to environmental characteristics and root exudates (Tian et al., 2013). However, the influences of these factors are lower than direct fertilizer application.

Our results demonstrated that the dominant bacterial species in these four groups belonged to eight phyla (*Table 2; Fig. 4*). The eight phyla identified were the same as those detected in rice soils by means of pyrosequencing (Ahn et al., 2012). Two of these were Gram-positive bacteria (*Firmicutes and Actinobacteria*), the others being Gram-negative. Hence, the predominant bacteria in rice rhizosphere were Gram-negative bacteria, especially *Proteobacteria*, irrespective of soil type and growth stage. Moreover, the microbial community possessing more Gram-negative bacteria was found to be more active as well as relatively stable and resilient (Peng et al., 2016). These findings are consistent with DGGE analysis results from Ikenaga et al. (2003).

Acidobacteria usually functions in biogeochemical cycling of carbon and is more abundant in acidic soils (Sait et al., 2006). The increase of *Acidobacteria* in fertilizer treatment groups compared to the control group may be explained by CF's decreased pH of soils that made them more acidic. Meanwhile, HRR and OCF increased soil organic carbon and its production. Previous studies have demonstrated that *Planctomyces* is a marine bacterium existing in habitats rich with organic nutrients and oxidizing ammonium. Our study demonstrated that *Planctomycete* did not exist at all in CF treated soils, further suggesting that inorganic components in CF are not suitable for this type of bacteria. Therefore, HRR and OCF but not CF provided organic matter that promoted *Planctomycete* growth. Moreover, compared to NF treated soil, organic fertilizers demonstrated significantly lower soil bulk density (Rasool et al., 2008), which make soils more suitable for the survival of *planctomycete.*

Last, the decline of the relative abundance of *Gemmatimonadetes* in HRR and OCF was probably due to their preference for drier soils. *Gemmatimonadetes* have been

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DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

found in a variety of arid soils, so it tends to be more dependent on the moisture availability than aggregation (DeBruyn et al., 2011). Organic matter lead to soil aggregation, and thus dryness-favored bacterial *Gemmatimonadetes* decreased. The moisture-favored bacterial *Planctomyces,* by contrast, increased in HRR and OCF.

Rice is the most important crop in China, thus, fertilization is an important agricultural practice for increasing crop yields. Traditionally, farmers have used organic fertilizer such as manure and crop residues to maintain soil fertility. However, chemical fertilizers have increasingly been applied to rice paddies in China as of the 1980s. This has been done in order to meet food demand of increasing populations, primarily because inorganic fertilizers directly boost crop yields and are more affordable. Consequently, fewer organic fertilizers are currently used, and large chemical fertilizer input has caused soil acidity major croplands (Guo et al., 2010).

Long-term fertility experiments provide insight into the consequences of land management strategies. In our study, compared to other fertilizers, OCF improves both grain yields and soil biomass; this is due to the combination of organic manure and chemical fertilizer, in which chemical P availability plays a vital role in improving rice productivity as what Ding et al. (2018) recently reported. Moreover, previous studies demonstrated that P, N, and MBC increased in soils with organic manure treatment, significantly improving rice yields and microbial activity (Zheng et al., 2016). However, the grain yields of CF (containing chemical P) and HRR (containing organic straw residuals) were not significantly different, indicating that the combination of both organic and inorganic fertilizers is the best method to improve grain yield.

Similarly, previous studies have demonstrated that chemical N increased in HRR but that chemical P did not change (Zheng et al., 2016). Further, it has been demonstrated that N fertilization promoted crop productivity only after following the supplement of P (Lv et al., 2011), which explains why CF or HRR did not promote significant grain yields. Although HRR has similar effects as chemical fertilizer (CF) on grain yield, HRR had more beneficial effects on soil sustainability, such as increased microbial diversity and biomass (*Table 3*).

Generally, soil microbial biomass plays an important role in maintaining soil structure, facilitating microbial metabolic processes, and regulating the release of nutrients. Our results suggest that OCF and HRR had similar MBC and MBN (*Table 3*), and thus similar microbial community structures (*Figs. 2* and *3*). However, our results also suggest that OCF has more advantages on improving grain yields, while HRR is more beneficial to soil quality and microbial structures.

In sum, chemical fertilizer plays an important role in increasing yield, but large fertilizer input in the field usually causes environmental problems. This has become a major concern for scientists, environmental groups, and agricultural policymakers worldwide. Figuring out how to assess and design healthy fertilizer practices that achieve optimum yields and maintain soil sustainability is challenging, and it will be our focus in future studies.

Conclusions

This study has reported the effects of three long-term fertilization treatments (CF, HRR, and OCF) on bacterial community structure and abundance at different rice growth stages of red paddy soils in southern China. It has also reported the corresponding microbial biomass and grain yields. PCR-DGGE results revealed that among the three fertilizer practices, both HRR and OCF showed positive and lasting effects on soil quality, but that the soil microbial community in HRR was steadiest and most diverse. The bacterial diversity of OCF was next after HRR, with OCF demonstrating lasting effects on the bacterial community with growth stages.

The bacterial species belonged to 8 different phyla, and the abundance of each phylum of bacteria changed with various treatments. *Planctomycete* was not present in the CF group, suggesting the lower bacterial diversity in CF. HRR and OCF treatments significantly increased soil micro biomass compared to NF, whereas CF did not. Furthermore, OCF with both inorganic and organic compositions promoted dry matter and total grain yield compared to any other treatment.

Overall, HRR is the fertilizer that best increases bacterial diversity in red paddy soils, whereas OCF is the best to promote crop yield. Broadly, this study has provided insights into the impact of different fertilizers on rhizospheric bacterial community structure, soil quality, and crop yield, providing critical information in selecting the optimal fertilization treatment. Further studies should focus on the application of combined inorganic and organic fertilizers, and balancing the economic and environmental benefits between crop yields and soil sustainability.

Acknowledgements. This research was funded by Shanxi Nonggu Establish Research Earmarked fund (No. SXNGJSKYZX201704), Shanxi Scholarship Council of China (No. 2016-068), and Shanxi Provincial Natural Science Foundation for Youths (No.201701D221181).

Conflicts of interests. The authors declare that they have no conflicts of interests.

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APPENDIX

Table A1. *Sequences of 16S rRNA gene fragments retrieved from DGGE bands*

16S rDNA fragments	Sequences
B1	CCTACGGGAGGCAGCAGTAAGGAATATTGGTCAATGGACGAAAGTCTGAACCAGCCATG CCGCGTGGAGGATGAAGGTCCTCTGGATTGTAAACTTCTTTTATATGGGACGAAAAAAG GCTTTTCCAAGTCGTCTGACGGTACCATATGAATAAGCACCGGCTAACTCCGTGCCAGCA GCCGCGGTAAT
B2	CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAAC GCCGCGTGGGGGATGAATGGCTTCGGCCCGTAAACCCCTGTCATTTGCGATCAAGGTGC CGGGGTAAATAATCCCGGTACTTGACGGTACTGTCAGAGGAAGCCCCGGCTAACTCCGT GCCAGCAGCCGCGGTAATA
B3	CCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCAAC GCCGCGTGGAGGATGAAGGTCTTTGGATTGTAAACTCCTGTCAGCGGCGGAGAAGGGAC TCGACCTCATAATCCCGGTACTTGACGGTACTGTCAGAGGAAGCCCCGGCTAACTCCGTG CCAGCAGCCGCGGTAAT
B4	ATTACCGCGGCTGCTGGCACGTAGTTAGCCGAGACTTATTCCTGGGATACTGTCCTTTCT CATCTCCCAGAAAAGTGCTTTACGATCCGAAGACCTTCGTCGCACACGCGGCGTTGCTGG GTCAGGCTTTCGCCCATTGCCCAATATTCCCTACTGCTGCCTCCCGTAGGA
B5	ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTCCTCCAGAGGTATCGTCATTATC GTCCCTCTAGACAAAGGTTTACGATCCGAAGACCTTCATCCCTCACGCGGCGTTGCTCGG TCAGGCTTGCGCCCATTGCCGAAAATTCCCTACTGCTGCCTCCCGTAGGA
B6	ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCCTCTGGTACCGTCAGTCCC CATAGAGATAGGGGTTTCGTCCCAAAGAAAAGAAGTTTACAACCCAGAGGGCCTTCATC CTCCACGCGGCATGGCTGGTTCAGACTTGCGTCCATTGACCAATATTCCTTACTGCTGCC TCCCGTAGGA
B7	CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGGGAAGAACAAGT ATCGGAGTAACTGCCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT GCCAGCAGCCGCGGTAATA
B8	ATTACCGCGGCTGCTGGCACCTTTTTGCCCTATTACCGCGGCTGCTGGCACGTAGTTAGC CGTGGCTTATTCCTCAGGTACTGTCCTTACTCCTCCCTGAGAAAAGAGGTTTACGACCCG AAGGCCTTCTTCCCTCACGCGGCGTCGCTGTGTCAGGCTTTCGCCCATTGCACAATATTC CTTGCTGCTGCCTCCCGTAGGA
B9	CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATG CCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGACGGAAAGAAATCGCC ATTCCTAATACGAGTGGTGGATAACGGTACCGTCATAAGAAGCACCGGCTAACTACGTG CCAGCAGCCGCGGTAATA

B10	CAGACGTATTATCTTCCCGGTGAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGC GGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAG GGCCG
B11	ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTTCAGAGGTACCGTCAGGTCG CCGACGTATTAGGTCGACGAGGTTTCGTCCCTCTTGACAGGGCTTTACGACCCGAAGGCC TTCATCACCCACGCGGCGTCGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCCCACT GCTGCCTCCCGTAGGA
B12	ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGGGCTTATTCTCCCGGTACTGTCATTATC ATCCCGGGTAAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCATTGCTGGA TCAGGCTTTCGCCCATTGTCCAATATTCGCCACTGCTGCCTCCCGTAGGA
B13	ATTACCGCGGCTGCTGGCACGTAGTTGGCCGGGGCTTCTTCTGCAGGTACCGTCAATTTC GTCCCTGCTGAAAGCGGTTTACAACCCAAAGGCATTCATCCCGCACGCGGCGTTGCTGCG TCAGGCTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGA
B14	CCTACGGGAGGCAGCAGTGAGGAATCTTGCGCAATGGGGGAAACCCTGACGCAGCAAC GCCGCGTGAGTGAGGAAGGCTTTCGGGTCGTAAAGCTCTGTCAGATGGGAAGAAATGGA TGGAAGCCAATACCTTTCATTCTTGACGGTACCATCAGAGGAAGCACCGGCTAACTCCGT GCCAGCAGCCGCGGTAATA
B15	CCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGCCGAAAGGCTGACGCAGCGAC GCCGCGTGTGGGATGAAGGTCTTCGGATCGTAAACCACTGTCGCGAGGGACGAAATTCT GACGGTACCTCGAAAGGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATA
B16	ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTCTGATGGTACCGTCAGACAC AGGGACTGTTTATCCCTATGCGGTTCTTTCCATCTGACAGAGCTTTACGATCCGAAGACC TTCCTCACTCACGCGGCGTTGCTGCGTCAGGGTTTCCCCCATTGCGCAAGATTCCTCACT GCTGCCTCCCGAGGA
B17	ATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTGCGGGTACCGTCATTATC GTCCCCGCTGAAAGAATTTTACAATCCTAAGACCTTCATCATTCACGCGGCATGGCTGCG TCAGGCTTTCGCCCATTGCGCAAGATTCCCTACTGCTGCCTCCCGTAGGA
B18	ATTACCGCGGCTGCTGGCACGTAGTTGGCCGGTGCTTCTTCTGTAGGTACCGTCACGTTA GCTTCGTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTTG CTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA
B19	ATTACCGCGGCTGCTGGCACAGAGTTAGCCGGGGCTTCCTCTGCGGGTACAATCAAGTCC CCGACGTATGAGGTCGAGTCCCTTTTTCCCCGCTGACAGGAGTTTACAATCCAAAGACCT TCATCCTCCACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAAATTCCCCACTG CTGCCTCCCGTAGG
B20	ATTACCGCGGCTGCTGGCACGAAATTATGCGGTGCTTATTCCTCACGTACCGTCAGCCCC ACCGGGTATTATCCGGGACTATTTCTCTCCTGAGAAAATAGGTATACAACCCGAAGGCCT CCATCCCTCACGCGGCGTTGCTGCTTCTAGCTTTCGCCCATTGAGCAAAATTCCCGACTG CTGCCTCCCGTAGGA
B21	CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAAC GCCGCGTGGAGGACGAAGGTCTTCGGATCGTAAACTCCTGTCAGACGGGACGAAGGTGC CTGGGTAAATAATCCAGGTGCTTGACGGTACTGTCAGAGGAAGCCCCGGCTAACTCCGT GCCAGCAGCCGCGGTAATA
B22	CCTACGGGAGGCAGCACTGAGGAATATTGGTCAATGGGCGGAAGCCTGAACCAGCCATC CCGCGTGCATGAAGACTGCCCTATGGGTTGTAAACCGCTTTTCCAGGGGGTGAATAGTCG CGACGTGTCGTGGCATGACGGTACCCTGGGAATAATCATCGGCTAACTCCGTGCCAACA GCCGCGGTAATAGGGCAAAAAGGTGGACATTGGGATCCTACCAGCATCCAGCAGCCGCG GTAATT
B23	ATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTCTTCTTACGGTACCGTCATCCCC ACCCGGTATTAGCGGATAGGATTTCTTTCCGTCCGAAAGAGCTTTACAACCCCAAAGACT TCTTCACTCACGCGGCATGGCTGGATC
B24	CCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGGAAGCCTGATCCAGCAAC GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGACGAAGCGCA AGTGACGGTACCTACAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATA
B25	TACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTATTCATCAGGTACCGTCATTATTCTT CCCTGATAAAAGAGGTTTACGACCCGAAGGCTGTCCTCCCTCACGCGGTGTTGCTGCGTC AGGCTTTCGCCCATTGCGCAAAATTCCCTGCTGCTGCCTCCCGTAA

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33533370

PROTECTIVE EFFECT OF CURCUMIN ON DIFFERENT TISSUES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* W., 1792) AGAINST EXPOSITION TO CHLORPHYRIFOS

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(Received 13th Dec 2018; accepted 20th Feb 2019)

Abstract. In this study, protective effects of Curcumin (CUR) against Chlorphyrifos (CPF)-induced oxidative stress on blood serum, liver and gill tissues of rainbow trout (*Oncorhynchus mykiss*) were investigated. In the first group, normal water and recommended diet were used as the control group. The second fish group was exposed to water containing 0.040 mg/l CPF. The third fish group received only 0.5% (CUR1) and the fourth group received only 1% CUR (CUR2). The fifth group was exposed to water containing 0.040 mg/l CPF and received only 0.5% (CPF + CUR1). The sixth group was exposed to water containing 0.040 mg/l CPF, giving and received only 0.1% (CPF + CUR2). Samples were taken from the blood, liver and gill tissues and total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI) were measured. The supplementation of CUR with CPF attenuated the adverse effects of CPF intoxication by reducing TOS and OSI and increased TAC in serum and liver. These results showed that CPF, alone, increased oxidative stress in rainbow trout but CUR reduced oxidative stress alone or when combined with CPF in the serum and liver, but not in the gill.
Keywords: *insecticides, turmeric, liver, gill, blood antioxidant, fish*

Introduction

Pesticides are synthetic or organic compounds used to destroy organisms that negatively affect human activities. The pesticides used in the struggle against weeds, insects and other harmful substances in the fields where the cultivated plants are grown pollute to the groundwater sources in various forms and can reach aquatic environments through infiltration (Karacan, 2007). Chlorpyrifos (CPF) [O, O-diethyl O- (3, 5, 6-trichloro-2-pyrrolidyl)-phosphorothioate)] is a broad-spectrum organophosphorus insecticide used to control insect and arthropod populations in agricultural areas (Girón-Pérez et al., 2006). CPF, which has low water solubility, directly affects neurotransmission through inhibiting acetylcholinesterase activity in the organism. Unlike other organophosphorous pesticides, CPF has a long lasting effect and accumulates in fat tissues (Kammerbauer and Moncada, 1998).

In aquatic environments, living creatures, especially fishes, are primarily affected by pesticides. In genotoxicity studies in aquatic environments, various tests that were prepared for mammals in order to determine the clastogenic effects of chemical

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33713385

substances, have been modified in different ways and used on aquatic creatures such as especially fish, mussels and other crustaceans (Burgeot et al., 1995; Sanchez-Galan et al., 1998). In acute toxicity studies, it has been reported that 50-70% mortality was seen in *Aulacomya ater* (Molina, 1782) (Bivalvia: Mytilidae) at 0.050 mg/l CPF levels and inhibited enzyme activity of 0.02-0.16 mg/l acetylcholinesterase (Fuhrer et al., 2012).

Curcumin (CUR) has many effects such antiinflammatory, antioxidant, anticancerogenic, antimutagenic, anticoagulant, antidiabetic, antibacterial, antiviral and nerve preservative effects in a broad spectrum which is made of turmeric/curcuma that is used as a spice and gives yellow color to the meals (Turmeric, *Curcuma longa*) (Naik et al., 2011). CUR has been extensively investigated for its protective effect on the cardiovascular system, as it facilitates the release of many reactive oxygen radicals, especially superoxide anions, nitrogen dioxide radicals and hydroxyl radicals (Sreejayan, 1997; Duan et al., 2012). In these studies, CUR has been shown to inhibit endotelial and smooth muscle cells from damage (Srivastava and Mehta, 2009), to prevent heart damage (Nirmala et al., 1999), to accelerate the restructuring of heart and vessels have been reported to improve oxidative damage (Morimoto et al., 2008; Bala et al., 2006).

Among all creatures living in aquatic environments, fish have a very important place in the use of these tests because they can be easily kept, fed and grown up in laboratory environments. Akdemir et al. (2016) investigated the effects of different rates of CUR on the growth performance of trout and reported that CUR extract reduced the negative impact of high stocking intensity on performance of rainbow trout. Cao et al. (2015) found that Carp (*Cyprinus carpio*) which are fed CUR at different doses for 60 days increased their oxidative tolerance to carbon tetrachloride-induced liver damage. Similarly, the protective effect of CUR on the *Anabas testudineus* (Bloch) was studied and for this purpose, fish were fed for 2 to 8 weeks with fish feed to which two doses of CUR (0.5% and 1%) was added. As a result of the research, the antioxidant status, protein content and texture of fish were investigated acutely and chronically. In all groups fed with CUR, the lipid peroxidation value was reduced or unaffected. The antioxidant enzyme activity level is increased as the glutathione level changes depending on the time and the dose. In addition, the protective effect of CUR was also determined in histological analyzes (Manju et al., 2012).

Organophosphates are the insecticides most commonly used worldwide in the pest control of crops. The global pesticide market is growing at around 1% per annum as tonnage. Herbicides are ranked first in agrochemicals with a share of 47%. This is followed by insecticides by 29% and fungicides by 19%. Herbicides and insecticides that make up more than 70% of agricultural drugs are distributed around the land use (Dağ et al., 2000). The effects of these pesticides are different according to the environment and amount of accumulation. CPF is commonly used in agricultural areas around the dam reservoir (EPDA, 2004).

Organophosphorus insecticide (OPI) has been reported to alter oxidative stress characteristics (Malkovics, 1995). Recent studies on pesticide poisoning have highlighted the oxidative stress caused by free radicals produced by pesticides. The generation of hydrogen peroxide (H_2O_2) is catalyzed by the dismutation reaction of superoxide dismutase (SOD) and superoxide anion (O_2^-). Hydrogen peroxide is catalytically decomposed by water (CAT) activation and molecular oxygen. If the free radical production crosses endogenous antioxidant levels, they can lead to cell damage or death (Malkovics, 1995; Zámocký et al., 1999).

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DOI: http://dx.doi.org/10.15666/aeer/1702_33713385

CPF intoxication has been shown to significantly reduce reduced glutathione (GSH), catalase (CAT) and glutathione S-transferase (GST) activities in the rat liver. As a result of zinc treatment, CPF-treated animals have been shown to have an elevation in the levels of GSH, catalase and GST, as well as a significant decrease in the levels of SOD (Goel et al., 2005).

Dimethoate, an organophosphate pesticide, has been reported to increase catalase, (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activity in hepatic cytochrome P450 enzymes, brain and liver lipid peroxidation in acutely exposed male rats (Sharma et al., 2005).

These results show that organophosphorus (CPF) may be an oxidant. This study is important because the treatment of CPF exposed fish and CUR is also important in preventing toxic effects. Therefore, it is aimed to determine the protective effect of CUR against the toxic effect of Chlorpyrifos. There was no information on this subject in the literature search.

In this context it is planned within three main objectives; 1) to investigate the oxidative stress of CPF in some tissues 2) to examine the effects of CUR alone on the oxidative mechanism 3) to determine the protective effects of CUR on the oxidative stress resulting from CPF toxicity. So, the protective effects of Curcumin against chlorpyrifos-induced oxidative stress on serum, liver and gill tissues of rainbow trout (*Oncorhynchus mykiss*) were investigated.

Materials and methods

Materials

Chemicals

CUR, CPF (96.5% purity (technical grade)), were purchased from Sigma Chemicals (Sigma-Aldrich, Turkey). All other chemicals were purchased from standard commercial suppliers (St Louis, MO, USA).

In the study; the establishment and implementation of the feeding experiment was carried out in concrete pools in the Department of Fisheries and Aquaculture at Harran University Bozova Vocational School for Higher Education for 30 days. 120 male rainbow trout (*O. mykiss*) (2 replicate) of 2 years old, with an average weight of 80-100 g, were obtained from Karacalar Food, Agriculture, Aquaculture Import and Export Limited Company. Water temperature, pH and dissolved oxygen were 15.0 ± 1.5 °C, 7.8 ± 0.9 and 8.8 ± 0.3 mg/L, respectively.

It is produced from marine raw materials and have high digestibility and high protein content supplied from a commercial company.

CPF was dissolved in acetone as stock solution (0.1%) and this stock solution was mixed into pools at defined ratios. It was stored at 4 °C and from these daily requirements is added to pools. The use of fish and experimental application protocols were supervised by the Dollvet A.Ş. Animal Experiments Local Ethics Committee (Şanlıurfa-Turkey). Analyzes of the tissues were carried out at Harran University Biology Department laboratories.

Methods

Rainbow trout was kept in 500 l pools with continuous aeration for 30 days. In the study, pellet trout feed was given two times a day as once in the morning and once in the evening for feeding fish in the pools which shall be ventilated continuously. The stock solutions of CPF, the pesticide, given to the environment where fish live, were prepared and the water in the pools was changed by 1/3 per day. CUR, which will be used in the study, is adjusted to 0.5% and 1.0% of the total weight of the fish (Manju et al., 2012). The CUR extract was dissolved in DMSO and stock solutions were prepared in PBS at pH 7.2 and given intraperitoneally to fish. The fish was placed in 12 pools as 10 fishes (2 replicates) in each pool. Fish were divided into 6 groups (control group, CPF, CUR1, CUR2, CPF + CUR1, CPF + CUR2). In the first group, normal water was used as the control group and the fish were given feed without additives (Control). The second fish group was exposed to water containing 0.040 mg/l CPF (Doğu et al., 2015). The third fish group received only 0.5% CUR (CUR1), received eight doses (two doses per week) of CUR by intraperitoneal injections (IP) and the fourth group received only 1% CUR (CUR2), received eight doses (two doses per week) of CUR by IP. The fifth group was exposed to water containing 0.040 mg/L CPF and given CUR (CPF + CUR1) at a rate of 0.5% of the total weight of the fish, received eight doses (two doses per week) of CUR by IP. The sixth group was exposed to water containing 0.040 mg/L CPF and given CUR (CPF + CUR2) at 1.0% of the total weight of the fish, received eight doses (two doses per week) of CUR by IP. Experimental conditions are summarised in *Table 1*.

Table 1. *Experimental design of the study*

	Experimental groups					
	Control	**CPF**	**CUR1**	**CUR2**	**CPF+CUR1**	**CPF+CUR**
CPF (mg/l)	-	0.040	-	-	0.040	0.040
CUR (%)	-	-	0.5	1	0.5	1

At the end of the study 0.6 mL/L 2-phenoxyethanol was administered as an anesthetic agent before taking blood samples from all experimental groups. Subsequently, blood samples from each fish were transferred individually to heparinized test tubes. The anesthetized fish were rinsed with distilled water prior to the dissections to avoid the external pesticide residue.

Fish were dissected and liver and gill tissue were collected and stored at -20 °C for examination. Blood from the fish was transferred to EDTA glass tubes and allowed to stand at +4 °C at 3000 rpm for 1 h at +4 °C before being centrifuged for 15 min by refrigerated centrifugation. After centrifugation, the plasma was separated from the erythrocytes and blood samples were stored at -20 °C until the enzymatic antioxidants were examined. Then the following tests were applied. Liver and gill were collected and perfused with isotonic salt solution (0.9% NaCl) to remove blood and others and used for preparation of tissue homogenate.

Preparation of tissues homogenate

The whole liver and gill tissues were homogenized in phosphate saline buffer (100 mM) containing EDTA (1 mM; pH 7.4; 1:10 w/v) and then centrifuged (12,000×g, 30 min, 4 °C). The supernatant was separated and used for biochemical analysis.

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Oxidative stress test

Total antioxidant status (TAC)

Intracellular total oxidant capacity (TAC) was measured using Rel Assay Diagnostics total oxidant capacity measurement kit (Gaziantep/Turkey). In this measurement method, 2,2' -azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS radical) is used. ABTS radical loses its blue and green color (faded) according to antioxidant concentration and antioxidant capacity. This color change is assessed by measuring the absorbance value at 660 nm. The principle of this measurement method is based on the fact that the ABTS molecule in the presence of hydrogen peroxide is oxidized to the ABTS+ molecule. The color of radical which is dark brown at 30 mmol/L acetate buffer and t pH: 3.6 is faded at 0.4 mol/L acetate buffer and pH: 5.8. There is an inverse relationship between color change and the amount of antioxidant in the sample. The reaction rate was expressed as Trolox equivalent/L by calibrating with the standard method Trolox (Erel, 2005).

Total oxidant status (TOS)

Intracellular total oxidant status (TOS) was measured by using Rel Assay Diagnostics total oxidant capacity measurement kit (Gaziantep/Turkey). In this method; Reagent 1: The main solution is prepared by dissolving 25 mM H_2SO_4 in 140 mM NaCl solution. The main solution is prepared by first dissolving 10% glycerol and then dissolving 250 µM xylenol orange in total volume. Reagent 2: Reagent is prepared by dissolving 10 mM o-Dianisidine dihydrochloride in the main solution and then dissolving 5 mM ammonium ferrous sulphate. Principle: The oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to the ferric ion. The glycerol in the medium accelerates this reaction to about three fold. Ferric ions form a colored complex with xylenol orange in acidic environment. The intensity of the color associated with the amount of oxidants present in the sample will be measured spectrophotometrically, in units of mol H_2O_2 Eqv./L (Erel, 2005).

Oxidative stress index (OSI)

The TOS: TAC ratio was used as the oxidative stress index (OSI). To perform the calculation, the units of TAC, mmol Trolox equivalent/L, was converted to µmol Trolox equivalent/L. OSI was calculated and expressed as Unit AU (Arbitrary Unit) and as shown the formula below (Erel, 2005):

$$OSI = (((TOS,\ \mu mol/L) / (TAC,\ \mu mol\ Trolox\ equivalent/L)) / 100)$$

Statistical analysis

Statistical analysis of the data was carried using SPSS 16.0 software. Analysis of variance (ANOVA) and Tukey' post hoc test was employed to compare the differences in TOS, TAC and OSI in the serum, liver and gill. A p value less than 0.05 was considered as statistically significant. All data are expressed as mean ± SE.

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Results

The results of the examined parameters of blood serum are given in *Figures 1, 2* and *3*. There were significant differences in serum TOS, TAC and OSI values ($p < 0.001$) in serum. Serum TAC was similar when compared to the control groups given CUR 0.5% and 1%. The serum TAC values of the CPF group were significantly lower than those of the CUR-1 group. When combined CUR with CPF, serum TAC values were significantly lower than those of the groups which were given only CUR-1. When compared with the control, the highest TOS was obtained in the CPF group, whereas the TOS values of the CUR-treated groups and CPF+CUR in both doses were similar. CUR (0.5% and 1.0% quantities) supplied with CPF significantly reduced the TOS values when compared to the CPF group in serum. Serum OSI were significantly higher in CPF groups compared to control group ($p < 0.001$). There were no significant differences between control and Curcumin groups, control and CPF+CUR groups in OSI, except CPF+CUR2 in OSI (*Fig. 3*).

The results of the examined parameters of liver tissue are given in *Figures 4, 5* and *6*. Significant differences were observed between the groups in liver TOS, TAC and OSI values ($p < 0.001$). Liver TOS values were lowest in the CUR group and the highest in the CPF group compared with the control group. In the CPF + CUR group, TOS were significantly lower than in the CPF group, although similar to the control group. Liver TAC was found to be at the highest level of CUR 1.0% when compared with the control group. The CPF group had the lowest TAC compared to the control group. Liver OSI was the lowest in the CPF group and the highest in CUR group compared to the control group, but similar in the other groups.

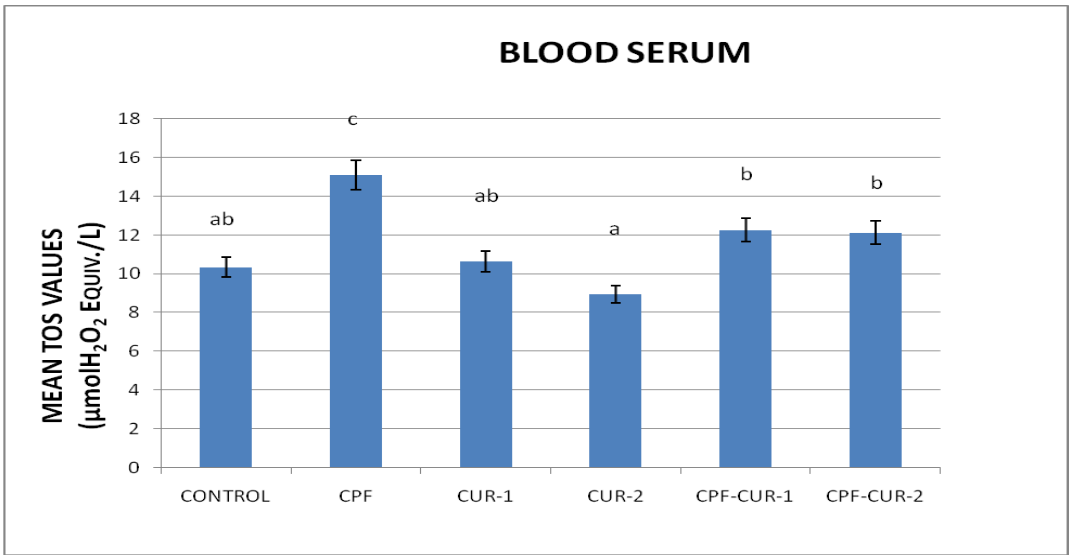

***Figure 1.** Effect of CPF and Curcumin on TOS values of blood serum in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

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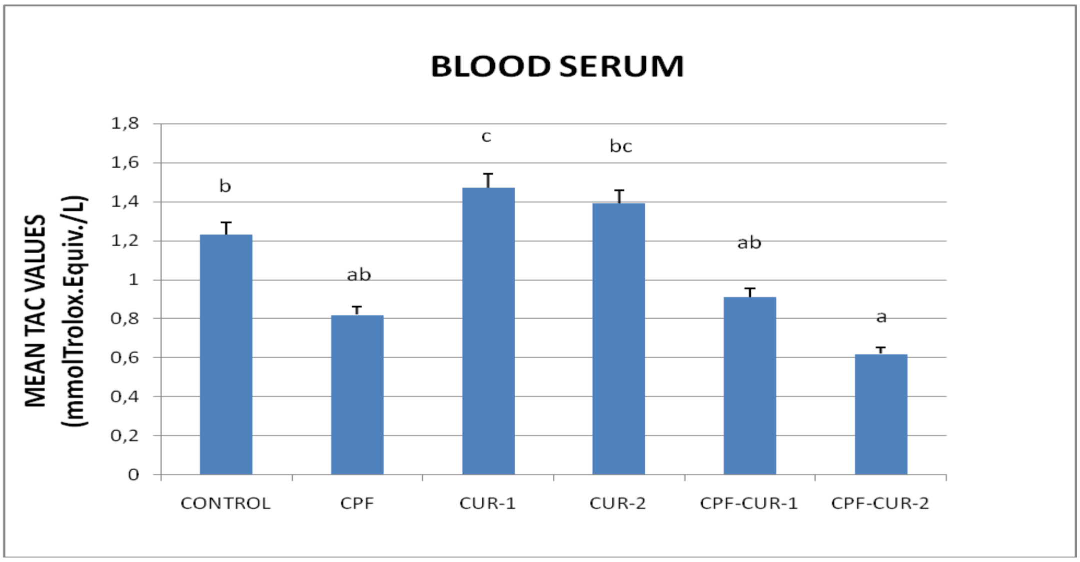

***Figure 2.** Effect of CPF and Curcumin on TAC values of blood serum in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

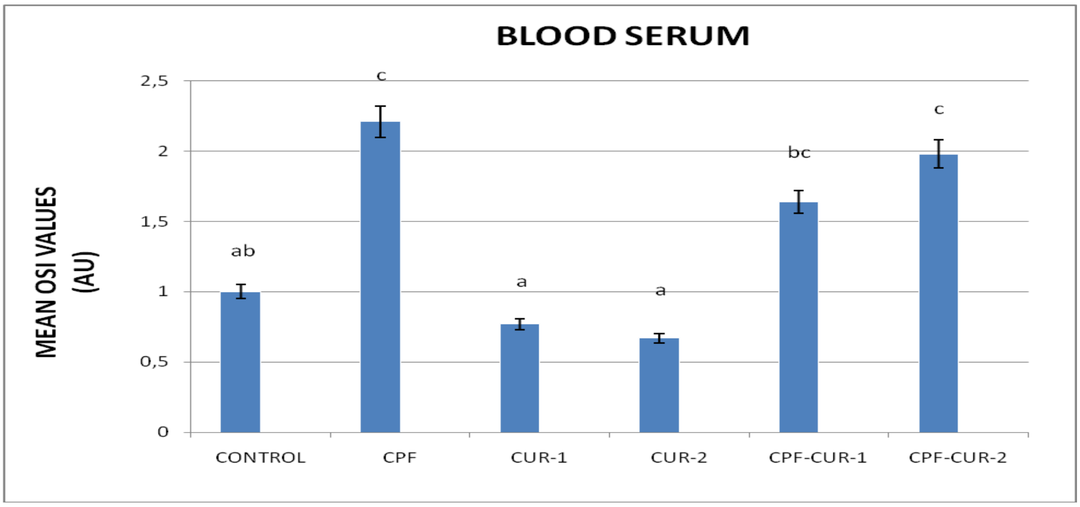

***Figure 3.** Effect of CPF and Curcumin on OSI values of blood serum in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

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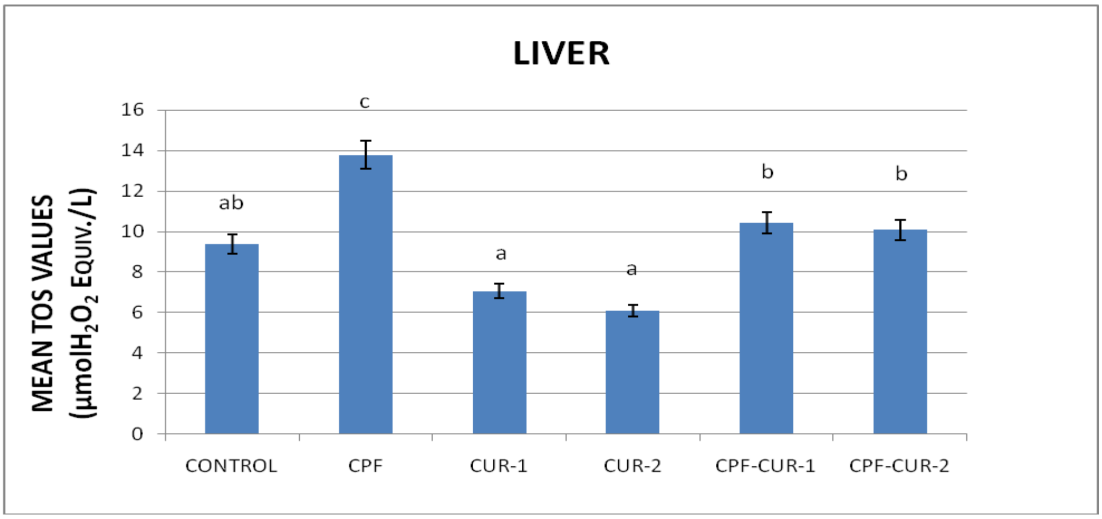

Figure 4. *Effect of CPF and Curcumin on TOS values of liver tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

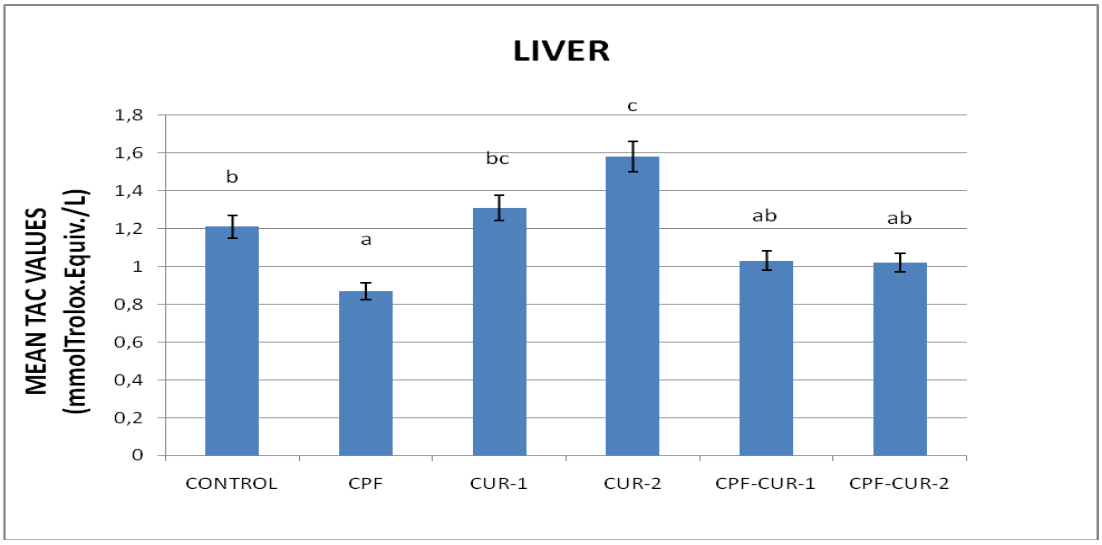

Figure 5. *Effect of CPF and Curcumin on TAC values of liver tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

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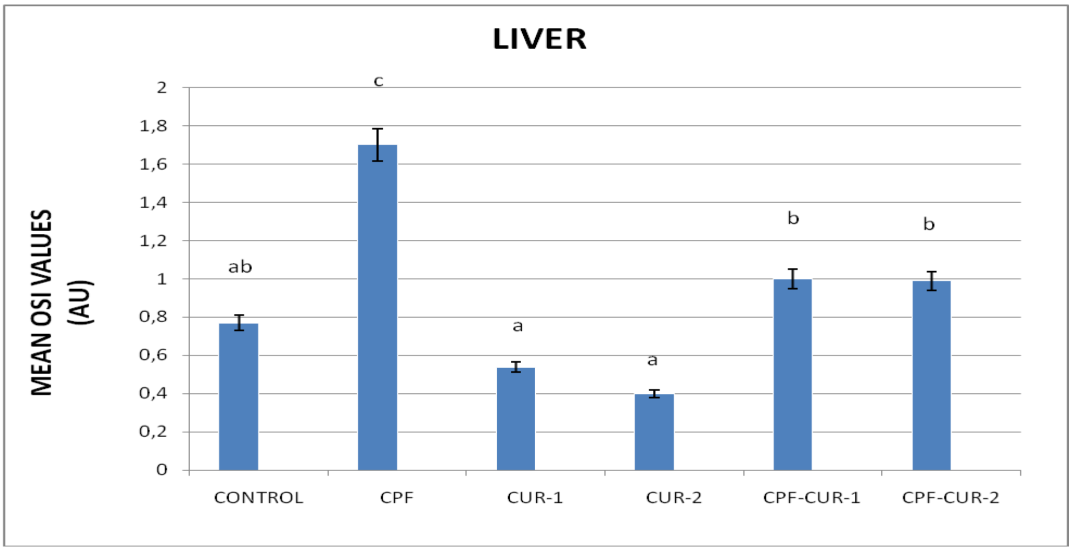

Figure 6. *Effect of CPF and Curcumin on OSI values of liver tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

The results of the examined parameters of gill tissue are given in *Figure 7, 8* and *9*. In the gills, the TOS were significantly higher ($p < 0.001$) in the CPF and CPF + CUR-1 groups when compared to the control. The TOS in both CUR and CPF + CUR-2 groups were similar to those of the control group. The TAC in the all groups in the gill was not found to be significant when compared to control group. Only the gill TAC value of the fish given CUR at % 1 was higher than the fish that received 0.5% CUR with CPF ($p < 0.05$).

The gill OSI values were significantly higher ($p < 0.01$) in the CPF + CUR-1 group when compared to the control. OSI values of fish fed both doses of CUR were significantly lower than that of fed the CPF + CUR-1 group ($p < 0.05$). The OSI in gill in fish fed at a dose of 0.5% of CUR were lower than the fed with CPF and CPF + CUR-1.

Discussion

CPF is one of the environmental toxicants, and hepatoxicity, genotoxicity, behavioral toxicity, neurotoxicity have been reported in fish (Deb and Das, 2013). Blood and liver is an important indicator for evaluating the physiological and pathological conditions in the organism when exposed to environmental pollution. Free radicals attack the membrane components of erythrocytes and cause a change in membrane structure and function (Prieto et al., 2006; Cunha-Oliveira et al., 2013; Mustafa, 2016). In order to test the oxidative stress in vivo, TOS, TAC and OSI were used in the blood serum, liver and gill tissues.

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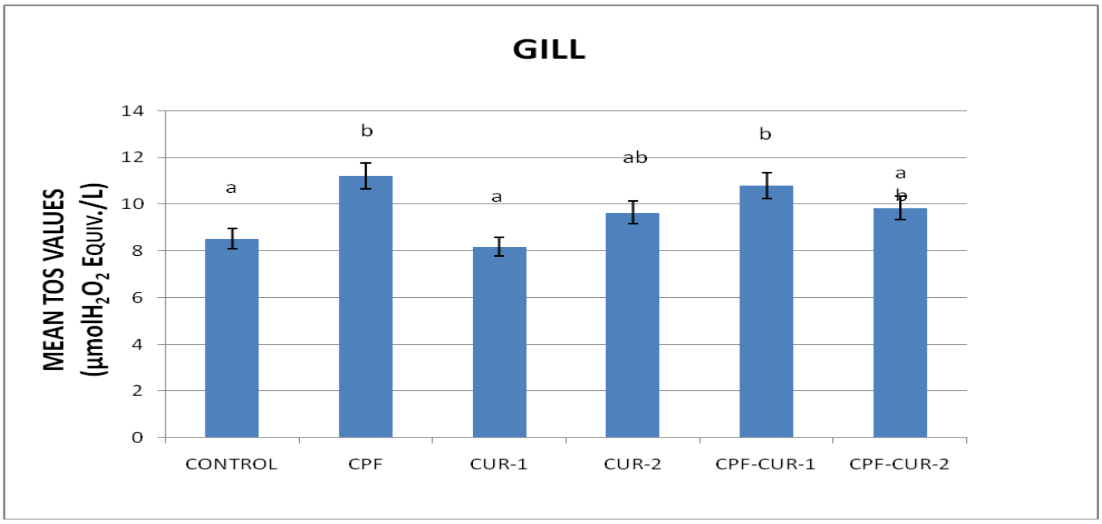

***Figure 7.** Effect of CPF and Curcumin on TOS values of gill tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

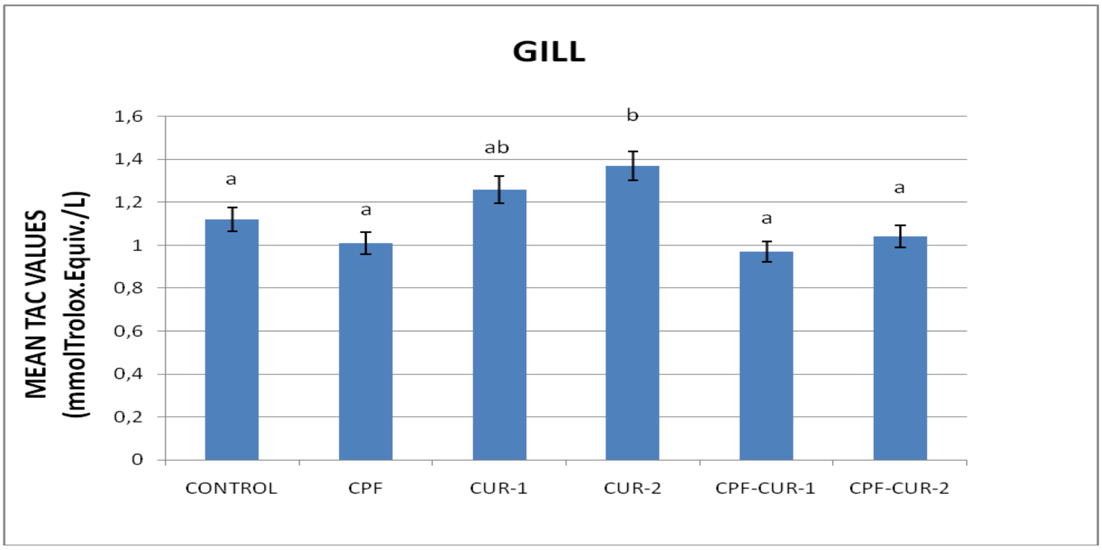

***Figure 8.** Effect of CPF and Curcumin on TAC values of gill tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

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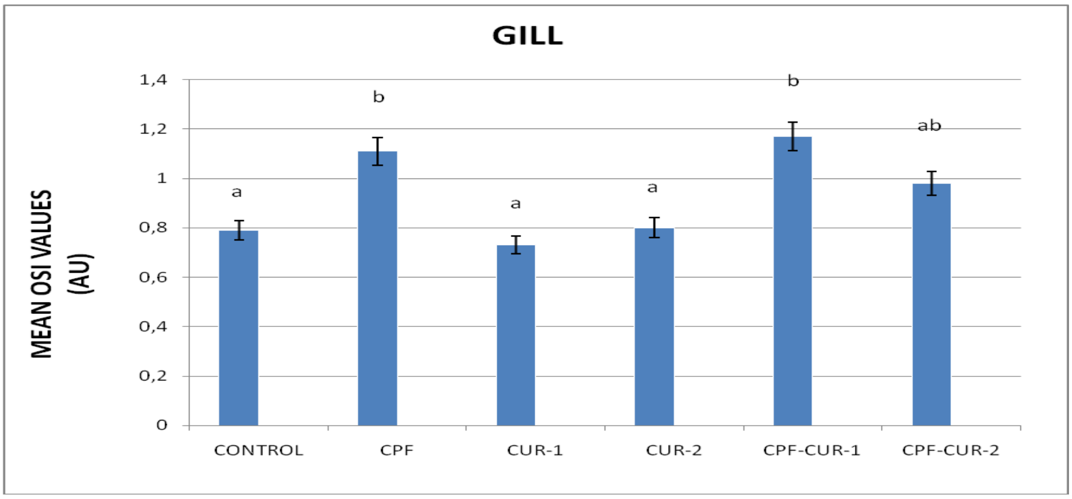

***Figure 9.** Effect of CPF and Curcumin on OSI values of gill tissue in O. mykiss (n = 10). Control: The fish was fed with pellet without additives. CFP (Chlorpyrifos): The group was exposed to water containing 0.040 mg/l CPF. CUR1: The group received only 0.5% Curcumin. CUR2: The group received only 1% Curcumin. CPF + CUR1: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at a rate of 0.5% of the total weight of the fish. CPF + CUR2: The group was exposed to water containing 0.040 mg/l CPF, giving Curcumin at 1.0% of the total weight of the fish. All data are presented in mean ± SE (n = 20). abc: Bars bearing common letters are not statistically different (p > 0.05). There was a significant difference between the groups (p < 0.001)*

The results showed that CPF increased levels of TOS and OSI in blood serum. When CUR was given alone and in combination with CPF, TOS values were similar to the control group. These results showed that CPF alone increased oxidative stress and CUR reduced oxidative stress when the combination of CPF and CUR is given. CUR given alone did not have a significant effect on TOS and OSI levels. Therefore, CUR may be thought to have oxidative stress-reducing effect caused by CPF. Previous studies have similarly reported that the toxic effects of various toxicants such as metal, solvent and 6-OHDA are weakened by CUR (Yadav et al., 2010; Jaisin et al., 2011; Yang et al., 2014). The superiority of TAC test is to show the antioxidant capacity of all the antioxidants in the sample (Kusano and Ferrari, 2008; Hismiogullari et al., 2015). In the present study, 0.5% and 1.0 levels of CUR did not change the level of blood serum TAC according to the control group. TAC level in CPF and CPF + CUR-1 groups were similar to control group. TAC level in CPF + CUR-2 groups were lower that of the control. These results indicate that CUR does not have a significant effect on blood antioxidant levels in fish at these levels in feed. However, it has been reported that CUR has antiradical/antioxidant activity and potential cell regenerative properties (Mustafa, 2016; Karahan et al., 2016). In fact, the combined delivery of CPF and 1.0% of CUR significantly reduced TAC levels compared to the control group. Thus, it can be said that when high levels of CUR combined with toxic substances reduces the level of antioxidants.

Oxidant and antioxidant levels in liver homogenates can be used as a marker of hepatocellular sensitivity. In the present study, liver TOS and OSI levels increased significantly while TAC levels decreased in CPF-treated fish compared to controls. Free

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radicals and ROS are key factors in cell damage. These factors have been shown to impair function and structural integrity in a few cells (Estebe et al., 2011; Karahan et al., 2016). Reactive oxygen species and lipid peroxidation lead to degradation in membranes and protein liver cells. Microcystin's (MCs) toxicity has been shown to increase lipid peroxidation as an indicator of oxidative damage in *Brycon amazonicus* liver homogenates (Martins et al., 2017). Furthermore, 0.5% and 1.0% levels of CUR reduce the TOS levels, while 1.0% level of CUR increased the TAC levels significantly. In addition, this is an indication of non-hepatotoxic potential of both doses of CUR. When both amounts of CUR (CPF + CUR-1 and CPF + CUR-2) were given in combination with CPF, the TOS level significantly decreased compared to the CPF alone. This shows that antioxidants derived from CUR are used in the liver to reduce the effects of oxidation of the liver caused by CPF. Furthermore, it may be suggested that CUR has a hepatoprotective effect against toxic substances such as CPF. The CUR antioxidant activity is due to 2 phenolic OH groups (Prasad et al., 2014. These phytochemicals may have increased levels of cellular antioxidants and increased levels of antioxidants (Dinkova-Kostova et al., 2000). Intraperitoneal application of CUR and dexmedetomidine in rats has been shown to reduce ischemia-reperfusion injury and oxidative stress in the kidney (Karahan et al., 2016).

The TOS values in the gill homogenate were higher only in the CPF and CPF + CUR-1 groups than in the control group. CUR did not cause significant changes in TOS values in the gills, compared to control. Similarly, TAC levels of all groups were similar to control groups. CPF increased TOS levels by causing oxidative damage in the gills. CUR has not been able to reduce oxidative damage in the gills and has not been associated with antioxidant levels. These results suggest that antioxidant activity is limited in the gills. Similarly, the gill GPx activity of *Brycon amazonicus* (Martins et al., 2017) and *O. niloticus* (Prieto et al., 2006) exposed to microcystin toxicity has been altered but significantly increased in the liver. Martins et al. (2017) reported oxidative damage to Microcystin toxicity in the gills of *Brycon amazonicus*. CUR has been shown to inhibit and stimulate apoptotic signaling (Khar et al., 1995; Somasundaram et al., 2002). CUR may induce oxidative damage by caspase-3 activation. However, the mechanism of CUR that causes the induction of ROS formation is not fully understood (Hsuuw et al., 2005).

As you seen in the findings, the OSI can better point out the oxidative stress status. For this reason, TAS and TOS could be determined and then OSI values should be calculated. This could be more beneficial for estimation of comprehensive oxidative stress status (Wu et al., 2017). Intraperitoneal application of CUR and dexmedetomidine in rats has been shown to reduce ischemia-reperfusion injury and oxidative stress in the kidney.

Conclusions

The results of the research show that the level of antioxidants varies among tissues and is highest in the liver. It was observed that the dose of 0.040 mg/l CPF at toxic level increased the oxidation level and lowered the antioxidant level of blood and liver in fish but not having any effect on the gill antioxidant level. CUR, given at a rate of 0.5% according to the live weight, increased the antioxidant level in the blood serum. In the presence of toxic substances such as CPF, CUR can also trigger oxidation in blood. The effect of CUR on tissues is not stable, but may increase the level of antioxidants or

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DOI: http://dx.doi.org/10.15666/aeer/1702_33713385

oxidants, depending on the presence of toxic substances such as CPF, tissue and the amount of toxic substances in feed. Also, our findings were showed that oxidative stress index could be often used as an indicator of oxidative stress in trout. Future studies should focus on molecular mechanism involved in protective effect of CUR on CPF-ınduced cell damage.

Acknowledgements. We would like to thank Harran University Scientific Research Coordination Unit [Project Number 16213] for supporting this research.

Ethics statement. All experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the Dollvet A.Ş. (Protocol No: 2014/61).

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BENTHIC MACROINVERTEBRATE DIVERSITY AND FUNCTIONAL FEEDING GROUPS IN RELATION TO PHYSICOCHEMICAL FACTORS IN SANJIANG PLAIN WETLANDS, NORTHEAST CHINA

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(Received 18th Dec 2018; accepted 14th Feb 2019)

Abstract. Sanjiang plain wetland is the largest freshwater ecosystem in northeast China, and with its richness in animal and plant species, it displays a high biodiversity. This study provides information about benthic macroinvertebrate diversity and functional groups in association with physicochemical parameters in wetland habitats of Sanjiang plain. Benthic macroinvertebrate samples were collected using a D-frame aquatic net. Shannon-Wiener, Simpson and Pileou's evenness indices were calculated in terms of abundance. The relationship between measured physicochemical variables and benthic macroinvertebrate functional feeding groups (FFGs) was explored using CCA. A total of 57 macroinvertebrate taxa were collected from the 16 sampling sites. Aquatic insects were the most abundant with 11 families, Dytiscidae, Chironomidae, Leptophlebiidae, Belostomatidae, Corixidae, Gerridae, Corduliidae, Gomphidae, Macromiinae, Libellulidae and Phryganeidae. Chironomids were the most diverse and abundant with 26 taxa. The metrics of abundance, Shannon-Wiener, Simpson and Pileou's evenness indices differed significantly among the 16 sampling sites ($p = 0.0163$, $p = 0.0092$, $p = 0.0474$, $p = 0.0222$, respectively). The findings showed that these 57 benthic macroinvertebrate taxa were categorized in six functional feeding groups, including 19 predators, 15 gathering-collectors, 7 scrapers, 6 filtering-collectors, 5 omnivores and 5 shredders. CCA results displayed that benthic macroinvertebrate functional feeding groups had strong relationships with the physicochemical characteristics in the wetlands of Sanjiang plain.
Keywords: *aquatic macroinvertebrate fauna, functional group composition, water parameters, wetland, China*

Introduction

Wetland ecosystems account for 5-8% of the Earth's surface area (Sui et al., 2017) with their characteristic mix of land and water. They display the richest biodiversity, highest productivity, and highest ecological impact of all terrestrial ecosystems (Sui et al., 2017). They are important for the reduction of greenhouse gas emission, conservation of biodiversity, and contribute to regulating the balance of the Earth's ecosystem (Galloway et al., 2004; Dentener et al., 2006). Aquatic ecosystems are mostly affected by the large-scale agricultural expansion which influences the overall quality of the ecosystems by directly altering habitat, channel structure, and water quality posing severe threats to aquatic biodiversity (Vitousek et al., 1997; Allan, 2004; Azrina et al., 2006; Dudgeon et al., 2006; Smith and Lamp, 2008; Carlson et al., 2013;

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DOI: http://dx.doi.org/10.15666/aeer/1702_33873402

Narangarvuu et al., 2014). Most aquatic macroinvertebrates reside in the benthic biotope for at least part of their life, relatively immobile, and very sensitive; therefore any disturbances in the aquatic environment may cause them to disappear or a reduction in their diversity (Hilsenhoff, 1988; Zamora-Muñoz et al., 1995; Morse et al., 2007; Narangarvuu et al., 2014). Hence, biomonitoring is considered as one of the alternatives for rapid assessment of the aquatic environment status (Tan and Beh, 2016).

Aquatic macroinvertebrates are widely used as biological indicators of the ecological conditions, their abundance and diversity being used to evaluate the occurrence of pollution (Abel, 1966; Hellawell, 1986; Metcafe, 1989; Lenat, 1993; Rosenberg and Resh, 1993; Rosenberg et al., 2008; Southerland et al., 2008; Shabani et al., 2016). While macroinvertebrates in wetlands share some of their attributes in streams and rivers useful such as ubiquity, sedentary nature, moderately long life cycles, environmental sensitivity (Rosenberg and Resh, 1993), they have many other attributes that reduce their ability to reflect environmental quality such as strong responses to weather and seasonality, and frequent dispersal into and out of habitats (Rosenberg et al., 2008).

In standing and flowing water ecosystems, aquatic macroinvertebrates are important elements in the ecological dynamics (Hynes, 1970), playing an important role in the cycle of materials and in trophic transfers (Cummins, 1974; Vannote et al., 1980; Cummins et al., 1989; Dunbar, 2010; Shabani et al., 2016; Safari et al., 2018). They are important animal organisms both economically and ecologically (Li et al., 2007).

Understanding the distribution and abundance of invertebrates in aquatic ecosystem has long been a goal of aquatic ecologists (Bergon, 1996). Immature stages of aquatic invertebrates are a vital link between primary producers and fish (Yapo et al., 2018). Most aquatic macroinvertebrates are sensitive to changes in pH, dissolved oxygen, temperature, salinity, turbidity, conductivity, availability of food and other changes in their biotope (Gage et al., 2004; Crisci-Bispo et al., 2007). They are useful surrogates of ecosystem attributes, and the relative abundance of functional groups reflects anthropogenic impact (Merritt et al., 2002; Cummins et al., 2005; Masese et al., 2014).

Sanjiang plain wetland is the largest freshwater wetland in northeast China (Zheng et al., 2013; Liu et al., 2014; Taufik et al., 2015; Wu et al., 2017), and with its richness in animals and plant species, it displays a high biodiversity. It is world-renowned for hosting many rare, large-sized water birds; several most notable ones are globally threatened cranes, such as the Oriental stork (*Ciconia boyciana*), white-naped crane (*Grus vipio*), and red-crowned crane (*G. japonensis*) (Zhang et al., 2009a). Its role in maintaining the ecological balance in the region is irreplaceable (Zheng et al., 2013; Liu et al., 2014). However, qualitative and quantitative studies of aquatic macroinvertebrates in the area remain generally scarce due to the lack of large-scale investigations and insufficient data. Thus, the conservation status of many benthic macroinvertebrates in the Sanjiang plain wetland is not yet assessed. In order to reach a rational and sustainable management of freshwater wetlands of Sanjiang plain, it is very crucial to implement a follow-up study program, to monitor and classify the freshwater macroinvertebrates (Shabani et al., 2016). The identification of species and their distribution patterns provide more information for monitoring and conserving these important ecosystems (Maneechan and Prommi, 2015). Community indices, diversity indices, and functional feeding groups (FFGs) were the most effective measures that can be used in water quality assessment, and have a response across a range of human influence (Rosenberg and Resh, 1993; Merritt et al., 1996, 2002; Karr and Chu, 1999;

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DOI: http://dx.doi.org/10.15666/aeer/1702_33873402

Masese et al., 2014). FFGs are a classification approach that is based on morpho-behavioral mechanisms of food acquisition rather than taxonomic group (Merritt et al., 1996). This study aimed to collect benthic macroinvertebrate fauna, and explore the relationships between macroinvertebrate FFGs and water physicochemical properties in the wetland environments of Sanjiang plain.

Materials and methods

Study area

The Sanjiang Plain is located in the northeast boarder part of the Heilongjiang Province in NE China (Zhang et al., 2009a; *Fig. 1*). It is in a low alluvial plain of Heilong River, Songhua River, and Wusuli River (Zhou and Liu, 2005; Zhang et al., 2009a; Jiang et al., 2011), from 129°11E to 135°05E and from 43°49N to 48°27N, covering a total area of 108,900 km^2 (Liu and Ma, 2002; Jiang et al., 2011; Du et al., 2018). The Sanjiang plain supports one of the largest freshwater wetland complexes in China, and its wetland areas are strongly affected by agriculture drainage and reclamation efforts (Wu et al., 2017). Its elevation in the southwest is higher than in the northeast. The climate in this area belongs to the temperate humid or sub-humid continental monsoon climate. The mean annual temperature ranges from 1.4-4.3 °C (Du et al., 2018), with average maximum of 21-22 °C in July and average minimum -18 °C in January (Wang et al., 2015). The mean annual precipitation is 500-650 mm and 80% of rainfall occurs between May and September (Jin et al., 2015). The frost-free period is 120-140 days (Yang et al., 2018). Most of the rivers in the area have the characteristics of the wetland river: the slight gradient and large channel curve coefficient. There are four main soil types: meadow soil, swamp soil, washed and black soil. They account for more than 95% of the whole area. Main types of vegetation in Sanjiang plain include *Phragmites communis*, *Carex lasiacarpa*, *Carex pseudocuraica*, *Carex meyeriana*, *Alnus sibirica*, *Betula fruticosa*, *Salix brachypoda*, *Lythrum salicaria*, *Calamagrostis anagustifolia* (Wang et al., 2018), and the Sanjiang plain wetland has been designated within list of wetlands of international importance (Zhang et al., 2009). Due to the relatively cold weather, deep surface waters, large marsh patches, and sparse population, reclamation of small Sanjiang plain marshes started relatively late, and the Honghe National Nature Reserve and Sanjiang National Nature Reserve were created (Zhang et al., 2009; Du et al., 2018).

Benthic macroinvertebrate procedures

Sixteen sampling sites were selected based on prospected freshwater ecosystems and accessibility (catchment areas including sites 1, 2, 3, 4, 5 and 6; ponds: sites 7 and 15; marshes: site 8; rivers: sites 9, 11, 12, 13 and 16; lake: site 10; ditches: site 14). The latitude and longitude of the 16 sampling sites were determined using a portable global positioning system (GPS, *Table 1*). Benthic macroinvertebrates were collected using a D-frame aquatic net (30 × 30 cm frame, 500 µm mesh) and the sampling gears were washed thoroughly between sites during three seasons: autumn, spring and summer of year 2016. In each sampling period, 3 replicate samples were collected at each site and the sampled specimens were sieved through a 500 µm mesh sieve in the field for approximately 30-40 min. Macroinvertebrate populations were separated from the sand, mud and substrates by hand. The specimens were grouped according to the sites and

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DOI: http://dx.doi.org/10.15666/aeer/1702_33873402

dates of sampling and placed in 100 ml labelled plastic containers, preserved in 95% ethanol, and then transported to the laboratory for further analysis.

In the laboratory, the macroinvertebrate communities were keyed to species or genus and counted using identification keys of Thorp and Covich (1991); Morse et al. (1994); Merritt et al. (1996); Tong (1996); Dudgeon (1999); Duan et al. (2010).

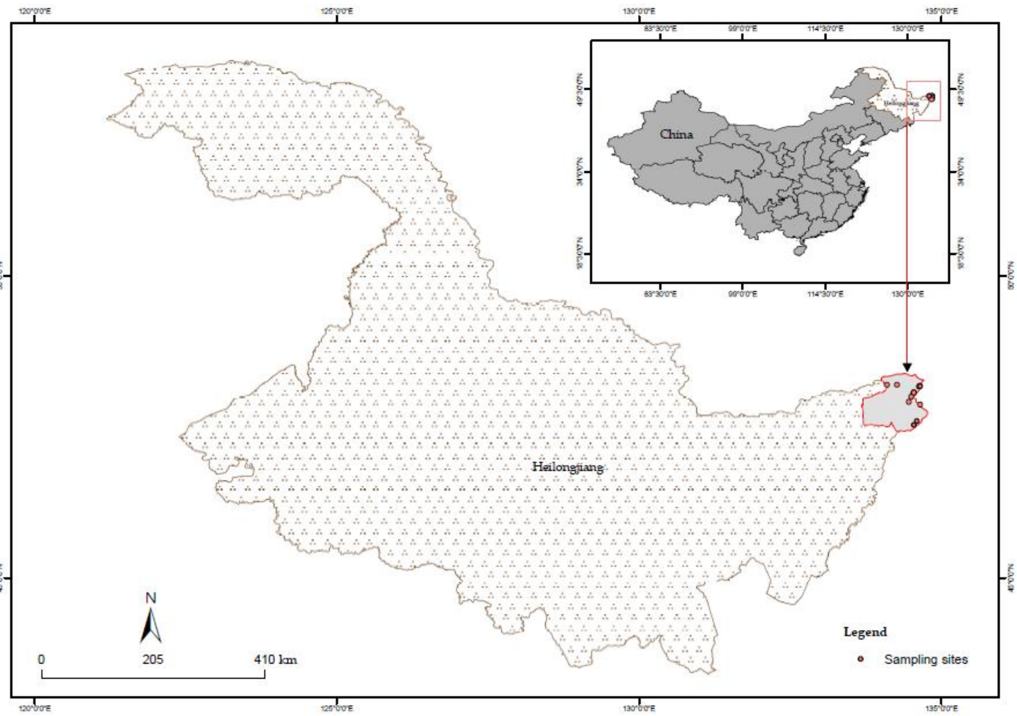

Figure 1. *The location of study area with sampling sites within China*

Functional group composition

Major functional feeding groups were assigned based on invertebrate morphological and behavioral adaptations for food acquisition (Merritt et al., 2002; Merritt et al., 1996) and 6 FFGs (shredders, scrapers, filtering-collectors, gathering-collectors, predators and Omnivores) that were identified. The FFGs are: 1) shredders (SH) feeding on coarse particulate organic matter >1 mm in size, either live aquatic macrophyte tissue or coarse terrestrial plant litter; 2) scrapers (SC) scraping off and consuming the organic matter attached to stones and other substrate surfaces, primarily live plant stems; 3) filtering-collectors (FC) sifting fine particulates 1000 to 0.45 µm from the flowing column of water; 4) gathering-collectors (GC) which gather fine particulates of organic matter from the debris and sediments on the stream beds; 5) predators (PR) feeding on other animals, i.e. live prey; and a sixth category, other, includes species that are omnivores (OM), or simply do not fit neatly into the other categories (Zhang et al., 2014; Masese et al., 2014; Fu et al., 2016).

Measurement of physicochemical factors

Simultaneously with benthic macroinvertebrate sampling, the water temperature (T °C), electrical conductivity (EC, µS/cm), pH, chlorine (Cl^-, mg/l), turbidity (NTU),

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chlorophyll a (Chl, mg/l) were measured using multi-parameter probe YSI Professional (YSI 06E2512AG). The transparency (cm) was measured with a Secchi disc. Water samples were collected from each sampling site in 500 ml labelled plastic containers. Chemical parameters such as chemical oxygen demand (COD_{Mn}, mg/l), biological oxygen demand (BOD_5, mg/l), total nitrogen (TN, mg/l), ammonia-nitrogen (NH_3-N, mg/l), nitrate (NO_3^-, mg/l), and total phosphorus (TP, mg/l) were analyzed in the laboratory based on standard methods procedures (APHA, 2012).

Data analysis

The Shannon-Wiener, Simpson, Pileou's evenness and alpha (α) biological diversity indices were calculated in terms of abundance using PAST software (Hammer et al., 2001; Hammer and Ryan, 2008). A principal component analysis (PCA) based on a correlation matrix among samples was used to analyze the physicochemical parameters. Canonical correspondence analysis (CCA) was also used to describe community trends in macroinvertebrate data and to identify physicochemical variables that best explained species distribution patterns at study sites. PCA and CCA were performed using RcmdrPlugin.FactorMineR and vegan packages, respectively in R software (version 3.5.1). One-way analysis of variance (ANOVA) test was used to test the significance of physicochemical factors on macroinvertebrate abundance and FFGs differences among the sixteen wetland sites. The non-parametric Kruskal-Wallis test was used if normality was not achieved after transformation (Zar, 1984). ANOVA and Kruskal-Wallis tests were computed using Rcmdr package in R software. Canonical correspondence analysis was used to select linear combinations of physicochemical variables that account for most of the variation in macroinvertebrate distributions (Niba and Sakwe, 2018).

Table 1. *Sixteen sampling site coordinates*

Site	Latitude	Longitude
1	N48°04'02"	E134°32'39"
2	N48°04'00"	E134°32'42"
3	N48°03'58"	E134°32'49"
4	N48°04'02"	E134°33'10"
5	N48°04'12"	E134°33'04"
6	N48°04'07"	E134°33'00"
7	N48°09'55"	E134°37’55"
8	N48°10'58"	E134°39'01"
9	N48°11'40"	E134°16'12"
10	N48°10'13"	E134°39'07"
11	N47°59'52"	E134°30'14"
12	N47°55'00"	E134°27'57"
13	N47°52'09"	E134°39'19"
14	N47°35'45"	E134°36'00"
15	N47°31'57"	E134°33'09"
16	N48°11'46"	E134°06'00"

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Results and discussion

Benthic macroinvertebrate communities

A total of 57 benthic macroinvertebrate taxa belonging to 24 families were collected from the sixteen sampling sites. Insects were most caught with 11 families Dytiscidae, Chironomidae, Leptophlebiidae, Belostomatidae, Corixidae, Gerridae, Corduliidae, Gomphidae, Macromiinae, Libellulidae and Phryganeidae. In general, Chironomids were the most diverse and abundant family, which possessed 26 taxa followed by Gomphidae with 4 taxa (*Table 2*). Among all populations, *Cipangopaludina ussuriensis* (Viviparidae) was the most abundant species representing up to 18.2% and occurring at almost each sampling site expect for sites 1, 2, 6 and 13. *Leander modestus* (Palaemonidae) was dominant on site 7, and *Cipangopaludina ussuriensis* on sites 16 and 10, respectively. Total taxon richness was higher in spring and at site 16. The Shannon-Wiener diversity index was higher at sites 8 and 14, respectively (*Fig. 2*). We observed that these sites were dominated abundantly by aquatic Poaceae vegetation. Aquatic vegetation plays an important role in structuring macroinvertebrate populations via the provision of living space (Angradi et al., 2001; Sipkay et al., 2005; Ali et al., 2007; Valinoti et al., 2011; Gleason et al., 2018) or the selecting species traits related to population dynamics and feeding habits (Céréghino et al., 2008).

Table 2. *Diversity broad outline and relative abundance of benthic macroinvertebrates collected in Sanjiang plain wetland*

Order	Family	Species	(%)
Arhynchobdellida	Haemopidae	*Whitmania laevis*	0.3
Coleoptera	Dytiscidae	*Cybister cimbatus*	0.3
Decapoda	Palaemonidae	*Leander modestus*	12.1
Diptera	Chironomidae	*Chinonomus lugubris*	1.3
		C. riparius	1
		C. salinarius	2.5
		C. anthracinus	0.2
		C. circumdatus	0.3
		C. flaviplumus	0.5
		C. kiiensis	0.3
		C. pallidivittatus	0.2
		C. plumosus	2.5
		Cricotopus anulator	0.2
		C. trifasciatus	4.1
		Cryptochironomus defectus	0.2
		Dicrotendipes tritomus	0.3
		D. nervosus	0.3
		Einfeldia dissident	1.8
		Glyptotendipes cauliginellus	1.3
		G. pauens	2.3
		G. tokunagaai	1.6
		Orthocladius roussellae soponis	0.2
		Parachironomus arcuatus	0.2

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		Polypedilum nubifer	0.5
		P. nubeculosum	2.5
		Propsilocerus sp.	2
		Stictochironomus maculipennis	0.5
		Tanypus formosanus	0.5
		Tanytarsus mendex	3
Ephemeroptera	Leptophlebiidae	*Leptophlebia* sp.	0.7
Erpobdelliformes	Erpobdellidae	*Erpobdella octoculata*	2
Eulamellibranchia	Sphaeriidae	*Sphaerium lacustre*	0.3
Heteroptera	Belostomatidae	*Kirkaldyia deyrollei*	0.7
	Corixidae	*Corixa substriata*	0.3
	Gerridae	*Ranatra chinensis*	0.5
Littorinimorpha	Bithyniidae	*Bithynia fuchsiana*	10
Hygrophila	Lymnaeidae	*Lymnaea stagnalis*	4.3
	Semisulcospiridae	*Semisulcospira amurensis*	2.3
Architaenioglossa	Viviparidae	*Cipangopaludina cathayensys*	0.3
		C. ussuriensis	18.2
		Viviparus chui	1.1
Odonata	Corduliidae	*Somatochlora* sp.	0.2
	Gomphidae	*Gastrogomphus* sp.	0.3
		Gomphinae sp.	0.5
		Ictinogomphus sp.	0.2
		Sinictinogomphus sp.	0.7
	Macromiinae	*Epophthalmia* sp.	0.8
	Libellulidae	*Brachythemis* sp.	0.2
Hygrophila	Planorbidae	*Gyraulus convexiusculus*	1.8
Rhynchobdellida	Glossiphoiidace	*Glossiphonia lata*	0.3
		Parabdella quadrioculata	1.1
Trichoptera	Phryganeidae	*Phryganea* sp.	0.2
Tubificida	Naididae	*Nais simplex*	0.5
	Tubificidae	*Branchiura sowerbyi*	0.2
		Limnodrilus amblysetus	1.1
		Limnodrilus hoffmeisteri	1
Unionoida	Unionidae	*Unio douglasiae*	7.2

According to the ANOVA results, the abundance, Shannon-Wiener, Simpson and Pileou’s evenness indices were significantly different ($p = 0.0163$, $p = 0.0092$, $p = 0.0474$, $p = 0.0222$, respectively) among the sixteen sampling sites. On the other hand, it appeared that there’s no significant difference in the total abundance and taxa richness of macroinvertebrate communities caught during spring, summer and autumn seasons ($p = 0.229$, $p = 0.7662$, respectively, *Fig. 2c*).

Our results agreed with the findings of Wang et al. (2007); Zhao et al. (2012); Zhang et al. (2014); Rosser and Pearson (2018), who reported that insects represented the most diverse group and Chironomids were the most abundant family. Macroinvertebrate biodiversity is mainly determined by the number of taxa and individuals, and higher

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diversity can be detected in complex habitats because of more living space or surface area (Shostell and Williams, 2007).

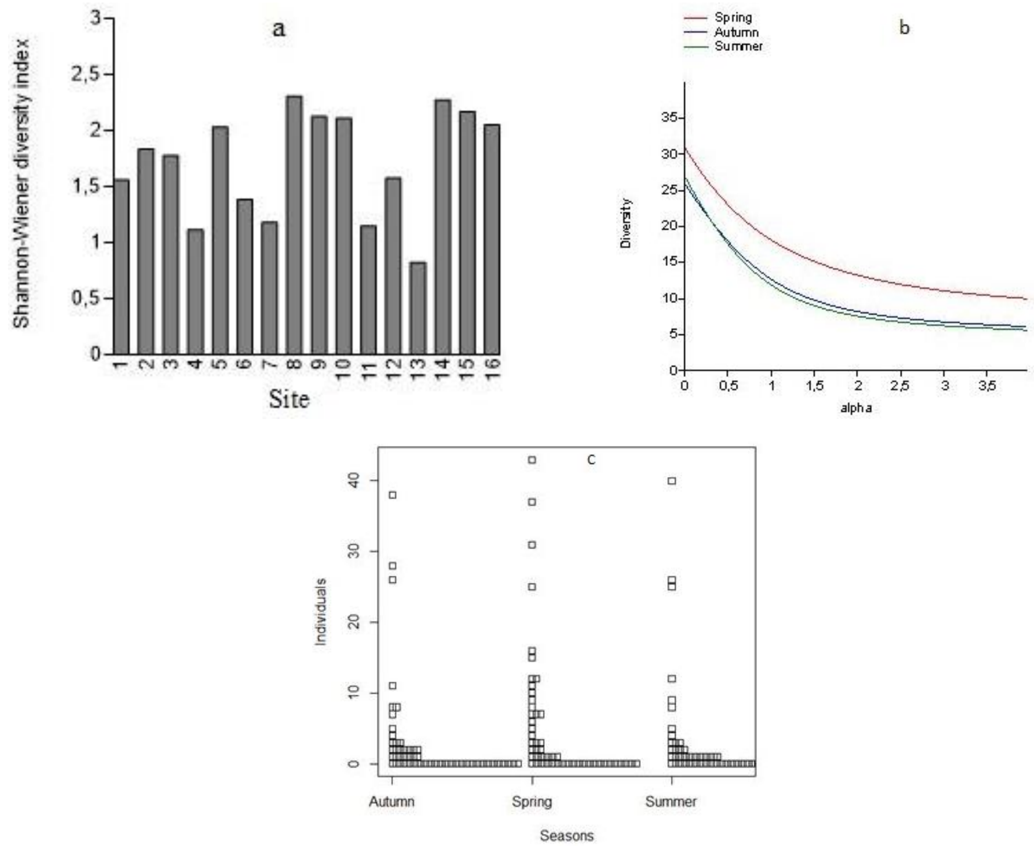

Figure 2. *Trends in Shannon-Wiener (a) and alpha (b) diversity indices, and comparison of mean values (c) of the total abundance for each sampling site during autumn, spring and summer seasons. Autumn (26 species, n_{ind} = 165), spring (31 species, n_{ind} = 287) and summer (27 species, n_{ind} = 158)*

Physicochemical variables at sites

As summarized in *Table 3*, some of the measured physicochemical characteristics changed with seasons. Temperature, chlorophyll a, TN, pH and COD_{Mn} (Kruskal–Wallis test, $p < 0.001$) and NO_3^-, TP, BOD_5 and turbidity (Kruskal–Wallis test, $p < 0.05$) significantly varied among the sampling sites during these three seasons. While conductivity, chlorine, NH_3-N and transparency did not vary significantly (Kruskal–Wallis test, $p > 0.05$) during autumn, spring and summer seasons. Temperature, conductivity, NO_3^-, transparency, TP, COD_{Mn} and BOD_5 increased during the summer, while chlorine, NH_3-N, turbidity and TN increased during the spring. Moreover, pH and chlorophyll increased during the autumn. The pH values indicating the waterbodies of Sanjiang plain tends to be neutral to alkaline. These pH values were nearly similar to those recorded by Narangarvuu et al. (2014) in the Xindian watershed, Taiwan. According to Grady et al. (2011), at this pH level, the denitrification process performs well and forms nitrate.

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The sixteen sites were different in terms of water quality (*Fig. 3*); the first two axes accounted for 62.24% of the cumulative variance. The sites 1, 4 and 6 were characterized by high concentrations of total nitrogen, temperature, chemical oxygen demand, BOD_5 and chlorophyll a. While the sites 9, 11, 12, 15 and 16 were characterized by high concentrations of ammonia-nitrogen, conductivity, chlorine, turbidity and total phosphorus. The site 10 was characterized by concentrations of NO_3^- and pH. There was also a significant positive correlation between pH and NO_3^-.

Table 3. *Means and standard deviations of the physicochemical variables at 16 sites*

Water parameters	Spring	Summer	Autumn	*p-value*
T °C	22.19 ± 1.74	31.36 ± 2.80	18.93 ± 1.81	0.0000***
Conductivity (µS/cm)	118.68 ± 73.41	119.53 ± 70.57	70.73 ± 40.21	0.0847
pH	7.19 ± 0.44	7.42 ± 0.72	7.78 ± 0.30	0.0006***
Cl^- (mg/l)	4.76 ± 4.21	2.83 ± 2.03	2.33 ± 1.02	0.4304
NH_3-N (mg/l)	0.43 ± 0.21	0.30 ± 0.11	0.41 ± 0.19	0.1122
NO_3^- (mg/l)	0.63 ± 0.48	1.18 ± 1.26	1.14 ± 0.37	0.0104*
Turbidity (NTU)	82.54 ± 83.08	15.00 ± 10.59	24.46 ± 17.58	0.0420*
Chlorophyll a (mg/l)	3.60 ± 3.69	3.65 ± 4.21	13.19 ± 9.75	0.0001***
Transparency (cm)	29.43 ± 19.79	35.40 ± 28.07	34.00 ± 24.71	0.6164
TN (mg/l)	8.67 ± 3.35	4.81 ± 1.96	3.98 ± 1.91	0.0001***
TP (mg/l)	0.20 ± 0.15	0.39 ± 0.24	0.33 ± 0.21	0.0127*
COD_{Mn} (mg/l)	6.47 ± 8.08	45.15 ± 36.94	18.27 ± 14.79	0.0006***
BOD_5 (mg/l)	1.04 ± 0.73	1.93 ± 1.31	1.813 ± 0.89	0.0153*

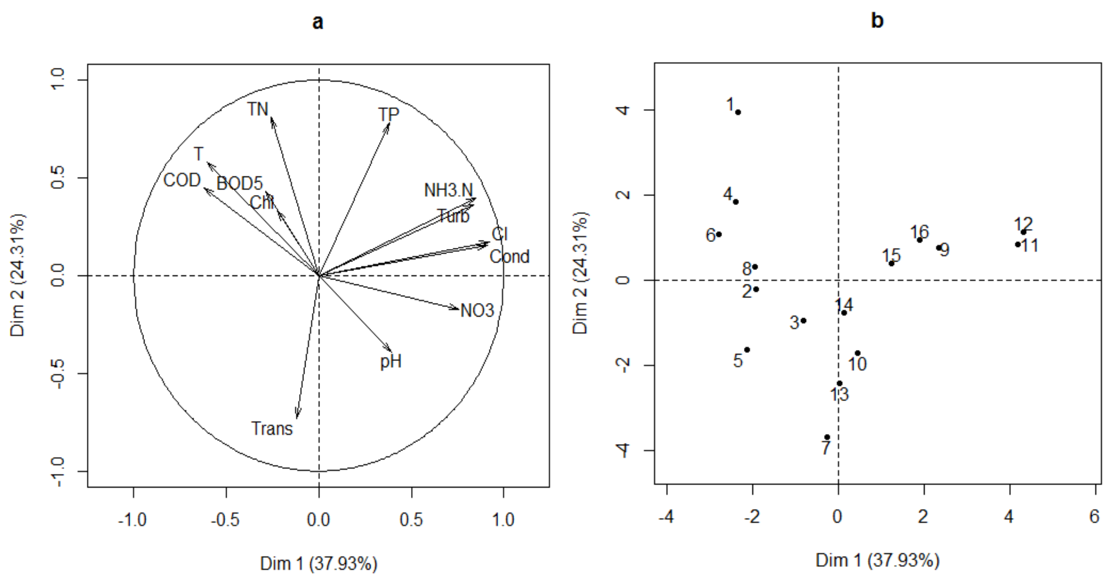

Figure 3. *Principal Component Analysis (PCA, a&b) of the first 2 principal components expressing the relationship sites and physicochemical parameters. T = temperature, Cond = conductivity, Turb = turbidity, Cl^- = chlorine, Chl = chlorophyll a, Trans = transparency, COD_{Mn} = chemical oxygen demand, BOD_5 = biological oxygen demand, TN = total nitrogen, NH_3-N = ammonia-nitrogen, NO_3^- = nitrate, TP = total phosphorus*

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Relationships between physicochemical variables and macroinvertebrate FFGs

The CCA identified 13 physicochemical variables that were significantly correlated with the benthic macroinvertebrate FFGs within the sites (*Fig. 4*). The first two CCA axes respectively explained 38.27 and 30.58% of macroinvertebrate FFGs variance, with eigenvalues of 0.33 and 0.27.

The 57 benthic macroinvertebrate taxa were categorized as follows: 19 predators, 15 gathering-collectors, 7 scrapers, 6 filtering-collectors, 5 omnivores and 5 shredders. According to the percentage contributions of the various macroinvertebrate FFGs (abundance data) within sites, scrapers dominated the benthic communities and accounted for 38.4% of the total abundance followed by omnivores (17.4%), filtering-collectors (15.7%), predators (11.1%), gathering-collectors (10.0%) and shredders (7.4%). Our findings are similar to the results from Zhang et al. (2014), scrapers were the most widely distributed and made up the largest proportion in streams and rivers of a highly developed region, lake Taihu basin, China. Fu et al. (2016) found that predators made up the most contributions to the FFGs (25 taxa) in the Dongjiang River basin, southeast China. Fu et al. (2016) added that shredders and predators mainly included insects, while scrapers were mainly gastropods.

As shown in the *Fig. 4*, the distribution of scrapers including *Bithynia fuchsiana Cipangopaludina cathayensys, Cipangopaludina ussuriensis, Gyraulus convexiusculus, Lymnaea stagnalis, Semisulcospira amurensis* and *Viviparus chui* at sites 8, 9 and 16, which consume algae and associated material were largely influenced by TP and BOD_5. Mccormick et al. (2004) reported that TP is an important factor controlling the macroinvertebrate FFG communities. Turbidity, conductivity, ammonia-nitrogen, nitrate, chlorine and TN were the most important variables to impact the distribution of the filtering-collector FFGs (*Glyptotendipes cauliginellus, G. pauens, G. tokunagaai, Sphaerium lacustre, Tanytarsus mendex* and *Unio douglasiae*), which collect FPOM from the water column using a variety of filters at sites 3, 11, 12 and 13.

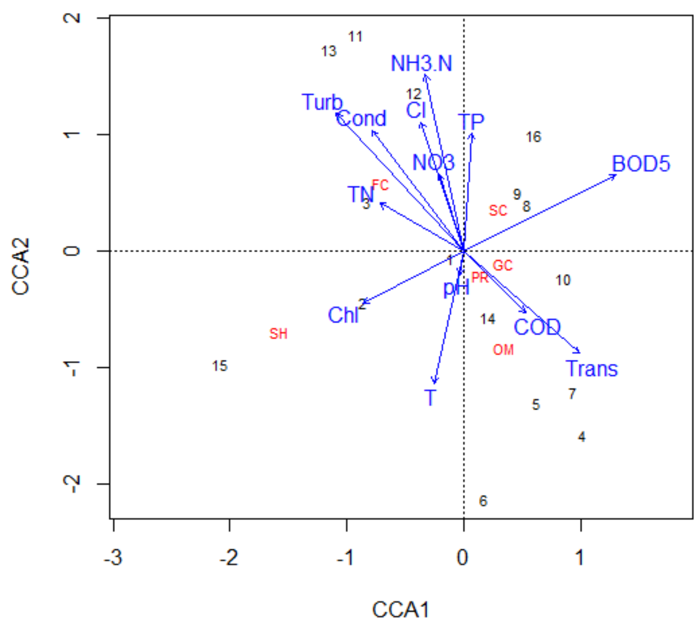

Figure 4. *Canonical Correspondence Analysis (CCA) lot relating the macroinvertebrate FFGs to physicochemical factors. SH = shredders, SC = scrapers, FC = filtering-collectors, GC = gathering-collectors, PR = predators, OM = omnivores*

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The distribution of gathering-collectors (*Branchiura sowerbyi*, *Chironomus anthracinus*, *C. circumdatus*, *C. flaviplumus*, *C. kiiensis*, *C. pallidivittatus*, *C. plumosus*, *Dicrotendipes tritomus*, *D. nervosus*, *Einfeldia dissident*, *Leptophlebia* sp., *Limnodrilus amblysetus*, *L. hoffmeisteri*, *Nais simplex* and *Orthocladius roussellae* soponis), predators (*Brachythemis* sp., *Corixa substriata*, *Cryptochironomus defectus*, *Cybister cimbatus*, *Epophthalmia* sp., *Erpobdella octoculata*, *Gastrogomphus* sp., *Glossiphonia lata*, *Gomphinae* sp., *Ictinogomphus* sp., *Kirkaldyia deyrollei*, *Parabdella quadrioculata*, *Parachironomus arcuatus*, *Propsilocerus* sp., *Ranatra chinensis*, *Sinictinogomphus* sp., *Somatochlora* sp., *Tanypus formosanus* and *Whitmania laevis*), and omnivores (*Chinonomus lugubris*, *C. riparius*, *C. salinarius*, *Leander modestus* and *Stictochironomus maculipennis*) at sites 4, 5, 6, 7, 10 and 14 was strongly associated with transparency and COD_{Mn}. Fu et al. (2016) found that many gathering-collectors are tolerant to disturbance and organic pollution, such as *Tubifex* spp., *Limnodrilus* spp., *Branchiura* sp., and *Chironomids*, which are abundant in polluted rivers. Niba and Sakwe (2018) suggested that benthic macroinvertebrate species populations differently respond to disturbance regimes, and many species have various habitat requirements at each stage of their developmental life cycle (Simaika and Samways, 2011).

The physicochemical factors, such as chlorophyll a, pH and temperature correlated with the composition of shredders (*Cricotopus anulator*, *C. trifasciatus*, *Phryganea* sp., *Polypedilum bubifer* and *P. nubeculosum*) which consume leaf litter or other CPOM, were ordinated on the negative part of CCA axis 1 (*Fig. 4*). Jonsson et al. (2001); Fu et al. (2016) noted that shredders are an important ecological guild in headwater streams, playing a vital role in the process of leaf litter decomposition. They facilitate the release of nutrients into stream ecosystems and provide other invertebrates with food sources in the form of coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM) (Li and Dudgeon, 2008, 2009; Yule and Gomez, 2009). Nutrient loads could influence macroinvertebrate community structure as a consequence of increased food availability where nutrients stimulate primary production (Gao et al., 2011; Wu et al., 2011; Zhang et al., 2014). Niba and Sakwe (2018) concluded that habitat patch level management along the aquatic ecosystem should aim at preserving sufficient indigenous vegetation categories (riparian, aquatic macrophytes and algae), especially during summer season when habitat requirements are optimal for most growth stages of benthic macroinvertebrate species. The results of Wang and Tan (2017) showed that macroinvertebrate assemblage variations were better explained by water quality factors than land use based on variance partitioning procedures.

Conclusion

During the three sampling seasons in the wetlands of Sanjiang plain, we recorded 57 taxa of benthic macroinvertebrates belonging to 6 FFGs, including predators, gathering-collectors, scrapers, filtering-collectors, omnivores and shredders. Insects were the most species-rich group, with 39 taxa and occurred at all sites during spring, summer and autumn. We found higher numbers of macroinvertebrate species and individuals in spring. The results of this study contributed the interesting information about benthic macroinvertebrates FFGs and their relationships with physicochemical parameters in the wetland ecosystems. It was found that predator and scraper FFGs may serve as potential candidates for assessing the health of wetlands. These findings might recommend a need for conservation and management to be targeted in the wetland environments. We

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suggest that further research should be complemented on the composition and structure of macroinvertebrate fauna with an evaluation of density and biomass of FFGs to observe changes from a functional perspective and to obtain an overview on the wetlands of Sanjiang region.

Acknowledgements. This work was supported by a fund from the Ministry of Science and Technology, National Key Research and Development Project (Fund N°. 2016YFC0500406). The authors thank the Ministry of Science and Technology for the realization of this work. Our thankful feelings are also addressed to Wenjie Bai from Sanjiang National Nature Reserve, who helped us collect samples during field work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_33873402

THE EFFECT OF ANNUAL TEMPERATURE CHANGES ON SOME IMPORTANT QUALITY TRAITS OF SOYBEAN *(GLYCINE MAX* L.*)* GENOTYPES

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(Received 19th Dec 2018; accepted 14th Feb 2019)

Abstract. The study was conducted to investigate the changes in yield, fat and protein ratios and micronutrient concentrations of 9 soybean *(Glycine max* L.*)* lines and 2 standard varieties in semi-arid climatic conditions during soybean production seasons in 2012 and 2013. The experiments were established in a randomized block design with three replicates and were started on 08 May and 06 May in 2012 and 2013, respectively. The plot length was 6 m and each plot had 4 rows. The interrow spacing was 70 cm and intra row spacing was 5 cm. Yield, fat and protein ratios and micronutrient concentrations in seeds of soybean varieties/lines were different in two growing seasons. Average temperature during the first growing season was higher than in the second season. The difference in temperature significantly affected the yield, fat and protein ratios and micronutrient concentrations in seeds. The Ca, Cu, Fe, K, Mg, Na P and Zn concentrations of soybean seeds in the first year were higher than those obtained in the second year. The differences in nutrient concentrations between years indicated the effects of temperature differences on mineral compositions of seeds in addition to the yield, fat and protein ratios.
Keywords: *micro element, yield, mineral composition, climate factors, fat and protein ratios*

Introduction

Soybean (*Glycine max* L.), which has an important place in human and animal nutrition, is an industrial legume crop with an average of 18-22% fat, 38-56% protein in seeds, growing upright, pile rooted, annual, summer crop and grown as main or second crop (Baydar and Erbaş, 2014). Soybean has a nitrogen-fixing symbiotic life with *Rhizobium japonicum* bacterium, therefore it is an extremely important plant to improve the physical and chemical structures of soils. Soybean is used as soybean oil, soybean sprout and flour in human nutrition as well as in the production of dough products, baby foods, confectionery products, hypoallergenic milk and dairy products, special dietary products, artificial meat products, dry/cold ready food blends. Soybean is used in the production of insecticides as well as to produce wax, soap, candle oil, biodiesel, glue, paper raincoats and plastic materials. Soybean pulp contains 40 to 46% protein, 1 to 6% fat, 30 to 31% carbohydrate and 5 to 6% cellulose, and is used in animal feeding (Singh and Shivakumar, 2010). Studies on soybean demonstrated that soybean is protective against cancer and individuals fed with soya and soy products are less likely to develop different types of cancers. Soybean has also positive effects on prevention of many other diseases (menopause, cholesterol, osteoporosis, cardiovascular and chronic diseases - coronary heart diseases) (Uesugi et al., 2002; Lovati et al., 1987; Messina, 1999; Xiao, 2008; Brouns, 2002).

Soybean is the most widely produced oilseed plant in the world (USDA, 2018). Although 30 °C temperature is considered optimum for dry matter accumulation of soybean plants (Raper and Kramer, 1987), soybean is grown in regions of temperate and tropical climate with various environmental conditions (Hasanuzzaman et al., 2016). According to the oldest records known in China, soybean had been known by mankind

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

since 11th century or a little earlier time (Hymowitz and Shurtleff, 2005). Soybean, originated from China, was brought from the old world to the new world in the middle of the 17th century, and began to gain importance all over the world in the early 20th century. The introduction of soybean to Turkey coincides to the World War I (Arıoğlu, 2007). The soybean in 2016 has been cultivated on approximately 121 million ha land throughout the world, and 335 million tons of soybean have been produced (FAOSTAT, 2017). Soybean consumption per person in the world is 40.64 kg year^{-1} considering that entire soybean produced in the world have been consumed by human being. However, a large part of the produced soybean is used in animal feeding. In Turkey, 150.000 tons of soybean have been produced in 34.000 ha land. Soybean ranks 4th with 1,073,757 tons among imported agricultural products by Turkey and processed soybean products ranks the 3rd with 1.0867.4 million tons (FAOSTAT, 2017).

Studies on cultivation and adaptation of soybean, which is important as a raw material in human nutrition and in the industry, are also of great importance. Studies indicated that climate, soil and other environmental factors have significant influences on yield and quality traits of soybean (Jumrani and Bhatia, 2018; Shah and Paulsen, 2003; Hasanuzzaman et al., 2016). This study was conducted to determine the yields, fat and protein ratios and micronutrient contents of different soybean genotypes grown under semi-arid climatic conditions and to investigate the relationships among soybean characteristics determined.

Materials and methods

The study was conducted during the main crop soybean production season in 2012 and 2013 using 2 standard soybean varieties (Ataem-7 and Türksoy) and 9 advanced soybean lines (6, 11, 13, 17, 24, 27, 834, 1021 and 1022) as plant materials. The pedigrees of lines were given in the *Table 1*.

Table 1. *Pedigrees of lines used in the study*

Line number	♂	♀
6	Wayne	Keller
11	Spancer	SGI 1308
13	Williams	Keller
17	Spancer	SGI 1308
24	Williams	Keller
27	Williams	Keller
834	Williams	Keller
1021	Spancer	SGI 1308
1022	Spancer	SGI 1308

The field experiments were carried out in Gündaş research station of GAP (Southeast Anatolian Irrigation Project) Agricultural Research Institute during soybean growing seasons of 2012 and 2013 in different plots. Gündaş station is located at 36044’ 05.22 N and 380 48’ 49.76 E within the borders of Akçakale town of Sanliurfa province, Turkey (*Fig. 1*). The summers are dry and hot, and the winters are relatively warm. The average

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

temperature in July and August is 40 °C, while in some nights the temperature goes above 30 °C (Turkish State Meteorological Service, 2017).

***Figure 1.** Location of experimental site within GAP Agricultural Research Institute*

The experiments were conducted in 2012 and 2013 at the same locations. Particle size distribution of soils were determined by water saturation method (Richards, 1954). Soil reaction (pH) was measured in saturation paste with a pH meter (Richards, 1954). Calcium carbonate content of soils was analyzed by using Scheibler calcimeter method (Çağlar, 1949), plant available phosphorus content was determined according to Olsen method (Olsen et al., 1954). Available potassium was extracted using 1 N ammonium acetate (pH = 7.0) solution (Pratt, 1965). Organic matter content of soils was determined by using modified Walkey-Black wet combustion method (Nelson and Sommers, 1982). Boron concentration of soils was determined by azomethine-H method (John et al., 1975). Micronutrient concentrations were extracted using diethylene triamine penta acetic acid (DTPA) and determined by an atomic adsorption spectrophotometer (Perkin Elmer Analyst 800) (Lindsay and Norvell, 1978). The results were presented in *Tables 2* and *3*.

***Table 2.** Soil characteristics of experimental sites*

Years	Water saturation (%)	pH	EC (dS m^{-1})	$CaCO_3$ (%)	P_2O_5 (kg ha^{-1})	K_2O (kg ha^{-1})	Organic matter (%)
2012	64	7.53	0.51	26.6	56	2490	2.32
2013	69	7.47	0.89	24.3	51.9	3000	2.45

***Table 3.** Micronutrient concentrations of soils in experimental sites*

Years	Cu	Fe	Mn	Zn	Ca	Mg	Na	K	B
	(mg kg^{-1})								
2012	1.461	9.386	26.79	0.508	7811	464.9	79.27	884.8	1.041
2013	1.915	9.572	15.3	0.641	7383	942.8	169.8	807.7	1.693

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DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

Soils were neutral in reaction (Ülgen and Yurtsever, 1995), non-saline (Richards, 1954), highly calcareous, low in phosphorus, high in potassium and moderate in organic matter content (Ülgen and Yurtsever, 1995). Soil water saturation (%), pH level, salinity (EC dS m^{-1}), calcium carbonate (%), plant available phosphorus (P, kg ha^{-1}), available potassium (K, kg ha^{-1}) and organic matter content of soils in experimental sites were not significantly different from each other (*Table 2*).

Concentrations of soil Cu, Mg, Na and B in the second year were significantly higher than that of the first year, while Fe and Zn concentrations were slightly higher in the second year. In contrast, Mn and Ca concentrations of soils in the second year were lower compared to that of the first year. Soils contained sufficient amount of Cu, Fe and Mn however, Zn concentration of soils was at critical level (Lindsay and Norvell, 1978). Boron concentration of soils was sufficient (Sürücü et al., 2018).

The average temperatures in June, July and August of 2012 which are the vegetative and generative development periods of soybean were higher compared to the temperatures in 2013 (*Table 4*).

Table 4. *Long-term and 2012-2013 average climate data for Akçakale Town of Şanlıurfa Province (Turkish State Meteorological Service, 2017)*

Months	Years	Monthly average min. temp. (°C)	Monthly average max. temp. (°C)	Monthly average temp. (°C)	Monthly average relative humidity (%)	Monthly total precipitation (mm = kg m^{-2})
April	2012	10.70	26.90	18.60	48.30	44.80
	2013	10.10	26.00	17.80	53.00	9.20
	LTA*	8.19	23.86	16.34	67.64	25.96
May	2012	14.60	29.80	22.00	49.80	43.20
	2013	15.60	31.60	23.30	47.20	43.40
	LTA*	13.00	30.20	22.56	56.42	22.82
June	2012	20.80	38.20	30.10	26.00	4.60
	2013	19.60	36.80	28.70	28.90	0.20
	LTA*	17.46	36.01	28.19	47.34	3.12
July	2012	23.10	40.40	32.10	26.30	0.00
	2013	21.90	39.60	31.30	27.20	0.00
	LTA*	20.73	39.90	31.41	46.23	2.89
August	2012	21.90	39.80	31.10	32.70	0.00
	2013	20.90	39.40	30.40	30.60	0.00
	LTA*	19.87	38.99	30.43	48.37	0.00
September	2012	17.80	36.30	26.80	37.80	3.80
	2013	16.50	33.70	25.10	38.70	0.00
	LTA*	15.28	34.44	25.39	52.11	0.55
October	2012	14.10	28.40	20.80	54.80	35.20
	2013	10.00	26.90	18.40	33.50	0.40
	LTA*	11.28	27.98	19.74	59.09	21.41

*LTA: long-term average (1927-2013)

The experiments were established in a randomized block design with three replicates on May 08 and May 06 in 2012 and 2013, respectively. The plot length was 6 m and

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DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

each plot had 4 rows. The interrow spacing was 70 cm and intra row spacing was 5 cm. Irrigation, hoeing and all other maintenance works of experimental sites have been carried out as needed. Plants were harvested between October 01 and 10, 2012 in the first year and October 05 and 10, 2013 in the second year. *Rhizobium japonicum* bacterial inoculation could not be carried out during planting. Soybean seed yield, fat ratio, protein ratio and seed micro nutrient concentrations which have significant effect on soybean seed quality (Kacar and İnal, 2008) were determined.

The data obtained were subjected to analyses of variance using JUMP statistic software package and average values for each of traits were grouped according to LSD test. The correlation test was also conducted to determine the relationship between the characteristics examined. The correlation test was also performed using JUMP statistical software. Since the study was not carried out under controlled climatic conditions, the effects of environmental factors were not analyzed.

Results

Average yield (kg ha^{-1}), fat ratio (%) and protein ratio (%) of varieties/lines were given in *Table 5*. Yield values (kg ha^{-1}) of different soybean varieties/lines in 2012 were significantly different ($p < 0.01$), while fat and protein ratios were not significantly different. Yield of varieties/lines varied between 2044.30 (line 11) and 2950.00 kg ha^{-1} (line 6). The highest fat ratio was obtained with line 1022 as 18.18%, while the lowest fat ratio (15.44%) was obtained with line 13. The highest protein ratio (37.46%) was obtained with line 13 and the lowest protein ratio (34.73%) was with line 24. Yield and fat ratio obtained for soybean varieties/lines in 2013 were significantly different ($p < 0.01$), while protein ratio values did not significantly change among soybean varieties/lines. Yield values of varieties/lines in 2013 ranged from 2892.90 kg ha^{-1} (line 24) to 4464.60 kg ha^{-1} (line 11). The fat ratio of varieties/lines varied between 18.49% (line 13) and 22.56% (line 1021). The highest protein ratio (37.43%) was obtained with line 13 and the lowest ratio was (34.30%) with line 1021.

Average soybean seed B, Ca, Cu, Fe, K, Mg, Mn, P and Zn concentrations in 2012 were significantly different ($p < 0.05$) among varieties (*Table 6*). In contrast, Na concentration of seeds was not significantly different. The concentrations of B, Ca, Cu, Fe, Mg, Mn, P and Na in 2013 were not significantly different, while Zn was significantly different ($p < 0.01$). The differences in Zn response is probably resulted from the differences in genetic structures of the genotypes. Boron concentration in seeds of varieties ranged from 2.11 to 10.04 mg kg^{-1} in 2012. Boron concentration of line 1021 was higher than the rest of the varieties/lines. Calcium concentration of varieties was between 1800.33 and 2601.00 mg kg^{-1}. Calcium concentrations of line 1021 and Ataem-7 variety were similar to each other and higher than other lines/varieties investigated. Cupper concentration of lines/varieties ranged from 19.80 to 26.58 mg kg^{-1}. Cupper concentrations of Türksoy variety and lines 27 and 1021 were similar to each other and higher compared to the other lines/varieties. Average seed Fe content was 65.81 mg kg^{-1} and the highest Fe content was obtained with line 1021. Potassium concentration in seeds of lines/varieties varied between 33798.33 and 38106.67 mg kg^{-1} and the highest K concentration was obtained with line 1021. Magnesium concentration in seeds of lines/varieties ranged from 1598.33 to 1901.67 mg kg^{-1} and line 1022 had the highest Mg concentration. Manganese concentration in seeds varied between 26.26 and 51.41 mg kg^{-1} and Mn concentrations of lines 1022 and 6 were similar to each other and

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DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

higher than the rest of the lines/varieties. Sodium concentration of lines/varieties ranged from 79.49 to 119.63 mg kg^{-1}. The highest Na concentration was obtained with line 13. Phosphorus and Zn concentrations of lines/varieties ranged from 3627.667 to 4825.33 mg kg^{-1} and from 29.74 to 46.93 mg kg^{-1}, respectively Phosphorus concentration of line 13 and Zn concentration of line 834 were higher compared to the other lines/varieties investigated. Boron concentration of all lines/varieties in 2013 increased, while Ca, Cu, K, Mg, Na and P concentrations decreased relative to that of 2012. The Fe concentration of Gapsoy-16 increased in the second year, while decreased in all other lines/varieties. The Mn concentration of lines 1022, 6 and 1021 decreased relative to the first year, whereas Mn concentration of other lines/varieties in the second year increased. The Zn concentration of lines 1022 and 24 increased in the second year, while decreased in other lines/varieties.

Table 5. *Average values and groups of traits investigated for different soybean varieties/lines in 2012 and 2013*

Years	Varieties/lines	Yield** (kg ha^{-1})		Oil rate** (%)		Protein rate (%)
2012	11	2950.00	a	15.89		36.97
	Ataem-7	2900.50	a	15.83		35.68
	13	2760.50	a	15.44		37.46
	834	2560.50	b	17.52		35.39
	27	2552.90	b	18.00		34.56
	17	2500.00	b	17.67		36.48
	1022	2433.30	b	18.18		35.78
	24	2413.80	b	16.18		34.73
	Türksoy	2150.00	c	17.20		35.28
	1021	2145.70	c	16.85		37.22
	6	2044.30	c	17.36		37.00
	Average	2492.00		16.92		36.05
	CV (%)	4.54		7.09		5.70
	LSD	192.70		NS		NS
2013	11	4464.30	a	20.80	ab	35.60
	13	4339.30	ab	18.49	c	37.43
	27	4214.30	bc	21.21	ab	35.61
	1021	4071.40	cd	22.56	a	34.30
	Ataem-7	4000.00	cd	19.73	bc	37.15
	1022	3982.10	d	21.87	a	35.31
	834	3928.60	d	21.13	ab	35.27
	Türksoy	3928.60	d	21.90	a	35.33
	17	3875.00	de	20.80	ab	35.03
	6	3678.60	e	21.02	ab	35.66
	24	2892.90	f	19.46	bc	36.29
	Average	3943.20		20.82		35.73
	CV (%)	3.38		5.08		4.81
	LSD	22.70		1.80		NS

**Means followed by different letters are significantly different to each other P < 0.05
NS: non-significant; CV: coefficient of variation; LSD: least significant differences

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DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

Table 6. *Microelement contents of soybean varieties/lines according to years in grain*

Years	Varieties/lines	B**	Ca**	Cu**	Fe**	K**	Mg**	Mn**	Na	P**	Zn**
		(mg kg^{-1})									
2012	**1022**	2.11 e	2174.67 c	23.64 bc	78.63 b	33798.33 e	1901.67 a	49.89 a	96.18	4426.00 c	37.87 g
	27	9.23 ab	2170.33 c	26.58 a	71.26 c	38106.67 a	1749.33 c	33.75 d	89.76	4683.67 b	43.70 b
	17	3.61 de	1800.33 f	21.81 de	64.55 d	36820.00 bc	1662.33 de	26.26 f	83.16	4424.00 c	41.20 de
	834	5.96 bcd	1923.67 d	24.06 b	54.33 f	37306.67 b	1802.00 b	32.78 d	79.99	4250.33 d	46.44 a
	6	4.82 de	1870.33 def	21.18 e	63.67 d	35426.67 d	1708.00 cd	51.41 a	99.34	3627.67 f	29.74 h
	Türksoy	6.08 bcd	2356.33 b	25.72 a	69.70 c	33983.33 e	1726.67 c	32.72 d	106.54	4684.00 b	43.22 bc
	1021	3.52 de	2601.00 a	25.93 a	85.47 a	36256.67 c	1799.33 b	41.63 b	79.49	4732.00 ab	42.12 cd
	24	8.53 abc	1842.67 def	22.63 cd	60.21 de	36220.00 c	1617.00 ef	29.84 e	93.29	4191.67 d	44.25 b
	Gapsoy-16	6.30 bcd	1829.00 ef	19.80 f	46.92 g	36266.67 c	1598.33 f	41.63 b	103.85	3896.67 e	39.01 fg
	Ataem-7	5.24 cde	2540.67 a	21.13 e	58.50 ef	37276.67 b	1665.33 d	39.52 c	95.01	4140.33 d	40.13 ef
	13	10.04 a	1892.00 de	23.90 b	73.75 c	37370.00 ab	1748.33 c	33.63 d	119.63	4825.33 a	46.93 a
	Average	5.95	2091	23.31	65.81	36257.42	1725.30	37.57	95.11	4352.88	41.33
	CV	34.46	1.85	2.65	4.31	1.28	1.56	2.82	18.56	1.87	2.14
	LSD	3.49	88.89	1.05	4.83	793.05	45.89	1.80	ns	138.70	1.50
	Varieties/lines	**B**	**Ca**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**Zn**
		(mg kg^{-1})									
2013	**1022**	50.39	1478.33	18.35	50.63	16796.67	1475.00	42.07	28.91	3054.00	37.98 bc
	27	49.32	1541.00	18.25	51.07	17170.00	1502.67	40.87	34.47	3108.33	36.61 bc
	17	48.94	1673.67	19.77	50.94	17180.00	1600.33	39.47	34.04	3148.00	38.98 bc
	834	50.00	1632.33	19.56	50.58	16796.67	1589.67	43.10	29.73	3064.33	39.99 bc
	6	50.12	1717.00	20.11	53.69	18436.67	1667.67	43.93	29.28	3325.33	40.77 b
	Türksoy	51.44	1544.33	19.99	50.08	17660.00	1673.33	40.76	27.81	3203.33	40.05 bc
	1021	53.33	1625.00	19.18	49.83	17726.67	1604.67	40.88	33.76	3268.00	37.17 bc
	24	50.47	1725.67	18.87	56.72	18056.67	1609.33	42.53	33.07	3483.33	46.12 a
	Gapsoy-16	54.11	1669.67	19.73	55.18	18970.00	1661.00	45.47	27.03	3507.67	36.37 c
	Ataem-7	54.57	1678.33	19.22	55.50	17900.00	1666.00	40.72	31.67	3409.67	39.31 bc
	13	51.26	1781.33	21.11	59.68	17753.33	1666.33	42.94	29.19	3308.33	37.55 bc
	Average	51.27	1642.42	19.47	53.08	17676.97	1610.55	42.07	30.82	3261.85	39.17
	CV	5.98	10.38	8.15	9.60	5.06	8.00	9.85	13.94	7.37	6.23
	LSD	ns	ns	ns	ns	ns	ns	ns	ns	ns	4.16

**Means followed by different letters are significantly different to each other $P < 0.05$
ns: not significant; CV: coefficient of variation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

Relationship between grain yield and micronutrient concentrations of lines/varieties

Correlation analysis was conducted to reveal the relationship between yield, fat ratio, protein ratio and seed micronutrient contents. In the first year, soybean yield had a significant positive correlation with K concentration and negative relationship with Cu and Fe concentrations. The relationship can be attributed to plant growth and explained by the dilution effects of plant nutrients. Although not statistically significant, yield had negative relationships with fat ratio, protein ratio, Ca, Mg, Mn and P concentrations, while yield had positive relationships with B, Na and Zn concentrations (*Table 7*). In the second year, yield had non-significant negative relationship with Ca, Fe, Na and P concentrations while yield had positive relationship with fat ratio, protein ratio, B, Cu, K, Mg and Mn concentrations. Yield had significant negative relationship with Zn concentration. The correlation analysis indicated that soybean yield had positive relationship with B and K contents of seeds in both years, while yield had significant positive relationship with Ca, Fe and P concentrations of seeds.

Table 7. *Correlation coefficients between traits investigated in 2012 and 2013*

Investigation features	2012			2013		
	Yield	**Fat ratio**	**Protein ratio**	**Yield**	**Fate ratio**	**Protein ratio**
Fat ratio	-0.327			0.076		
Protein ratio	-0.094	-0.364*		0.051	-0.552**	
B	0.228	-0.301	0.166	0.115	0.088	-0.109
Ca	-0.152	0.010	-0.071	-0.08	-0.391*	0.325
Cu	-0.374*	0.287	-0.215	0.097	-0.006	-0.01
Fe	-0.493**	0.263	-0.021	-0.081	-0.361*	0.147
K	0.506**	-0.162	-0.085	0.013	-0.181	-0.028
Mg	-0.310	0.353*	-0.020	0.076	-0.157	0.095
Mn	-0.197	0.078	0.173	0.013	-0.103	0.245
Na	0.136	-0.425*	0.272	-0.159	-0.158	0.23
P	-0.0429	0.0920	0.0314	-0.095	-0.322	0.114
Zn	0.3254	-0.1690	0.2327	-0.769	-0.225	-0.047

**Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

Relationship between micronutrient contents of soybean seeds and fat and protein ratios

The relationship between fat ratio and Na, B, K and Zn contents in 2012 was negative, which was only statistically significant for Na content. In contrast, correlations between fat ratio and Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn concentrations were positive, which was only statistically significant for Mg content. Positive correlation between fat ratio and Mg content indicated that increasing Mg content in soybean seed favors the fat accumulation in seed. The relationship between fat ratio and Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn contents in the second year was negative, which was statistically significant for Ca and Fe contents. Fat ratio had positive relationship between B content in the second year and the relationship was not statistically significant.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

The relationships between protein ratio and micronutrient contents of soybean seeds were not statistically significant in both 2012 and 2013. Protein ratio had positive relationship with B, Mn and Na in the first year and negative relationship with Ca, Cu, Fe, K and Mg contents. In the second year of the study, non-significant positive relationship was obtained between protein ratio and Ca, Fe, Mg and Mn contents, while the relationship was negative for B, Cu and K contents.

Discussion

Average seed yield obtained in the first year (2492.00 kg ha^{-1}) was lower than that of the second year (3943.20 kg ha^{-1}). Higher maximum, minimum and total temperatures during growing season (June, July, August, September) of the first year compared to the second year may cause lower seed yield in the first year (*Table 3*). Increasing temperature during growing season leads to water stress in soybean plants (Jumrani and Bhatia, 2018; Shah and Paulsen, 2003; Kırnak et al., 2010; Dornbos and Mullen, 1992), which has negative impact on fertilization of the flowers and also causes the flowers to fall down.

The results revealed that genetic structure of lines/varieties, temperature stress and micronutrient contents of soils have significant effects on soybean yield and micronutrient content of seeds. Significant impacts of environmental factors on chemical composition of soybean seeds in addition to genetic structures of varieties have also been reported by Jumrani and Bhatia (2018) and Shah and Paulsen (2003).

Fat ratio is also an important trait of soybean along with high yield. Differences of genotypes in response to environmental factors indicated that fat ratio of seeds was significantly affected by environmental factors in addition to the genetic characteristics. High temperatures in 2012 caused decreasing the fat ratio of seeds in addition to the decline in yield. Similar results have been reported from studies conducted to investigate the effects of environmental factors on fat ratio of soybean seeds (Piper and Boote, 1999; Wang et al., 2015).

The difference in protein ratio between years was not significant, though protein ratio obtained in the first year was higher than the second year. In contrast to yield and fat ratio, protein ratio did not respond to the changes in temperatures, and protein ratio did not change with changes in temperatures. However, negative correlation between soybean yield and protein ratio in seed indicates that protein ratio decreases as soybean yield increases. Therefore, impact of environmental factors on soybean yield indirectly affects the protein ratio of seed, which is not directly affected by the environmental factors. Similar results have also been reported by Rotundo et al. (2009) and Kumar et al. (2006).

The Ca, Cu, Fe, K, Mg, Na P and Zn concentrations of soybean seeds in the first year were higher than those obtained in the second year. The differences in nutrient concentrations between years indicated the effects of temperature differences on mineral compositions of seeds in addition to the yield, fat and protein ratios. Contrasting results have been reported by studies investigating the effects of temperature differences during growing period on mineral composition of seeds. Kresović et al. (2017) reported significant effect of temperature on mineral composition, while Gibson and Mullen (2001) found increases and decreases in nutrients of soybean seeds. Researchers also indicated that differences in temperatures have indirect effect on quality of food produced by using soybean (Lim et al., 1990; Gibson and Mullen, 2001).

DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

Conclusion

Yield, fat ratio, protein ratio and seed mineral nutrient composition of soybean seeds were determined to investigate the performances of different soybean genotypes grown under arid conditions in two different growing seasons (2012-2013). Yield, fat ratio, protein ratio and some of important mineral nutrients of soybean lines/varieties obtained in two different seasons were different from each other. Temperatures over optimum temperature requirements of soybean had negative effect on soybean yield. High temperatures had also negative impact on fat ratio which is an important quality criterion for soybean, while protein ratio was not directly affected by high temperatures. The results also revealed that micronutrient contents of soybean seeds have been affected by the increasing temperature.

Significant impact of growing conditions on yield and quality criteria of soybean indicates the necessity to develop regional varieties. Therefore, further studies under controlled climatic conditions may increase the chance of success. The adaptation studies also should be carried out to determine the appropriate varieties prior to the initiation of soybean production in a region.

Acknowledgements. This study was conducted by the supports of General Directorate of Agricultural Research and Politics affiliated to the Turkish Republic Ministry of Food, Agriculture and Livestock within the scope of Soybean Breeding Project (TAGEM/TA/09/05/01/0014). Soybean line 11 was registered as GAPSOY-16 in 2016 by the Directorate of Seed Registration and Certification Center affiliated to the Ministry of Food, Agriculture and Livestock of Turkey.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34033414

DIFFERENCES AMONG CAMİYANI BLACK PINE (*PINUS NIGRA* ARNOLD. SUBSP. *PALLASIANA*) CLONES IN SEED ORCHARD BECAUSE OF *RHYACIONIA BUOLIANA* (DEN. & SCHIFF) DAMAGES IN TURKEY

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(Received 19th Dec 2018; accepted 14th Feb 2019)

Abstract. This study examined the level of damage caused by pine shoot moth (*Rhyacionia buoliana* (Den. & Schiff) between 2014 and 2016 in the seed orchards of Camiyanı Black Pine (*Pinus nigra* Arnold. *subsp. pallasiana* (Lamb.) Holmboe). When the variance analysis (ANOVA) was applied, and observations and counts of the seed orchard were evaluated, a statistically significant difference was found between the clones in terms of number of individuals affected by $P < 0.001$. When Duncan Range Test results were examined, 20 groups were formed in total, with a $P < 0.05$ confidence level in terms of the average number of individuals damaged by the pine shoot moth. In this respect, it was determined that the clones which had the highest damage in the seed orchard number 70, clones 21, 25, 26 and 29 were damage the most, while the clones that had the least amount of damage are clones 2, 6, 8 and 23. The differences in the average number of clones which the pine shoot moth damage were investigated. When the ANOVA results were examined, it was determined that there was a statistically significant difference in $P < 0.001$ confidence level between 2014, 2015 and 2016 in terms of the mean number of clones exposed to pine shoot moth damage. There was no significant difference between the blocks in terms of the mean number of clones which the pine shoot moth damaged. When the results of regression analysis were analyzed, strong relationships were found between clone and number of damaged individuals and the number of clones damaged by pine shoots for years.
Keywords: *Camiyanı Black Pine, seed orchard, clone, Rhyacionia buoliana, Turkey*

Introduction

Forests are special ecosystems that carry out important tasks in protecting the health and ecological balance of the products and services they provide. Therefore, it is important to ensure that the forest resources develop and shape under completely open field conditions, and are protected from harmful biotic and abiotic factors, to ensure the future of humanity. First of all, forests play an important role in the prevention of global warming by mitigating carbon emissions in particular, which is the most relevant environmental problem of our day. Forests transform carbon dioxide through photosynthesis, and store carbon. Thus, it creates an important balance between carbon dioxide and oxygen gases under natural conditions. For example, a well-developed 100-year-old beech can absorb up to 40 million m^3 of air through photosynthesis, turning 1200 m^3 of carbon dioxide in this air to 6 tonnes of carbon as a biochemical transformation. On the other hand, it is reported that a forest can bind 13-30 tons of carbon dioxide for annual compulsory dry biomass production (Görücü and Eker, 2009). However, in today's modern forestry approach, forest resources being used as a multi-faceted collective of services (recreation, erosion prevention, hydrological

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34153426

resources, non-wood forest products etc.) are decreasing rapidly both in our country and in the world. As a matter of fact, according to the latest statistical figures, it is reported that the average annual forest loss in the less developed and developing countries has reached approximately 18% of the world total forest area (FAO, 2016). Our country has valuable ecological, economic and silvicultural forest ecosystems on different elevation levels in terms of tree species type and stand structure characteristics with the effect of different ecological conditions (Saatçioğlu, 1969; Ata, 1995). However, these valuable forest resources, which are estimated to be 22 million hectares in our country, have been significantly damaged by the impact of biotic and abiotic factors such as inadequate technical applications, illegal cuttings, fires, storms, insects and other harmful factors, (GDF, 2016). On the other hand, as a result of the damage caused by these factors in forest resources, 6.7% of amphibians, birds, mammals and reptiles in the forests, approximately 3.9% of plant communities, including endemic plants in Turkey are threatened (Kırış and Toprak, 2009). In this context, preserving forest resources is obligatory for the products and services obtained from these sources and for the protection of biodiversity. Successful natural and artificial regeneration promoting methods and afforestation studies are needed to achieve this (Genç, 2004). For this purpose, the use of seedlings produced from genetically modified seed sources is very important in terms of increasing the quality and health of new forests (Tunçtaner, 2007). In this respect, these seed sources are of the highest genetic quality. Seed orchards are important facilities established by high amounts labor, time and money (Ürgenç, 1982). However, these seed sources need to be protected against biotic and abiotic factors to ensure the continuity of genetically improved seed yields from seed orchards. One of the factors that have a negative impact on seed orchards is insect damage. It can reduce seed production by up to 50% especially in seed orchards with primary pests (Tunçtaner, 2007). The pine shoot moth (*Rhyacionia buoliana* (Den. & Schiff) is one of these primary pests that cause significant damage (Çanakçıoğlu, 1993). Especially *Pinus contorta* var. *latifolia* orchards in Sweden, this pest was determined that it was one of the pest caused significant damage (Lindelöw and Björkman, 2001).

In this study, wide area of usage and economically valuable ecotype of the Camiyanı Anatolian black pine (*Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) was used. The level of damage on the clones was examined both years, and the most resistant clones were determined.

Materials and methods

The seed orchard is located in Karabük-Yenice region and is established with cuttings taken from Bakraz region in Yenice of black pine ecotype called Camiyani black pine (*Fig. 1*). 30 saplings were taken from Bakraz provenance and were planted with an 8 × 8 m spacing distance in 1990 in 70 numbered divisions. The seed orchard has a size of 11.3 ha and its elevation is 100 m with a land slope of 10%. The general exposure of the seed orchard is southern. The soil structure in the seed orchard has clay texture and the pH varies between 6.06 and 7.80 (Ertekin, 2006). The average annual temperature in the research area is 12.8 °C, with a maximum temperature of 42.8 °C, and a minimum temperature of -18.6 °C, while the average annual precipitation is 1040.5 mm with an average relative humidity of 73.6% (Anonymous, 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34153426

Figure 1. *Research area*

The seed orchard, which forms the research area, consists of 30 clones and 3 blocks. In this context, in order to determine the damage of pine shoot moth in each block in accordance with the purpose of the study, individuals belonging to all clones were examined. In the research, it was accepted that insect damage occurred in clone in case of damages in single shoot of trees representing clone. In this context, in the 3 blocks of seed orchard, observations and counts were started to be made with the start of vegetation period randomly in the individuals belonging to all clones in the 3-year period covering 2014-2016, and the individuals whose pine shoot moth damage was detected were separated each year by marking a different color with a spray paint and in this way, the damage occurred in both years is determined and the damage at the level of clones were tried to be determined. In this respect, clones and individuals were identified, while any damage in the height of the tree in a single exile was determined that the clone was considered damaged. Likewise, the same method was applied in similar studies on this subject (Fabre et al., 2004; Turgeon et al., 2004). Thus, the damage caused by the pine shoot moth in the seed orchard has been revealed in the most delicate and detailed manner.

In the study, various statistical analysis methods were used to reveal the pine shoot moth damage in terms of clones, years and blocks in the seed orchard. For this purpose, Kolmogorov-Simirnov (K-S) test was used to determine whether the data obtained from the determinations and counts carried out in the seed orchard during the 3-year period covering 2014-2016. Then, a multi-way ANOVA was applied in order to compare the pine shoot moth losses that occurred due to clones, years and blocks of seed orchard. In the case of a statistically significant difference due to any factor as a result of the variance analysis, Duncan Test was performed, observation years and blocks were required. For this purpose, the mean of the data obtained after observations and determinations for pine shoot moth damage was used (Ercan, 1997). In addition, stepwise regression analyzes were applied to correlate the number of clones with the pine shoot moth. All statistical analyzes were conducted with SPSS 22.0 Statistical Package Program.

Results

Damages at the clones level

Figure 2 shows the distribution of the pine shoot moth damages in 2016 as a result of observations and determinations in the seed orchard where the research was carried out.

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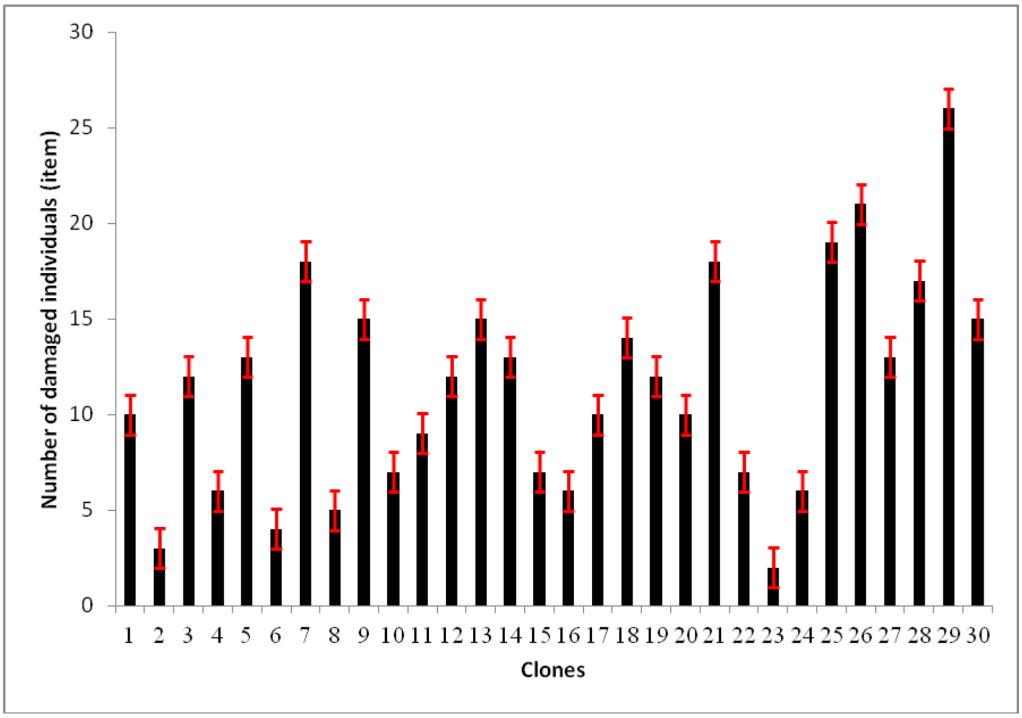

Figure 2. *Distribution of number of damaged individuals by clones*

As a result of observations and determinations carried out in 2016, it has been determined that the average number of individuals exposed to pine shoot moth damages is between 2.3 and 26.2. In this context, variance analysis was performed to determine whether there was a statistically significant difference between clones in terms of the average number of individuals in which insect damage occurred (*Table 1*).

Table 1. *Results of ANOVA*

Variance sources	Sum of squares	df	Mean square	F	Sig.
Between clones	2857.567	29	98.537	5495.639	*0.000****
Within clones	1.076	60	0.018		
Total	2858.643	89			

***P < 0.001 significance level

When ANOVA results in *Table 1* were evaluated, it was determined that there was a statistically significant difference between the clones in terms of $P < 0.001$. In this context, Duncan Range Test was applied to group clones in terms of the number of individuals in which the pine shoot moth was damaged (*Table 2*).

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Table 2. *Results of Duncan range test*

Clones	Number of individuals damaged	Significance level
1	10.13^{l}	
2	3.13^{t}	
3	12.10^{j}	
4	6.13^{p}	
5	13.10^{i}	
6	4.10^{s}	
7	18.10^{e}	
8	5.13^{r}	
9	15.10^{g}	
10	7.12^{o}	
11	9.22^{m}	
12	12.20^{j}	
13	15.16^{g}	
14	13.23^{i}	
15	7.46^{n}	$P < 0.05$
16	6.10^{p}	
17	10.20^{kl}	
18	14.23^{h}	
19	12.10^{j}	
20	10.36^{k}	
21	18.46^{d}	
22	7.19^{o}	
23	2.1^{u}	
24	6.10^{p}	
25	19.20^{c}	
26	21.13^{b}	
27	13.20^{i}	
28	17.16^{f}	
29	26.10^{a}	
30	15.20^{g}	

When the Duncan Test results in *Table 2* were examined, 20 groups were formed in total $P < 0.05$ confidence level in terms of the mean number of individuals damaged by pine shoot moth. In terms of the number of injured individuals, clone 29 constitutes the first group alone and clone 23 constitutes the first group. In this respect, it can be said that the clones having the highest damage in the seed orchard 70 are the clones which are the most damaging pine shoot moth.

Damage level according to years

The change in the mean number of clones that the pine shoot moth has caused damage in the seed orchard in the period of 2014-2016 in which the research was carried out is shown in *Figure 3*.

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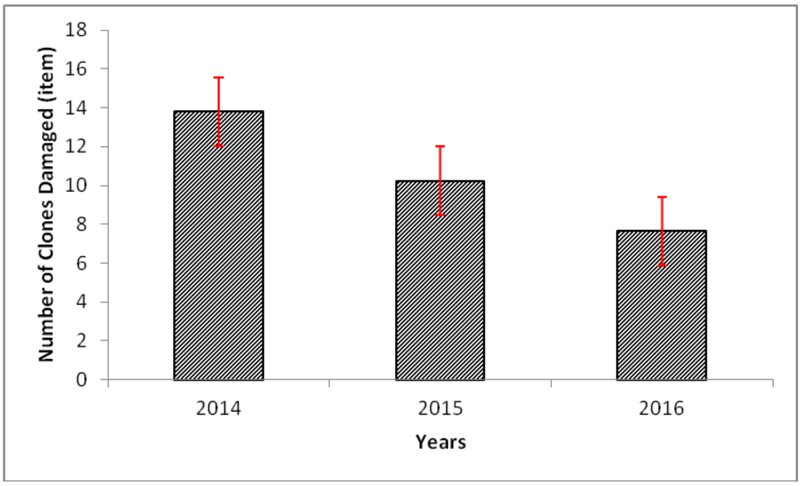

Figure 3. *Mean number of clones damaged over the years*

According to *Figure 3,* there is decreasing trend towards 2014 from the year 2016, when the study was conducted in terms of the average number of clones in which pine shoot moth was damaged. According to this, the average number of clones was 13.78 in 2014, 10.23 in 2015 and 7.64 in 2016. Variance analysis was performed to determine if there were any statistical differences in terms of the average number of clones in which pine shoot moth damage occurred (*Table 2*).

Table 2. *Results of ANOVA*

Variance sources	Sum of squares	df	Mean square	F	Sig.
Between clones	2996.314	2	97.271	6987.118	*0.000****
Within clones	0.075	87	0.023		
Total	2996.389	89			

****P < 0.001* significance level

When the ANOVA results in *Table 2* were examined, it was determined that there was a statistically significant difference in $P < 0.001$ confidence level between 2014, 2015 and 2016 in terms of the mean number of clone number exposed to pine shoot moth damage. In this context, Duncan Range Test was applied at $P < 0.05$ confidence level and the results are given in *Table 3*.

Table 3. *Results of Duncan range test*

Years	Number of clones damaged	Significance level
2014	13.78[a]	
2015	10.23[b]	***P < 0.05***
2016	7.64[c]	

As a result of the Duncan Range Test, 3 different groups have emerged in terms of the average number of clones in the $P < 0.05$ confidence level. Accordingly, 2014 was the first group in which the most damages occurred during the 3-year period during

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which the survey was carried out, while this group was followed by the second group formed by 2015 alone, and 2016 was the third group alone (*Table 3*).

Damages level according to blocks

The seed orchard, which forms the research area, consists of three random blocks of experimental design. Therefore, investigations and evaluations have been made in terms of the mean number of clones in which pine shoot moth has caused damage. In this context, the change in the average number of clones that have been damaged in 2016 by blocks is shown in *Figure 4*.

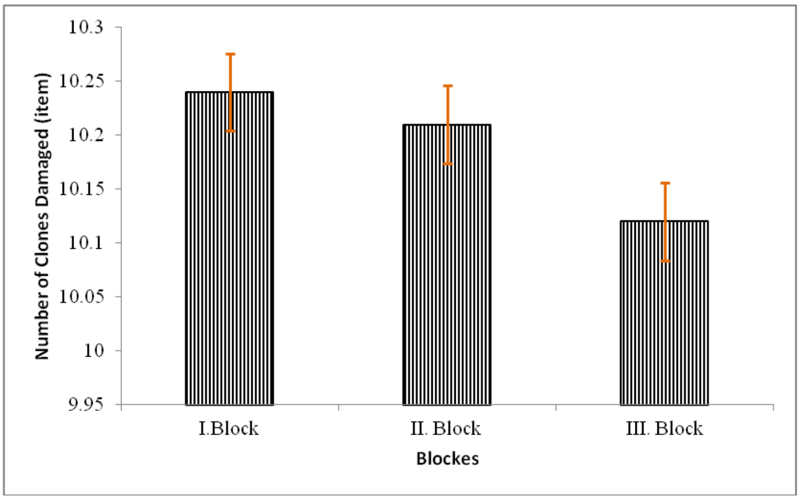

Figure 4. *Mean number of clones damaged by blocks*

When *Figure 4* is examined, it has been determined that there is a tendency towards the decrease in the number of average clone numbers that have been damaged by pine shoot moth as of 2016. According to this study, the average number of damaged clones determined in block I 10.24, block II 10.21 and block III 10.12, respectively. In this context, ANOVA was performed to determine whether there were any statistically significant differences in terms of the mean number of clones exposed to pine shoot moth damage between the blocks (*Table 4*).

Table 4. *Results of ANOVA*

Variance sources	Sum of squares	df	Mean square	F	Sig.
Between blocks	1023.017	2	0.021	0.001	*0.998*^{ns}
Within blocks	0.042	87	32.857		
Total	1023.059	89			

ns: non significant

According to the analysis of variance in *Table 4*, it was determined that there were no statistically significant differences between the blocks in terms of the average number of clones in the pine shoot moth.

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The relationships between the variables examined in the research

In the research, the level of the damage caused by the pine shoot moth 70 in the division seed orchard was determined. For this purpose, detection, counting and observations were made in 3 blocks of seed orchard and 30 clones in the 3 year period of 2014-2016. As a result of detection, counting and observations performed; the number of individuals affected by the clone, the number of clones in which the damage occurred and the number of blocks and the number of clones damaged were also investigated (*Table 5*). In this respect, stepwise regression analysis method was used.

Table 5. *The type, model and relationship degrees of the relationships between variables*

Variables	Type of relation	Model	R^2
Number of clone-damaged individuals	Second order polynomial	$y = 0.0171x^2 - 0.2474x + 9.9537$	0.95
Years-damaged clones	Linear	$y = 3.07x + 4.41$	0.99
Blocks-damaged clones	Linear	$y = -0.06x + 10.31$	0.92

When the results of the double regression analysis in *Table 5* are examined; a second-order polynomial relationship was found to be positively influenced by the number of individuals affected by the clone. On the other hand, the relationship between the clone and the number of individuals affected by the clone was found to be quite strong ($R^2 = 0.95$). In the study, the relationship between the number of clones damaged by pine shoot moth factor and the years has been investigated. According to this analysis, it was found that there was a strong positive correlation between these two variables ($R^2 = 0.99$) and a positively effective linear relationship. Another double relationship examined in the study is between the blocks and the number of clones damaged by the pine shoots moth. As a result of the study, it was determined that there was a strong ($R^2 = -0.92$) and a negative effective relationship between these two variables.

Discussion

In this study, pine shoot moth damage in Yenice-Bakraz provenance of seed orchard which is located in Bartın region were investigated. In research process, there are no determinants of natural factors affecting insect damage. As a result of the determinations and counts conducted between 2014 and 2016, the pine shoot moth has different effects on 30 clones that constitute the seed orchard. For this purpose, according to 2016 data, the average number of individuals exposed to pine shoot moth damage was found to vary between 2.3-26.2 units (*Fig. 2*). As a result of the variance analysis performed in this context, a significant difference was found between the clones in terms of the number of individuals affected by $P < 0.001$ (*Table 1*). As a result of this study, 30 clones of $P < 0.05$ confidence level were sequenced and grouped according to the number of individuals affected by pine shoot moth (*Table 2*). Accordingly, a total of 20 groups were formed as a result of Duncan Range Test. In terms of the number of damaged individuals, clone 29 constitutes the first group alone and clone 23 constitutes the first group. In this respect, it can be said that the clones which have the highest damage in the seed orchard 70 number division which is the research subject of the pine shoot moth are clones with 21, 25, 26 and 29 clones and the

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clones with the least damage are clones with 2, 6, 8 and 23 (*Table 2*). As a matter of fact, there is a *Pinus contorta* var. *latifolia* plantations in Sweden have caused significant damages, and in this context, the clonal production of the species and the need to determine the origin of local clones resistant to these and similar pests are emphasized (Lindelöw and Björkman, 2001). On the other hand, a study conducted at Bosnian Pine (*Pinus helderichii* H. Christ. (1863) syn. *Pinus leucodermis* Ant. (1864)) found that pine shoot moth caused different rate of damage in the natural distribution area of the Bosnian pine and the seed sources established at the origin level (Vendramin et al., 2003). A study conducted in New Zealand on *Pinus radiata* found that some insect species, such as pine shoot moth, as well as snow, frost and drought damages caused significant damages in plantations and genetically modified seed sources; therefore, it has been reported that clones and origins are obtained from these damaging factors by intra-site crossover studies (Dungey et al., 2003). In this context, in the light of the results obtained from this research carried out in the Camiyanı black pine, it can be said that the clones, which the pine shoot moth has created the least damage during the 3-year period, constitute an important potential in terms of resistance improvement studies against this pest. There are significant benefits in monitoring the temporal course of damage to examine the effects of bacterial, fungus and insect on forest resources. In this study, the damage caused by the pine shoot moth on clones in the seed orchard in 2014, 2015 and 2016 was investigated. According to the results of 3-year observations and determinations, the average number of clones in 2014 was 13.78, in 2015 it was 10.23 and 7.64 was in 2016 (*Fig. 3*). As a result of the variance analysis, it was found that there was a statistically significant difference in $P < 0.001$ confidence level between the years in terms of the average number of clones that the pine bend-twist caused by membrane (*Table 2*). In this context, Duncan Range Test was applied at $P < 0.05$ confidence level. As a result of the Duncan Range Test performed, 3 different groups emerged in terms of the average clone number variable damaged by pine shoot moth at $P < 0.05$ confidence level. Accordingly, 2014 was the first group in which the most damages occurred during the 3-year period during which the survey was carried out, while this group was followed by the second group formed by 2015 alone, and 2016 was the third group alone (*Table 3*). Accordingly, it is possible to say that there is a significant decrease in the level of the damage caused by the pine shoot moth in the seed orchard. As a matter of fact, in a study conducted in Canada, pheromone traps placed in natural forest area, special seed sources and city centers were determined to show significant increases in monthly and yearly periods of pine shoot moth population in periods when the average temperature was above -2.2 °C (Heeley et al., 2003). On the other hand, in a study conducted in China, it has been determined that 39 insect species detected in native coniferous species are continuous and if the necessary measures are not taken, the severity of this situation increases. As a matter of fact, in the same study, it was found that there were many insect in the fir trees of spruce, douglas fir and hybrid species in China (26 species), and that the same insects caused significant damages in the seeds of 72 broad leaf species in the world in a very short time (Roques et al., 2003). In another study, it has been tried to determine the damages caused by *Rhyacionia frustrana* (Comstock) which is a different species of pine shoot moth in the seed orchard of the Turkish Red Pine (*Pinus brutia* Ten.) it has been determined that the pine shoot moth, especially in green cones, has caused damage that has a high rate of severity in different periods of different years (Can and Özçankaya, 2006).

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In the seed orchard with the necessary examination and determinations in the study, 3 random blocks were formed depending on the experimental design in the organization and the average clone numbers were determined in the blocks in which the pine shoot moth was damaged (*Fig. 4*). As of 2016, pine shoot moth has been damaged in terms of the average number of clones in Block I, III. A downward trend towards the block was determined. Accordingly, the average number of damaged clones determined in Block I as a result of the findings and counts performed in 2016 was 10.24, II. The average number of damaged clones detected in the block was 10.21 and III. The average number of damaged clones detected in the block was 10.12. In this context, variance analysis was performed to determine whether there were any statistically significant differences in terms of the mean number of clones exposed to pine shoot moth damage between the blocks (*Table 4*). As a result of the analysis of variance, no statistically significant difference was found between the blocks in terms of the average number of clones in which the pine shoot moth was harmful (*Table 4*). As a matter of fact, no studies have been carried out in Sweden about the difference in damage caused by pine shoot moth between blocks in seed orchard (Lindelöw and Björkman, 2001). In another research carried out in the *Pinus brutia* seed orchard in Turkey, there was no significant difference between the blocks in terms of the average number of clones exposed to the damage caused by the pine shoots (Can and Özçankaya, 2006). In this study, which was carried out over a 3-year period covering the years 2014-2016, the relationship between the number of individuals affected by the clone, the number of clones damaged by years, and the number of blocks and the number of clones damaged were also investigated with the help of binary regression analysis (*Table 5*). When the results of the binary regression analysis are examined; a second-order polynomial relationship was found to be positively influenced by the number of individuals affected by the clone. On the other hand, it was found that the relationship between the clone and the number of individuals damaged was quite strong (R^2 = 0.95). In the study, the relationship between the number of clones damaged by pine shoot moth factor and the years has been investigated. According to this analysis, it was found that there was a strong positive relationship (R^2 = 0.99) between these two variables. Another double relationship examined in the study is between the blocks and the number of clones damaged by the pine shoots moth. As a result of the study, it was found that there was a strong (R^2= -0.92) and negative relationship between these two variables (*Table 5*). These strong relationships have also enabled the creation of some models and use in practice in determining the extent of the damage to be caused by the pine shoot moth in the seed production (*Table 5*). The strong relationships between the variables investigated in this study were obtained in a similar study of *Pinus radiata* seed sources (Dungey et al., 2003).

Conclusions

In this research carried out in the seed orchard number 70 of Camiyani black pine, an important ecotype of black pine, which has a wide natural distribution area and geographical variation in Turkey, significant time, effort and money were spent in established, were damaged by the *Rhyacionia buoliana* level of the damage was investigated between 2014-2016. In this study, although no determinations and determinations have been made about the effects of clones on seed efficiency, which caused a particularly high level of damage, a research was carried out which is

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important in terms of the damage caused in the seed orchard and the determination of clones that can be resistant to this damage. In accordance with the information obtained from this research, the level of damage caused by pine shoot moth should be closely monitored in the seed orchard which constitutes the research object. More detailed studies should be determined precisely and the chemical and biological control should be started in a planned way without any loss of time in order to prevent these pests from causing damages that cause greater losses in the seed orchard. On the other hand, in accordance with the findings obtained from the research, it was determined that the clones that were affected from the shoot moth caused by the environmental conditions of the seed orchard were the least affected clones 2, 6, 8 and 23 respectively. Accordingly, planned vegetative reproduction of these clones, which are determined to be resistant to this pest, in the form of residual breeding techniques and use in afforestation studies of individuals obtained by clonal production with cuttings taken from clones, biotic and abiotic causes caused by the change of environmental conditions of afforestation and artificial regeneration studies. Resistance to pests will significantly improve your success.

Acknowledgements. We would like to thank the General Directorate of Forestry, Forest Trees and Seed Breeding Research Institute for their support and contribution at every stage of this research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34153426

THE FIRST PARASITOID RECORD OF *GARELLA MUSCULANA* (NOLIDAE, LEPIDOPTERA) FROM TURKEY; *PIMPLA SPURIA* (ICHNEUMONIDAE, HYMENOPTERA)

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(Received 26th Dec 2018; accepted 14th Feb 2019)

Abstract. This research was conducted within the border of Bartın province between the years of 2015-2018 for making detailed research on the biology of the important walnut pest *Garella musculana* identified for the first time in Turkey. The pupas collected from the study area were brought to the laboratory and monitored. It was observed that some pupas were parasitic, and the type of parasitic species that emerged from these pupae was also recorded and the species were diagnosed. As a result of the diagnosis, the parasitoid species were identified as *Pimpla spuria* (Gravenhorst, 1829). This was the first parasitoid recorded on *Garella musculana* in Turkey. This situation should be helpful of biological control on *Garella musculana* in walnut orchards.
Keywords: *Garella musculana, walnut, Pimpla spuria, parasitoid, first record, Turkey*

Introduction

Walnut (*Juglans regia*), an income-generating species, has a large distribution area and is a very old and important fruit of Turkey. Turkey's walnut production is the fourth in the world (FAO, 2016). *Garella musculana*, one of the important pests of the walnut plant, was determined for the first time in Turkey in the studies carried out in Bartın province in 2015.

Garella musculana is one of the most important pests of walnut fruit and young shoots in infected countries. Larvae damage fruit as a result of nutrition, and usually there are 1 larvae in the fruit, while sometimes 2 and rarely 3 larvae have been observed. In fruit, larvae are only nourished in the green shell (pericarp). As a result of the damage of larvae, fruits become deformed and there is no normal fruit development. As a result of the damage of the species 70-80% yield decrease can occur in the nuts. In addition to the loss of product in Walnut, this insect can cause seed distress for regeneration of natural *Juglans regia* forests. In the years of low fruit production, larval nutrition in shoots causes the death of shoots and this is usually a more serious danger for young trees (Orozumbekov and Moore, 2007).

One of the reasons for the rapid spread of invasive species in the area where they first infect is the absence of natural enemies. Therefore, in this study we tried to determine the natural enemies of *Garella musculana* in Bartın province.

Townes et al. (1965) reported that most of the species belonging to the Ichneumonidae family parasite Lepidoptera larvae and some species parasite Coleoptera

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DOI: http://dx.doi.org/10.15666/aeer/1702_34273431

larvae. Especially *Pimpla* (F.) species belonging to the type of polyphagous pests many species under pressure has been recorded. Borror et al. (1981) indicate that species belonging to the Ichneumonidae family have a large host distribution.

Ichneumonids, as hosts, use a range of insects and arachnids can play an important role in the normal functioning of terrestrial ecosystems. They were also successfully used as biocontrol agents (Gupta, 1991).

Ichneumonidae species are present as internal and external parasites in larvae and pupae of species belonging to Lepidoptera, Coleoptera and Hymenoptera (Oğurlu, 2000).

In this study conducted between the years of 2015-2018, parasitoid species of *Garella musculana* were investigated and *Pimpla spuria* was determined as the first parasitoid record for the related pest which can be used as a biological control agent.

Materials and methods

In Bartın province, in order to carry out detailed studies about pest, pupas under the bark (*Fig. 1*) from the study areas of different localities such as Karasu, Akıncılar were brought to the laboratory and monitored after the adult exits. Preparation of the parasitoid species derived from some parasitoid pupas was made and photographed under microscope and sent to the diagnosis and species was identified.

***Figure 1.** Pupae cocoons of Garella musculana*

Results and discussion

In this study, parasitoid species *Pimpla spuria* (F.) (Hymenoptera: Ichneumonidae) was obtained from pupae cocoons of *Garella musculuna.* This parasitoid is reported for the first time on *G. musculuna* (*Fig. 2*).

Pimpla spuria is a common species that is widely distributed in the Holarctic/Palaearctic and Oriental regions (Kolarov et al., 2014). In Turkey, this parasitoid has been identified in Adana, Adıyaman, Ankara, Afyon, Artvin, Balıkesir, Bilecik, Burdur, Bursa, Çanakkale Denizli, Edirne, Erzincan, Erzurum, Gaziantep, Giresun, Hatay, Isparta, Mersin, Istanbul, Kars, Kırklareli, Kocaeli, Konya, Manisa, Muğla, Rize, Şanlıurfa, Tekirdağ, Tunceli, Trabzon, Uşak and Yalova so far (Konca, 2015; Çoruh et al., 2014; Kolarov et al., 2014; Coruh and Kolarov, 2010). This is the first time Bartın has been added to its distribution area.

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***Figure 2.** Pimpla spuria. **a** General view of adult, **b** wings, **c** antenna, **d** head*

As the hosts of *Pimpla spuria*; *Ostrinia nubillis* (Özdemir, 1981) and *Lobesia botrana* (Özdemir and Kılınçer, 1990) were recorded in Turkey. Aslan (2015) identified *Pimpla spuria* as the most densest species in a study of *Lobesia botrana* parasitoids that harmful in vineyards in Gaziantep province.

Yurtcan and Beyarslan (2005) identified 21 species of Pimpline and Polysphinctinini (Hymenoptera: Ichneumonidae: Pimplexae) in the Trakya region and *Pimpla implemporator* (Müller, 1776) and *P. spuria* (Gravenhorst, 1829) as the most common species.

Aydoğdu (2014) *P. spuria* has been detected as parasitoid of *Archips rosana* (L., 1758) (Lepidoptera: Tortricidae) in organic cherry orchards the first time. And also, in the cherry orchards of Sultandağı Basin, including *Pimpla spuria*, 30 species from the Ichneumonidae family were identified (Özdemir and Güler, 2009).

Pimpla spuria was identified as pupa parasitoid of *Sesamia nonagrioides* (Lepidoptera:Noctuidae) in Cukurova region. At the same time, Oztemiz et al. (2004) reported as pupa parasitoid of *Sesamia nonagrioides* (Lepidoptera:Noctuidae) and *Ostrinia nubilaris* (Lepidoptera:Pyralidae) of the same species.

In Bulgaria, *Pimpla spuria* (Gravenhorst, 1829) was identified as parasitoid of *Cydia pomonella* and *Grapholita funebrana* species. *Grapholita funebrana* was given to the new host of *P. spuria* (Velcheva and Atanassov, 2016).

The natural enemies of *G. musculana* can play an important role in regulating its populations. Sixteen species of parasitoids and predators belonging to Ichneumonidae, Braconidae, Pteromalidae, Torymidae, Trichomammatidae, Carabidae, Raphididae and Formicidae are recorded as *G. musculana*. The most common of these, *Trichogramma*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34273431

sp. and *Pimpla instigator*. Sometimes, caterpillars are infected by bacteria *Bacillus thuringiensis* or fungi *Beauveria bassiana* (Degtyareva, 1964; Dzhaparov, 1990).

Conclusion

Garella musculana which is harmful to walnut trees causes severe damage and brings about economic loss since first detected in Turkey. In order to control *G. musculana,* added to the A2 list in 2003 by the European and Mediterranean Plant Protection Organization (EPPO), some biological control methods are investigated against this pest. In our study conducted within the border of Bartın Province; it was observed that some pupas collected from the study areas were parasitic. As a result of the diagnosis, the parasitoid species were identified as *Pimpla spuria.* The importance of this identifying is*; P. spuria* was the first parasitoid recorded on *Garella musculana* in Turkey.

The abundance and effectiveness of *Pimpla spuria*'s host diversity is thought to play an active role in control of the important walnut pest *Garella musculana*.

Acknowledgements. We are thankful to Oleksandr Varga for the identification of this Ichneumonidae species.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34273431

SIDE EFFECTS OF DIFFERENT DOSES OF AZADIRACHTIN ON PREDATORY MITE *METASEIULUS OCCIDENTALIS* (NESBITT) (ACARI: PHYTOSEIIDAE) UNDER LABORATORY CONDITIONS

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(Received 4th Jan 2019; accepted 14th Feb 2019)

Abstract. *Metaseiulus* (=*Galendromus* or *Typhlodromus*) *occidentalis* (Nesbitt) (Acari: Phytoseiidae) is an important and effective predator of pest mites in agricultural crops and is commercially available in the United States. Azadirachtin is a biorational pesticide, which contains azadirachtin A (10 g/l) as the basic active compound. The active compound has several properties useful for insect control (repellency, feeding and oviposition deterrence) and is considered safe for the environment. With the use of natural compound like azadirachtin A in integrated pest management programs, it is important to know whether the compounds have any side effects on predatory mite such as *M. occidentalis.* Therefore, the current study aimed to examine the toxic effect of different doses (0.1 ml (NZ-1*2), 0.05 ml (NZ-1), 0.025 ml (NZ-2), 0.01 ml (NZ-3), 0.005 ml (NZ-4) and 0.001 (NZ-5)/ 10 ml water) of NeemAzal-T/S (a.i. 10 g/l azadirachtin A) on *M. occidentalis.* The mortality rate of *M. occidentalis* was 77.5% 24 h after application of NeemAzal-T/S at 0.1 ml/10 ml water dose. The mortality caused by the recommended dose (0.05 ml/10 ml water) of NeemAzal-T/S was 62.31%, 70% and 73.07%, 24, 48 and 72 h after application, respectively. It is concluded that NeemAzal-T/S is moderately toxic against the predatory mite *M. occidentalis.* Therefore, the use of NeemAzal-T/S to control the pest mites in agricultural crops might affect the populations of predatory mites.
Keywords: *azadirachtin, spider mite, Metaseiulus occidentalis, side effect, pesticide*

Introduction

Plant protection strategies, pest management in particular, have focused on minimizing the use of pesticides in the recent years. The use of organic pesticides, i.e., derived from plants or living organisms has gained attention in this regard. However, the evaluation of the side effects of organic pesticides is required to know whether or not these pesticides are fatal to the natural enemies.

Agricultural crops are infested by several pests, some of them may be controlled by using pathogens, predators, and parasitoids in addition to pesticides. However, selective pesticides are required to achieve a satisfactory control of complexes of pests (Cloyd et al., 2006) when using living organisms for pest control. Most of the biological control agents, including predators and parasitoids can provide excellent management of many pests. There is great potential for reducing pesticide use in pest management through exploiting natural enemies of the pests (Kavousi and Talebi, 2003) as alternatives to pesticides. Alternative pest management approach includes the use of predator mites along with pesticides instead of using pesticides or biocontrol agent alone (Sato et al., 2000).

It is very important to determine the positive/negative impacts of selective insecticides/acaricides that could be used in combination with biological control agents (Martin and Woodcock, 1983) for pest management. Phytoseiidae mites are one of the most important biological control agents effective on different kinds of pests on various

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

greenhouse plants and orchards (Schmutterer, 1990). *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae) is one of these mites. It is a predatory mite species, specialized in the phytophagous red spider mite species (Tetranychidae) in North America (Hoy et al., 1982; Hoy, 2011). This predatory mite species has been adapted to semi-arid conditions, can produce large populations in low relative humidity environments, thus suppresses spider mites (Mc Murtry, 1982; Mc Murtry and Croft, 1997). The length of time taken from egg to adult is aproximately 8.5 days. The average eggs laying capacity of adult female is 2.2 eggs/day and capable of laying 34 eggs during the entire life cycle, longevity of *M. occidentalis* females is approximately 20 days (Laing, 1969). The length of time from egg to adult varies greatly with temperature and as the temperature increases, the development time is shortened with increasing temperature. While the length of time from egg to adult is 7-10 days at 24 °C, it become 8.7, 7.4 and 5.2 days at 27 °C, 29 °C, and 32 °C respectively (Tanigoshi et al., 1975).

Phytoseilus persimilis and *Neoseiulus californicus* are commercially available and actively used in the biological control of two-spotted spiders. *Metaseiulus occidentalis* is a predator mite belonging to the Type II group and is feed with Tetranychidae mite species and rust mites (McMurtry and Rodrigues, 1987).

One of the botanical pesticides is Azadirachtin (10 g/L). Azadirachtin (10 g/L) is the commercially available plant orginated pesticide; It has been reported that it is effective on more than 200 plant pests, such as aphids, White flies, leafminers, Thrips, Colarado potato beetle, woolly Apple aphid, scale insects, mealy bugs, leafhopper, Noctuids, Coleopters, Yponometua species, American White fly, Pine processionary caterpillar, Oriental fruit moth, Olive fruit fly, Cherry fruit fly, leaf tortrix moths, and spider mites. It is also widely used in organic farming (Schmutterer et al., 1981; Isman, 2006). The active substance diffuses into the leaves and is partly systemically dispersed in the plant; insects take this active substance by mouth (by sucking or chewing) during feeding. Azadiractin inhibits the feeding and molting of the exposed inscets. At the same time, it decreases the fecundity of insect and may causes infertility (Isman, 2006).

This study was aimed at evaluation of different doses of Azadiractin on *Metaseiulus occidentalis*.

Materials and methods

Tetranychus urticae

The adults, nymphs and especially eggs of *Tetranychus urticae* are needed to feed the predator mites. Therefore *T. urticae* was reared on green bean plants, *Phaseolus vulgaris*. The bean plants used in the experiment were grown at 25 ± °C and 16:8 h L:D photoperiod in the growth chamber (*Fig. 1*).

a **b** **c**

Figure 1. a *Predatory mite Metaseiulus occidentalis culture.* ***b, c*** *View from experiments*

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DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

Neem formulation

Neem Azal is a commercial formulation of neem seed kernel extract containing10 g/LAzadirachtin.

Effect of azadirachtin on Metaseiulus occidentalis

The predatory mites *Metaseiulus occidentalis* were obtained from Emeritus Prof. Dr. Marjorie Hoy, University of Florida, USA and fed with *T. urticae*. *M. occidentalis* is nonnative to Turkey; therefore, necessary permission was obtained from Turkish Agricultural Ministry to work with this predatory mite species. The experiments were carried in Petri dishes (90 mm in diameter) by placing ten adult females of *M. occidentalis* per Petri dish. Dry film method was used to determine the toxicity of azadirachtin on *M. occidentalis* (Birah et al., 2008). Inner sides of Petri dishes were coated with 60 µl solution of six different doses (0.1, 0.05, 0.025, 0.01, 0.005, 0.001 ml/10 ml of water) of azadirachtin (10 g/l) (*Table 1*). The solution was spread uniformly and allowed to dry at room temperature for 30 min. Ten adult females of *M. occidentalis* were released onto the film in each Petri dish and the dishes were wrapped with parafilm to prevent evaporation. Thereafter; the predatory mite was exposed to the solution for a period of 24 h. Thereafter the adult females were transferred to bean leaf disk (2 cm in diameter) which was placed inside a petri dish on moist cotton. The experiments were carried out at 25 ± 2 °C and 16:8 h L:D photoperiod and 45 ± 5% humidity in the growth chamber. Each treatment was replicated 5 times. The experiments were repeated two times. The number of living and dead *M. occidentalis* adults was monitored 24, 48, 72, 96, 120 and 144 h after exposure to azadirachtin. The number of eggs laid by each female after 24 h period was recorded to investigate the effects of tested doses of azadirachtin on eggs laying capacity of the female.

Table 1. *Different doses of azadirachtin used in the study*

Treatments doses (ml per 10 ml water)	
Control (C)	Distilled water
NZ - 1*2	0.1
NZ - 1	0.05
NZ – 1/2	0.025
NZ - 3	0.01
NZ - 4	0.005
NZ - 5	0.001

The results were expressed as percent mortality with correction for untreated mortality using Abbott’s formula (Abbott, 1925). The data were subjected to ANOVA tests and the means were compared by Tukey test, using SPSS 17.0 software program (SPSS, 2008).

Results and discussion

One of the practical and environmentally safe biological pesticides are plant-derived products, due to their bio-degradability and target-specific nature (Birch, 2011). The

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DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

combined effect of both botanical pesticides and predators or parasitoids as biological control agents is expected to have greater effects on target insects than both approaches opted alone. The predatory mites can be combined with safe doses of botanical pesticides, which have minimum negative impacts on natural enemies and provide enough control over target pests. Therefore, the knowledge of safe dose of a botanical pesticide is required to maintain the pest population below an economic threshold level without effecting the population of predatory mite. Toxicities of different doses of azadirachtin (10 g/l) on predatory mite *M. occidentalis* were evaluated in the current study and results are summarized in *Table 2*.

Table 2. *Mean mortality rates (% ± SE) of predatory mite (Metaseiulus occidentalis) 24-144 h after exposure to azadirachtin (10 g/l)*

Doses	Mean mortality % ± SE					
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**
C	6 ± 2.45a*	6 ± 2.45a	10 ± 3.16a	17.78 ± 3.64a	23.33 ± 3.72a	28.89 ± 5.12a
NZ -1*2	77.5 ± 4.53e	82.50 ± 5.26e	86.25 ± 4.60e	82.5 ± 4.79d	85 ± 2.89d	92.50 ± 2.50d
NZ - 1	62.31 ± 3.23d	70 ± 2.53de	73.08 ± 3.47de	77.78 ± 6.19cd	80 ± 6.67cd	84.44 ± 6.26cd
NZ - 2	48.46 ± 2.49c	58.46 ± 3.37cd	62.30 ± 3.78cd	70 ± 5.27cd	77.78 ± 6.40cd	84.44 ± 6.48cd
NZ - 3	46.92 ± 2.37c	53.08 ± 3.08cd	61.54 ± 3.37cd	61.11 ± 4.55cd	71.11 ± 6.55cd	76.67 ± 7.26cd
NZ - 4	40.83 ± 1.48bc	42.5 ± 1.79bc	49.17 ± 3.12bc	53.75 ± 4.98bc	53.75 ± 4.98bc	61.25 ± 7.18bc
NZ - 5	32.14 ± 3.17b	35.71 ± 3.43b	36.43 ± 3.25b	35 ± 2.89ab	37.50 ± 2.50ab	40 ± 4.08ab

*Means followed by the same letter in each column are not significantly different (P < 0.05)

Females of *M. occidentalis* were more sensitive to double (0.1 ml/10 ml water) and recommended (0.05 ml/10 ml water) doses of azadirachtin than the other doses tested in the study. The female mortality was decreased with decreasing dose. At double dose, the mortality rates were 77.5, 82.5 and 86.25% after 24, 48, and 72 h of exposure, respectively. Similarly, recommended dose of azadirachtin caused a significant reduction in the viability of *M. occidentalis* 24 to 72 h after exposure, highlighting the negative effect on predatory mite. Mortality of *M. occidentalis* ranged from 62.31 to 73.8% at 0.05 ml/10 ml water concentration 24 to 72 h after exposure (*Tables 2* and *3*). These results are consistent with the findings of Mourao et al. (2004) who reported that neem cake caused 88% mortality of *Iphiseiodes zuluagai* Denmark and Muma (Acari: Phytoseiidae) adults. Several studies showed the compatibility of neem-based products with predatory mites, e.g., azadirachtin at 4.5% concentration was found compatible with *Phytoseiulus persimilis* (Bernardi et al., 2013; Duso et al., 2008; Cote et al., 2002). The effects of neem-derived compounds can vary, depending on the predatory mite species, formulation of pesticide or the plant part used.

Our study indicates that mean longevity of alive adult females of *M. occidentalis* was also decreased with increasing dose of azadirachtin. While the longevity of female adults was 7.55 days in control treatment and with the same category at 0.01, 0.005 and 0.001 ml/10 ml water concentrations statistically, at 0.01, 0. 05 and 0.025 ml/10 ml concentrations it was 3.42, 3.62, 5.62 days respectively ($F = 114.49$; $df = 6$; $P < 0.001$) (*Table 4*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

Table 3. *Anova tables of mean mortality rates (%±SE) of predatory mite (Metaseiulus occidentalis) 24-144 h after exposure to azadirachtin (10 g/l)*

	Between groups				
	Sum of squares	**df**	**Mean square**	**F**	**Sig.**
24 h mortality	22459.029	6	3743.172	38.661	.000
48 h mortality	27587.784	6	4597.964	38.479	.000
72 h mortality	28517.353	6	4752.892	32.208	.000
96 h mortality	24014.423	6	4002.404	20.096	.000
120 h mortality	24398.825	6	4066.471	15.499	.000
144 h mortality	25171.474	6	4195.246	12.846	.000

Table 4. *Effects of azadirachtin on mean longevity of alive adult females of Metaseiulus occidentalis*

Doses	**Mean adult female longevity (day) mean ± SE**
C	7.55 ± 0.10c*
NZ - 1*2	3.42 ± 0.19a
NZ – 1	3.62 ± 0.16a
NZ – 2	5.22 ± 0.13b
NZ – 3	7.46 ± 0.11c
NZ – 4	7.50 ± 0.22c
NZ – 5	7.31 ± 0.24c

*Means followed by the same letter in each column are not significantly different (P < 0.05)

Fecundity of female adults of the mite was significantly reduced as the total number of eggs laid per female were decreased at 0.1 and 0.05 ml/10 ml water concentrations, which were significantly lower than the control treatment (*Table 5*) (F = 45.25; df = 6; P < 0.001). These results are consistent with the findings of Irigaray and Zalom (2006), who reported significant influence of acequinocyl on fecundity of *M. occidentalis* females. Additionally, there was significant difference in the mean number of eggs deposited per mite/per day. The female exposed to double (0.1 ml/10 ml water) and recommended (0.05 ml/10 ml water) doses of azadirachtin deposited significantly lower number of eggs than the females in control treatments (p ≤ 0.05) (0.29, 0.61 and 1.30/per day/per female, respectively) (F = 26.24; df = 6; P < 0.001) (*Table 6*).

Table 5. *The effects of Azadirachtin on fecundity (total number of eggs laid/per female) of Metaseiulus occidentalis*

Doses	**Mean egg number/adult female mean ± SE**
C	10.15 ± 0.48d*
NZ - 1*2	1.08 ± 0.83a
NZ – 1	2.28 ± 0.21ab
NZ – 2	4.00 ± 0.28b
NZ – 3	8.46 ± 0.51cd
NZ – 4	6.92 ± 0.55c
NZ – 5	7.26 ± 0.63c

*Means followed by the same letter in each column are not significantly different (P < 0.05)

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DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

Table 6. *Effects of Azadirachtin on fecundity (average number of eggs laid/per day/per female) of Metaseiulus occidentalis*

Doses	Mean daily egg number/adult female mean ± SE
C	1.30 ± 0.05e*
NZ - 1*2	0.29 ± 0.03a
NZ – 1	0.61 ± 0.05b
NZ – 2	0.77 ± 0.05bc
NZ – 3	1.08 ± 0.06de
NZ – 4	0.88 ± 0.06cd
NZ – 5	0.94 ± 0.07cd

*Means followed by the same letter in each column are not significantly different (P < 0.05)

Hatched larvae to reaching the adulthood stage of predatory mite ranged between 66.67 and 100%, b ut at 0.001 ml/10 ml concentration, there was no larval hatching (*Table 7*). Yanar and Hoy (2017), found that surviving females held for 2 and 4 f week cold storage laid eggs at the same rate as control females of *M. occidentalis*. Nicoli and Galazzi (1998) found that survival of predatory mites during storage decreased but the surviving females show greater longevity, fertility and fecundity.

Table 7. *Effects of azadirachtin on larval hatching and reaching adulthood from larva of Metaseiulus occidentalis*

Doses	Emerging larvae %	Larvae which can reach adulthood %
C	38.89	95.24
NZ – 1	40.00	87.50
NZ – 2	40.91	66.67
NZ – 3	50.00	100.00
NZ – 4	42.86	83.33

Conclusion

The data obtained from this study will be useful for commercial producers and pest management specialists wishing to use these predatory mites in integrated pest management programs. Considering the results of present study, it can be concluded that recommended and half doses of azadirachtin (10 g/L) should not be used in IPM programs where *M. occidentalis* is used to control pest mites. Lower concentrations (0.01 and 0.005 ml/10 ml water) can be used in combination with *M. occidentalis,* reducing the selection pressure and development of resistance (Marcic, 2007). Herron et al. (1993) and Cheon et al. (2007) suggested that acaricides at reduced rates might be used to adjust the predator/prey ratio.

Acknowledgements. The results of this study was presented as an oral presentation at Turkey 6th Plant Protection Congress 5-8 September 2016, Konya, Turkey and abstract was published in congress proceedings book. I would like to thank Agricultural Engineer Havva Öz Tural for her help during experiments.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34333440

DETERMINING FACTORS IN FOOD AWAY FROM HOME EXPENDITURE OF TURKISH HOUSEHOLDS

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(Received 22nd Jan 2019; accepted 14th Feb 2019)

Abstract. The aim of this study was to determine the effects of socio-demographic and economic factors of households and household heads on monthly expenditures of food away from home (FAFH) in Turkey. The sample size was determined as 11290 households. The data were analyzed by generalized Heckman type sample selection models. The results showed that both the probability and monthly spending levels of household FAFH increased with male household heads, household income, whilst the married household heads, the households that receive cash income aids from the government and private sector, decreased both the probability and spending levels of FAFH in Turkey. The findings in the study may provide useful information for more effective identification and implementation of the marketing strategies of the stakeholders operating in the sector and also help the relevant public and private institutions determine more effective nutritional policies towards families with a certain profile.
Keywords: *eating out, consumption, expenditure patterns, Heckman Sample Selection Model, Turkey*

Introduction

Many countries have experienced changes in eating habits over the last decades in terms of individual consumers and households. These changes generally were from home consumption towards food away from home (Stewart et al., 2004; Akbay and Boz, 2005; Gül et al., 2007; Drescher and Roosen, 2013; Bozoglu et al., 2013; Niyonzima et al., 2017; Mottaleb et al., 2017). Changes were generally derived from convenience and increasing food availability, but demographic changes such as a higher share of working women and an increasing number of single households contributed as well (Nayga and Capps, 1992; Robson et al., 2016). One of the discouraging factors on the preparing the meal at home was reported to be working longer hours (Blick et al., 2017). In many studies, it was stated that globalization, urbanization, income, education, marketing, religion, culture, tourism women's participation in the labor force and consumer attitudes increases in FAFH consumption (Nayga, 1995; Ma et al., 2006; Bozoglu et al., 2013; Liu and Niyongira, 2017).

Worldwide, food consumption trends have been moving towards FAFH in many countries (Mancino et al., 2009; Bozoglu et al., 2013; Liu et al., 2013). For instance, the ratio of FAFH in household food expenditure of the United States of America (USA) increased twofold from 25% in 1954 to 50% in 2014 (USDA, 2018). This rate rose to 32% in 1992 in Canada (Jensen and Yen, 1996). FAFH ratio in 2002 was 24% reaching to 31% in 2008 in Brazil (Bezerra et al., 2013). In the last two decades, the percentage of FAFH rose to 25% in 1996 and 30% in 2006 in Spain (Mutlu and Gracia, 2006; Angulo et al., 2007). Again, the period of 2004-2010 experienced an increase from 20% to 25% FAFH consumption in Slovakia (Cupak et al., 2016), while in the 1987-1999 period FAFH consumption rate rose from 13% to 23% in Ireland (Keelan et al., 2009).

Turkey has also experienced similar changes in the pattern of food consumption expenditures mainly due to changes in the socio-economic structure of Turkish

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

population in the last two decades. Composition and size of households, consumer lifestyles, per capita income, age distribution of the population and urbanization had a significant effect in FAFH. Thus, more efficient marketing strategies are required in the sector. Money spent of food, including non-alcoholic beverages as a share of total household spending continued to shrink, from 26.7% in 2002 to 19.5% in 2016, while expenditures on hotels, restaurants, and pastry shops (FAFH) as a share of total household spending increased from 4.4% in 2002 to 6.4% in 2016 (TURKSTAT, 2018).

The current work has identified the effects of socio-demographic and economic factors of households and household heads on their FAFH consumption expenditures using a Double Hurdle (DH), Heckman Sample Selection (HSS) and Log-Heckman Sample Selection (LHSS) models. The study, which is rich in terms of household socio-demographic and economic profiles, also presented marginal impacts of exogenous variables on spending of FAFH. The presentation of such marginal effects may provide useful information for more effective identification and implementation of the marketing strategies of the stakeholders operating in the industry. This study will also help the relevant public and private institutions determine more effective nutritional policies towards families with a certain profile.

Materials and methods

Turkish Statistics Institute (TSI)' Household Budget Surveys data of 2015 was used as main material of this study. These data are regularly collected by TSI on the annual basis so as to cover the period between January 1 and December 31 and cover 10 thousand households on average across the country. The sample size was determined as 11290 family observations after deleting incomplete data and outliers.

Table 1 shows the descriptive statistics for sample. The results of this study showed that 77.3% of households had monthly expenditures on food away from home. In some studies conducted in different parts of Turkey, this ratio was reported as 55.4% (Gül et al., 2007), 83.6% (Uzunöz et al., 2011), 74.5% (Bozoglu et al., 2013) and 68.4% (Traş and Şengül, 2017). The reported figures for some countries, were as follows: Slovakia, 62.0% (Cupak et al., 2016), Brazil, 40.0% (Bezerra et al., 2013), China, 83.0% (Liu et al., 2015), Egypt, 38% (Fabiosa, 2008) and Malaysia, 71.9% (Heng and Guan, 2007). Monthly average FAFH consumption expenditures of households were found to be 180.14 Turkish Liras (TL). These figures were as follows for different parts of Turkey: 137.66 TL (Bozoglu et al., 2013), 371.00 TL (Onurlubaş et al., 2015) and 131.82 TL (Traş and Şengül, 2017).

Three censored models competing with one another and at the same time compatible with the maximization of the utility function of consumers were considered. The mathematical representation of each econometric model were made here while referring to the textbooks on how these models are derived from the utility function under the good in question (e.g., food away from home). Before moving on to the presentation of each econometric model, it is worth mentioning a very important point here. Some families may not be able to consume FAFH and such observations are often reported as zero. Food away from home may not have been consumed, either because its current price is too high or because the family income is not affordable for the consumption or because of some health concerns and psychological factors. The Heckman sample selection (HSS) model is characterized by a selection equation (d_i) (e.g., the decision on

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

the expenditure of the food away from home) and level equation (y_i) (e.g., monthly expenditure level for the food away from home) as follows (*Eq. 1*):

$$\begin{aligned} d_i &= 1 \quad if \quad z_i'\alpha + u_{1i} > 0 \\ &= 0 \quad if \quad z_i'\alpha + u_{1i} \leq 0 \\ y_i &= x_i'\beta + u_{2i} \quad if \quad z_i'\alpha + u_{1i} > 0 \\ &= 0 \quad if \quad z_i'\alpha + u_{1i} \leq 0 \\ &(can\ be = unobserved) \ if \quad z_i'\alpha + u_{1i} \leq 0 \end{aligned} \tag{Eq.1}$$

where z and x are sets of exogenous factors affecting the household decision and expenditure levels on food away from home, respectively whilst α and β are vectors of associative parameters to be estimated for each equation, respectively. u_1 and u_2 are error terms unknown to researchers. The error terms (u_1, u_2) are distributed as truncated bivariate normal with zero means, standard deviations (1, σ), correlation ρ, and covariance ρσ, all indicating:

$$\begin{bmatrix} u_{1i} \\ u_{2i} \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} 1 & \rho\sigma \\ \rho\sigma & \sigma^2 \end{bmatrix} \right)$$

The corresponding sample likelihood function is (*Eq. 2*)

$$L = \prod_{d_i=0} \{1 - \Phi(z_i'\alpha)\} \prod_{d_i=1} \left\{ \frac{1}{\sigma} \phi\left(\frac{y_i - x_i'\beta}{\sigma} \right) \Phi\left(\frac{z_i'\alpha + \frac{\rho}{\sigma}(y_i - x_i'\beta)}{(1-\rho^2)^{1/2}} \right)^{-1} \right\} \tag{Eq.2}$$

where Φ and ϕ are the univariate standard normal cumulative and probability distribution functions, respectively. The log-transformed Heckman SSM (LHSS) is characterized by replacing y_i with log y_i in *Equation 2* with its corresponding likelihood function as (*Eq. 3*):

$$L = \prod_{d_i=0} \{1 - \Phi(z_i'\alpha)\} \prod_{d_i=1} \left\{ y_i^{-1} \frac{1}{\sigma} \phi\left(\frac{\log y_i - x_i'\beta}{\sigma} \right) \Phi\left(\frac{z_i'\alpha + \frac{\rho}{\sigma}(\log y_i - x_i'\beta)}{(1-\rho^2)^{1/2}} \right)^{-1} \right\} \tag{Eq.3}$$

The same distributional assumption for error terms (u_1, u_2) as in Heckman-SSM are maintained and y_i^{-1} is the Jacobian of transformation from log y_i to y_i. Now, augmenting the binary decision $z_i'\alpha + u_{1i}$ with an additional mechanism $x_i'\alpha + u_{2i}$ the double-hurdle (DH) model can be stated as follows (*Eq. 4*):

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

$$\begin{aligned} y_i &= x_i'\beta + u_{2i} \quad if \quad z_i'\alpha + u_{1i} > 0 \text{ and } x_i'\beta + u_{2i} > 0 \\ &= 0 \qquad\qquad\; if \quad z_i'\alpha + u_{1i} \le 0 \text{ and } x_i'\beta + u_{2i} \le 0 \end{aligned} \tag{Eq.4}$$

The error terms (u_1, u_2) in the double-hurdle model are distributed as the bivariate normal as in Heckman's (1979) sample selection model (HSS) without truncation. The sample likelihood function is (*Eq. 5*):

$$L = \prod_{d_i=0}\left\{1-\Phi_2\left(z_i'\alpha, \frac{x_i'\beta}{\sigma}, \rho\right)\right\}\prod_{d_i=1}\left\{\frac{1}{\sigma}\phi\left(\frac{y_i - x_i'\beta}{\sigma}\right)\Phi\left(\frac{z_i'\alpha + \frac{\rho}{\sigma}(y_i - x_i'\beta)}{\left(1-\rho^2\right)^{1/2}}\right)\right\} \tag{Eq.5}$$

where Φ2 is the standard bivariate cumulative distribution function. By imposing parametric restriction ρ = 0 in model, The HSS, LHSS and DH models reduce to their two-part models. This can be done via the conventional tests such as Wald, Likelihood Ratio, or Lagrangian Multiplier (LM). On the other hand, the choice between the three competing models can be done by a non-nested Vuong's specification test.

While the probability of both the HSS and LHSS models are the same, their conditional and unconditional, expenditure level equations vary greatly (*Eq. 6*):

$$\Pr\left(y_i > 0\right) = \Phi\left(z_i'\alpha\right) \tag{Eq.6}$$

Conditional and unconditional means of y_i of the HSS model are respectively (*Eq. 7*):

$$E\left(y_i \mid d_i = 1\right) = x_i'\beta + \rho\sigma\frac{\phi\left(z_i'\alpha\right)}{\Phi\left(z_i'\alpha\right)} \text{ and } E\left(y_i \mid d_i = 1\right) = \Phi\left(z_i'\alpha\right)x_i'\beta + \rho\sigma\phi\left(z_i'\alpha\right) \tag{Eq.7}$$

While the conditional and unconditional mean levels for the LHSS model is respectively (*Eq. 8*):

$$E\left(y_i \mid d_i = 1\right) = \exp\left(x_i'\beta + \sigma^2/2\right)\frac{\Phi\left(z_i'\alpha + \rho\sigma\right)}{\Phi\left(z_i'\alpha\right)} \text{ and } E\left(y_i \mid d_i = 1\right) = \exp\left(x_i'\beta + \sigma^2/2\right)\Phi\left(z_i'\alpha + \rho\sigma\right) \tag{Eq.8}$$

However, the probability, conditional and unconditional mean levels of monthly household food away from home product in DH is, respectively (*Eq. 9*):

$$\begin{aligned} &\Pr\left(y_i > 0\right) = \Phi_2\left(z_i'\alpha, \frac{x_i'\beta}{\sigma}; \rho\right) \\ &E\left(y_i \mid y_i > 0\right) = x_i'\beta + \sigma\Phi_2\left(z_i'\alpha, \frac{x_i'\beta}{\sigma}; \rho\right)^{-1}\left\{\phi\left(\frac{x_i'\beta}{\sigma}\right)\Phi\left[\frac{z_i'\alpha - \rho x_i'\beta/\sigma}{\sqrt{1-\rho^2}}\right] + \rho\phi\left(z_i'\alpha\right)\Phi\left[\frac{x_i'\beta/\sigma - \rho z_i'\alpha}{\sqrt{1-\rho^2}}\right]\right\} \\ &E\left(y_i\right) = \Pr\left(y_i > 0\right)E\left(y_i \mid y_i > 0\right) \end{aligned} \tag{Eq.9}$$

Marginal effects can be obtained by differentiation each equation with respect to z and x variables. Delta method is used to construct the variance-covariance of marginal impact estimates.

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

Table 1. *Variable definition and sample means*

Variables	Definition	Mean	VIF
	Dependent variables		
Food away from home	Expenses per month (TL)	180.14 (235.58)	-
	% consuming	77.3%	-
	Independent variables		
	Continuous explanatory variables		
Children 0-5	Number of kids aged 0-5	0.316 (0.637)	-
Children 6-14	Number of kids aged 6-14	0.569 (0.917)	-
Children 15-18	Number of kids aged 15-18	0.215 (0.481)	-
Adult 19 +	Number of adults aged over 18 years	2.476 (1.085)	-
Total expenditure	Household total expenditure (1000 TL/month)	2.782 (2.047)	-
Household size	Household size	3.576 (1.900)	1.758
Education	Household head education in years	7.392 (4.801)	1.913
House feature index	Index created by house characteristics	7.270 (2.236)	2.499
Number of autos	Number of automobiles owned	0.449 (0.497)	1.272
Number of properties	Number of properties owned	1.025 (1.156)	-
	Binary explanatory variables: Household head and household characteristics		
Male	Gender is male	0.862 (0.345)	2.223
Age < 30	Age < 30 (Reference)	0.076 (0.265)	-
Age 30-50	30 < Age ≤ 50	0.457 (0.498)	4.213
Age > 50	Age > 50	0.467 (0.499)	5.399
No diploma	No diploma (Reference)	0.128 (0.330)	-
Primary school	Primary school education	0.445 (0.497)	-
Secondary school	Secondary school education	0.122 (0.328)	-
High school	High school education	0.163 (0.370)	-
College school	College school education	0.142 (0.349)	-
Compulsory insurance	Has compulsory health insurance	0.840 (0.367)	1.517
Married	Married	0.835 (0.371)	2.298
Employed	Employed	0.666 (0.472)	1.926
Manager	Manager	0.047 (0.211)	1.110
Retired	Retired	0.320 (0.467)	2.072
Entrepreneurial income	Families with entrepreneurial income	0.339 (0.473)	1.432
State cash aids	Receives cash income from government	0.304 (0.460)	1.484
State in-kind aids	Receives in-kind type help income from state	0.105 (0.307)	1.306
Private cash aids	Receives cash income from private	0.131 (0.337)	1.185
Private in-kind aids	Receives in-kind type income from private	0.096 (0.295)	1.170
Foreign income	Has abroad retired, scholarship or in-kind aid	0.024 (0.152)	1.033
Apartment	Resides in an apartment	0.493 (0.500)	2.096
Renter	Resides in rental house	0.230 (0.421)	2.187
Homeowner	Resides in own house	0.632 (0.482)	2.248
Combi	Resides in a house warmed up with a combi	0.321 (0.467)	2.435
Stove	Resides in a house warmed up with a stove	0.547 (0.498)	3.736
Income 1	Monthly income < 2000 TL (Reference)	0.374 (0.484)	-
Income 2	Monthly income 2000 – 5000 TL	0.494 (0.500)	1.725
Income 3	Monthly income > 5000 TL	0.132 (0.338)	2.019
Internet	Has home internet access	0.350 (0.477)	1.442
One child	Family with only one children	0.195 (0.396)	1.358
Two child	Family with only two children	0.197 (0.398)	1.529
Three and more child	Family with three and more children	0.141 (0.348)	1.611
Sample size		11290	

Standard deviations are in parentheses

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Results and discussion

Specification tests and maximum-likelihood estimates

Before discussing the marginal impacts of the preferred model, some specifications applied to the data and models were taken into consideration. First, in each model under consideration, the two-part models were rejected using Wald statistic (W = $\frac{\rho^2}{Var(\rho)}$, df = 1, where Var (ρ) is the estimated variance of the correlation coefficient, ρ, between the decision to participate at food-away from home and its expenditure level). Therefore, the error terms generating the relationship between the decision to spend and the spending levels on FAFH are statistically interrelated, affecting one to other equations. In addition, Vuong non-nested tests discriminating competed models were used (see *Table 2*). In all pair comparison, the error-dependent log-Heckman sample selection (LHSS) model outperforms over its competitors (e.g., the double-hurdle and conventional error-dependent Heckman models). Most likely, the logarithmic nature of the model correcting outliers and minimizing the persistent heteroscedasticity in the data gives an advantage to the LHSS model to outperform the other two competing models (e.g., HSS and DH). In this case, all correlation coefficients are statistically significant, indicating after controlling exogenous variables in models, uncontrollable factors that affect the decision to spend also significantly affect the spending level on FAFH. The correlation coefficient in the LHSS model is negative, indicating that the uncontrollable factors that boost the likelihood to spend also reduce expenditure level or vice-versa. Also sings of most of the estimated parameters echoed with the economic theory. Since these parameters do not indicate the direct marginal effects of regressors on both the probability and the spending level, the marginal impacts derived from *Equations 6–9* were discussed in subsequent section.

Table 2. *Some specification tests comparing independence and used models*

Specification tests	Test-statistic
Independence	
Heckman SS	Wald 491.3661, df = 1, p < 0.0001
Log-Heckman SS	Wald 1009.967, df = 1, p < 0.0001
Double- hurdle	Wald 196.0345, df = 1, p < 0.0001
Vuong's non-nested test	
Double-hurdle versus Heckman SS	z = 54.3073, p < 0.0001
Double-hurdle versus Log-Heckman SS	z = -14.0318, p < 0.0001
Heckman SS versus Log-Heckman SS	z = -30.2837, p < 0.0001

Note: The null hypothesis under Vuong's test for non-nested models is that the expected value of competing log-likelihood ratios equals zero, indicating the competing pair models are equally away from the data being modelled. Under this test, if z > 1.96 the first listed model is preferred, while if z < -1.96 the second listed model is chosen. However, if |z| < 1.96 then no decision is made among the competing pairs

Marginal effects

Marginal effects of explanatory variables on the probabilities, conditional level, and unconditional level of FAFH households are presented at *Table 3*. According to these results, it was observed that various socio-demographic and economic factors of

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

households and heads of households had significant effects on FAFH expenditures. The subsequent discussion will continue only on statistically significant variables for the preferred LHSS model.

Table 3. *The error-dependent Log-Heckman sample selection (LHSS) model both with maximum likelihood (MLE) and marginal impact estimates*

Variables	MLE estimates				Marginal effects					
	Probability		Level		Probability		Conditional		Unconditional	
	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value
Constant	-0.164	-1.337	4.837***	34.399						
Male	0.280***	5.099	-0.092	-1.392	8.321***	4.750	25.179***	3.002	34.390***	4.792
Age 30-50	-0.019	-0.279	-0.163**	-2.373	-0.512	-0.278	-33.116***	-2.623	-27.676**	-2.324
Age > 50	-0.309***	-4.303	-0.060	-0.798	-8.495***	-4.275	-27.042***	-4.255	-61.628***	-4.919
Married	-0.199***	-3.723	-0.249***	-4.036	-5.124***	-3.967	-87.301***	-7.193	-83.125***	-7.658
Employed	0.218***	5.769	0.345***	7.728	6.164***	5.649	90.769***	13.853	83.169***	14.237
Manager	-0.121	-1.643	0.018	0.223	-3.476	-1.574	-14.686	-1.091	-17912	-1.456
Compulsory insurance	0.131***	3.271	0.203***	4.207	3.712***	3.160	-52.520***	-8.458	48.052***	8.864
Retired	0.090**	2.262	-0.346***	-7.359	2.437**	2.290	-49.084***	-6.922	-35.426***	-5.575
Entrepreneurial income	-0.132***	-3.809	-0.473***	-11.867	-3.680***	-3.769	-100.33***	-16.24	-86.892***	-15.64
State cash aids	-0.120***	-3.670	-0.258***	-6.586	-3.358***	-3.604	-62.514***	-11.27	-55.990***	-11.44
State in-kind aids	-0.029	-0.671	-0.003	-0.052	-0.789	-0.665	-4.781	-0.609	-5.309	-0.784
Private cash aids	0.064	1.479	-0166***	3.342	1.724	1.511	-20.759***	-2.858	-13.780**	-2.102
Private in-kind aids	0.012	0.249	-0.024	-0.431	0.322	0.250	-2.700	-0.315	-15.582	-0.207
Foreign income	0.042	0.523	-0.166*	-1.709	1.121	0.532	-23.497*	-1.762	-17.111	-1.456
Apartment	-0.064	-1.553	-0.064	-1.425	-1.762	1.553	-21.647***	-2.840	-20.775***	-2.948
Renter	0.109**	2.089	0.053	0.943	2.926**	2.144	27.248***	2.602	27.914***	2.860
Homeowner	-0.110**	-2.338	0.014	0.280	-2.969**	-2.370	-13.912	-1.608	-16.905**	-2.096
Combi	-0.033	-0.646	0.038	0.689	-0.898	-0.643	2.210	0.225	0.091	0.010
Stove	0.048	0.888	-0.045	-0.752	1.322	0.887	-1.231	0.124	1.488	0.163
Internet	0.107***	2.787	0.094**	2.328	2.897***	2.839	34.529***	4.345	33.612***	4.478
One child	0.222***	5.279	0.093**	2.017	5.719***	5.657	54.084***	5.791	56.268***	6.452
Two child	0.167***	3.785	0.018	0.371	4.370***	3.968	29.211***	3.271	32.543***	3.928
Three and more child	0.057	1.207	-0.016	-0.302	1.538	1.229	5.511	0.600	7.396	0.886
House feature index	-0.012	-1.254	0.003	0.283	-0.331	1.254	-1.227	-0.649	-1.611	-0.939
Number of autos	0.041	1.331	-0.085**	-2.401	1.116	1.333	-9.708*	-1.709	-5.751	-1.122
Primary school	0.198***	5.719			5.360***	5.743	29.807***	5.632	34.272***	5.653
Secondary school	0.206***	4.190			5.249***	4.499	31.791***	4.052	36.759***	4.045
High school	0.306***	5.391			7.625***	5.969	47.668***	5.144	55.183***	5.143
College school	0.209***	3.027			5.357***	3.262	32.333***	2.933	37.377***	2.925
Number of properties	-0.041***	-3.217			-1.112***	-3.215	-6.100***	-3.202	-7.009***	-3.206
Children 0-5	-0.105***	-5.135			-2.882***	-5.119	-15.813***	-5.107	-18.170***	-5.117
Children 6-14	-0.077***	-5.132			-2.094***	-5.133	-11.492***	-5.097	-13.205***	-5.103
Children 15-18	0.029	1.200			0.797	1.199	4.374	1.199	5.026	1.200
Adult 19 +	0.088***	7.304			2.400***	7.243	13.169***	7.228	15.132***	7.257
Total expenditures	0.221***	27.238			6.038***	26.295	33.137***	20.347	38.076***	21.590
Education			-0.025***	-5.404			-4.657***	-5.323	-3.758***	-5.321
Household size			0.103***	11.157			19.391***	10.821	15.650***	10.762
Income 2			0.477***	14.476			90.648***	13.336	73.161***	13.238
Income 3			0.062***	14.056			233.042***	9.705	188.085***	9.655
σ			1.532***	105.393						
ρ			-0.982***	-96.855						
Log-likelihood value		-60266.65								

Statistical significance *** at the 1% level; ** at the 5% level; * at the 10% level

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

Because of culinary skills, households under the supervision of the male are reported to have higher FAFH expenditures (Byrne et al., 1996). Our results confirmed that hypothesis. Households with a male headed were 8.32% more likely to consume FAFH and spend 25.18 TL more per month than households with a female headed Bozoglu et al. (2013) found that male household heads tended to 13.84% more likely to consume FAFH and spend 51.05 TL more per month than their female peers. Previous findings is consistent with our findings (Byrne et al., 1996; Ham et al., 2004; Binkley, 2006; Angulo et al., 2007; Keelan et al., 2009; Fanning et al., 2010; Liu et al., 2013; Blick et al., 2017).

Heng and Guan (2007) reported that the young differed from the old as regards tastes, food preferences, lifestyle, eating habits and opportunities to socialize. In the study, there was a negative relationship between the age of the head of household and the monthly average expenditure on FAFH. Household with households heads whose ages between 30-50 and over 50 years tended 0.51% and 8.50% points less likely to consume FAFH and they spent 33.12 TL and 27.04 TL less than households whose heads aged below 30 years. These negative effects of ages were also reported in previous studies conducted in Turkey (Akbay et al., 2007; Gül et al., 2007), in South Africa (Blick et al., 2017), in China (Min et al., 2004; Ma et al., 2006), in USA (Stewart and Yen, 2004; Binkley, 2006), in Ireland (Keelan et al., 2009), in Slovakia (Cupak et al., 2016), in Egypt (Fabiosa, 2008), in Brazil (Rezende and Avelar, 2012), and in Spain (Mutlu and Gracia, 2006; Angulo et al., 2007).

Households with a married head were 5.12% points less likely to consume FAFH and spent 87.30 TL less per month than single headed households. The results indicated that married households were less likely to consume FAFH and spent less than single headed households. Bozoglu et al. (2013) found that married household heads tended to 4.14% points less likely to consume FAFH and spent 18.60 TL less per month than that of unmarried peers. Our results were consistent with the earlier findings (Ham et al., 2004; Keelan et al., 2009; Drescher and Roosen, 2013; Piekut, 2016; Traş and Şengül, 2017).

Compulsory health insurance played a positive role in the probability of consume FAFH, but negatively affected the expenditure levels. Households whose heads had a compulsory health insurance were 3.71% points more likely to consume FAFH but spent 52.52 TL less per month than those without insurance. Employment played a role on FAFH, households with an employed head were 6.16% points more likely to consume FAFH and spent 90.77 TL more per month than their unemployed counterparts. The findings showed that households with employed head in Turkey had significantly higher probability of entering the FAFH market and they spent more on FAFH. Bozoglu et al. (2013) and Traş and Şengül (2017) reported a positive employment effect on FAFH in Turkey. These positive effects of employment were also reported for USA (Jensen and Yen, 1996; Stewart and Yen, 2004), for Spain (Angulo et al., 2007), for Mexico (Langellier, 2015) and for Slovakia (Cupak et al., 2016).

Households with a retired head were 2.43% points more likely to consume FAFH but spent 49.08 TL less per month than their not retired counterparts. Retired played a positive role in the probability of consume on FAFH, but negatively affected expenditure levels. These results were in line with the expectations because retired household head, on average might be older than those not retired and similar health and/or financial reasons in the retired group might result in less consumption of FAFH. Piekut (2016) found that in Poland, households with a retired head were less likely to

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

consume FAFH and spent 15.96 PLN per month than those not retired households head. Negative effects of retired household head on FAFH were also reported in earlier studies (Jang et al., 2007; Drescher and Roosen, 2013).

In the study, the households receiving financial aid from the state resources had lower probability of consumption and related expenditure levels by 3.39% points and 62.51 TL, respectively, in comparison to those who were not receiving financial aid from public resources. This finding was in line with findings of Binkley (2006). The finding was in accordance of expectations. The major reason could be income levels and high quality life due to financial support provided by the government. Therefore, it is expected that the expenditures on such households on FAHF would be lower.

Households residing in rental house were 2.93% more likely to consume FAFH and spent 27.25 TL more per month than those residing state apartments. This result was consistent with Bozoglu et al. (2013), who reported a positive relationship between households residing in rental house and FAFH expenditures. Home ownerships had a higher income compared to no home ownership due to high income and low cash flow effects, which is contradictory, the effects of homeowner status on FAFH is reported to be unclear (Yen, 1993). This means that homeowner would show a less probability of FAFH and less expenditure on FAFH in comparison of hypothesized a lack of homeowner. Homeowners were 2.97% points less likely to consume FAFH and spent 16.91 TL less per month than those residing state apartments, similar to findings by Ham et al. (2004), but contradicts to those by Jensen and Yen (1996), Mutlu and Gracia (2006), Jang et al. (2007) and Keelan et al. (2009).

Having an internet connection at home increased the probability of consuming FAFH (2.90%) and it also had a significant and positive effect on FAFH expenditures for households (34.53 TL). This result was consistent with Bozoglu et al. (2013) who reported a positive relationship between having an Internet connection at home and FAFH expenditures.

Households with only one and two children were 5.72% and 4.37% points more likely to consume FAFH and spend 54.08 TL and 29.21 TL more per month than households without children, respectively. These results agreed with the expectations. Piekut (2016) found that in Poland, households with a children are more likely to consume FAFH and spent 16.49 PLN per month than childless households. Similar findings were also reported by Stewart and Yen (2004) and Bozoglu et al. (2013).

Education level increases the probability of the FAFH decision and also increases expenditures on FAFH. Households with a primary, secondary, high and college school head were 5.36%, 5.25%, 7.63% and 5.36% points more likely to consume FAFH, and spent 29.81 TL, 31.79 TL, 47.67 TL and 32.33 TL more per month than their illiterate counterparts, respectively. The findings were consistent with the previous findings (Jensen and Yen, 1996; Mihalopoulos and Demoussis, 2001; Stewart and Yen, 2004; Angulo et al., 2007; Zan and Fan, 2010; Langellier, 2015; Cupak et al., 2016; Piekut, 2016; Traş and Şengül, 2017).

Households who had pre-school children is reported to have spent less FAFH expenditures compared to other families mainly because of difficulties feeding children in public places (Heng and Guan, 2007). Our results confirmed this expectation. In the study, households with a young children aged 0–5 and aged 6-14 were 2.88% and 2.09% less likely to consume FAFH and they spent 15.81 TL and 11.49 TL less than their counterparts without such young children, respectively. Presence of children (aged 0–5 and aged 6–14) decreased FAFH expenditure of households. Negative effects of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

pre-school (aged 0-5) and school children (aged 6-14) on FAFH were also reported in the earlier studies (Mihalopoulos and Demoussis, 2001; Keelan et al., 2009; Chang and Yen, 2010; Drescher and Roosen, 2013; Leschewski et al., 2018; Rezende and Avelar, 2012).

As the total expenditure of households increased, the probability of consuming FAFH increased by 6.04% points and spending increased by 33.14 TL per month. These positive effects of total expenditure might be related to the increased income because household's total expenditures are generally viewed as proxy for income. The positive effects of total expenditure of households on FAFH expenditures were consistent with the earlier studies (Mihalopoulos and Demoussis, 2001; Fabiosa, 2008; Traş and Şengül, 2017).

Level of FAFH expenditure and the probability of consumption in the FAFH market were affected positively from household size. Each additional household member increased the probability of consuming FAFH by 0.10% point, the conditional expenditure by 19.39 TL per month. While these positive effects of household size on FAFH was in consistent with the findings of some earlier studies (Mihalopoulos and Demoussis, 2001; Ham et al., 2004; Chang and Yen, 2010; Cupak et al., 2016), it contradicted with some other findings of Stewart and Yen (2004), Heng and Guan (2007), Akbay et al. (2007), Drescher and Roosen (2013) and Mottaleb et al. (2017).

Households with an average monthly income between 2000 and 5000 TL and over 5000 TL spent 90.65 TL on FAFH and 233.04 TL more than households with an average monthly income below 2000 TL. This was consistent with the applied theoretical framework and other empirical studies investigating the link between income and FAFH spending (Byrne et al., 1996; Min et al., 2004; Ma et al., 2006; Zan and Fan, 2010; Liu et al., 2013; Cupak et al., 20016; Piekut, 2016; Blick et al., 2017; Traş and Şengül, 2017). The results were as it is expected. The major reasons could be households with relatively high income would tend to have more expenditure on products and services including dinning (Bozoglu et al., 2013).

Conclusions

In the study, the characteristics of households and the heads of households had a significant effect on determining the probability of consuming FAFH and the related expenditure. The findings were supported by analyses. Results showed that 77.3% of Turkish households participated in the FAFH market.

The results also showed that both the probability and monthly spending levels of household FAFH increased with male household heads, decreasing in age of household heads, educational levels of household heads, working household heads, household size, household income, reside in rental house, use of internet and the number of adults in a family, whilst the female headed households, married household heads, the households receiving cash income aids from the government and private sector, homeowner, the number of properties in family and families with children 0-5 and 6-14 years old, decreased both the likelihood and spending levels of food away from home in Turkey.

These findings are very important especially for the companies and enterprises operating in the non-home food industry. Because knowing the factors that increase and decrease the food consumption expenditures of households FAFH will provide important clues to the companies and enterprises operating in this sector for what kind of services they will develop for households. For example, children's play areas for

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households with children, car parking for car-owners, low-income households and large households multiple alternative options will have a positive impact on household consumption expenditures. Increasing demand for FAFH will probably ensure further growth in industry. In these days, industry provides employment for many people and creates demand for other food chains from farmers to retailers.

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APPENDIX

Table A1. *Heckman sample selection model both with maximum likelihood (MLE) and marginal impact estimates*

Variables	MLE estimates				Marginal effects					
	Probability		Level		Probability		Conditional		Unconditional	
	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value
Constant	-0.221*	-1.661	215.201***	9.450						
Male	0.346***	5.476	-2.965	-0.267	9.790***	4.980	15.371	1.479	32.973***	3.737
Age 30-50	-0.067	-0.954	-27.485***	-2.905	-1.696	-0.952	-30.720***	-3.304	-29.141***	-3.218
Age > 50	-0.392***	-5.164	-11.463	-1.037	-10.049***	-5.104	-30.606***	-2.838	-46.905***	-4.607
Married	-0.199***	-3.295	-26.184***	-2.592	-4.733***	-3.532	-35.336***	-3.669	-40.740***	-4.648
Employed	0.341***	8.230	55.888***	7.308	9.096***	7.912	73.090***	10.117	78.014***	12.713
Manager	-0.183**	-2.387	9.372	0.987	-5.00**	-2.229	-0.043	-0.005	-10.944	-1.134
Compulsory insurance	0.202***	4.529	29.880***	3.291	5.443***	4.273	40.150***	4.678	43.671***	6.180
Retired	0.051	1.159	-54.394***	-7.033	1.277	1.168	-51.953***	-6.907	-40.556***	-6.014
Entrepreneurial income	-0.261***	-6.884	-86.305***	-14.198	-6.875***	-6.697	-99.334***	-17.242	-95.024***	-18.881
State cash aids	-0.144***	-3.954	-39.807***	-5.610	-3.733***	-3.857	-46.896***	-6.923	-46.343***	-8.015
State in-kind aids	-0.050	-1.071	7.799	0.765	-1.301	-1.051	5.324	0.550	1.523	0.187
Private cash aids	0.060	1.248	-24.532***	-2.776	1.497	1.276	-21.662**	-2.534	-14.945**	-1.971
Private in-kind aids	0.007	0.135	-5.876	-0.569	0.181	0.135	-5.531	-.0559	-4.203	-0.490
Foreign income	0.010	0.118	-17.252	-1.072	0.260	0.119	-16.755	-1.089	-13.377	-0.999
Apartment	-0.111**	-2.463	-13.794**	-2.052	-2.803**	-2.462	-19.142***	-2.969	-21.982***	-3.666
Renter	0.095*	1.680	19.611**	2.231	2.350*	1.721	24.118***	2.839	25.439***	3.210
Homeowner	-0.137***	-2.657	4.682	0.577	-3.400***	-2.708	-1.829***	-0.234	-8.948	-1.244
Combi	0.012	0.233	2.819	0.390	0.313	0.234	3.416	0.486	3.521	0.516
Stove	0.071	1.213	-0.845	-0.101	1.794	1.209	2.577	0.324	6.047	0.815
Internet	0.219***	5.509	20.924***	3.560	5.366***	5.718	31.230***	5.589	38.072***	7.218
One child	0.254***	5.615	8.590	1.203	5.954***	6.116	20.127***	2.914	30.386***	4.713
Two child	0.175***	3.712	-4.280	-0.595	4.211***	3.917	3.840	0.557	12.466*	1.956
Three and more child	0.049	0.954	-16.169*	-1.921	1.225	0.970	-13.823*	-1.720	-8.919	-1.237
House feature index	-0.018*	-1.697	0.118	0.0733	-0.446*	-1.693	-0.733	-0.466	-1.581	-1.031

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Number of autos	0.019	0.555	-15.215***	-2.793	0.469	0.555	-14.320***	-2.745	-10.866**	-2.302
Primary school	0.185***	4.115			4.638***	4.135	8.868***	4.020	17.506***	4.112
Secondary school	0.131**	2.178			3.152**	2.285	6.074**	2.240	12.059**	2.246
High school	0.289***	4.534			6.643***	5.025	12.925***	4.753	25.764***	4.855
College school	0.228***	3.112			5.329***	3.397	10.336***	3.280	20.583***	3.300
Number of properties	-0.046***	-3.181			-1.155***	-3.176	-2.205***	-3.154	-4.350***	3.009
Children 0-5	-0.110***	-4504			-2.790***	-4.492	-5.324***	-4.415	-10.505***	-4.399
Children 6-14	-0.088***	-5.022			-2.235***	-5.019	-4.266***	-4.948	-8.417***	-4.852
Children 15-18	0.013	0.443			0.332	0.443	0.633	0.442	1.249	0.436
Adult 19 +	0.081***	6.102			2.059**	6.057	3.929***	5.685	7.752***	5.839
Total expenditures	0.273***	34.493			6.908***	30.998	13.183***	17.034	26.009***	23.503
Education			-4.686***	-6.835			-4.686***	-4.903	-3.888***	3.762
Household size			14.972***	9.799			14.972***	9.577	12.423***	9.391
Income 2			74.116***	10.226			74.116***	10.226	61.497***	10.210
Income 3			202.437***	20.766			202.420***	20.765	167.956***	20.614
σ			228.437***	104.540						
ρ			-0.551***	-22.167						
Log-likelihood value		-64110.05								

Statistical significance *** at the 1% level; ** at the 5% level; * at the 10% level

Table A2. *Double hurdle model both with maximum likelihood estimates (MLE) and marginal impact estimates*

Variables	MLE estimates				Marginal effects					
	Probability		Level		Probability		Conditional		Unconditional	
	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value	Parameter	t-value
Constant	-0.374	-0.782	48.099**	2.027						
Male	0.566***	3.950	28.305**	2.355	3.884**	2.303	14.693**	2.437	20.699**	2.422
Age 30-50	-0.130	-0.562	-29.081***	-2.795	-3.891***	2.787	-15.315***	-2.808	-21.539***	-2.806
Age > 50	-0.549**	-2.163	-42.829***	-3.599	-5.734***	-3.590	-22.581***	-3.624	-31.736***	-3.622
Married	-0.216	-1.487	-40.553***	-3.643	-5.205***	-3.788	-22.064***	-3.543	-30.819***	-3.568
Employed	0.011	0.088	84.973***	10.857	11.712***	10.470	43.494***	11.211	61.352***	11.210
Manager	0.327	0.317	-5.029	-0.484	-0.674	-0.480	-2.630	-0.484	-3.706	-0.484
Compulsory insurance	-0.029	-0.218	55.831***	5.811	7.800***	5.562	28.250***	6.078	39.962***	6.052
Retired	-0.241*	-1.741	-34.611***	-4.268	-4.692***	-4.199	-18.037***	-4.340	-25.410***	-4.329
Entrepreneurial income	0.217	1.618	-108.756***	-17.095	-15.077***	-16.361	-55.261***	-17.603	-77.935***	-17.678
State cash aids	0.211*	1.811	-58.331***	-7.814	-8.000***	-7.570	-30.014***	-8.026	-42.352***	-8.010
State in-kind aids	0.129	0.935	-6.801	-0.637	-0.913	-0.632	-3.562	-0.640	-5.017	-0.639
Private cash aids	-0.114	-0.842	-6.223	-0.651	-0.836	-0.648	-3.276	-0.657	-4.608	-0.656
Private in-kind aids	0.311**	2.059	-9.562	-0.856	-1.287	-0.847	-4.992	-0.862	-7.036	-0.861
Foreign income	11.503***	3.169	-40.803**	-2.434	-5.709**	-2.332	-20.606**	-2.542	-29.174**	-2.530
Apartment	0.098	0.723	-22.648***	-3.119	-3.021***	-3.114	-11.944***	-3.123	-16.797***	-3.123
Renter	0.349**	2.048	19.427**	2.004	2.555**	2.033	10.389**	1.986	14.568**	1.990
Homeowner	0.254*	1.689	-21.971**	-2.471	-2.905**	-2.492	-11.654**	-2.453	-16.372**	-2.457
Combi	-0.576***	-2.852	16.769**	2.145	2.212**	2.162	8.850***	2.122	12.450**	2.128
Stove	-0.302	-1.411	18.962**	2.078	2.53^{3**}	2.074	9.971**	2.080	14.032**	2.080
Internet	-0.444***	-3.067	50.534***	8.105	6.590***	8.230	27.091***	7.973	37.953***	8.015
One child	-0.133	-0.939	36.789***	4.773	4.753***	4.924	19.895***	4.664	27.829***	4.692
Two child	-0.179	-1.056	20.551***	2.667	2.693***	2.715	10.984***	2.632	15.407***	2.640

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DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

Three and more child	0.115	0.597	-6.870	-0.775	-0.922	-0.770	-3.600	-0.779	-5.070	-0.778
House feature index	-0.105***	-3.346	1.226	0.695	0.163	0.340	0.641	0.590	0.904	0.690
Number of autos	-0.022	-0.192	-7.504	-1.285	-1.002	-1.285	-3.959	1.287	-5.567	1.286
Primary school	0.253**	2.436			0.001	0.925	0.015	0.994	0.015	0.974
Secondary school	0.373**	2.085			0.001	0.953	0.014	1.025	0.014	1.004
High school	0.225	1.035			0.001	0.796	0.010	0.836	0.010	0.825
College school	-0.012	-0.045			0.000	0.044	-0.001	-0.044	-0.001	-0.044
Number of properties	-0.146***	-2.650			-0.001	-0.044	-0.009	-0.009	-0.009	-0.012
Children 0-5	-0.079	-0.799			0.000	0.003	-0.005	-0.006	-0.005	-0.006
Children 6-14	-0.197***	-3.198			-0.001	-0.008	-0.012	-0.020	-0.012	-0.016
Children 15-18	0.097	0.768			0.001	0.004	0.006	0.018	-0.006	-0.007
Adult 19 +	0.175***	2.805			0.001	0.008	0.010	0.008	0.010	0.009
Total expenditures	1.852***	13.728			0.011	0.058	0.110	0.166	0.110	0.120
Education			-3.598***	-4.853	-0.480*	-1.672	-1.899	-1.579	-2.761***	-2.896
Household size			9.178***	5.476	1.224***	2.994	4.845***	4.723	6.812***	4.704
Income 2			102.321***	14.305	13.567***	13.897	54.127***	14.480	75.862***	14.549
Income 3			247.599***	24.818	24.101***	31.178	157.816***	21.519	207.372***	23.213
σ			242.332***	165.359						
ρ			-0.868***	-37.169						
Log-likelihood value	-61885.49									

Statistical significance *** at the 1% level; ** at the 5% level; * at the 10% level

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34413455

THE EFFECT OF GROWTH BIOSTIMULATORS AND HERBICIDE ON THE CONTENT OF SUGARS IN TUBERS OF EDIBLE POTATO (*SOLANUM TUBEROSUM* L.)

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(Received 8th Jan 2019; accepted 14th Feb 2019)

Abstract. Currently, new solutions are searched for in crop production that will provide plants with favourable conditions for growth and development, ensuring high consumer quality of harvested products further to being friendly for the environment. One of the most important features negatively affecting the quality of consumer quality of edible potato tubers is the increased content of total sugars and especially reducing sugars. Therefore, the aim of the experiment was to determine the impact of five methods of care using the growth biostimulators and herbicide on the content of total sugars and reducing sugars in tubers of three edible potato varieties. A series of three-year field experiments was carried out in the years 2015-2017 in the region of Eastern Poland (52°02'N; 23°07'E). The experiments were assumed with random sub-blocks method in three repetitions, on light acidic soil. The examined factors were: I factor – three early edible potato varieties (Owacja, Bellarosa, Vineta), II factor – five methods of care using growth biostimulators: GreenOk – Universal Pro and Asahi SL and their combination with Avatar 293 ZC herbicide. As a result of the studies, it was concluded that genetic features of the cultivated varieties, as well as methods of care applied in the experiment did not have a significant impact on the content of total sugars and reducing sugars in tubers of potato. The value of these characteristics substantially depended only on the course of thermal-precipitation conditions in the given years of research. Significantly higher content of total sugars (an average 0.74%) was shown by the tubers harvested in a warm and dry growing season of the year 2015, whilst the highest content of reducing sugars in tubers was recorded in a cool, wet growing season of 2017 (an average of 0.39%).
Keywords: *Solanum tuberosum L., anti-nutritional components, GreenOk-Uniwersal Pro, Asahi SL, Avatar 293 ZC*

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important cultivated plants not only in Poland (potato consumption in Poland amounts to approximately 100 kg/person/year) but it also constitutes the basis of diet of a growing number of people worldwide – it is grown in about 160 countries. Current consumption of fresh potatoes has a decreasing trend, however the processed products have become more and more popular; especially fried potatoes (French fries, crisps), dried, pasteurized, blanched, as well as sliced and subjected to an initial heat treatment (Camire et al., 2009; Ezekiel et al., 2013; Grużewska et al., 2016).

The nutritional value of potato results mainly from the chemical composition of the tubers. The components that are of large importance in human nutrition include: starch, complete protein, fibre, vitamins and minerals. Contrary to the prevailing opinion, Potato tubers are low-calorie and easily digestible. The caloric value of the tubers is approximately 50-90 kcal/100 g and is comparable to the energy value of apples (54

DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

kcal) or milk (62 kcal) and is 10 times less than the caloric value of chocolate (563 kcal) (Navarre et al., 2009; Zarzecka et al., 2013; Zgórska, 2013; Wegner et al., 2015).

In the edible potato production, particular attention should be paid to the contents of sugars: total (glucose + fructose + sucrose) and reducing sugars (glucose + fructose), which are important features indicating the usefulness of tubers intended for direct human consumption, frying, or drying. In the weight of fresh tubers intended for direct consumption and processing, the total sum of sugars should not exceed 1% and reducing sugars 0.5%. Whist within the potatoes used for the production of French fries and crisps the content of reducing sugars should not be greater than respectively 0.25 and 0.15%. Increased content of total sugars (>1%) gives the tubers sweet taste and causes them to darken faster, which results in deterioration of the culinary quality of tubers (Cropp et al., 2000; Finaly et al., 2003; Zgórska and Sowa-Niedziałkowska, 2005; Storey, 2007; Grudzińska et al., 2014).

In potato tubers, the increased content of reducing sugars is especially undesirable and dangerous to human health (Hebeisen et al., 2005). Their negative impact is revealed during the preparation of potato products, fried foods (French fries, crisps), as well as baked or roasted ones (at temperature >120 °C). Reducing sugars with amino acids are involved in *Maillard's reactions* (this is a series of chemical reactions, which occur frequently under the influence of heat between amino acids and reducing sugars). They adversely affect the colour, taste and smell of food products, as well as give rise to acrylamide (C_3H_5NO) very harmful for our health – a compound with neurotoxic and carcinogenic action. According to Mojska et al. (2008) and Żyżlewicz et al. (2010) in French fries and crisps there may be significant content of acrylamide (from 50 to 2400-2600 µg/kg), whilst French fries prepared at home tend to have a higher content of acrylamide than those prepared in industrial conditions.

The use of raw material with low (0.1-0.2%) concentration of reducing sugars is considered the primary factor limiting the formation of acrylamide toxicity (Amrein et al., 2004; Biedermann-Brem et al., 2003; De Wilde et al., 2006; Wójcik-Stopczyńska et al., 2012).

The content of total sugars and reducing sugars in tubers of potatoes depends on numerous factors, such as a genetic factor of the cultivated varieties, climatic conditions, as well as the applied agrotechnical treatments (Grudzińska and Zgórska, 2008, 2010; Grudzińska et al., 2014; Zarzecka et al., 2017).

In plant production, including also in potato agro-technology, all sorts of preparations friendly to agro-eco-systems have been increasingly applied, which are referred to as biostimulators. They restrict the use of agrochemicals and thus contribute to the development of sustainable crop production methods (Du Jardin, 2012; Calvo et al., 2014; Baranowska et al., 2017). Biostimulators are synthetic or natural preparations which enhance stimulating of the life processes of plants and increase the resistance of plants to stress conditions (Ziosi et al., 2013; Matyjaszczyk, 2015). Their chemical composition includes a whole range of bioactive compounds such as humus substances, algae, microorganisms, mineral elements, vitamins, amino acids, nitrophenols, cytokinins, auxins and other substances classified as plant hormones (Bulgari et al., 2015).

However, the possibility of using biostimulators in farming practice is not yet fully discovered, and in Poland so far there have been no scientific studies on the impact of GreenOk-Uniwersal Pro on more important qualitative features of potato tubers. Therefore, the aim of the experiment was to determine the impact of the five methods of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

care using growth biostimulators and herbicide on the content of total sugars and reducing sugars in tubers of three edible potato varieties.

Materials and methods

Experiment and plant material

The three-year field experiment was executed in the years of 2015-2017 in the region of Eastern Poland, in Biała Podlaska municipality (52°02'N; 23°07'E), in the Lublin Voivodeship on light, acidic soil (*Fig. 1*). The soil was characterized by a very high content of assimilable forms of phosphorus and potassium and high to very high form of magnesium.

Figure 1. *One of the experiment objects*

The experiment took place in the split-plot system in three repetitions. The impact of the two factors was studied (*Table 1*).

Table 1. *Factors of the experiment*

I. Factor – Three early edible potato cultivars: Bellarosa, Owacja, Vineta	
II. Factor – Five methods of treatment with the application of growth biostimulators and herbicide:	
1.	Standard object – mechanical treatment (without biostimulators and herbicide).
2.	From sprouting of potato plants – mechanical treatment and after sprouting – GreenOK Universal–PRO bioactivator, three times to leaves: at a dose of 0.10 dm^3 ha^{-1} (phase BBCH 13-19) + 0.15 dm^3 ha^{-1} (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} (phase BBCH 51-55).
3.	From sprouting of potato plants – mechanical treatment, and after sprouting – Asahi SL bioactivator, three times to leaves at a dose of 0.50 dm^3 ha^{-1} (phase BBCH 13-19) + 0.50 dm^3 ha^{-1} (phase BBCH 31-35) + 0.50 dm^3 ha^{-1} (phase BBCH 51-55).
4.	From sprouting – mechanical treatment, and after the final shaping of ridges and just before sprouting Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05). After sprouting – three applications of GreenOK Universal–PRO bioactivator at a dose of 0.10 dm^3 ha^{-1} (phase BBCH 13-19) + 0.15 dm^3 ha^{-1} (phase BBCH 31-35) + 0.15 dm^3 ha^{-1} (phase BBCH 51-55).
5.	From sprouting – mechanical treatment, and after the final shaping of ridges before sprouting of potato plants – Avatar 293 ZC herbicide at a dose of 1.5 dm^3 ha^{-1} (phase BBCH 00-05).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

The characteristics of potato varieties and preparations used in the experiment have been presented in *Tables 2* and *3*.

***Table 2.** Characteristics of varieties of potatoes grown in the experiment. (Source: Plant Breeding and Acclimatization Institute – National Research Institute, Poland 2015)*

Variety	Year of inscription to the variety register	Breeder of variety	Usefulness of varieties for food processing
Bellarosa	2006	Europlant, Pflz. GmbH, Germany	Cooked products, French fries
Owacja	2006	PMHZ Strzekęcino, Poland	Puree, pancakes, cooked products
Vineta	1999	Europlant, Pflz. GmbH, Germany	Canned goods, frozen goods, salads, cooked products

***Table 3.** Characteristics of preparations used in the experiment. (Source: Information disclosed by agents' manufacturers)*

Name of preparation	Content of active substances
Avatar 293 ZC herbicide	Clomazone 60 g dm^3 (5.13%), metribuzin 233 g dm^3 (20.64%)
GreenOK Universal–PRO bioactivator	Humus substances ≥ 20 g dm^3, (NPK 0.13-0.09-0.7), dry mass 22 g dm^3, humidity 96%, organic substances 3%, pH 7-9
Asahi SL bioactivator	Sodium para-nitrophenolate 3 g dm^3 (0.3%), sodium orto-nitrophenolate 2 g dm^3 (0.2%), sodium 5-nitroguaiacolate 1 g dm^3 (0.1%)

During each year of the experiment, in autumn, the manure fertilization at a dose of 25 t ha^{-1} and phosphatic fertilization P: 44.0 kg ha^{-1} kg and potassium K: 124.5 kg ha^{-1} were applied, and in spring – fertilization with nitrogen at a dose of N: 100 kg ha^{-1}. Tubers of potato were planted in the second decade of April (in 2015 and 2016) and the third decade of April (in 2017). Treatments for protection against pests (diseases and pests) were used in accordance with the recommendations of the plant protection. The plantation was protected by applying spraying with the following insecticides: Actara 25 WG (thiamethoxam 250 g kg^{-1}) at a dose of 0.08 kg ha^{-1}, Calypso 480 SC (thiacloprid 480 g dm^3) at a dose of 0.1 dm^3 ha^{-1} and fungicides: Copper Max New 50 WP (copper – 500 g kg^{-1}) at a dose of 2.0 kg ha^{-1}, Dithane Neo Tec 75 WG (mancozeb 750 g kg^{-1}) at a dose of 2.0 kg ha^{-1}. Tuber collections were carried out in the phase of technological maturity.

Chemical analysis methods

In order to perform chemical analyses of all experimental objects, the tubers from 10 randomly selected plants of potato were sampled (with the exception of the marginal plants). Chemical analyses were performed in three repetitions for each combination of the experiment (90 tests in total). The content of reducing sugars (glucose + fructose) and total sugars (total sugars) were marked in the fresh tuber mass not selected by means of the method of *Schoorl-Luff* (Krełowska-Kułas, 1993). The results were expressed as percentage of fresh mass.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

Statistical analysis

Results of research were statistically elaborated by means of the analysis of variance (one-way and two-way Analysis of Variance ANOVA). The significance of the variation sources was studied through test F of Fisher-Snedecor, and the assessment of significance of differences at the significance level 0.05 between the compared averages using multiple compartments of Tukey (post hoc test).

Weather conditions

Weather data came from The Weather Station of the Cultivars Research Centre (COBORU) in Słupia Wielka and were prepared for the Experimental Station of the Evaluation of Varieties in Cicibór Duży, localized within 6 km from Biała Podlaska. The weather conditions in growing seasons of 2015-2017 were described on the background of the multi-year period 1981-2010 based on deviations of average monthly air temperature (°C) and total precipitation (mm) and of hydrothermal Sielianinov coefficient (Chereszkowicz, 1979; Skowera, 2014).

The precipation-thermal conditions during the research were varied (*Table 4*). The total rainfall in the year 2015 reached approx. 63 mm lower than average rainfall during the multi-year period (1981-2010), while the average temperature was about 1.1 °C higher than the average long-term air temperature. The biggest deficiency of precipitation was specific for the months of June, July and August. The hydrothermal Sielaninov coefficient (K = 0.99) indicates that the growing season of the year 2015 was a dry season. During the execution of the experiment, the moistest and coolest growing season was in 2017. In this season, the rainfall was higher by 74 mm from the average rainfall from the respective period of the multiannual period, and hydrothermal Sielianinov coefficient was the highest in relation to the rest of the years of research and was 1.50. In 2016, during the period of the largest potato demand for precipitation, the conditions of thermal runoff were more favourable compared to the growing season of 2015 and 2017 (*Table 4*).

Results and discussion

One of the most important characteristics that determine the usefulness of potato tubers for direct consumption as well as for processing is the content of total sugars and reducing sugars, which in fresh tuber mass should not exceed 1% (Grudzińska et al., 2016).

As a result of the executed research, it was found that the content of total sugars in tubers of potato was on average 0.659% while reducing sugars 0.321% (*Tables 5* and *6*). The methods of care applied in the experiment, as well as the genetic features of the varieties, did not have a significant impact on the content of total sugars and reducing sugars in tubers of potato. The value of these characteristics depended on the course of thermal-precipitation conditions in the relevant years of research (*Tables 5* and *6*).

Treatment methods using growth biostimulators and herbicide (object 2-5) have contributed to a slight increase of the content of total sugars and reducing sugars compared to the control object that uses only mechanical treatment (without growth biostimulators and herbicide) (object 1) (*Tables 5* and *6*). Also, Trawczyński (2014) after the application of amino acid biostimulators in the cultivation of potatoes did not note their significant impact on the content of reducing sugars in tubers. Similar test

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

results were obtained also by Maciejewski et al. (2007) by applying foliar feeding of potato plants with Asahi SL and SL Atonik biostimulators. Furthermore, Rudzińska-Mękal (2000) has proved that the growth biostimulators caused a significant increase in the content of reducing sugars in tubers of potato, which may have resulted from inhibition of the biosynthesis of oligo-and polysaccharides due to the decrease in the concentration of potassium in the tubers of potato.

Table 4. *Meteorologic conditions according to the Research Centre for Cultivar Testing (COBORU) in Słupia Wielka Poland*

Study year	Deviations from the long-term (1981-2010) mean						
	IV	**V**	**VI**	**VII**	**VIII**	**IX**	**IV-IX**
Rainfall (mm)							
2015	+1	+32	–36	–21	–60	+21	–63
2016	0	–37	+18	+51	–39	–42	–49
2017	+27	–19	+40	+1	–26	+51	+74
Air temperature (°C)							
2015	+0.4	–1.1	+0.1	+0.8	+3.7	+2.4	+1.1
2016	+1.8	+1.1	+1.8	+1	+0.8	+2.5	+1.5
2017	–0.2	–0.1	+1.4	–0.3	+2	+2	+0.8
Sielianinov hydrothermal coefficient *(K)							
2015	1.43	2.28	0.59	0.80	0.11	1.66	0.99
2016	1.19	0.47	1.53	1.97	0.48	0.26	1.00
2017	2.68	0.93	1.97	1.24	0.66	2.37	1.50

*Sielianinov hydrothermal coefficient *(K)* – formula as in research methodology;
**Month's classification according to: Chereszkowicz (1979) and Skowera (2014): extremely dry $K \leq 0.4$, very dry $0.4 < K \leq 0.7$, dry $0.7 < K \leq 1.0$, quite dry $1.0 < K \leq 1.3$, optimal $1.3 < K \leq 1.6$, quite humid $1.6 < K \leq 2.0$, humid $2.0 < K \leq 2.5$, very humid $2.5 < K \leq 3.0$, extremely humid $K > 3.0$

In the studies of Gugała et al. (2013) and Sawicka and Pszczółkowski (2005), the applied herbicides, as well as their mixtures did not have a significant impact on the content of sugars in tubers, however, they caused a slight increase in the content of total sugars and reducing sugars. In contrast, Zarzecka et al. (2017) have shown that herbicides: Plateen 41.5 WG (metribuzin + flufenacet) and Racer 250 EC (fluorochloridone) significantly increased the content of total sugars, and herbicide Plateen 41.5 WG increased the content of reducing sugars. Zarzecka and Gugała (2018) after applying herbicide Sencor WG 70 (metribuzin) and growth regulator Asahi SL have found a significant increase in the content of total sugars and reducing sugars in tubers of potato.

Also, the potato varieties cultivated in the study did not differ significantly in terms of discussed quality features. The varieties were characterised by a similar concentration of total sugars – an average from 0.635 to 0.681% and reducing sugars, on average from 0.314 to 0.345% (*Tables 5* and *6*), what is confirmed in studies carried out by Sawicka and Pszczółkowski (2005), who found no significant effect of the genetic characteristics of varieties on the content of total sugars and reducing sugars. However, close relationship of the sum of sugars and reducing sugars in cultivated tubers of potato was recorded in the works of Jarych (2004), Zgórska and Grudzinska (2012) and Bhattacharjee et al. (2014). In studies of Maciejewski et al. (2007) after application of biostimulators Asahi SL and Atonik SL, the variety Ditta resulted in the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

reduction of the content of reducing sugars, while the variation of Satina gathered more of them.

Table 5. *The average content of total sugars in tubers of potato, % of fresh mass, depending on the methods of treatment and years of research*

Methods of treatments*	Cultivar			Years			Mean
	Bellarosa	Owacja	Vineta	2015	2016	2017	
1	0.626	0.632	0.660	0.717	0.607	0.594	0.639
2	0.631	0.669	0.683	0.738	0.626	0.620	0.661
3	0.641	0.665	0.684	0.740	0.630	0.621	0.664
4	0.640	0.667	0.675	0.743	0.622	0.616	0.661
5	0.639	0.677	0.702	0.767	0.637	0.615	0.673
Mean	0.635^{a}	$0.662^{a,b}$	0.681^{b}	0.741^{c}	0.624^{d}	0.613^{d}	0.659

Year $F(2, 80.33) = 150.51$, $p = 0.00000$**
Cultivar $F(2, 85.22) = 3.07$, $p = 0.05159$
Methods $F(4, 130) = 0.73$, $p = 0.57537$

[a,b,c,d]Different letter in the index indicates statistically significant variation
[*]As in the research methodology:
1. Standard object (without biostimulators and herbicide)
2. GreenOK Universal–PRO 0.10 $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$
3. Asahi SL 0.50 $dm^3 ha^{-1}$ + 0.50 $dm^3 ha^{-1}$ + 0.50 $dm^3 ha^{-1}$
4. Avatar 293 ZC 1.5 $dm^3 ha^{-1}$ + GreenOK Universal–PRO 0.10 $dm^3 ha^{-1}$ + 0.15. $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$
5. Avatar 293 ZC 1.5 $dm^3 ha^{-1}$

[**]Significant difference

Table 6. *The average content of reducing sugars in tubers of potato, % of fresh mass, depending on the methods of treatment and years of research*

Methods of treatments*	Cultivar			Years			Mean
	Bellarosa	Owacja	Vineta	2015	2016	2017	
1	0.323	0.285	0.299	0.214	0.322	0.370	0.302
2	0.354	0.311	0.314	0.229	0.347	0.403	0.326
3	0.347	0.310	0.320	0.226	0.342	0.409	0.326
4	0.350	0.316	0.328	0.244	0.341	0.409	0.331
5	0.351	0.296	0.310	0.229	0.342	0.387	0.319
Mean	0.345	0.304	0.314	0.228^{a}	0.339^{b}	0.395^{c}	0.321

Year $F(2, 81.44) = 371.97$, $p = 0.0000$**
Cultivar $F(2, 85.33) = 2.97$, $p = 0.05639$
Methods $F(4, 130) = 0.55$, $p = 0.70018$

[a,b,c]Different letter in the index indicates statistically significant variation
[*]As in the research methodology:
1. Standard object (without biostimulators and herbicide)
2. GreenOK Universal–PRO 0.10 $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$
3. Asahi SL 0.50 $dm^3 ha^{-1}$ + 0.50 $dm^3 ha^{-1}$ + 0.50 $dm^3 ha^{-1}$
4. Avatar 293 ZC 1.5 $dm^3 ha^{-1}$ + GreenOK Universal–PRO 0.10 $dm^3 ha^{-1}$ + 0.15. $dm^3 ha^{-1}$ + 0.15 $dm^3 ha^{-1}$
5. Avatar 293 ZC 1.5 $dm^3 ha^{-1}$

[**]Significant difference

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Also, the potato varieties cultivated in the study did not differ significantly in terms of discussed quality features. The varieties were characterised by a similar concentration of total sugars – an average from 0.635 to 0.681% and reducing sugars, on average from 0.314 to 0.345% (*Tables 5* and *6*), what is confirmed in studies carried out by Sawicka and Pszczółkowski (2005), who found no significant effect of the genetic characteristics of varieties on the content of total sugars and reducing sugars. However, close relationship of the sum of sugars and reducing sugars in cultivated tubers of potato was recorded in the works of Jarych (2004), Zgórska and Grudzinska (2012) and Bhattacharjee et al. (2014). In studies of Maciejewski et al. (2007) after application of biostimulators Asahi SL and Atonik SL, the variety Ditta resulted in the reduction of the content of reducing sugars, while the variation of Satina gathered more of them.

The implemented statistical calculations have shown that changing weather conditions prevailing in the years of research substantially modified the content of total sugars and reducing sugars in tubers of potato.

The largest total sugar content – an average of 0.741% was characterized by tubers harvested in 2015, in which during the period from June to August there was a high deficit of precipitation and air temperature in August (before the harvest of tubers) was higher by 3.7 °C from the average multiannual temperature (*Tables 4* and *5*). While most reducing sugars – an average of 0.395% were recorded in 2017, when the lowest air temperature and rainfall compared to the other years occurred (*Tables 4* and *6*). These results are consistent with the research of Gugała et al. (2013) and Zarzecka et al. (2017), who reported a significant effect of diverse weather conditions on the content of total sugars and reducing sugars in tubers of potato. Also according to other authors, Rodriguez et al. (2010), Murniece et al. (2011) and Brazinskiene et al. (2014), the content of sugars in tubers of potato is not stable and is subject to significant changes in individual years of research.

Sawicka and Pszczółkowski (2005) noted the highest content of total sugars and reducing sugars in the growing season with the most intense sunlight and rainfall. According to Grudzińska et al. (2014), a decisive influence on the content of reducing sugars in the tubers of potato is triggered by air temperature and total precipitation for ten days before harvesting and during the time of harvesting tubers. The authors, by studying 268 potato varieties over the course of fourteen years, found that the higher the air temperature and rainfall deficiency before harvest tubers, the less content of reducing sugars in tubers; whilst Frydecka-Mazurczyk and Zgórska (2002) stated that the potatoes, which before the harvest experienced autumn overcooling, collected reducing sugars more intensely. According to Nourian et al. (2003), this may be due to the activity of the enzyme which catalyses sucrose to glucose and fructose.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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An in-depth analysis revealed a significant effect of interaction of weather conditions and genetic features of varieties on the content of total sugars and reducing sugars in edible potato tubers (*Table 7*).

Bellarosa variety cumulated substantially less total sugars than the remaining varieties in humid and cool growing season of 2017, whilst in the favourble growing season of 2016, less total sugars were gathered by the tubers of Owacja variety.

In the season of 2017 also a significant effect of genetic features of varieties was reported on the content of reducing sugars. The majority of reducing sugars was cummulated by the tubers of Bellarosa variety, whilst the least –tubers of Owacja variety. No substantial differences in terms of the discussed features were noted in the warm and dry growing season of 2015 (*Table 7*).

Table 7. *Impact of weather conditions on the average content of sugars in fresh potato tuber mass, considering genetic features of varieties (%) (in-depth analysis)*

Cultivar	Total sugars			Reducing sugars		
	Year			Year		
	2015	2016	2017	2015	2016	2017
Bellarosa	0.740^{a}	0.639^{b}	0.528^{d}	0.230^{a}	0.348^{b}	0.457^{d}
Owacja	0.737^{a}	0.599^{c}	0.651^{b}	0.230^{a}	0.333^{b}	0.348^{b}
Vineta	0.747^{a}	0.635^{b}	0.661^{b}	0.225^{a}	0.336^{b}	0.381^{c}
Anova results	Cultivar: $F(2, 126)=23.64$, $p=0.0000$ Year: $F(2, 126)=226.54$, $p=0.0000$ Cultivar xYear : $F(4, 126)=33.39$, $p=0,0000$			Cultivar: $F(2,126)=30.13$, $p=0.0000$ Year: $F(2, 126)=470.20$, $p=0.0000$ Cultivar x Year: F(4,126)=19.66, $p=0.0000$		

[a,b,c,d]Indicates a homogeneous group (the same letter indicates no significant differences between the averages)

Conclusions

Currently, an increasing interest in agricultural practice and teachings has been inspired by preparations referred to as growth biostimulators. Therefore, it is very important to familiarize oneself with their impact on the more important quality characteristics of crops, including potato, which occupies an important place in human diet and which has been one of the most frequently cultivated plants (in the global crop production it occupies the fourth place after wheat, rice and maize).

The content and quality of biological substances included in the chemical composition of potato decide about the dietary value of tubers. In tubers, however, there can be substances, whose increased content affects the consumer quality. The priority size of the tubers for food processing is the content of total sugars and reducing sugars. Understanding the impact of agricultural science treatments on the value of these features is a challenge for research not only in Poland but also on the international level.

In own studies, the treatment methods using growth biostimulators as well as cultivation of potato varieties do not have a significant impact on the content of total sugars, as well as the reducing sugars compared to the control object (on which growth biostimulators and herbicide were not applied). The value of these characteristics depended only on the course of weather conditions in the years of research. More total sugars were collected by tubers in a warm and dry growing season of 2015 while reducing sugars – in humid and cool growing season of 2017.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34573468

ANALYSIS ON ENRICHMENT OF AQUATIC PLANTS RESPONSE TO DIFFERENT HEAVY METAL IONS IN POLLUTED WATER-TAKING DUCKWEED AS AN EXAMPLE

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(Received 15th Jun 2018; accepted 2nd Aug 2018)

Abstract. In order to improve the quality of water, the enrichment of different heavy metal ions by aquatic plants is studied—taking duckweed as an example. The site of the experiment is in Sichuan University of China, mainly from the following aspects of research: the heavy metal content in water, the heavy metal content in duckweed, chlorophyll content, adsorption capacity of Cadmium ions in different concentrations of nitric acid solution, enrichment capacity of Cadmium ion and the relationship between heavy metal content in duckweed and that of water. The study shows that the aquatic plants such as duckweed have a strong enrichment to Cu, Pb and Zn in the complex water body of heavy metals Cu, Pb and Zn, and their enrichment ability is higher than that of the water plants. With the increase of Cd^{2+} concentration, the content of chlorophyll a and b in duckweed decreased significantly, and the decline is increased with the increase of Cd^{2+} concentration. In 3 mg/L Cadmium nitrate solution, duckweed has a maximum absorption rate of Cd^{2+} reaching 87%, the adsorption of duckweed decreased with increasing concentration and the enrichment of Cd^{2+} in duckweed showed a nonmonotonic curve with the increase of treatment time. The concentration of Cd^{2+} in the duckweed of 8d reached the maximum value (1.6 mg/g), and the concentration of Cd^{2+} is reduced in the 9-12d duckweed, the concentration of heavy metals in water is significantly correlated with the concentration of Cu, Pb, Zn and Mn in aquatic plants duckweed. The method can effectively analyze the supposed enrichment. The sensitivity of duckweed can be used to detect changes of heavy metals in water, which can be used as an bioindicator of environmental pollution. At the same time, the accuracy of heavy metal determination by this method is high.
Keywords: *duckweed, heavy metal ions, enrichment, chlorophyll content, determine*

Introduction

Since 1970s, with the development of industry and agriculture, all kinds of untreated sewage have been discharged into lakes and rivers, and the amount of heavy metals in the water increased. Most of these have the characteristics of enduring hazards, geochemical recycling and ecological risk. Aquatic plants such as duckweed are part of the ecosystem, and they play an important role in material circulation, migration of elements and purification of sewage. A large number of studies have shown that the heavy metal content in water has a positive correlation with the heavy metal content in aquatic plant, the amount of many of elements in duckweed are hundreds of times as much as these in the water, therefore, the content of heavy metals in duckweed is used to reflect the level of heavy metals in water (Li and Wang, 2016). The heavy metals absorbed by aquatic plants are mainly derived from their growing environment. If the concentration of heavy metals in water increases, the content heavy metals in aquatic plants will also increase. That is to say, the higher the heavy metals level in the pollution, the higher the concentration of heavy metals in plants. The toxic effect of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34693482

metals on duckweed has been studied by scholars. After sampling, different heavy metal ions were analyzed to compare the different enrichment of duckweed. The analysis basis for sewage treatment is provided and improves the quality of water (Unsal and Soylak, 2015).

Tang et al. in order to screen duckweed varieties which can efficiently remove heavy metal Cadmium from water and accumulate biomass rapidly, 12 duckweed varieties with tolerance to 30 mg/L Cd^{2+} concentration are screened again at 0.5 mg/L and 10 mg/L Cd^{2+} concentrations. The best variety of *Landoltia punctata* ZH0049 is obtained. At the same time, the relationship between the change of chlorophyll content and the concentration of Cadmium stress are analyzed. However, the results of this method were not accurate (Tang et al., 2015a); Fan et al. take duckweed as the research object and applied phosphate to study the response mechanism of antioxidant enzyme system and the enrichment ability of Uranium under different concentration of Uranium stress. There is a problem that the research time is too long (Fan et al., 2018). In view of the above problems, the enrichment of different heavy metal ions by aquatic plants is studied, taking duckweed as an example.

Materials and methods

Materials

Duckweed samples of size 1-2 mm (1 mm × 1 mm) are collected in the lake water, specifically in TaiHu Lake (120° 36’ E and 31° 22’ N), JiangSu province. The samples are collected by duckweed fishing vessel with a sample quantity of 5 kg. 150 clean plastic drums with a volume of 14 L are selected. The human nutrient solution is injected into each barrel without heavy metal ions to be measured, and an open foam plate is placed on the barrel as the growth carrier of the plant. Duckweed is transferred into three round holes of foam carrier, and its root is immersed in 1/10 Hoagland solution. The growth and management of plants are cultivated in the open air, watered every 3 days and supplemented with 1/10 Hoagland solution, which kept the plants well ventilated and illuminated. Plant samples are collected after 15 days.

Finally, the collected samples will be rinsed repeatedly with tap water and distilled water. After sucking the water with the absorbent paper, the fresh weight is measured. Then it was dried in a ventilated and dry place, and the dry weight was measured after drying. The crushed and mixed samples were kept in grinding bottles and jars. The site of the experiment is in Sichuan University of China, the GPS coordinates of the experimental site are 104° 08’ E and 30° 63’ N.

Methods

Determination of heavy metal content in water

The sealed water samples are brought back to the laboratory, and the content of heavy metals in water is measured by flame atomic absorption spectrometry, repeated three times.

Determination of heavy metal content in duckweed

The nitrate and perchloric acid (HNO_3 40 mL, $HCLO_4$ 40 mL) added to 0.3 g duckweed powder were digested and heated on the electric heating board, and set up to

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100 mL by deionized water. The atomic spectrophotometer is used to determine the content by air acetylene flame (Zhao and Shi, 2015).

Determination of chlorophyll content in duckweed

Plants with good growth and uniform leaf size are selected for continuous cultivation of 3d in plastic pots containing tap water (Park and Kim, 2013). They are transferred to 1/10 Hoagland solution for 1 week and treated with Cadmium.

$CdCl_2$ is added to the 1/10 Hoagland solution, and 7 concentration gradients of Cd^{2+} are set up as 0 (control), 1, 2, 4, 6, 8 and 10 mg/L. Each treatment set up 3 parallel samples with 20 plants. The growth of the plant must be observed during the treatment. The duckweed is cultured in the PGX multi section light culture box. When the light of 4500 lux is irradiated for 12 h/d, the temperature was (25 ± 1) °C, the chlorophyll fluorescence parameters of duckweed after 24, 48, 72 and 96 h are determined, respectively.

The chlorophyll fluorescence parameters are determined by underwater luminoscope. Before measuring, add duckweed that is sucked out of water into a Petri dish wrapped in tin foil. After 10 mins, the fibers are aligned to the colorimeter and the modulation light is opened to determine the minimum fluorescence (F0). Then the saturation pulse is opened and the maximum fluorescence (Fm) is measured to calculate the maximum light chemical quantum yield (FV/Fm); The photochemical light of setting intensity continuous irradiation time is 3 min, and a saturated pulse (4000 mu mol/m^2 s) with a time interval of 20 s and a duration of 0.8 s is opened, then the quenching analysis is carried out. After the determination of the fluorescence parameters, the stable fluorescence value was obtained and analyzed (Lalau and Mohedano, 2015). The calculation formula of chlorophyll fluorescence parameters is as follows (*Eqs. 1–7*):

$$F_V / F_m = (F_m - F_0) / F_m \quad \text{(Eq.1)}$$

$$\Phi_{PSII} = (F'_m - F_t) / F'_m \quad \text{(Eq.2)}$$

$$qP = (F_m - F_t) / (F_m - F_0) \quad \text{(Eq.3)}$$

$$rETR = \Phi_{PSII} \times PAR \times 0.5 \times 0.84 \quad \text{(Eq.4)}$$

$$F_V / 2 = (F_m - F_0) / 2 \quad \text{(Eq.5)}$$

$$qN = (F_m - F'_m) / (F_m - F_0) \quad \text{(Eq.6)}$$

$$F_0 / F_V = F / (F_m - F_0) \quad \text{(Eq.7)}$$

Where F'_m the maximum fluorescence output is obtained when the saturation pulse is opened; F_t represents the fluorescence output measured at any time; PAR indicates the light and effective radiation intensity (μ mol/m^2 s) of the incident to the samples.

It should be made clear that the bioaccumulation coefficient is used in the determination. Bioaccumulation coefficient is the ratio of the concentration of certain

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elements or refractory compounds in an organism to the concentration of the substance in the environment in which it exists, and can be used to indicate the degree of biological concentrations. The calculation formula is as follows (*Eq. 8*):

$$B = \frac{\text{Concentration of refractory compounds}}{\text{Total concentration of substances in the environment}} \tag{Eq.8}$$

The determination of chlorophyll content:

After 96 h, the centrifuge tube containing 0.5 g samples is frozen in the fridge for 1 H, then the 10 mL 95% ethanol heated to 50 °C in the water bath is added into, and the content of chlorophyll (Tang et al., 2015b) is determined by taking the supernatant in the dark cabinet and taking the supernatant in the dark cabinet. After Shaking and keeping quiet for 3 hours in dark room, the chlorophyll content is measured.

The chlorophyll absorbance A (Wan and Wu, 2018) is determined by spectrophotometer, and the chlorophyll concentration is calculated according to the following formulas (*Eqs. 9* and *10*):

$$Chla = 12.7A_{663} - 2.69A_{645} \tag{Eq.9}$$

$$Chlb = 22.9A_{645} - 4.68A_{663} \tag{Eq.10}$$

Where *Chla* and *Chlb* represent the concentration of chlorophyll a and b (mg/L) respectively; A_{663} and A_{645} mean the absorbance of chlorophyll solution at 663 nm and 645 nm respectively. The experimental data are analyzed and processed by SPSS15.0 software.

Enrichment of duckweed response to Cadmium ions in different concentrations of nitric acid solution

The lethal concentration and the actual situation of duckweed in Cd^{2+} solution are studied. The solution of 1 mg/L, 3 mg/L and 5 mg/L $Cd(NO_3)_2$ is disposed by the solid of Cadmium nitrate. The duckweed with good long potential, similar size and fresh weights 1.6 g is added into the 200 mL solution of 1 mg/L, 3 mg/L, 5 mg/L $Cd(NO_3)_2$, and the is added to the experiment (Xiong and Jiao, 2014). The other two groups are reserved, repeat it.

Enrichment of duckweed response to Cadmium ions in different periods of time

The solution of 3 mg/L $Cd(NO_3)_2$ is prepared with Cadmium nitrate solution. Duckweed with good growth, similar size and 7 g fresh weights is placed in the 3 glass cylinders containing 1 L solution of 3 mg/L $Cd(NO_3)_2$, respectively. A glass cylinder containing 1 L solution of 3 mg/L $Cd(NO_3)_2$ without duckweed was also used as a control. The concentration of Cadmium, the chlorophyll content in the duckweed, and the content of Cadmium in duckweed (Allende and Mccarthy, 2014) is measured in turn in the 3 cylinders solution after 4, 8 and 12d. The other two groups are reserved, repeat it.

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Determination of relation between heavy metal content in aquatic plants and heavy metal content in water

Duckweed with good growth, similar size and 4.5 g fresh weights is respectively placed in the 2 beakers containing 600 mL solution of lake water. Another 600 mL water beaker without duckweed is used as a control group. The two groups is added with nutrient solution and placed in the place with adequate light and suitable environment (Chen and Liang, 2016). After 4 days, the concentration of Cu, Zn, Ni and Cr in the solution (Chaudhary and Sharma, 2014) and the chlorophyll content in the duckweed is measured. Another group was prepared, repeat triple times.

Results

Results of heavy metal content in water

The contents of Cu, Pb, Zn, Cd and Mn in water samples are compared with the standard of Surface Water Environmental Quality (GB3838-2002). The contents of Cu, Pb, Zn, Cd and Mn in the waters of Taihu Lake (120° 36' E and 31° 22' N) are described in *Table 1*.

Table 1. *Water quality and heavy metal contents in water and surface water V (mg/L)*

Item	Cu	Pb	Zn	Cd	Mn
Water sample	7	1.02	5	4	4.38
V class standard	1	0.1	2	0.01	1

Analysis of heavy metal content in aquatic plants such as duckweed

The bioaccumulation coefficient is an important index for measuring the accumulation capacity of heavy metals in plants, the greater the BCF, the stronger the enrichment capacity (Jain and Verma, 2015). The heavy metal content and the BCF of the aquatic plants are described by *Table 2* and *Figures 1* and *2*.

Table 2. *Heavy metal content and enrichment factor of aquatic plants such as duckweed (mg/kg)*

Name of seed	Position	Cu(BCF)	Pb(BCF)	Cd(BCF)	Zn(BCF)	Mn(BCF)
Water turtle	Root	82.04(9.12)	33.74(33.41)	3.21(0.80)	161.59(26.93)	440.26(1.01)
	Stem and leaf	23.30(2.59)	5.05(5.00)	1.18(0.30)	54.33(9.06)	104.28(0.24)
Alternantheraphiloxeroides	Root	37.71(4.19)	8.20(8.12)	0.90(0.23)	55.05(9.18)	325.52(0.74)
	Stem and leaf	11.83(1.31)	7.32(7.25)	0.84(0.21)	31.47(5.25)	150.22(0.34)
Chinese arrowhead	Root	34.16(3.80)	28.80(28.51)	2.59(0.65)	68.28(11.38)	338.30(0.77)
	Stem and leaf	12.75(1.42)	8.27(3.74)	0.62(0.16)	27.62(4.60)	63.65(0.15)
Reed	Root	26.95(2.99)	7.06(6.99)	0.68(0.17)	49.94(8.32)	12.95(0.03)
	Stem and leaf	10.91(1.21)	3.78(3.74)	0.36(0.09)	25.75(4.29)	34.72(0.08)
Zizania	Root	54.90(6.10)	16.34(16.18)	1.15(0.29)	60.13(10.02)	73.30(0.17)
	Stem and leaf	14.24(1.58)	5.88(5.82)	0.42(0.11)	28.38(4.73)	71.07(0.16)
Typha	Root	22.87(2.54)	5.88(5.82)	0.75(0.19)	41.69(6.95)	24.79(0.06)
	Stem and leaf	9.19(1.02)	9.21(9.12)	1.04(0.26)	18.97(3.16)	62.35(0.14)
Duckweed	Whole plant	74.25(8.25)	19.35(19.16)	0.90(0.23)	70.15(11.69)	68.30(0.16)
The lotus root	Root	27.20(3.02)	11.21(11.10)	1.12(0.28)	31.02(5.17)	76.99(0.18)
	Stem and leaf	9.21(1.02)	2.12(2.10)	0.32(0.08)	24.75(4.13)	74.12(0.17)

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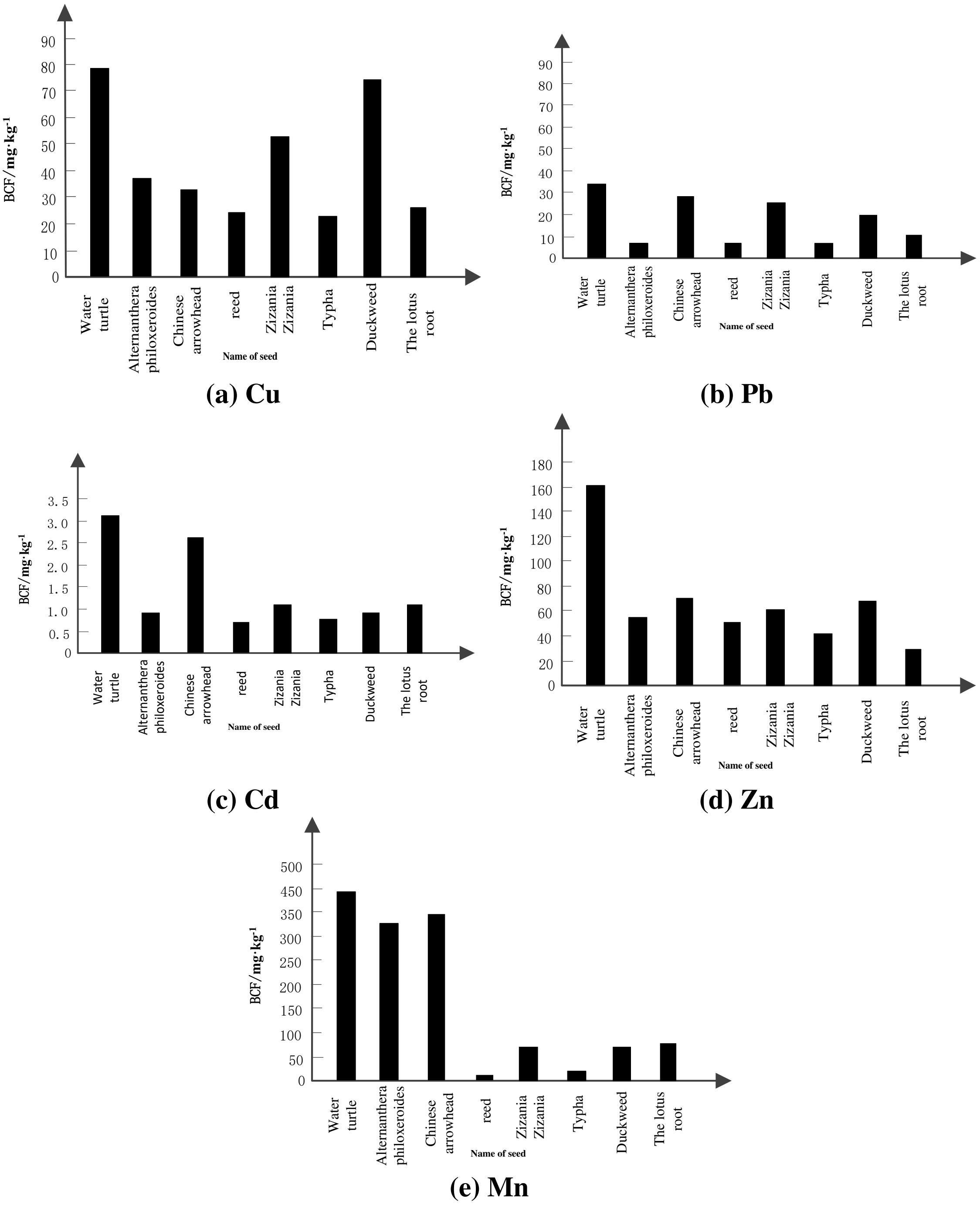

Figure 1. *Contents of heavy metals in the roots of duckweed and other aquatic plants*

The relationship between heavy metal content and BCF of the aquatic plants can be seen from *Table 2* and *Figures 1* and *2*. The uptake of heavy metals by different plants is quite different. The accumulation of Cu, Pb and Zn in aquatic plants is relatively large, and the BCF of roots, stems and leaves is all more than 1. It can be seen that the aquatic plants have strong accumulation ability of Cu, Pb and Zn, and the accumulation ability of heavy metals in duckweed is generally higher than that of floating plants. According to *Table 2* and *Figures 1* and *2,* compared with other organisms, the contents

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of heavy metals in leaves of duckweed are higher, which indicated that the enrichment ability of leaves of duckweed is stronger.

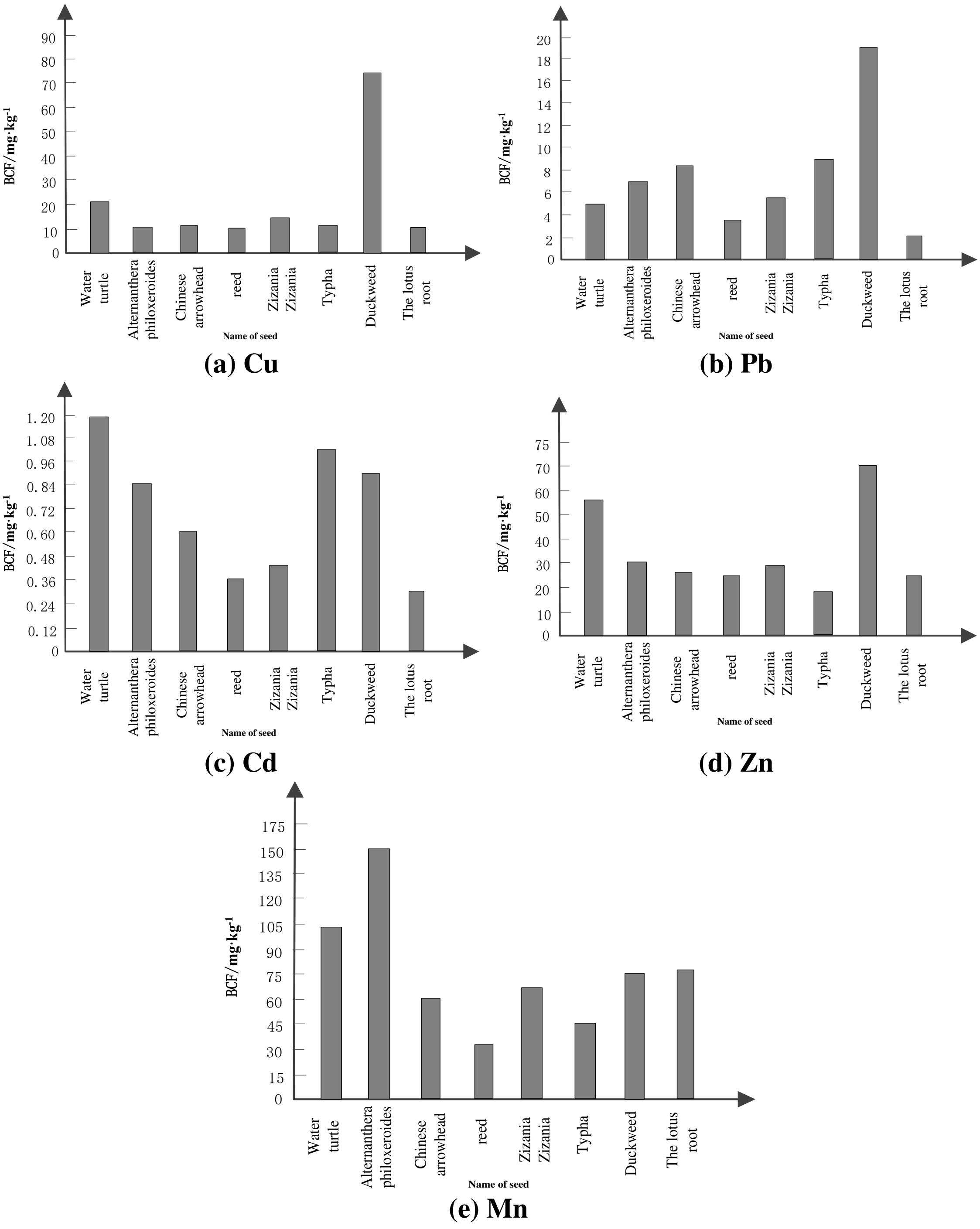

Figure 2. *Contents of heavy metals in leaves and stems of duckweed and other aquatic plants*

Determination of chlorophyll content in duckweed

In order to avoid the inhomogeneity of light in the experiment and the error produced by the instrument debugging (Qiao and Wang, 2015), the data are expressed as relative

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values, that is, the ratio of the chlorophyll fluorescence value measured by the daily Cadmium stress (the following is represented by 0 h). In fluorimetric analysis, the maximum photochemical quantum yield F_V / F_m of PSII is the most commonly used parameter. It reflects the initial light energy conversion efficiency of PSII (PSII apparatus). In normal physiological state, the change is smaller, but it will fall after the plant is stressed (Park and Kang, 2014). Therefore, it is an important parameter for the good environment of plant growth, and could indicate the damage to photosynthesis by Cadmium ions in the short time of the plant being broken. The effects of different concentrations of Cadmium ions on the growth of duckweed are shown in *Figures 3* and *4*.

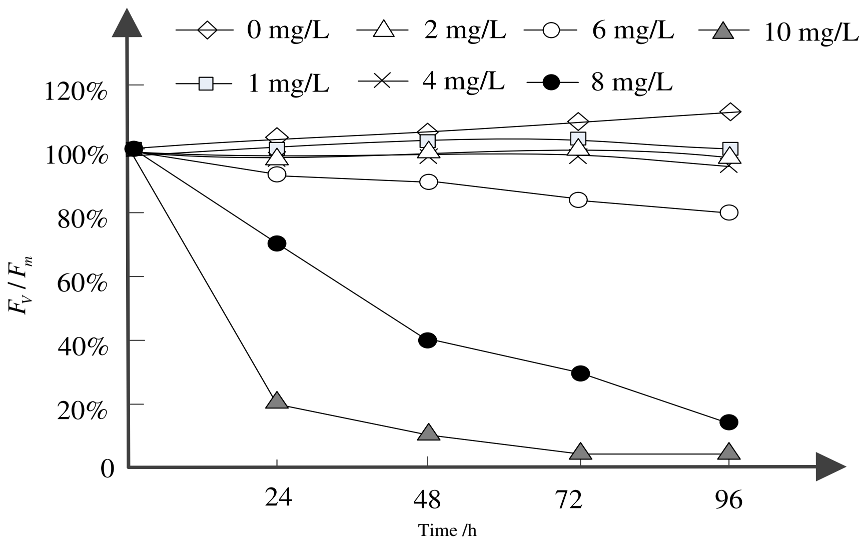

Figure 3. *Effect of Cadmium on duckweed* F_V / F_m *with different concentrations of Cadmium*

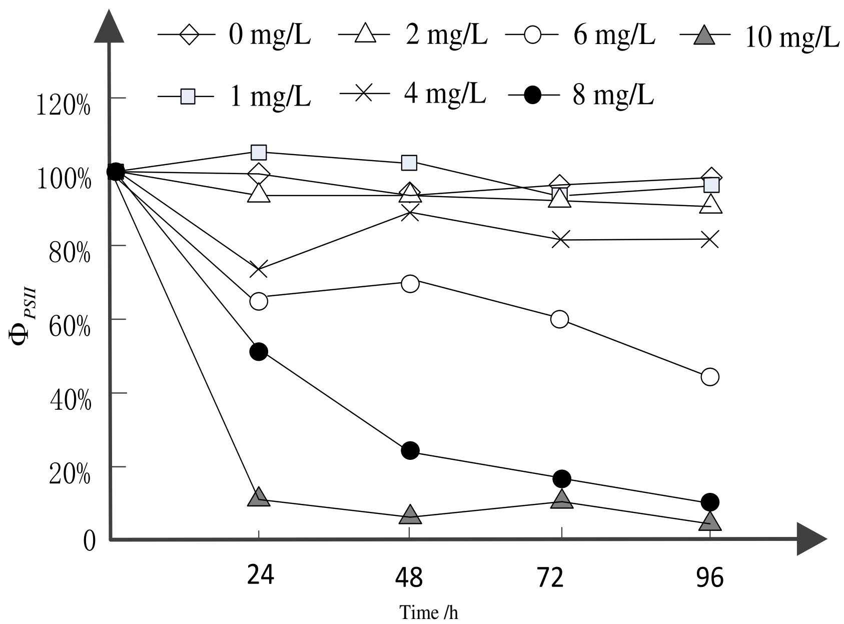

Figure 4. *Effect of Cadmium on duckweed* Φ_{PSII} *with different concentrations of Cadmium*

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In the experiment, the chlorophyll content of duckweed in different concentrations of Cadmium ion is shown in *Figure 5*.

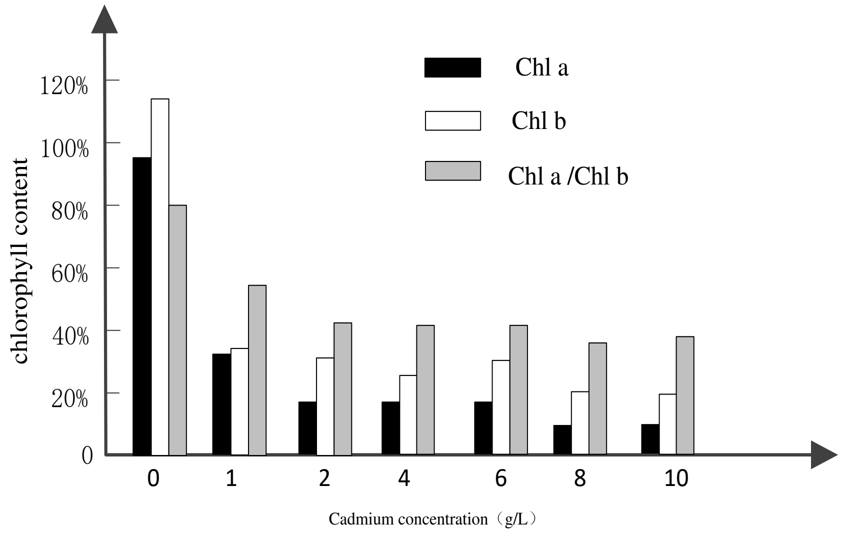

Figure 5. *Chlorophyll content of duckweed under different concentrations of Cadmium ions*

Enrichment of duckweed in different Cadmium nitrate solutions

The duckweed is placed in Cadmium nitrate solution of 1 mg/L, 3 mg/L and 5 mg/L respectively, the experimental results were described in *Figure 6*.

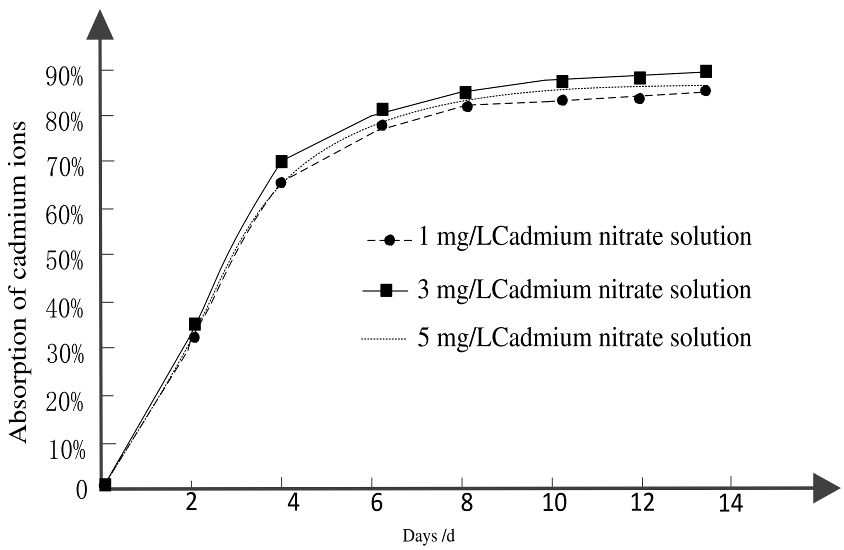

Figure 6. *Adsorption of duckweed on Cadmium nitrate solution with different concentrations*

Enrichment situation of duckweed response to Cadmium ions in different periods of time

The concentration of Cadmium ions in the 3 cylinders after 4d, 8d and 12d are measured by the experiment (Teixeira and Bini, 2017). The relationship between the

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number of experimental days and the enrichment of Cadmium ions is shown by *Figure 7*.

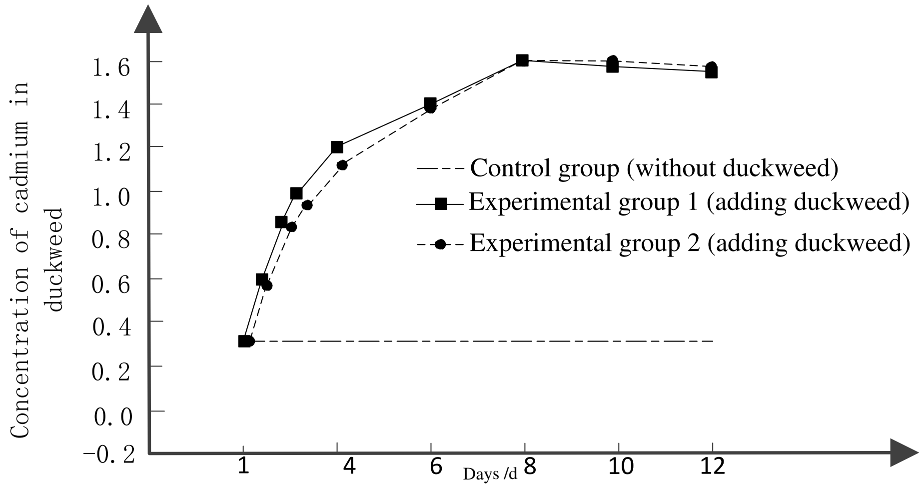

***Figure 7.** The relationship between concentration of Cadmium and time in duckweed*

Relationship between heavy metal contents in aquatic plants such as duckweed and heavy metal contents in water

Table 2 shows that the average content of heavy metals in different aquatic plants is calculated, and the correlation with the concentration of heavy metals in the water is analyzed (Küsters and Gerhartz, 2015; Gu, 2017), which is described in *Table 3*.

***Table 3.** The correlation between the average content of heavy metals and the concentration of heavy metals in aquatic plants such as duckweed and other aquatic plants*

Name of seed	Position	Correlation coefficient (r)				
		Cu	Pb	Cd	Zn	Mn
Water turtle	Root	0.999*	0.997*	0.982	0.999*	0.996
	Stem and leaf	0.999**	0.971	0.996	0.999*	0.694
Alternantheraphiloxeroides	Root	0.999*	0.922	0.786	0.919	0.983
	Stem and leaf	0.945	0.839	0.786	0.919	0.548
Chinese arrowhead	Root	0.875	0.996	0.945	0.689	0.949
	Stem and leaf	0.984	0.96	0.945	0.782	0.429
Reed	Root	0.998	0.885	0.945	0.835	0.631
	Stem and leaf	0.969	0.953	0.945	0.897	0.202
Zizania	Root	0.999**	0.885	0.655	0.646	0.999*
	Stem and leaf	0.997*	0.961	0.655	0.722	0.566
Typha	Root	0.999*	0.999**	0.945	0.945	0.952
	Stem and leaf	0.945	0.999*	0.655	0.757	0.666
Duckweed	Whole plant	0.980	0.971	0.945	0.803	0.979
The lotus root	Root	0.655	0.971	0.999**	0.909	0.993
	Stem and leaf	0.907	0.971	0.655	0.982	0.016

*p < 0.05, **p < 0.01

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Discussion

Discussion on the determination of heavy metal content in water

In *Table 1,* the test results of the heavy metal content in water indicated that the content of Cd^{2+} and Mn^{2+} in water body is obviously greater than that in other aquatic plants, but the content of Mn^{2+} in the root of soft shelled turtle is slightly higher than that of water. This is due to the continuous oxidation of plant rhizosphere. the root surface and the root outer body are oxidized to form iron and manganese film, preventing the toxicity caused by excessive absorption of Cd^{2+} and Mn^{2+}, resulting in the lower content of Cd^{2+} and Mn^{2+} in the aquatic plants of duckweed and other aquatic plants. In order to prevent the toxicity caused by excessive absorption of Cd^{2+} and Mn^{2+}, the iron and manganese films might be formed on the root surface and the root outer body, which led to the lower content of Cd^{2+} and Mn^{2+} in the aquatic plants.

Discussion on the determination of chlorophyll content in duckweed

In *Figure 3,* when the concentration of Cadmium stress is low (1, 2, 4 mg/L), the change of F_V / F_m is smaller. With the increase of Cadmium stress (6, 8, 10 mg/L), the change of F_V / F_m becomes sensitive. The F_V / F_m values of 8 and 10 mg/L Cadmium stress are decreased by 28.97% and 75.95% at 24 h, respectively, and are declined by 74.86% and 95.95% at 48 h, respectively. While the concentration of Cadmium is 6 mg/L, it decreased by 14.95% at 72 h.

Figure 4 showed the changes of Φ_{PSII}. The Φ_{PSII} changes in the 0, 1 and 2 mg/L groups are small in 24 h, and the Φ_{PSII} of 4, 6, 8 and 10 mg/L groups respectively, while that are decreased by 25.49%, 33.33%, 55.16% and 85.79%, compared with 0 h. Therefore, the declined of the Φ_{PSII} value increased with the increased of Cd^{2+} concentration, the magnitude of the decreased.

As can be seen from *Figure 5,* with the increasing of Cd^{2+} concentration, the contents of chlorophyll a and chlorophyll b are declined significantly, compared with the control group, and the declining of chlorophyll a content is remarkable than that of chlorophyll b, but also the ratio of chlorophyll a to chlorophyll b is decreased.

This indicated that the decline of chlorophyll a is larger than chlorophyll b, which is similar to previous studies (Zhao and Shi, 2015).

Discussion on enrichment of duckweed response to Cd^{2+} in nitric acid solutions with different concentration

From *Figure 6,* it can be seen that the adsorption of Cd^{2+} on duckweed decreased with increasing concentration. These suggested that Cd^{2+} is more toxic to duckweed and affects the normal growth of it.

Discussion on enrichment of duckweed response to Cd^{2+} in different periods of time

In *Figure 7,* the concentration of Cd^{2+} in Cadmium nitrate solution gradually decreased with the increased of treatment time, and the reduction rate of Cd^{2+} concentration slowed down gradually.

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The concentration of Cadmium in duckweed did not show a monotonous curve with the increase of the processing time, and the concentration of Cadmium in the duckweed reached the maximum (1.6 mg/g) at 8 days.

Discussion on relationship between heavy metal contents in aquatic plants and heavy metal contents in water

In the *Table 3,* the concentration of heavy metals in water is significantly correlated with the concentration of Cu, Pb, Zn and Mn in aquatic plants duckweed. The heavy metals can be absorbed and accumulated by duckweed. Therefore, the metals sensitivity of duckweed can be used to detect changes of heavy metals in water, which can be used as a bioindicator of environmental pollution.

To verify the effectiveness of this method, the accuracy rate of heavy metal determination in duckweed is compared with Tang et al. (2015a) and Fan et al. (2018). The results are shown in *Figure 8.*

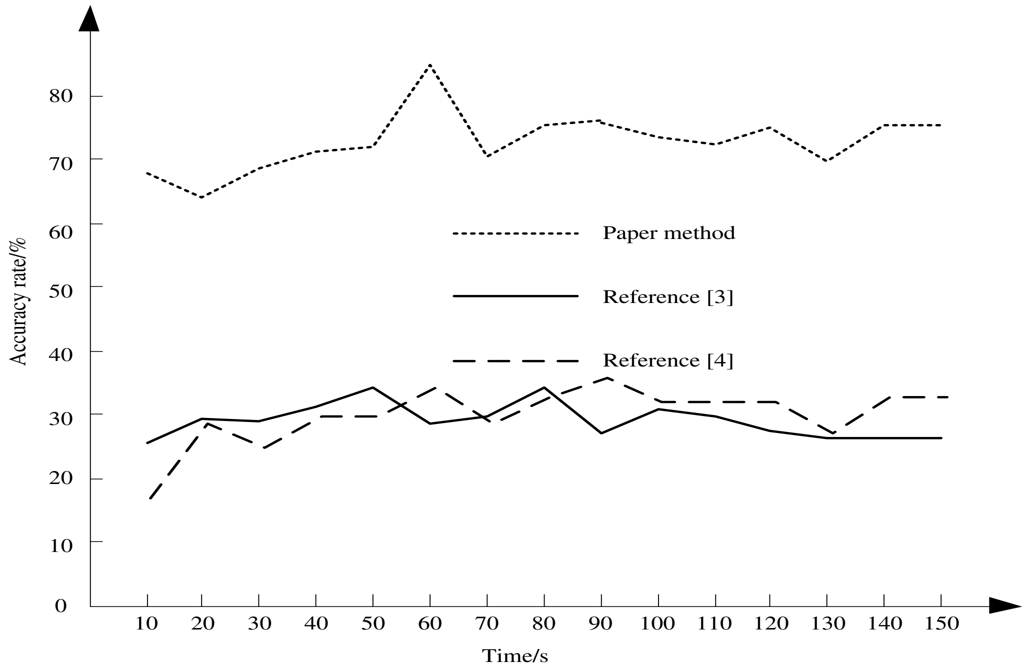

Figure 8. *Comparison of accuracy of different methods*

According to *Figure 8,* the accuracy of this method is much higher than that of Tang et al. (2015a) and Fan et al. (2018), and the average accuracy is more than 70%, while the accuracy of Tang et al. (2015a) and Fan et al. (2018) is less than 30%.

Conclusions

In the paper, the enrichment of duckweed response to different heavy metal ions in water is analyzed from the following aspects: the heavy metal content in water, the heavy metal content in duckweed, chlorophyll content, adsorption capacity of Cadmium ions in different concentrations of nitric acid solution, enrichment capacity of Cadmium ion and the relationship between heavy metal content in duckweed and that of water.

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The content of heavy metals in water is measured by flame atomic absorption spectrometry; the atomic spectrophotometer is used to determine the content by air acetylene flame atomic absorption spectrophotometry; the chlorophyll fluorescence parameters and chlorophyll content in duckweed are measured by grinding and underwater luminoscope. Different concentrations of Cd^{2+} solution are used to observe the lethal concentration and actual situation of duckweed.

Duckweed is cultured in the same concentration of Cadmium nitrate solution, and the enrichment of duckweed response to Cadmium ions in different periods of time is studied.

The average content of heavy metals in different aquatic plants is calculated, and the correlation with the concentration of heavy metals in water is analyzed by setting up the control group.

In short, in order to prevent the toxicity caused by excessive absorption of Cd^{2+} and Mn^{2+}, the iron and manganese films are formed on the root surface and the root outer body, which led to the lower content of Cd^{2+} and Mn^{2+} in the aquatic plants. The aquatic plants have strong accumulation ability of Cu, Pb and Zn, and the accumulation ability of heavy metals in duckweed is generally higher than that of emergent plants. With the increased of Cd^{2+} concentration, the content of chlorophyll a and chlorophyll b significantly decreased, compared with the control group, and the declined of chlorophyll a, but the chlorophyll b content increased. The absorption rate of Cadmium nitrate solution at 3 mg/L is the largest, reaching 87%, and the adsorption of Cd^{2+} on duckweed decreased with increasing concentration. The concentration of Cadmium in duckweed did not show a monotonous curve with the increased of the processing time, and, the concentration of Cadmium in the duckweed reached the maximum (1.6 mg/g) at the 8 days. The concentration of heavy metals in water is significantly correlated with the concentration of Cu, Pb, Zn and Mn in duckweed. The heavy metals can be absorbed and accumulated in the duckweed, and the absorption of heavy metals varies with the change of water environment.

As a new green pollution control technology, phytoremediation is still in the experimental research stage. There is still much work to be done in order to be widely used in wild.

(1) Strengthen the mechanism of metal removal by microbes and plants. The ecological and physiological characteristics of plant communities combined with plant roots, the role of root exudates in the selection of microbial communities and the mechanism of interaction between plant roots and rhizosphere microbial communities need to be further studied.

(2) In the future, genetic factors of superaccumulative plants or microbes growing in heavy metal contaminated environments will be applied to non-superaccumulative plants or microbes through transgenic technology; therefore, it is possible to develop super-enriched plants with strong adaptability, large biomass and enrichment ability to various target pollutants.

Acknowledgements. The authors acknowledge financial support received from the National Key Technology R&D Program of China (No. 2015BAD15B01); The Projects of International Cooperation of Ministry of Science and Technology of China (No. 2014DFA30680); Key Laboratory of Environmental and Applied Microbiology, Chengdu Institute of Biology, Chinese Academy of Sciences (No. KLCAS-2014-02); West Light Foundation of the Chinese Academy of Sciences (Y2C5021100).

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RESEARCH ON RUNOFF SIMULATION IN NINGXIA SECTION OF THE YELLOW RIVER BASIN BASED ON IMPROVED SWAT MODEL

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(Received 29th Aug 2018; accepted 7th Nov 2018)

Abstract. In recent years, the Ningxia Section of the Yellow River Basin of China has become water-stressed due to the reduction of the upstream precipitation, to the serious water and soil loss in the midstream, to the increased water consumption in industrial and agricultural areas and to other reasons. To fully understand the runoff variation of the Yellow River in the Ningxia Section of China and to rationally carry out the water dispatching and the water resource management, the applicability of the SWAT model for water balance in this basin is explored. Based on the improved SWAT model, the distributed hydrological model of the Ningxia Section of the Yellow River Basin of China has been constructed. The meteorological and hydrological data of the Ningxia Section of the Yellow River Basin of China from 1990 to 2017 have been used for simulation. The relative error R_e, the correlation coefficient R^2 and Nash-Suttcliffe coefficient Ens have been used as the standards, while the sensitive parameter of the measured monthly runoff from 2005 to 2011 to the model used for the calibration, and the model have been validated by the measured monthly runoff from 2012 to 2017. The research results show that the simulation results basically meet the model evaluation requirements, indicating that the SWAT model is applicable to the runoff simulation of the Ningxia Section of the Yellow River Basin of China, and it can provide decision-making basis for water resource management in the Ningxia Section of the Yellow River Basin of China.
Keywords: *SWAT model, Yellow River Basin in Ningxia Section, runoff simulation, hydrological modeling, water resource*

Introduction

The water quality and quantity play a very important role in the aquatic ecosystem. To protect the river ecosystem, the scientific hydrological knowledge is required (Armanini et al., 2011); a Canadian Ecological Flow Index was developed based on benthic macroinvertebrate flow sensitivity index for Canadian rivers. The river runoff plays a pivotal role in water quality and ecology, including phytoplankton, zooplankton and Bacterial communities (Godlewska et al., 2003; Sokal et al., 2010; Wu et al., 2015), and also including hydrological indicators and their application to ecological management and research, such as basin or river health assessments (US EPA, 2015; Biggs et al., 2002).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

Hydrological systems are complex and difficult to understand. To overcome these difficulties, hydrologists have studied hydrological systems with a simpler approach that is the hydrological model (Xu, 2002; Woessner, 2012). To research the different influencing factors of hydrological systems, the hydrological model has been continuously improved (Chow et al., 1988). In order to clarify some of the characteristics of the hydrological system, the hydrological model is used to simulate hydrological systems in the basin under climate change conditions, such as climate change, land use and soil (Leavesley et al., 1983; Viessman et al., 1989).

The SWAT (Soil and Water Assessment Tool) model was used to simulate the hydrological processes in the research area as they are influenced by the input data, namely slope, soil, land cover and climate. The SWAT model is the one used for simulating hydrological processes, and related to the input data, soil, climate and land use, as well as other factors. The SWAT model is a distributed watershed hydrological model developed by the Agricultural Research Service (ARS) of US Department of Agriculture (USDA) (Arnold et al., 1998). It is currently a relatively advanced international watershed hydrological model, and this model is mainly used to simulate the water quality and quantity of surface water and groundwater. It can predict the impact of the land management measure on the yield of hydrology, sediment and agricultural chemicals in large and complex watersheds with various soils, land use and management conditions.

The SWAT model is widely used in the world. Schuol et al. (2008) have verified the applicability of the model in the Lunnehe Basin under different hydrometeorological and underlying conditions. Arnold et al. (1998) have verified the applicability of the model in the Mississippi tributary, etc. under different hydrological conditions, different topographic features and different time and space. Fohrer et al. (2017) have found that the land use change had the highest impact on runoff by simulating the Aare River Basin in Germany. Joel and Jiben (2012) have clarified the impacts of various land use patterns on runoff by studying the land use and cover change of the Wami Basin 13a in Tanzania. Nie et al. (2011) have studied the relationship between land use pattern and water balance in the special years of the San Pedro Basin, and found that the urbanization is the biggest influencing factor. Lin et al. (2015) have studied landuse change impacts on catchment runoff using different time indicators based on SWAT model. Lotz et al. (2018) have studied factors of runoff generation in the Dongting Lake basin based on a SWAT model and implications of recent land cover change. Liu et al. (2017) researched the impacts of manure application on SWAT model outputs in the Xiangxi River watershed, Tamm et al. (2018) studied the effects of land use and climate change on the water resources in the eastern Baltic Sea region using the SWAT model. The SWAT model has been modified and adapted to simulate the changes in landuse, fertilizer management and tillage management (Amon-Armah et al., 2013; Maharjan et al., 2016; Liu et al., 2013, 2014). With the best parameters, the SWAT model will be used to simulate the pollution conditions in the selected watersheds (Dechmi et al., 2012; Liu et al., 2016).

In this paper, to fully understand the runoff variation of the Yellow River in the Ningxia Section and to rationally carry out the water dispatching and the water resources management, the applicability of the SWAT model for water balance in this basin is explored. Based on the improved SWAT model, the distributed hydrological model of the Ningxia Section of the Yellow River Basin has been constructed.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

Material and methods

Research area

The Yellow River has the entrance in the Xiaheyan in Zhongwei City, Ningxia, China. It flows through Zhongning County, Qingtongxia City, Litong District, Lingwu City, Yongning County, Yinchuan City, Helan County, Pingluo County, Dawukou District and Huinong District, and flows into Inner Mongolia in Shizuishan. There are three hydrological stations on the main stream of the Yellow River, including: Xiaheyan, Qingtongxia and Shizuishan Hydrological Stations. The Yellow River Basin in Ningxia Section is located in the north latitude 36°0′-39°23′ and east longitude of 104°17′-107°39′, and the drainage area is 51400 km^2. Its landform type is the alluvial plain of the Yellow River, and its terrain is flat; its ditches are vertical and horizontal, and its elevation is between 1100 and 2500 m. It is a temperate arid zone with sufficient sunshine. The average annual sunshine hour in the Basin is 2750-2950 h, and the annual average wind speed is 1.7-2.5 m/s. Its temperature difference is large; the heat is rich, and the frost-free period is longer. The Basin is continental climate, with drought and little rain, and its average annual precipitation is 180-220 mm. The annual precipitation changes are weakened from the north to the south. The monthly average difference of precipitation in its Yellow River irrigation area is relatively significant, and the distribution in the year is extremely uneven. The precipitations in arid area in central Ningxia and the southern mountainous areas are relatively flat during the year, showing the seasonal changes. The precipitation in the Basin is unevenly distributed during the year, and the dry and wet seasons are obvious. The precipitation in July-September accounts for 60%-70% of the annual precipitation, and its evaporation is strong, with an average annual evaporation of 1100-1600 mm. The land use types mainly include grassland and cultivated land accounting for 33.74% and 48.76% of the basin area respectively, while the forest land accounts for 3.60% of it, the water area 1.43% of it, the urban and rural industrial and mining resident land 1.46% of it, and the unused land 11.01% of it.

Theory of the SWAT model

The SWAT model has a sub-module for each link of the water cycle, and its design idea is advanced. Such design method is very beneficial for the expansion and application of the model. The model establishes an important connection between the river basin process and the land use activity. Therefore, the applicability of various decisions in the basin management can be evaluated. Different sub-models can be selected and used based on different research purposes. It is also very unique in its operation mode. The SWAT model uses a specific command code control method to control the evolution of water flow in the river network and between sub-basins.

According to the river network system, SWAT divides the studied basin into multiple sub-basins, and maintains the spatial relationship between the geographic location of basin and the sub-basin. There are different meteorological, hydrological, land use, soil, agricultural management measures and pesticide application in each sub-basin. Then, the sub-basin is divided into multiple HRUs (hydrologic response units) based on factors, such as land use type and soil type and their common attributes. The hydrologic response unit is used as the minimum hydrological simulation unit to simulate the various parts of the water cycle and its quantitative transformation relationship, and then the sedimentation, runoff and non-point source load of each hydrologic response unit in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

the sub-basin are summarized; finally, the water balance of the basin is obtained through the river network convergence calculation.

The SWAT model is essential for water balance in the basin simulation process. The hydrological simulation of the basin is divided into two parts: one is the land phase of the water cycle, which is mainly used to control the water, sediment, nutrients and pesticide amount; the other part is the calculation phase of the water cycle, which is used to define the movement of water, sand and other substances through the basin water network to the outlet part of the basin.

The SWAT model simulates the land phase of water cycle by using the following water balance expression:

$$SW_t = SW_0 + \sum_{i=1}^{t}(R_{day} - Q_{surf} - E_a - w_{seep} - Q_{gw}) \quad \text{(Eq.1)}$$

where, SW_t is the final soil water content (mm); SW_0 is the initial soil water content (mm); t is the time step (day); R_{day}, Q_{surf}, E_a, w_{seep}, Q_{gw} are respectively precipitation, surface runoff, evaporation, lateral stream flow of soil aquifer and infiltration amount, groundwater content (mm) of the i^{th} day.

The land phase of the water cycle of the SWAT model is mainly composed of meteorology, hydrology, sedimentation, vegetation growth, soil temperature, pesticides, nutrients and agricultural management, etc., while the calculation phase of the water cycle of the SWAT model is divided into two parts of the main channel and the reservoir, and mainly determines the transport movement of water, sand and other substances from the basin river network to the basin exit. The SWAT model has a number of modules that can be run separately or in combination with several of them depending on the object and content being simulated. The structural design adopted by the SWAT model is modular, which fully reflects the specific links from the beginning of precipitation to the formation of runoff in the basin.

Construction of SWAT model database

Before the simulation with the SWAT model, it is necessary to prepare relevant digital elevations, land use and soil maps and related database files to facilitate the generation of SWAT model input data sets. Considering the artificial disturbances, such as irrigation channels, drainage channels and river channels in the plain irrigation area, the SWAT model is improved in the extraction method of the ditch river network, the sub-basin and the division module of the hydrologic response unit. Specific practice includes that: according to the spatial distribution of natural river channels and artificial drainage ditch networks in the study area, the “burn-in” algorithm is used to sag the DEM. The principle of this method is to convert the digital river system and the drainage ditch network into the grid form, and its grid cell size is consistent with that of the original DEM. These grid cells are transformed into the unified coordinate system after the projection, and overlaid into the original DEM through the superposition. Keep the elevations of all grid cells in correction channel unchanged, and increase the elevations of the grid cells in non-correction channel perpendicular to the river direction by a small value, so that the elevation of the grid of the river channel is lower than the coastal elevation. Then, the modified DEM is processed again to generate a new basin

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

river network. For the artificial water distribution channel, the DEM is processed by the principle of the elevation increment superposition algorithm. To make the sub-basin of the SWAT model in the irrigation area consistent with the irrigation area covered by the dry-drainage system in the irrigation area, and to avoid that too many sub-basins generated in the process of spatial discretization result in excessive spatial data conversion and calculation, the areas covered by the backbone channel and the drainage ditch are used as the dividing standards to set reasonable minimum channel catchment area threshold to divide the sub-basin, and then to divide each sub-basin according to the consistency of land use and soil type. Later, considering different crop planting structure, the farmland is refined to form the hydrologic response unit.

DEM data

The digital elevation model is essential when the SWAT model is used for basin division, water system generation and hydrological process simulation. The most common DEM map is grid type. Using DEM data can not only calculate the slope and slope length parameters of each sub-basin, but also define the basin river network. The channel slope, slope length and width of the basin river network and other characteristics are extracted from the DEM data. The basin river network can be used to determine the quantity and distribution of the sub-basin.

Most parts of DEM are relatively smooth terrain surface models, but for the error and the existence of some special terrain, there are some concave areas on the surface of DEM, which leads to low-precision water flow direction and makes the original DEM data unable to meet the research needs. Therefore, before most of simulation experiments, the original DEM data will be filled in depression by the hydrological analysis model of ArcGis Software, and finally the non- depression DEM data meeting the research requirements will be obtained.

The high-resolution DEM data SRTM (Shuttle Radar Topography Mission) 90-meter resolution data used in this paper can be downloaded from the International Scientific Data Service Platform of the Chinese Academy of Sciences (http://datamirror.csdb.cn/index.jsp), and cropped within the scope of the study area, see *Fig. 1*.

Figure 1. *DEM Map of Ningxia Section of the Yellow River Basin*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

Land use data

Using the SWAT model to simulate water resources in the basin and the land use map are very important. Humans will not easily change the topography and soil in the basin, but the land use will be changed by humans. Therefore, it is recommended to use the latest land use map for simulation. For the projection coordinate system of the land use distribution map, in case it is different from that set in the research, the projection module Projections of ArcToolbox needs to be used for projection transformation. The categories for land use map definition should be re-classified, and based on the classification determined by the SWAT's land cover/vegetation type database and the city database. Then a lookup table in the required format is generated to determine the code of each land use type on the land use map in the SWAT model (where 4 characters are required).

The national land use map collected in this paper is in shp format. It is first cut based on the boundaries of the basin, and then converted into the Grid format. Since the land use classification system of the SWAT model is classified based on the US classification system and is different from China's land use classification system, it is necessary to reclassify the land use types and convert the code in the land use map into the one that the SWAT model could identify. Refer to the Land Use Status Classification Standard (GB/T21010-2007) and the land use attribute database of the SWAT model, and then establish the land use database for the Yellow River irrigation area. The land use types reclassified are shown in *Table 1*.

Table 1. *Reclassification Code Conversion of Land Use Types of the Basin*

<table>
<tr><th colspan="2">Original classification and coding</th><th colspan="3">Reclassification and coding</th><th rowspan="2">Area (km²)</th><th rowspan="2">Percentage of total area (%)</th></tr>
<tr><th>No.</th><th>Land use type</th><th>Coding</th><th>Type in SWAT</th><th>SWAT code</th></tr>
<tr><td>11
12</td><td>Irrigated paddy fields
Dry land</td><td>1</td><td>Agricultural Land Generic</td><td>AGRL</td><td>3750</td><td>45</td></tr>
<tr><td>21
22
23
24</td><td>Wood land
Shrubbery lands
Sparsely forested woodland
Other forest land</td><td>2</td><td>Forest-Mixe</td><td>FRST</td><td>217</td><td>2.6</td></tr>
<tr><td>31
32
33</td><td>Natural grass land
Improved grass land
Man-made grass land</td><td>3</td><td>Pasture</td><td>PAST</td><td>1117</td><td>13.4</td></tr>
<tr><td>41
42
43
46
64</td><td>River surface
Lake surface
Reservoir surfac
Beaches and flats
Wet land</td><td>4</td><td>Water</td><td>WATR</td><td>708</td><td>8.5</td></tr>
<tr><td>52
51
53</td><td>Residential quarters in rural areas
Areas of cities and town
Specially-used land</td><td>5</td><td>Urban</td><td>URBAN</td><td>393</td><td>4.7</td></tr>
<tr><td>61
62
63
65
66
67</td><td>Sandy land
Gobi
Saline-alkali land
Bare land
Exposed rock and shingle land
Others</td><td>6</td><td>Bare</td><td>BARE</td><td>2154</td><td>25.8</td></tr>
</table>

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

Soil data

The soil type charts collected in this paper is shp format. Same with the land use type, it is firstly cut based on the basin boundary, and then converted into the Grid format required for the model.

The soil types in Ningxia section of the Yellow River Basin mainly include the Light sierozems, Neo-alluvial soils, Aeolian soils, Calcic skeletal soils, Fluro-aquic soils, Wet fluro-aquic soils, Salinized fluro-aquic soils, Surface-rusted fluro-aquic soils, Cumulated There are 12 categories of irrigated fluro-aquic soils, Meadow solonchaks, Aquic irrigation silting soils, Surface-rusted irrigation silting soils, among which Light sierozems is the most widely distributed, accounting for 35.66% of the total area, followed by Aeolian soils and Neo-alluvial soils, Yellow River. The soil types and SWAT model codes of Ningxia section of the Yellow River Basin are shown in *Table 2*.

Table 2. *Soil type and code table of Ningxia section of the Yellow River Basin*

Soil type	Original code	SWAT code	Soil type involved	Area (10^4hm^2)	Proportion
Light sierozems	23113112	DHGT	Sierozems	77.2	35.66
Neo-alluvial soils	23115122	XJT	Neo-alluvial soils	36.8	17.00
Aeolian soils	23115143	FSHT	Aeolian soils	39.7	18.36
Calcic skeletal soils	23115194	GZHCGT	Skeletal soils	18.5	8.53
Fluro-aquic soils	23116141	CHT	Fluro-aquic soils	2.6	1.20
Wet fluro-aquic soils	23116144	SHCHT	Fluro-aquic soils	1.7	0.77
Salinized fluro-aquic soils	23116145	YHCHT	Fluro-aquic soils	3.8	1.76
Surface-rusted fluro-aquic soils	23116146	BXCHT	Fluro-aquic soils	1.7	0.80
Cumulated irrigated fluro-aquic soils	23116147	GYCHT	Fluro-aquic soils	3.2	1.48
Meadow solonchaks	23118101	CDYT	Solonchak	8.7	4.00
Aquic irrigation silting soils	23119113	GCHYT	Irrigation silting soils	9.2	4.25
Surface-rusted irrigation silting soils	23119114	BXGYT	Irrigation silting soils	13.4	6.19

When the SWAT model is simulated, the soil data required mainly includes two types: chemical attribute data and physical attribute data. The chemical attribute is optional data and chemical attributes of the soil are mainly used to assign the initial values to the model; the physical attribute is required, the physical attributes of the soil play an important role in water cycle of the hydrologic response unit, which determines the movement conditions of water and air in the soil profile. Before simulation, the hydrological and water conductivity attributes of various soils should be input into the SWAT model and divided into two parameter types, which are input by the soil type and soil layer. The soil particle size composition, soil layer and organic matter content used in the research can be found in Ningxia Soil. The international system standards and Kaczynski system are used for soil texture in China, and the soil particle size standard used in the SWAT model is the American system, therefore, the domestic data cannot be directly used in the SWAT model and the soil particle size should be converted from the international system to American system. The two classification criteria are shown in *Table 3* and the conversion is required. There are different methods

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

for soil texture conversion. Generally, the soil particle size distribution model in the parametric form is more suitable for general model, because it is more convenient for establishment of standard procedures and comparison and unification of particle size analysis data from the different sources. The empirical logic growth model of the two-parameter correction is used to convert the soil texture in this paper. The Levenberg-Marquardt+ general global optimization algorithm of the nonlinear fitting program in the 1STOPT software is used, and the values of the parameters *u* and *c* are obtained by the relevant regression iteration, and the content of clay, silt, sand, rock (gravel) is finally determined. Other soil parameters such as soil bulk density, effective field capacity and saturated hydraulic conductivity are calculated using the Soil-Water-Characteristics (SWCT) module in the soil water properties software SPAW developed by Washington State University. The soil erosivity factor can be calculated based on the known soil organic carbon and particle composition data and by using the soil erodibility factor K estimation method in the EPIC model.

Table 3. *Soil particle size classification*

International system		**American system**	
Particle size range (mm)	**Name**	**Particle size range (mm)**	**Name**
>2	Rock	>2	Rock
0.2-2	Coarse sand	0.05-2	Sand
0.02-0.2	Fine sand	0.002-0.05	Silt
0.002-0.02	Silt	<0.002	Clay
<0.002	Clay		

Weather data

The daily weather data required for SWAT model stimulation includes the following: maximum temperature, minimum temperature, rainfall, wind speed, solar radiation and relative humidity etc. If the measured data is partially missing, the SWAT model defines the weather generator used to supplement the missing data. The weather generator requires input of meteorological data related to the basin and monthly meteorological data for at least 20 years, mainly including monthly average maximum temperature (°C), monthly average minimum temperature (°C), maximum temperature standard deviation, minimum temperature standard deviation, monthly average rainfall (mm), monthly average rainfall standard deviation, rainfall skewness coefficient, monthly number of dry days (dry d), monthly number of wet days (wet d), average rain days (d), dew point temperature (°C), monthly average solar radiation [KJ/(m^2.d)], monthly average wind speed (m/s) and maximum half-hour rainfall (mm).

The daily observation data of Ningxia Zhongning, Yinchuan, Huinong and Taole for 23 consecutive years from 1995 to 2017 is used in this paper, including daily maximum/minimum temperature, rainfall, wind speed, relative humidity, etc. The above data is processed in .dbf format required for SWAT model. If the weather generator is used to calculate the parameters one by one, the workload is complicated; however, the simple and easy-to-operate calculation program SwatWeather.exe saves a lot of working time for the model users. As long as the files are input in required format, the required data can be calculated and saved according to the prompts.

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DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

Division of hydrological response unit

When the SWAT model was used for simulation in the past, the hydrological response units were divided basically based on natural basins; however, the irrigation network in Ningxia section of Yellow River Basin was completely completed by artificial canals and the irrigation districts along Yellow River currently have 17 large and medium-sized diversion main canals and main canals. In this paper, based on the actual situation of the Ningxia section of the Yellow River Basin, the artificial canal network is used as the water system to divide the Ningxia section of the Yellow River Basin into several hydrological response units based on the division principles of hydrological response units.

Results

The SWAT model needs to perform the sensitivity analysis according to the actual situation of the research area, and analyze and judge which input parameter values are sensitive to the output results. The analog value of the model is close to the measured value by adjusting these parameter values. The SWAT model parameter analysis module is used to continuously adjust the parameters in the model. The adjustment process should analyze and determine which parameters are sensitive to the results and the degree of influence on the simulation results based on the actual situation and the parameter thresholds, which will make the simulation more accurate. After the sensitivity analysis on the model input parameters, the measured series data is subsequently divided into two parts, one part is used for model parameter calibration and the other part is used for model validation. After completing the model parameter calibration, the measured series data and simulated data are analyzed and compared to judge whether the simulation accuracy meets the requirements; the model applicability and reliability is validated, if the simulation value meets the requirements, the model can be used for hydrological simulation of the Ningxia section of the Yellow River Basin. In the basin simulation, the parameters sensitive to the runoff simulation results include the soil evaporation compensation coefficient $ESCO$, the number of runoff curves CN_2 and the available water amount in the soil.

In the SWAT model simulation process, the relative error R_e, correlation coefficient R^2 and Nash-Suttcliffe coefficient Ens are generally selected to evaluate the model applicability. The relative error calculation formula is as below:

$$R_e = \frac{Q_p - Q_0}{Q_0} \times 100\% \qquad \text{(Eq.2)}$$

where R_e represents the relative error of model simulation; Q_0 represents the measured value; Q_p represents the stimulated value. If $R_e > 0$, it indicates that the model simulation value or predicted value is too large; if $R_e < 0$, it indicates that the model simulation value or predicted value is too small; if $R_e = 0$, it indicates that the simulation result matches with the measured value. The correlation coefficient R^2 is obtained by using the linear regression method in Excel. If $R^2 = 1$, it indicates that the results are very matched; when $R^2 < 1$, the smaller the value, the lower the degree of

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data matching.

The calculation formula of Nash-Suttcliffe coefficient Ens is as below:

$$Ens = 1 - \frac{\sum_{i=1}^{n}(Q_0 - Q_p)^2}{\sum_{i=1}^{n}(Q_0 - Q_{avg})^2} \quad \text{(Eq.3)}$$

where Q_0 is the measured value; Q_p is the stimulated value; Q_{avg} is the measured average value; n is the number of measured data. When $Q_0 = Q_p$, it indicates that the measured value is well matched with the stimulated value; if $Ens < 0$, it indicates that the model simulation average value has a lower reliability than directly using the measured average value.

Model parameter calibration

The SWAT model parameter calibration can be conducted by using the "Brute Force" method in two stages. In the first stage, the step size to be calibrated is estimated and the parameter value is appropriately adjusted based on the estimated step size and within the value range, subsequently the parameter is calibrated and the parameter optimal value range is determined by calculating and evaluating the system values; in the second stage, the initially estimated parameter adjustment step size is further refined. As some variables have the great impacts on the results at the initial model operation period, for example, the initial value of soil water content is zero, which has a great impact on the simulation results. Therefore, for this case, the initial simulation period is used as the model operation start period to reasonably estimate the initial variables of the model. The digital filtering technique is used to divide the measured total runoff into the base flow and the direct runoff. The difference between the model simulation value and measured value and its percentage with the measured average value (relative error) should be within the specified range and the evaluation coefficient R^2 and Ens of the monthly average stimulated value should also meet the specified accuracy standards. If the measured value and stimulated values meet the calibration requirements, but R^2 and Ens do not meet the requirements, the inspection should be conducted and the full consideration should be taken on spatial non-uniformity of simulated rainfall and correct stimulation of plant growing season. For the runoff parameter calibration, the annual average error between the simulated and measured values should be less than 20% of the measured value, and the evaluation coefficient of the monthly average value is $R^2 > 0.6$ and $Ens > 0.5$; after the base flow parameter calibration, the same evaluation method is used to the surface runoff for parameter calibration. Considering that the total runoff parameter adjustment will affect the base flow, the base flow should be re-inspected continuously in the adjustment process.

In this paper, the measured monthly runoff data of Qingtongxia Hydrological Station and Shizuishan Hydrological Station from 2005 to 2011 are used for model parameters adjustment. *Figs. 2 and 3* show the comparison between the measured and simulated values of the monthly runoff of the Qingtongxia Hydrological Station and the Shizuishan Hydrological Station in the calibration period.

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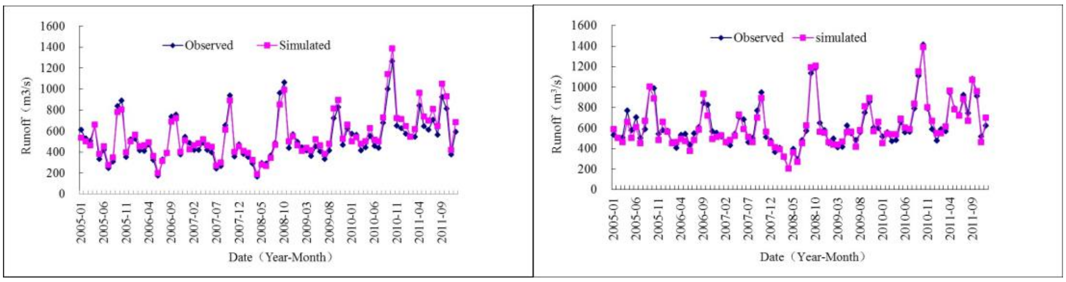
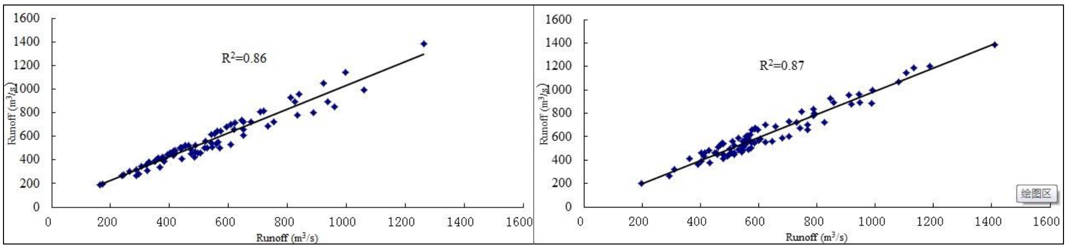


a, Qingtongxia Station *b, Shizuishan Station*

Figure 2. *Comparison of stimulated and observed monthly runoff for the calibration period*

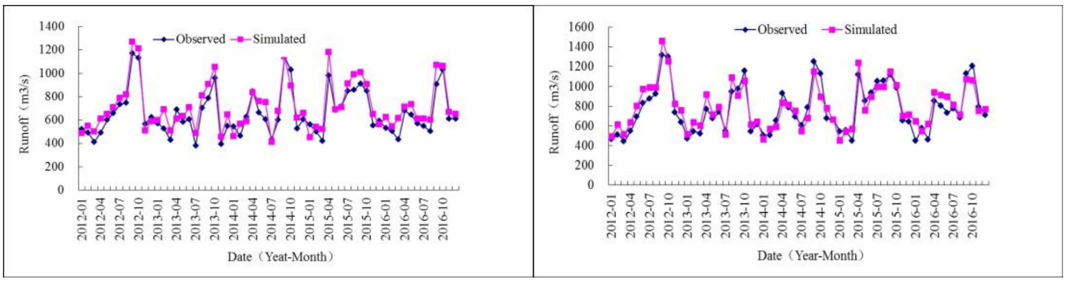


a, Qingtongxia Station *b, Shizuishan Station*

Figure 3. *Scatter diagram of stimulated and observed monthly runoff for the calibration period*

Model validation

The observed monthly runoff data of Qingtongxia Hydrological Station and Shizuishan Hydrological Station from 2012 to 2017 are used for model validation, the parameters obtained in the model parameter calibration process are used and the relative error R_e, correlation coefficient R^2 and Nash-Suttcliffe coefficient Ens are used to evaluate the model validation results. *Figs. 4 and 5* show the fitting comparison of the observed values and simulated values of monthly runoff of Qingtongxia Hydrological Station and the Shizuishan Hydrological Station in the validation period.



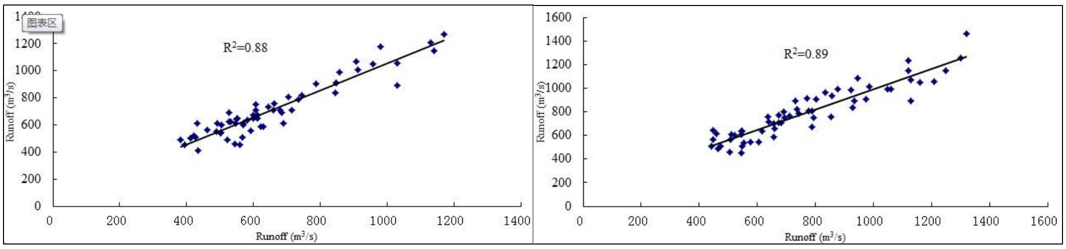
a, Qingtongxia Station *b, Shizuishan Station*

Figure 4. *Comparison of stimulated and observed monthly runoff for the validation period*

The relative error R_e is between -13.23% and 14.67%; the monthly average runoff correlation coefficient R^2 and The Nash-Suttcliffe coefficient Ens are 0.88 and 0.86 respectively. For the fitting comparison of the observed values and simulated values of monthly runoff of the Shizuishan Hydrological Station, the relative error R_e is between

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

-12.09% and 13.97%; the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are 0.89 and 0.85 respectively. The simulated and observed values of the model have a small difference and meet the simulation requirements.



a, Qingtongxia Station *b, Shizuishan Station*

Figure 5. *Scatter diagram of stimulated and observed monthly runoff for the validation period*

Discussion

In the calibration period, as can be known from the *Figs. 2 and 3*, the simulated and measured values of the Qingtongxia Hydrological Station are approximately in the same change trend, the peak value has the higher matching degree and the relative error R_e is between -12.98% and 14.58%; the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are 0.86 and 0.83 respectively. The change trend of simulated value and measured value of the monthly runoff of Shizuishan Hydrological Station is well matched, the peak value has a higher marching degree and the relative error R_e is between -11.98% and 13.14%; the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are respectively 0.87 and 0.84. Both meet the simulation evaluation requirements.

In the validation period, for the fitting comparison of the observed values and simulated values of monthly runoff of the Qingtongxia Hydrological Station, the relative error R_e is between -13.23% and 14.67%; the monthly average runoff correlation coefficient R^2 and The Nash-Suttcliffe coefficient Ens are 0.88 and 0.86 respectively. For the fitting comparison of the observed values and simulated values of monthly runoff of the Shizuishan Hydrological Station, the relative error R_e is between -12.09% and 13.97%; the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are 0.89 and 0.85 respectively. The simulated and observed values of the model have a small difference and meet the simulation requirements.

Conclusion

In this paper, the GIS software is used for to cutting, projection transformation and reclassification of DEM, land use and soil data for Ningxia Yellow River Irrigation District, and convert these data formats into Grid format required for the model; based on the actual situation of Ningxia Yellow River Irrigation District, a attribute database such as meteorological, hydrological, and soil physical attributes required for the SWAT model is established. The constructed SWAT model is used for hydrological

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

simulation of Ningxia section of the Yellow River Basin, the observed monthly runoff data of Qingtongxia Hydrological Station and Shizuishan Hydrological Station from 2005 to 2017 are used for model parameters sensitivity analysis, calibration and validation and continuous adjustment, the relative error R_e, correlation coefficient R^2 and Nash-Suttcliffe coefficient Ens are used for model applicability evaluation. The results show that the observed values and stimulated values of monthly runoff of Qingtongxia Hydrological Station and Shizuishan Hydrological Station in the calibration period are approximately in the same change trend, the peak value has the higher matching degree, for Qingtongxia Hydrological Station, the relative error R_e is between -12.98% and 14.58%, the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are 0.86 and 0.83 respectively. For Shizuishan Hydrological Station, the relative error R_e is between -11.98% and 13.14% and the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are respectively 0.87 and 0.84. The observed values and stimulated values of monthly runoff of Qingtongxia Hydrological Station and Shizuishan Hydrological Station in the validation period have the small difference, for Qingtongxia Hydrological Station, the relative error R_e is between -13.23% and 14.67% and the monthly average runoff correlation coefficient R^2 and The Nash-Suttcliffe coefficient Ens are 0.88 and 0.86 respectively. For Shizuishan Hydrological Station, the relative error R_e is between -12.09% and 13.97% and the monthly average runoff correlation coefficient R^2 and the Nash-Suttcliffe coefficient Ens are 0.89 and 0.85 respectively. The simulated and observred values of the model have a small difference and meet the simulation requirements. The constructed SWAT model is suitable for the hydrological process simulation of the Ningxia Yellow River Irrigation District, which lays the foundation for the subsequent water supply prediction.

Acknowledgements. The study was completed with the support of the First-class Major Foundation of Ningxia Institutions of High Education in China. (NXYLXK2017A03).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34833497

EMPIRICAL STUDY ON RECREATION SUITABILITY EVALUATION OF SUBURBAN FOREST PARK – A CASE STUDY OF BAIYUN FOREST PARK IN LISHUI CITY, CHINA

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(Received 12th Oct 2018; accepted 16th Jan 2019)

Abstract. Nowadays, suburban forest recreation is one of the most important forms of daily leisure activities of urban residents; therefore, it is of great significance to study the recreation suitability of suburban forest parks. On the basis of previous research results and in combination with the recreation suitability characteristics of the present suburban forest park, this paper uses the analytic hierarchy process (AHP) and the expert scoring method to build a suitability evaluation model. The results show that the model includes three criteria layers, 7 first-level index layers and 31 second-level index layers of resource conditions, basic conditions, and development and utilization conditions. In the distribution of index weight, that of resource conditions is the highest, followed by basic conditions and development and utilization conditions. Taking the Baiyun Forest Park in Lishui City, China, as an example, the scoring criteria of fuzzy comprehensive evaluation are adopted to evaluate its recreation suitability. The evaluation results show that the overall evaluation of recreation suitability of Baiyun Forest Park reaches the level of "good" and has a strong recreational nature. "Forest Resource", "Environment Quality" and "Location Condition" are rated excellent, while "human resources" and "supporting facilities" are rated average. Suggestions on developing forest natural resources, protecting the environment, earning positive cultural connotations, improving infrastructure and defining the tourist source market to enhance the suitability of recreation in parks are put forward on the basis of the evaluation result.
Keywords: *forest park, recreation suitability, evaluation index system, analytic hierarchy process (AHP), fuzzy comprehensive evaluation method, Lishui City in China*

Introduction

Recreation, a kind of entertainment method which allows people in modern society to relax themselves both physically and psychologically, refers to the leisure activities which individuals or groups take part in to feel pleased and satisfied in their spare time (Zhang, 2001) and the effect of the relaxation is amplified, when people can establish a positive connection to nature (Park et al., 2015). Forest recreation has become a tendency and fashion of modern ecological leisure activities. A forest park, due to its ecological system, mainly provides extraordinary environmental resources, health and fitness protection functions, sightseeing, recreation, sports and positive experiences to promote the soundness of body and mind for tourists. Recently, with the advance of industrialization and urbanization, many problems arise in human settlements. The suburban forest park is not only an important part of the urban ecological system, which provides ecological benefits and a pleasant landscape, but also promotes healthiness and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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fitness for people's body and mind while serving as a recreational site for the residents. Therefore, more and more cities have begun to construct suburban forest parks as "living" ecological systems (Wang et al., 2017) and the suburban forest park has become an important commodity for urban residents to form a healthy and civilized lifestyle.

With the recreation function of the suburban forest park gradually becoming the research focus, many scholars have done research and evaluation on the forest's recreation. America, as the most developed country in the aspect of forest recreation industry in the world at present, is the center of this research field, and the special research institutes are set up and the relevant managerial laws and regulations are formulated there (Qi et al., 1995). The foreign scholars think that the forest recreation is a summary of all the outdoor recreation forms which happen in the forest area, make statements about the planning, management, scientific research and selection of recreation location etc., learn about the tourists' needs about the urban forest recreation and the types of recreation activities and propose the suggestions on how to build a healthier and more comfortable environment (Douglas, 2000; Hunter, 2001). In recent years, some foreign scholars started from the forest's recreational value and did research on the leisure and entertainment value the forest can provide through the space analysis method (Baerenklau et al., 2010; Bestard and Font, 2010). Other foreign scholars analyzed the forest's spatial heterogeneity and the heterogeneity of tourists' preference through the research on the forest's property (Abildtrup et al., 2013; Termansen et al., 2013). The research into the forest recreation in China started a little late in early 1980s and afterwards, the construction and theoretical research into the forest recreation are gradually promoted (Liao, 2003). The State Forestry Administration of PRC has issued a series of laws and regulations successively to ensure the development of suburban forest parks (Chen and Wang, 1999). The scholars also do the research into the forest recreation from different aspects and angles. The research contents include: (1) The concept, characteristics and evaluation methods of forest recreation and so on (Chen and Shen, 2000); (2) The forest environment, animals and plants, and waters etc. such as monitoring the effect of the anion density and atmosphere quality inside the recreation area (He et al., 2015; Cai and Gu, 2005); (3) The forest's recreation value etc. (Yang and Zhang, 2010). To analyze the level of all the aspects of suburban forest park comprehensively and accurately in the research, all the dynamic factors inside the park need monitoring and the comprehensive evaluation model such as the principal component analysis method (PCA), clustering analysis method, fuzzy comprehensive evaluation method and landscape visual evaluation method are used to make objective analysis (Wang et al., 2017; Qi et al., 2015).

There needs the further research on how to evaluate and analyze the recreation suitability of suburban forest park. The Baiyun Forest Park in Lishui City, China belongs to the typical suburban forest park with outstanding geographic position and good recreation conditions. The paper chooses the Baiyun Forest Park of Lishui as the empirical research subject for analysis and combines the expert scoring method, AHP and FCE together to evaluate and discuss the recreation suitability of suburban forest park. It aims to provide the beneficial reference for the development of leisure recreation function of the suburban forest park and offer some guidance for further improving and promoting the recreation activities of the suburban forest park.

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Materials and methods

General description and data source of research area

Baiyun Forest Park is 2 km away from the northern part of Lishui urban area in the mountain area of Southern Zhejiang and middle reaches of Oujiang River and is bounded by the urban area with superior location and convenient traffic. It mainly serves the residents living in and around the city with the total area of 26.71 km^2. The geographic coordinates are east longitude 119°52'~119°58' and northern latitude 28°23'~28°27'.

The hilly area in the park belongs to Xianxia Ridge remaining vein which rise amid permanent peaks with ups and downs. The highest altitude is 1073.2 m. The geotectonic element of the park area belongs to Southern-east Zhejiang belt of folded strata which is mainly Longquan–Suichang broken rock with partial distribution of Cretaceous red sandstone and granite. There are a dozen of streams in the park which belong to Oujiang water system and among them, the largest one is Liyang pit and its total length is 9.5 km. Besides, its length in the park is 5.3 km and the drop in elevation is 520 m. The climate is mid semi-tropical monsoon climate with four distinctive dry and wet seasons as well as abundant rainfall. The average yearly temperature is 18.1 °C, the highest temperature of the year is 38 °C (the extreme highest temperature in the urban area is 43.2 °C) and the lowest temperature of the year is -7.7 °C (the extreme lowest temperature of Taishan is -14 °C). The forest in the park is covered with lush vegetation and rich vegetation types and the coverage rate reaches 97.2%. Besides the abundant natural resources, there are also profound human history and religious beliefs in the park.

The research data mainly come from the field investigation of Baiyun Forest Park with the method of recording, consulting the material on the Internet and questionnaire. First, do the field investigation in the park to have a rough understanding of the research area's topography, water system, landscape characteristics, vegetation planting and animals and plants resources etc. and then do the detailed survey and record the plant types, quantity, road condition and infrastructure condition etc. inside the research area in detail. Meanwhile, hand out the questionnaire to investigate the tourists' feelings about the park's recreation suitability. Lastly, sort out the field investigation condition and collected data and make analysis for the further research. The statistics in this paper come from the relative news website such as Lishui Statistics Information Network (http://tjj.lishui.gov.cn/sjjw/tjnj/201811/t20181119_3477511.htm; http://www.lsnews.com.cn/images/gg_banmian/lyp_baiyunshang090915/gg_bys090915.html).

Research method

Expert scoring method

Expert Scoring Method, also called "Delphi Method", refers to judging the current status and future trend of events by collecting experts' opinions and now it has been widely used in different subjects and organizations (Loo, 2002). The paper, on the basis of referring to a lot of research results about the related evaluation system and doing the field investigation, combines the own characteristics of suburban forest park and the conditions suitable for the recreation, summarizes the various analysis and research, concludes the primary index system of affecting the recreation suitability of suburban

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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forest park and makes the questionnaire for 30 experts from related fields to judge and score. The related fields refer to the forest recuperation, forest health care-based tourism, ecotourism, town and country planning, and garden landscape. Experts are asked to assign the value to the importance “Score 1 for the Unimportant, Score 2 for the Less Important, Score 3 for the Important, Score 4 for the More Important, Score 5 for the Most Important” and screen the primary index. Through the multiple rounds of experts scoring and after consulting experts’ comments, accepting the feedback and making adjustments multiple times, the index factors are screened, deleted and revised to perfect the whole index system and confirm the final evaluation index system (*Table 1*). The system is divided into the target layer, criterion layer, seven first-level index layers and 31 second-level index layers.

Table 1. *Evaluation system for recreation suitability of suburban forest park*

Target layer	Criterion layer	First-level index	SN	Second-level index
Recreation suitability evaluation of Baiyun Forest Park (A)	Resource condition (B1)	Forest resource (C1)	D1	Forest coverage rate
			D2	Forest impressions
			D3	Forest stand types
			D4	Forest canopy density
		Landscape resource (C2)	D5	Landscape diversity
			D6	Landscape attraction degree
			D7	Landscape rarity
			D8	Landscape configuration
			D9	Wild animals
		Human resources (C3)	D10	Human landscape
			D11	Cultural relics
			D12	Folk customs
			D13	Science education value
	Basic condition (B2)	Environment quality (C4)	D14	Air quality
			D15	Negative ion content
			D16	Surface water quality
			D17	Climate comfort level
			D18	Sanitary condition
			D19	Noise condition
			D20	Environment bearing capacity
		Supporting facilities (C5)	D21	Perfectness
			D22	Safety
			D23	Suitability
			D24	Humanization
	Development and utilization condition (B3)	Location condition (C6)	D25	Geographic position
			D26	Regional economic level
			D27	Tourist source condition
			D28	Publicity degree
		Traffic condition (C7)	D29	External traffic system
			D30	Internal traffic system
			D31	Parking lot

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DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

Analytic hierarchy process

Analytic Hierarchy Process (AHP), which is proposed by T. L. Saaty, an American operational research expert in the early 1970s, is a systematic and hierarchy analysis method of confirming the weight factor by combining the qualitative and quantitative together (Chiclana et al., 1998). The method is applied to multiple research fields, such as commercial investment analysis (Aragonés-Beltrán et al., 2014), exploration planning (Rahmati et al., 2015) and multi-criteria decision analysis (Xu and Liao, 2014). The paper determines the evaluation index factor weight of suburban forest park recreation suitability with AHP.

Build the structure model

The research confirms the index factor after taking a lot of research data in the literature, the comments from the related experts and field investigation into consideration, unfolds the evaluation system of suburban forest park recreation suitability with Analytic Hierarchy Process (AHP) level by level and forms a multi-layer and multidimensional index system with 4 layers and 31 evaluation index factors, as shown in *Figure 1*.

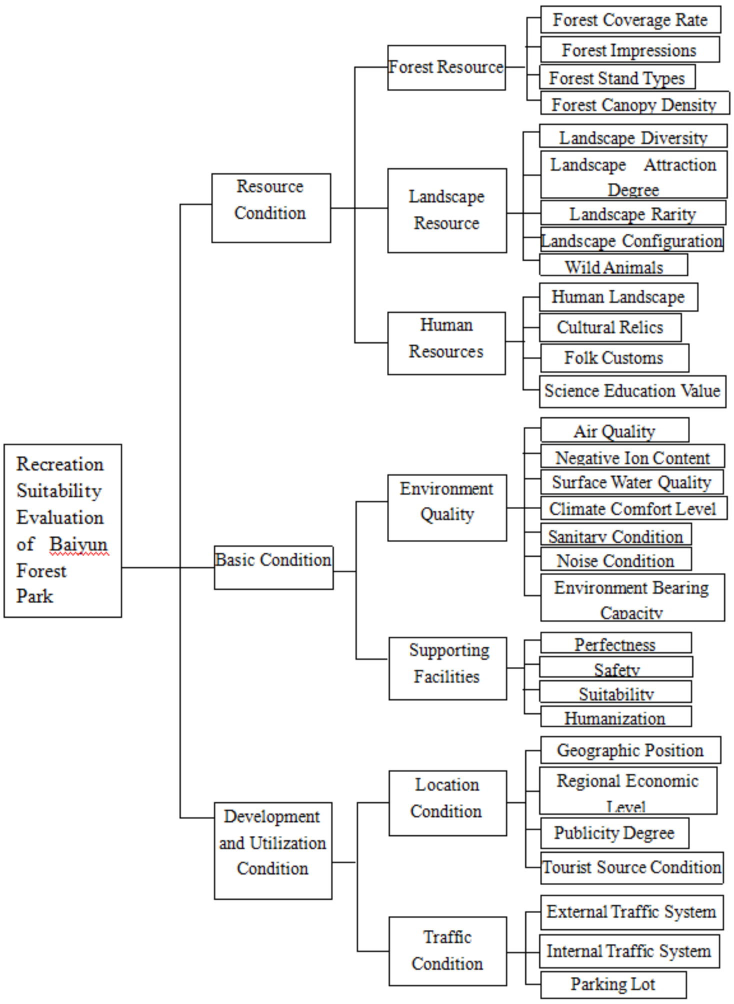

***Figure 1.** Hierarchical structure model of recreation suitability evaluation of suburban forest park*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

Build the judgement matrix

The judgement matrix, which is built based on the above established evaluation model, is a prerequisite of doing the weight calculation and an important reference standard for sequencing. When building the judgement matrix, collect the related experts' comments about the index importance by the 1-9 proportional scaling method, get the average value of the obtained experts' valuation and build the judgement matrix initially and at the same time, make appropriate adjustments by referring to the research results of related fields at home and abroad. Lastly, the judgement matrix in the paper is built after combining the scores in the questionnaire and the related research.

Weight value calculation and conformity verification

After building the judgement matrix of each index, calculate the weight value. In this paper, the square root method is used to calculate the maximum characteristic root λ_{max}, obtained by *Equation 1,* in which W is the characteristic vector corresponded with λ_{max}. The characteristic vector is gained by the normalization processing of the vector. To get the rational and effective weight value, do the consistency verification test to the judgement matrix. Random consistency ratio *CR* is obtained by *Equation 2* and *CI* can be obtained by *Equation 3,* in which n is judgement matrix order and RI is average random consistency ratio. When $CR = 0$, the judgement matrix has the complete consistency; When $0 < CR < 0.1$, the judgement matrix conforms to the consistency requirement; When $CR \geq 0.1$, the consistency of the judgement matrix is not acceptable and all the factors of the matrix need adjusting again until it meets the requirement of consistency verification (Liu, 2012).

$$AW = \lambda_{\max} W \tag{Eq.1}$$

$$CR = \frac{CI}{RI} \tag{Eq.2}$$

$$CI = \frac{\lambda_{\max} - n}{n-1} \tag{Eq.3}$$

Do the normalization processing to the weight of all the index factors according to the above method, get the weight value of each index through calculation and make the importance sequencing, namely, overall ranking (*Table 2*).

Fuzzy comprehensive evaluation method

The fuzzy comprehensive evaluation method is used to do the quantitative comprehensive evaluation to the recreation suitability of Baiyun Forest Park and the final score obtained in this way will determine the recreation suitability of Baiyun Forest Park. It is highly scientific and reliable to use the fuzzy comprehensive evaluation method, which combines the fuzzy theory and math model (Jiao et al., 2016) and does a quantitative analysis to the fuzzy object so as to get a comparatively scientific result (Ye, 2010). First, build a fuzzy comprehensive evaluation set of evaluation factors and then the judgement set V = (V1, V2, V3...Vm). Usually, the fuzzy five-point scale scoring system is used and it is classified into five levels: V1

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

stands for “Excellent”, V2 for “Good”, V3 for “Average”, V4 for “Bad” and V5 for “Very Bad” (Wang et al., 2017; Xue, 2011). The fuzzy scoring standard of each index factor in the evaluation system is made in accordance with the above requirement, as shown in *Table 3*.

Table 2. *Evaluation index weight of recreation suitability of suburban forest park*

Target layer	Criterion layer	Weight	Factor layer	Weight	Index layer	Weight	Sequencing
Recreation suitability evaluation of suburban forest park A	Resource condition B1	0.625	Forest resource C1	0.405	Forest coverage rate D1	0.211	1
					Forest impressions D2	0.082	2
					Forest stand types D3	0.081	3
					Forest canopy density D4	0.031	11
			Landscape resource C2	0.144	Landscape diversity D5	0.026	14
					Landscape attraction degree D6	0.047	6
					Landscape rarity D7	0.033	9
					Landscape configuration D8	0.025	15
					Wild animals D9	0.013	21
			Human resources C3	0.076	Human landscape D10	0.020	18
					Cultural relics D11	0.039	7
					Folk customs D12	0.009	25
					Science education value D13	0.006	28
	Basic condition B2	0.238	Environment quality C4	0.179	Air quality D14	0.056	5
					Negative ion content D15	0.034	8
					Surface water quality D16	0.014	20
					Climate comfort level D17	0.028	12
					Sanitary condition D18	0.012	22
					Noise condition D19	0.032	10
					Environment bearing capacity D20	0.008	26
			Supporting facilities C5	0.060	Perfectness D21	0.023	17
					Safety D22	0.024	16
					Suitability D23	0.005	29
					Humanization D24	0.007	27
	Development and utilization condition B3	0.137	Location condition C6	0.034	Geographic position D25	0.017	19
					Regional economic level D26	0.004	30
					Tourist source condition D27	0.010	24
					Publicity degree D28	0.003	31
			Traffic condition C7	0.103	External traffic system D29	0.066	4
					Internal traffic system D30	0.027	13
					Parking lot D31	0.011	23

After building the factor set, quantify every factor of the evaluation subject. According to the evaluation standard, design the questionnaire to investigate the tourists and the related working staffs of Baiyun Forest Park and carry out a statistical analysis on the investigation results to get the fuzzy relationship judgement matrix:

$$R=\begin{Bmatrix} r_{11} & r_{12} & \cdots & r_{1m} \\ r_{21} & r_{22} & \cdots & r_{2m} \\ \cdots & \cdots & \cdots & \cdots \\ r_{n1} & r_{n2} & \cdots & r_{nm} \end{Bmatrix}.$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

Then get the factor weight W = (W_1, W_2, W_3...W_m) according to the weight value of each index confirmed by the AHP, work out the comprehensive judgement result in accordance with *Equation 4* and then gain the final evaluation level according to the maximum membership principle.

$$Vi = Wi * Ri \quad \text{(Eq.4)}$$

Table 3. *Fuzzy scoring criteria for evaluation factors of recreation suitability of Baiyun Forest Park*

Target layer	Data source	Index score				
		5	4	3	2	1
Forest coverage rate	Statistical data	≥90%	80%≤X<90%	70%≤X<80%	60%≤X<70%	<60%
Forest impressions	Questionnaire	More beautiful	Beautiful	Average	Bad	Very bad
Forest stand types	Statistical data	More	Many	Average	Few	Less
Forest canopy density	Questionnaire	Higher	High	Average	Low	Lower
Landscape diversity	Questionnaire	Higher	High	Average	Low	Lower
Landscape attraction degree	Questionnaire	Higher	High	Average	Low	Lower
Landscape rarity	Questionnaire	Higher	High	Average	Low	Lower
Landscape configuration	Questionnaire	More beautiful	Beautiful	Average	Bad	Very bad
Wild animals	Statistical data	≥50 Types 种	40≤X<50	30≤X<40	20≤X<30	<20
Human landscape	Questionnaire	More beautiful	Beautiful	Average	Bad	Very bad
Cultural relics	Statistical data	National level with huge influence	Provincial level with comparatively big influence	Municipal level with average influence	Not in the protection list with small influence	With no influence
Folk customs	Questionnaire	Much dense	Comparatively dense	Average	Thin	Thinner
Science education value	Questionnaire	Higher	High	Average	Low	Lower
Air quality	Questionnaire	Excellent	Good	Average	Poor	Poorer
Negative ion content	Statistical data	≥12000	8000≤X<12000	5000≤X<8000	2000≤X<5000	<2000
Surface water quality	Statistical data	I Type	II Type	III Type	IV Type	V Type
Climate comfort level	Statistical data	≥300 Days	250≤X<300	150≤X<250	100≤X<150	<100
Sanitary condition	Questionnaire	Excellent	Good	Average	Bad	Very bad
Noise condition	Statistical data	≤40 dB	40≤X<50	50≤X<60	60≤X<70	≥70
Environment bearing capacity	Statistical data	≥5000 People	4000≤X<5000	3000≤X<4000	2000≤X<3000	<2000
Facility perfectness	Questionnaire	Excellent	Good	Average	Bad	Very bad
Facility safety	Questionnaire	Excellent	Good	Average	Bad	Very bad
Facility suitability	Questionnaire	Excellent	Good	Average	Bad	Very bad
Facility humanization	Questionnaire	Excellent	Good	Average	Bad	Very bad
Geographic position	statistical data	The distance between it and the central city is ≤10 km	10<X≤30	30<X≤50	50<X≤70	>70
Service facility	Questionnaire	Excellent	Good	Average	Bad	Very bad
Publicity degree	Questionnaire	Bigger	Big	Average	Small	Smaller
Tourist source condition	Statistical data	Richer	Rich	Average	Not rich	Poor
External traffic system	Statistical data	There is direct public transportation and it is much convenient to get there	Comparatively convenient	Average	There is difficulty in arriving there directly and it is inconvenient	Terrible traffic conditions
Internal traffic system	Questionnaire	Excellent	Good	Average	Bad	Very bad
Parking lot	Questionnaire	More	Many	Average	Few	Less

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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To show the recreation suitability of Baiyun Forest Park more clearly and directly, it is necessary to figure out the final scores of each index factor and the whole respectively of recreation suitability. First, assign the value $V_1 = 5, V_2 = 4, V_3 = 3, V_4 = 2, V_5 = 1$ to each judgement set in the $V = \{V_1, V_2, V_3, V_4, V_5\}$ respectively and get the scores of each factor according to *Equation 5*.

$$W_A = \sum_{k=1}^{n} B_{Ak} V_k \qquad \text{(Eq.5)}$$

in which, B_{Ak} (k = 1,2,3,4,5) is the kth membership of B_A.

Results and analysis

Analysis on the suburban forest park index factor weight

Through the above calculation and statistical analysis, it can be concluded that in the evaluation system of suburban forest park recreation suitability, the total weight value of criterion layer B is 1. The result shows that the sequence of the three index factors in the criterion layer is resource condition (0.625) > basic condition (0.238) > development and utilization condition (0.137) and the resource condition accounts for most, which indicates that in the suburban forest park recreation, the resource condition is the most important and it is also an essential factor of attracting the tourists. Whether the forest park has the suitable condition for the tourists to have recreation largely depends on whether it has the excellent resource condition or not. The basic condition and the development and utilization condition rank second and third and the weight difference between the two is not big, which means that in the recreation suitability, there is a lot of upside potential for the basic condition and the development and utilization condition, which also are the indispensable condition in the suburban forest park recreation suitability evaluation system.

In the criterion layer, the importance order of index factor in the resource condition (B1) is forest resource (0.405) > landscape resource (0.144) > human resources (0.076), and the forest resource accounts for a large part, which indicates that in the suburban forest park resource condition, the forest resource is the most important, next is the landscape resource, which is followed by human resources. This shows that in the forest park, the natural and ecological forest resource can attract a lot of tourists to come here for recreation and the human resources have the smallest influence in the forest park. In the forest resource, the weight value (0.211) of forest coverage rate is the largest index weight and also the factor whose weight ranks first in the whole evaluation index system. This reflects that the higher forest coverage rate in the suburban forest park is of great significance to attract the tourists.

In the basic condition (B2), the importance order of index factor is environment quality (0.170) > supporting facilities (0.060). For the suburban forest park, the outstanding environment quality still appeals to many tourists, which directly affects the recreation suitability. Since the environment quality has a big influence on the people's body and mind and nowadays people have a preference for the health care-based tourism, the weight ratio of forest park environment quality is comparatively big and that of the supporting facilities is relatively small. In the development and utilization condition (B3), the importance order of index factor is traffic condition (0.103) >

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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location condition (0.034). The suburban forest park needs to have the superior geographic position. The traffic condition has the direct limitation and influence on the number of tourists, so the traffic condition is of great importance to the park recreation. Besides, the location condition is also important, because if the suburban forest park is in the remote and inaccessible area, there will be fewer tourists, it will be not suitable for recreation and it is also hard to give a full play to its original value.

Analysis on the recreation suitability of Baiyun Forest Park

The calculation result obtained according to the fuzzy comprehensive evaluation method is the final score of Baiyun Forest Park recreation suitability. The evaluation level can be gained according to the corresponding evaluation index factor. From the above evaluation result, get all the levels of Lishui Baiyun Forest Park recreation suitability and the specific evaluation level of each evaluation factor, as shown in *Table 4*.

Table 4. *Summary of fuzzy comprehensive evaluation results of Baiyun Forest Park*

Target layer	Evaluation score	Criterion layer	Evaluation score	First level index	Evaluation score	Second level index	Evaluation score
Recreation suitability evaluation of Baiyun Forest Park A	3.809	Resource condition B1	3.842	Forest resource C1	4.104	Forest coverage rate D1	4.32
						Forest impressions D2	4.27
						Forest stand types D3	3.74
						Forest canopy density D4	3.28
				Landscape resource C2	3.509	Landscape diversity D5	4.46
						Landscape attraction degree D6	4.04
						Landscape rarity D7	3.03
						Landscape configuration D8	3.45
						Wild animals D9	1.90
				Human resources C3	3.166	Human landscape D10	3.41
						Cultural relics D11	3.11
						Folk customs D12	2.35
						Science education value D13	3.52
		Basic condition B2	3.967	Environment quality C4	3.992	Air quality D14	4.58
						Negative ion content D15	4.61
						Surface water quality D16	3.08
						Climate comfort level D17	4.05
						Sanitary condition D18	4.39
						Noise condition D19	4.39
						Environment bearing capacity D20	4.12
				Supporting facilities C5	3.091	Perfectness D21	3.04
						Safety D22	2.86
						Suitability D23	3.65
						Humanization D24	3.57
		Development and utilization condition B3	3.735	Location condition C6	3.745	Geographic position D25	4.08
						Regional economic level D26	3.91
						Tourist source condition D27	3.56
						Publicity degree D28	2.34
				Traffic condition C7	3.736	External traffic system D29	3.98
						Internal traffic system D30	3.45
						Parking lot D31	2.97

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

It is concluded from the evaluation result that the final score of Baiyun Forest Park recreation suitability is 3.809 and the whole evaluation result is “Good”. Based on the divided evaluation set score, the recreation suitability of Baiyun Forest Park belongs to the good level with strong recreation suitability. The evaluation result complies with the actual fact of the park. In the criterion layer, the basic condition is rated as “Excellent” and the resource condition and development and utilization condition are rated as “Good”; In the first level index, the forest resource, environment quality and location condition are rated as “Excellent”, the landscape resource and traffic condition are rated as “Good” and the human resources and supporting facilities are rated as “Average”; In the second level index, such factors as forest coverage rate, landscape diversity, air quality, negative ion content, sanitary condition, noise condition, geographic position and external traffic system are rated as “Excellent”, the factors such as forest impressions, forest stand types, landscape attraction degree, landscape configuration, climate comfort level, environment bearing capacity, regional economic level and tourist source condition are rated as “Good” and the factors such as forest canopy density, landscape rarity, human landscape, cultural relics, science education value, surface water quality, facility suitability, facility humanization, internal traffic system and parking lot are rated as “Average”; the factors such as folk customs, facility safety and publicity degree are rated as “Bad” and the only factor that is rated as “Very Bad” is wild animals. Based on the result, in the suburban forest park, the most important things are still the rich forest resource, outstanding environment quality and superior location condition. The fact that Baiyun Forest Park is in the suburb makes the unique location condition, which increases the park’s recreation suitability.

Resource condition evaluation

Based on the evaluation result, the general evaluation result of Baiyun Forest Park in the aspect of resource condition is “Good”. In it, the “Forest Resource” is rated as “Excellent”, which indicates that Baiyun Forest Park has rich forest resource with good quality, high forest coverage rate, good forest impressions, causing the strong overall recreation suitability. “Landscape Resource” is rated as “Good”, which shows the whole landscape is beautiful. However, “Landscape Rarity” is rated as “Average”, which means that many tourists think the landscape in the park is too common with no distinctive characteristics; “Wild Animals” is rated as “Very Bad”, indicating that there are nearly no wild animals. “Human Resources” is rated as “Average”, which is unsatisfactory. For example, the insufficient cultural deposits, the unattractive human landscape, few cultural relics, thin folk custom atmosphere and low science education value, lowering the recreation suitability relatively. Some profound historical cultural value is not utilized effectively, showing that the park needs to strengthen the preservation and exploitation in these aspects, especially the development of culture history and science education.

Basic condition evaluation

Based on the evaluation result, the general evaluation result of Baiyun Forest Park in the aspect of basic condition is “Excellent”. In it, the comprehensive evaluation result of “Environment Quality” is “Excellent” and the level of air quality and negative ion content is the highest in the evaluation layer of each item; the sanitary condition and noise condition are rated as “Excellent”; the climate comfort level and environment

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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bearing capacity are rated as “Good”, showing the environment quality of Baiyun Forest Park is good, which is beneficial for the health care and leisure recreation as well as the well-being of people’s body and mind. The surface water quality is rated as “Average” and it is found that the water body surface in the park is partially polluted; therefore, the improvement of surface water quality in the park should be one of the most important problems to be solved. The support facilities are rated as “Average”. The public toilet, road signs, shopping and accommodation and catering service are imperfect and not all of tourists’ needs can be met. Whether the supporting facilities are perfect or not will affect the park’s recreation suitability to a large extent, therefore, the perfectness, safety and humanization of supporting facilities in the park need further improving.

Development and utilization condition evaluation

Based on the evaluation result, the general evaluation result of Baiyun Forest Park in the aspect of development and utilization condition is “Good”. “Location Condition” is rated as “Excellent”. In it, the geographic position is rated “Excellent”, indicating that Baiyun Forest Park is a typical suburban forest park with clear location advantage. The superior geographic position strengthens the park’s recreation suitability. The tourist source condition and geographic position have mutual effect on each other. Because the park’s geographic position is superior, the tourist source condition is rated as “Good”; the publicity degree is rated as “Bad”. Although all the conditions of Baiyun Forest Park are good, the publicity is far from enough, therefore, it ss necessary to strengthen the publicity degree; “Traffic Condition” is rated as “Good” and the external traffic system is rated as “Excellent”, which means that people have easy access to Baiyun Forest Park. It is found in the investigation that there are many buses in the downtown by which people can get to the park gate directly and this also strengthens the park’s recreation suitability. The internal traffic system and the parking lot are rated as “Average”, indicating they cannot meet tourists’ requirements and need further improving.

Discussion and conclusion

1. The paper, by using the expert scoring method, systematically builds the suburban forest park recreation suitability evaluation system which includes 4 layers with 31 index factors after multiple rounds of screening and adjusting. The system is made up of three criteria layers, including the resource condition, basic condition, development and utilization condition, in which the resource condition and basic condition are the prerequisite and material guarantee and development and utilization condition is the factor affecting the coordination of forest park recreation suitability.

2. Confirm the weight value of all the index factors with the AHP and, on this basis, adopt the fuzzy comprehensive evaluation method to do the empirical study to Baiyun Forest Park in the Lishui city. Based on the evaluation result, the score of Baiyun Forest Park is 3.809 out of 5, indicating that the recreation suitability belongs to the good level. With the superior natural resource condition, environment quality and geographic position, the park has obvious tourism location advantage and high recreation suitability. Based on the fuzzy comprehensive evaluation result, the factors rated as “Excellent” include the “Forest Resource”, “Environment Quality” and “Location Condition”, showing that Baiyun Forest Park has rich forest resource, excellent environment, fresh air, which are important characteristic resources of Baiyun Forest

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Park and are beneficial for the recreation suitability. Besides, Baiyun Forest Park has the superior geographic position and increasing the accessibility to the park will increase the tourist amount. “Human Resources” and “Supporting Facilities” are rated as “Average”, indicating that problems exist in these two aspects of Baiyun Forest Park and they need further improving.

3. Due to the limitation of its own resource condition, there is difficulty in improving the recreation suitability greatly for Baiyun Forest Park. In the recreation development, put much emphasis on the forest health care function, make full use of the advantage of rich forest resource, strengthen the appreciation of multi-seasonal colored plants, keep the environment not being polluted continuously, clarify the park’s tourist source market, mainly base the development on the Lishui city as well as the surrounding counties and cities, dig the inherent cultural resource, rebuild the characteristic landscape, strengthen the publicity degree and meanwhile, improve the bearing capacity of recreation infrastructure to increase the park’s recreation suitability.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_34993512

EVALUATING THE SPREAD OF 10 INVASIVE WEEDS IN CHINESE NATURE RESERVES UNDER CLIMATE CHANGE SCENARIOS IN CONSIDERATION OF DIFFERENT SCALES

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(Received 5th Nov 2018; accepted 8th Feb 2019)

Abstract. Species distribution models are powerful tools for predicting species distributions and for assessing whether particular areas are at risk from invasive weeds, but they may produce different results when different climate data scales are used in the estimate. The results of species distribution models were compared across different spatial scales, and then evaluated the spread of invasive weeds in Chinese nature reserves under several models of climate change. We used Maxent software to estimate the potential spread of 10 phylogenetically diverse alien weeds in the largest 333 Chinese nature reserves. The estimates of invasive weed spread in nature reserves were not stable against changes in spatial scale. The 2.5 arc-minute data was selected to evaluate the ability of invasive weeds to spread in Chinese nature reserves under climate change. Nature reserves with a high risk of invasive weed spread were mainly distributed in southern China. We found a significant relationship between increased invasive weed spread and low and high concentration scenarios, suggesting we should prioritize the prevention and control of invasive weeds now to lessen their impact on nature reserves in the future. It is suggested that other studies may benefit from integrating different scales into the distribution models of invasive weeds.
Keywords: *ALIEN weeds, China, climate change, plant spread risk, Maxent, nature reserves, scale effect*

Introduction

Species distribution models (SDMs) are powerful tools for predicting species distributions and thus they support biological conservation and risk assessment of biological invasion in nature reserves (Alagador et al., 2011; Araújo et al., 2011; Elith et al., 2011; Velásquez-Tibatá et al., 2013; Wan and Wang, 2018). These models have used climate data to assess the distributions of invasive weeds (Chejara et al., 2010; Costa et al., 2013; Sheppard, 2013; Qin et al., 2014). Because invasive weeds represent introduced plant species with generally broad physiological niches and/or some special traits and may respond quickly to changing environmental conditions (Stratonovitch et al., 2012), climate change may increase the possibilities for invasive weeds to invade nature reserves and subsequently damage the efficacy of nature reserves for conservation (Ingwell and Bosque-Pérez, 2015; Thalmann et al., 2015; Merow et al., 2017). Hence, the management of invasive weeds in nature reserves is urgent (Foxcroft et al., 2017). The use of SDMs in biological invasion gives us the new insights into the prevention and control of invasive weeds in nature reserves.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

However, there are still many technical challenges associated with the use of SDMs in the prediction of invasive weeds. Understanding the effects of input data on SDM outputs may increase the precision of the models, thereby improving their usefulness to the risk management of invasive weeds in nature reserves (Elith et al., 2011; Merow et al., 2013).

One notable challenge with SDMs is that their predictive accuracy may vary at the different scales of input data (Rahbek and Graves, 2001; Wang et al., 2012). Some studies have demonstrated that SDMs at finer scales may reduce the uncertainty of the model output (Franklin et al., 2013; Bean et al., 2014). However, a particular scale of input localities may not meet the requirements of the model due to spatial bias in the species distribution data. This would have the potential to strongly distort our view of large-scale biodiversity patterns (Beck et al., 2014). For instance, some studies have shown that databases such as Global Biodiversity Information Facility (GBIF) have a spatially biased dataset due to uneven effort of sampling, data storage and mobilization (Beck et al., 2013, 2014). These studies have found that the most robust estimates of potential species distributions use the data at coarse resolutions (Beck et al., 2013, 2014). SDMs at coarser scales may over-estimate the size of a species distribution in the present and under different climate scenarios (Bean et al., 2014; Suárez-Seoane et al., 2014). These findings suggest that selecting the appropriate spatial scale is important for researchers to accurately estimate robust distribution models (Franklin et al., 2013).

Climate change studies have found that distribution patterns and the variables that determine distribution ranges vary when different spatial scales are used (Rahbek and Graves, 2001; Wang et al., 2012; Porfirio, 2014; Wan et al., 2016). One reason for this variation may be that the scale effect may be particularly pronounced in ecologically complex situations such as climate change (Rahbek and Graves, 2001). On the other hand, as a species expands its area of distribution, the explanatory power of climate variables may also increase, while the explanatory power of habitat heterogeneity and human activities may decrease (Wang et al., 2012). Hence, spatial scale affects estimates of the potential distribution of species under climatic change and the most appropriate scale to model the species distribution must be identified. Franklin et al. (2013) proposed selecting the appropriate scale by finding the smallest bias between results from different scales. There is usually a linear relationship between SDM model estimates at fine and coarse scales (Franklin et al., 2013). This relationship suggests that SDMs need to balance data scale with distribution estimate accuracy (Metzger et al., 2005; Wan et al., 2016).

Previous studies have used different scales (from 0.5 to 10.0 arc-minutes) to model the potential species distributions in nature reserves and did not consider the effect of spatial scales (Araújo et al., 2011; Elith et al., 2011; Jiménez-Alfaro et al., 2012; Thalmann et al., 2015). Not only that, few studies used SDMs to evaluate the risk of weed spread in nature reserves by predicting the potential distributions of invasive weeds at a large spatial scale. Thus, a challenging question is to predict whether and how invasive weeds spread in nature reserves that have been established to protect threatened native species, habitats and ecosystems under future climate change (Vanderhoeven et al., 2011). Therefore, we propose a method to integrate different spatial scales into SDMs in order to assess the vulnerability of Chinese nature reserves to invasive weeds under three climate change scenarios. To address the issue of spatial scale, a simple method was developed to improve SDM estimates for nature reserves

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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using data at several different spatial scales, and to identify the appropriate scale with which to model potential distributions of invasive weeds.

The main aim of our study is to evaluate the spread of 10 invasive weeds in Chinese nature reserves under climate change scenarios in consideration of different scales. For achieving this aim, we used 10 important invasive weeds in 333 Chinese nature reserves as the study cases. The Maxent software was used to model the current and future potential distributions of 10 invasive weeds across four spatial scales (grid resolution ranged from 1 to 256 km^2; Crall et al., 2015) and quantified the ability of invasive weeds to spread in nature reserves. Our approach is useful to identify the appropriate data scale for SDMs and our method can be especially useful to assess the spread of invasive weeds.

Materials and methods

Nature reserves in China

In 2012 China had 2,588 nature reserves covering a total area of ca. 149 million km^2 and representing ca. 14.17% of the land area (www.nre.cn). The world database of protected areas (WDPA; www.protectedplanet.net) was used to identify nature reserves (IUCN I–VI) in China with areas greater than 256 km^2 and thus, covering at least one grid cell of 256 km^2.

Invasive plant data

We modelled the potential spread of 10 invasive weeds including Bidens pilosa, Amaranthus spinosus, Cassia mimosoides, Conyza Canadensis, Daucus carota, Euphorbia hirta, Medicago sativa, Physalis angulate, Sonchus oleraceus and Vicia sativa in 333 nature reserves in China (Li, 1998; Xu and Qiang, 2011; *Table A1* in the Appendix). The species were chosen for this study according to four criteria: (1) the species had the most distribution records in China based on the study of Xu and Qiang (2011) and on the Chinese Virtual Herbarium (CVH; www.cvh.org.cn), (2) species occurrence records were dense enough to support a robust SDM (Phillips and Dudík, 2008), (3) species were widely distributed in China (Xu and Qiang, 2011) and (4) species have the negative impact on a variety of endangered plant species and ecosystem (Xu and Qiang, 2011). Occurrence records for the 10 invasive weeds, especially herbarium specimens or recorded sightings, were compiled from GBIF (www.gbif.org) and CVH (www.cvh.org.cn; Bird et al., 2014; Crall et al., 2015). We used descriptions of species locations in CVH to determine the localities within Google Earth and ArcGIS 10.2 (Bird et al., 2014; Zhang and Zhang, 2014; ESRI, 2014; *Table A1*). The occurrence records of 10 invasive weeds can cover the distribution range of species in China.

Bioclimatic data

The current potential distributions of invasive weeds in nature reserves were modelled using 19 bioclimatic variables available on the WorldClim database (averages from 1950-2000; www.worldclim.org). We removed those with absolute Pearson correlation coefficients > 0.8 in order to eliminate multi-collinearity effects in the parameter estimates of species distribution models (Sheppard, 2013; Porfirio, 2014). The resulting eight bioclimatic variables (the same as future bioclimatic

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variables) can influence the distribution and physiological performance of invasive weeds (Sheppard, 2013; *Table A2*). We used the average values of four global climate models for the 2080s (2071-2099; GCMs; i.e., bcc_csm1_1, csiro_mk3_6_0, gfdl_cm3 and mohc_hadgem2_es) and two greenhouse gas concentration scenarios, i.e., Representative Concentration Pathways (RCPs): 4.5 (mean: 780 ppm; range: 595 to 1005 by 2100) and 8.5 (mean: 1685 ppm; range: 1415 to 1910 by 2100; IPCC 5th Assessment Report) to model the future potential distributions of invasive weeds in the 2080s (2071-2099; www.ccafs-climate.org; Liang and Fei, 2014). RCP 4.5 is different from RCP 8.5 in that RCP 8.5 has a greater cumulative concentration of carbon dioxide than RCP 4.5. Thus, RCP 8.5 predicts a different climate due to anthropogenic accumulation of greenhouse gases and other pollutants. RCP 8.5 and RCP 4.5 were used as the high and low concentration scenarios, respectively (http://www.ipcc.ch/). We used bioclimatic variables at four levels of resolution (0.5, 2.5, 5.0 and 10.0 arc-minutes, namely, 1-256 km^2) because these are the most commonly used data types in SDMs.

Species distribution modelling

The Maxent software (ver. 3.3.3k; http://biodiversityinformatics.amnh.org/open_source/maxent/) was used to model the current and future potential distributions of the 10 invasive weeds across four spatial scales (0.5, 2.5, 5.0 and 10.0 arc-minute resolutions; Franklin et al., 2013). Maxent estimated the function of the potential distributions of the 10 invasive weeds based on maximum entropy and then modeled the geographic locations of the distributions based on environmental variables (Phillips and Dudík, 2008; Elith et al., 2011). Pixels in the Maxent results map with a value of 1 have the highest possibility of the species being located there, while pixels with a value of 0 have the lowest possibility of the species being located there (Phillips and Dudík, 2008; Elith et al., 2011). The pixel value reflects the potential distribution that was used to evaluate the risk of invasive weeds for nature reserves (Hoffman et al., 2010; Bean et al., 2014).

Climatic variables at four arc-minute resolutions were used as environmental input layers in Maxent. We used a 4-fold cross-validation approach to divide the presence dataset into 4 approximately equal partitions, and used 75% of the occurrence points for each species to train the model and the remaining 25% were used to test the model (each run used a different random sample of points; Merow et al., 2013). We set the regularization multiplier (beta) to 2.0 to produce a smooth and general response (Radosavljevic and Anderson, 2014). Auto features were used and other values were kept at default settings of Elith et al. (2011). The importance of bioclimatic variables was tested using the jackknife method (Phillips and Dudík, 2008; Elith et al., 2011).

The receiver operating characteristic (ROC) curves evaluated each value of the prediction result as a possible judging threshold. We assessed the performance of the Maxent model using the area under the ROC curve (AUC; Phillips and Dudík, 2008). This statistic regards each value of the estimate as a possible threshold based on the corresponding sensitivity and specificity when randomly selected background points are removed from the dataset. To ensure the high precision of SDM on four spatial scales, we only used SDMs with AUC values greater than 0.7 (Phillips and Dudík, 2008; Elith et al., 2011; Suárez-Seoane et al., 2014).

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Evaluating the spread of invasive weeds in nature reserves

Alagador et al. (2011) used a fixed threshold to match plant species with a nature reserve when the data were at different resolutions of environmental data. However, some studies have indicated that thresholds are problematic and can produce bias in predictions (Calabrese et al., 2014; Merow et al., 2013). The method of Alagador et al. (2011) and Calabrese et al. (2014) was used to evaluate the possibility of the potential distribution of all 10 invasive weeds in each pixel at the scales of 0.5, 2.5, 5.0 and 10.0 arc-minutes in ArcGIS 10.2, respectively (ESRI, 2014) (*Eq. 1*):

$$E_j = \sum_{k=1}^{k} p_{i,k} \quad \text{(Eq.1)}$$

where E_j represents the potential for invasive weeds to be present in each pixel j; k is the number of species in pixel j; i is species i; and $P_{i,k}$ is the probability of the appropriate potential distribution for species i in pixel j.

We also assessed the ability of the 10 invasive weeds to spread in each nature reserve in ArcGIS 10.2 as follows (Araújo et al., 2011; Calabrese et al., 2014; ESRI, 2014) (*Eq. 2*):

$$S_t = \sum_{j=1} X_j Y_j \quad \text{(Eq.2)}$$

where S_t is the ability of all 10 invasive weeds to spread in nature reserve t; X_j represents the potential for the presence of invasive weeds in each pixel j in nature reserve t; Y_j is the distribution area percentage of all invasive weeds in nature reserve t.

Several studies have shown that the scale of the data can potentially affect the SDM estimate (Pineda and Lobo, 2012; Franklin et al., 2013; Bean et al., 2014). There is a significant linear relationship between the potential distributions of species and fine and coarse spatial scales of the input data, and the medium prediction results computed by the scales would be stable (Kunin, 1998; Wilson et al., 2004; Franklin et al., 2013). Here, the medium results (S_t) was selected to assess the change in the ability of invasive weeds to spread within a nature reserve under climate change (Franklin et al., 2013).

We calculated the change in the ability of invasive weeds to spread within a nature reserve between the current scenario and the 2080s (in the low and high concentration scenarios) (*Eq. 3*):

$$A_t = \frac{S_{Future} - S_{Current}}{S_{Current}} \quad \text{(Eq.3)}$$

where A_t is the change in the ability of invasive weeds to spread in nature reserve t and S_{Future} and $S_{Current}$ are the future and current ability of invasive weeds to spread in nature reserve t.

Finally, we assessed the aggressiveness of each invasive weed by calculating the average values of the potential distribution possibilities of pixels within 333 studied nature reserves in China at medium scales.

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Results

The WDPA identified 333 Chinese nature reserves with an area greater than 256 km^2 that we sampled for our study (*Fig. 1*). There was no significant correlation between the number of invasive weed locations and AUC ($P > 0.05$). However, AUC measurements of SDM accuracy were greater than 0.9 (from 0.9030 to 0.9816; *Table A1*), indicating highly accurate predictions (*Fig. 2*). The most important variables for the 10 invasive weeds across all of the spatial scales were temperature seasonality and mean diurnal range (*Table A3*). We found no significant differences in the importance of bioclimatic variables for any of the species associated with changes in the spatial scales (correlation coefficient (R) > 0.935 across all the scales; $P < 0.001$; *Table A3*). However, the response of all the species to particular bioclimatic variables differed between scales (*Table A3*). For example, the average temperature seasonality range changed quite a bit from 0.5 to 10.0 arc-minutes for all the invasive weeds (from 26.660 ± 12.994 to 30.527 ± 13.388; *Table A3*).

The average ability of invasive weeds to spread in nature reserves would logically increase by using a coarser spatial scale (e.g., from 0.5 to 10.0 arc-minutes) (*Fig. 3*). Invasive weeds were able to increase their distribution the most using a data scale of 10.0 arc-minutes, and they increased their distribution the least using a scale of 0.5 arc-minutes (*Fig. 3*). We found that Maxent predictions of the spread of invasive weeds were unstable. In other words, they fluctuated when using different data scales (*Figs. 3* and *A1* in the *Appendix*). Jiaxi is a good example to show the various results of different data scales (*Fig. A1*). We found that using 2.5 arc-minute data could have the medium results to estimate invasive weed distributions in nature reserves at all data scales in the present and future (*Fig. 3*). Therefore, we selected the 2.5 arc-minute data as the appropriate data scale to evaluate the risk of invasive weed spread in Chinese nature reserves under climate change.

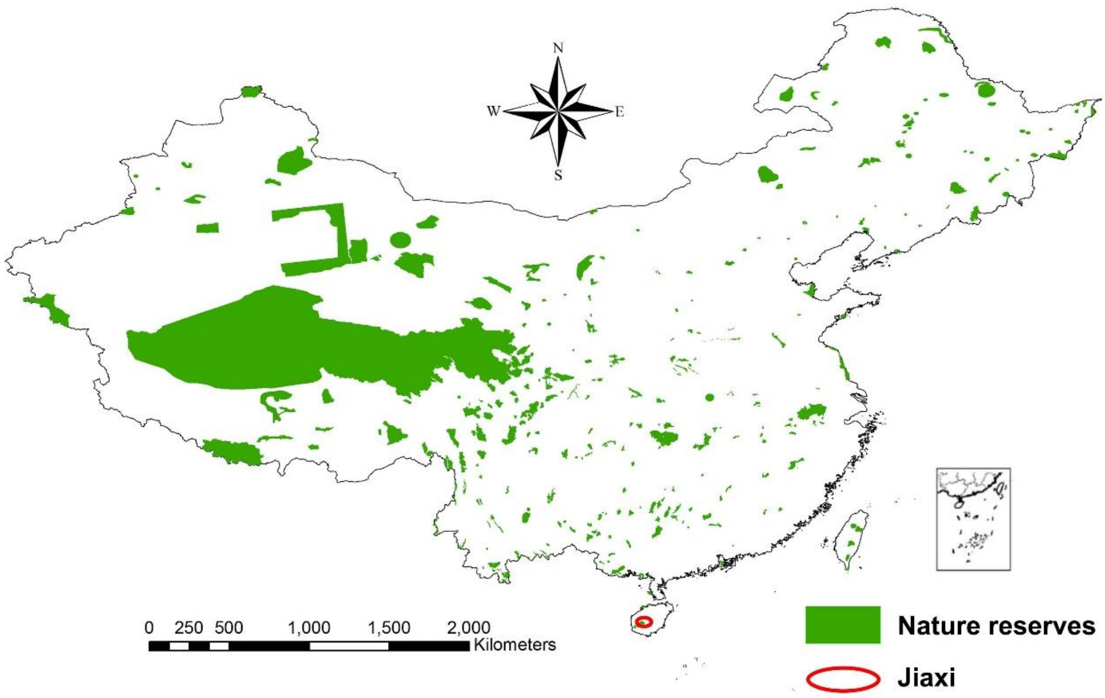

Figure 1. *Locations of the sampled nature reserves in China*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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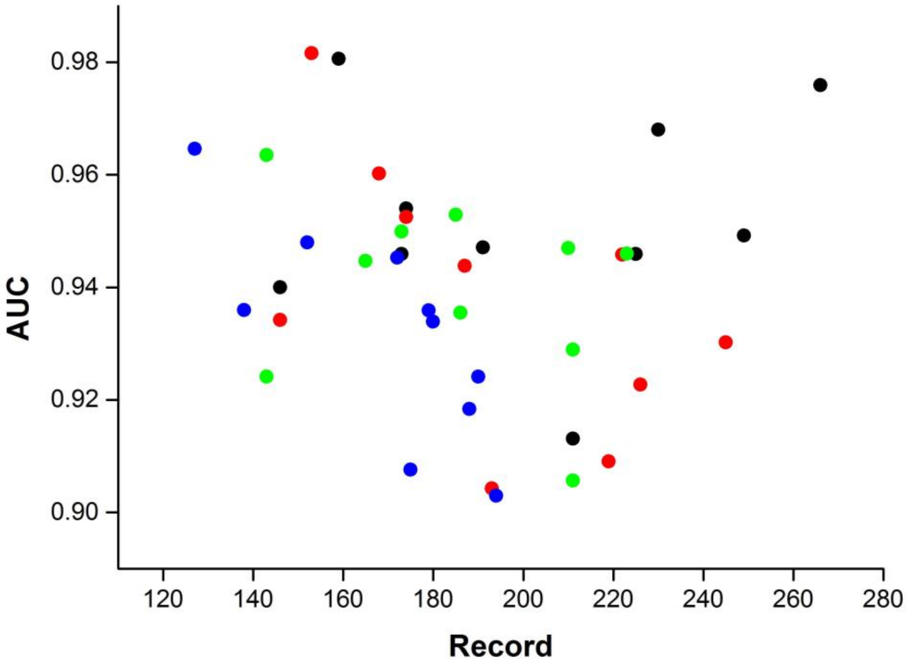

Figure 2. *The number of occurrence records and AUC values for 10 invasive weeds when using these different data scales. The black points represent 0.5 arc-minutes; the red points represent 2.5 arc-minutes; the green points represent 5.0 arc-minutes; the blue points represent 10.0 arc-minutes*

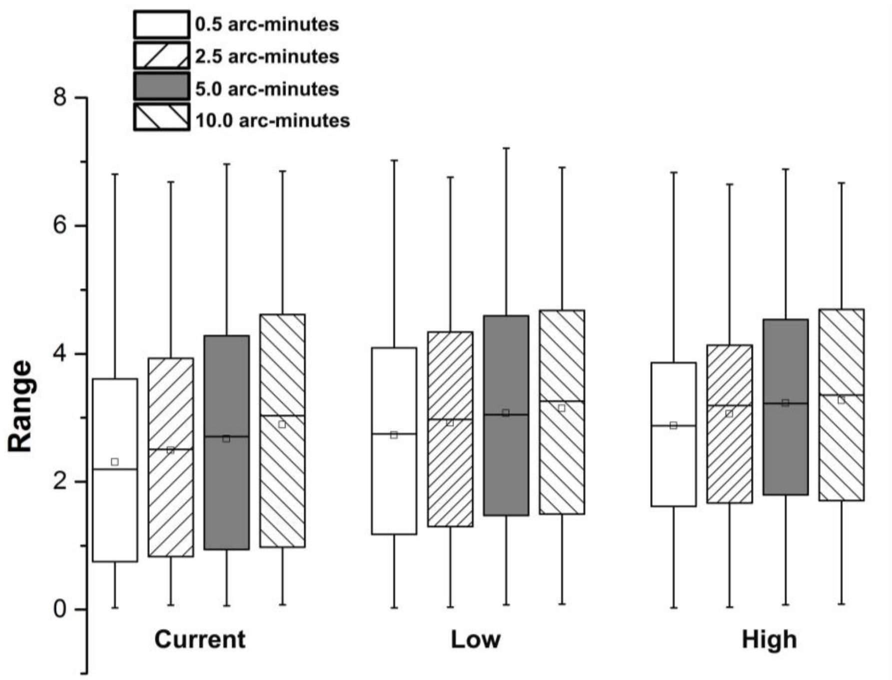

Figure 3. *The ability of invasive weeds to spread in nature reserves modeled using different spatial scales in current, low and high gas-concentration scenarios. Range: the ability of invasive weeds to spread in nature reserves bounded by horizontal bars; Current: present day; Low: low-gas-concentration scenario predicted into the future; High: high-gas-concentration scenario predicted into the future. The block point of the box is the mid-value value of the range and the line of box is the mean value of the range*

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There was a significantly positive relationship between the spread of invasive weeds in nature reserves with low and high concentration scenarios, suggesting pressure from invasive plants will continue at a similar rate even when different data scales are considered at either low and high concentration scenarios ($R^2 > 0.943$; $P < 0.001$; R^2 of 2.5 arc-minutes: 0.9618; $P < 0.001$). Furthermore, the average increase in the ability of invasive weeds to spread within nature reserves was larger in the high concentration scenario than the low concentration scenario (+70.25% (high) *vs.* +37.08% (low); *Table A4*). Hence, we used a high concentration scenario to map the spread risk of invasive weeds in nature reserves.

We found that *M. sativa* had the largest ability to spread within nature reserves, and *A. spinosusin* had the smallest spread ability in current and high concentration scenarios (*Table 1*). Meanwhile, *D. carota* and *M. sativa* would have the most significant increasing trends of spread risk under climate change (*Table 1*). Nature reserves with a high risk of invasive weed spread (e.g., Wuzhishan, Jiaxi, Jianfengling (Hainan province) and Tawushan (Sichuan province)) were mainly distributed in southern China (*Fig. 4a; Table A4*). These nature reserves are currently dominated by invasive weeds and our estimates predict many of them will continue to be so in the future (*Fig. 4a* and *b; Table A4*). We found that 291 of 333 nature reserves would be at higher risk for all 10 invasive weeds in the high concentration scenarios than in the present day (*Fig. 4; Table A4*). In addition, 303 of 333 nature reserves would be at higher risk of all 10 invasive weeds in the low concentration scenarios (*Table A4*). We found that nature reserves that had the highest increases in their risk for invasive weeds in the high concentration scenario were distributed in southwestern, northwestern and northeastern China (*Fig. 4c; Table A4*). In southern China, nature reserves had increased risk of invasive weeds, but the change in risk was not as large as in the rest of the country (*Fig. 4*). Jiaxi and Wuzhishan had the highest risk of all 10 invasive weeds in the current and future concentration scenarios (*Table A4*), and Kekexili and Aerjinshan had the highest increases in their risk for invasive weeds under climate change (*Table A4*).

Table 1. *Potential risk of the spread of 10 invasive weeds in Chinese nature reserves in the present day and high gas-concentration scenario at a spatial scale of 2.5 arc-minutes. Current indicates the spread of 10 invasive weeds in the nature reserves in the present days. High indicates the spread of 10 invasive weeds in the nature reserves in the high concentration scenario. High-change indicates the changes in the ability of invasive weeds to spread in the nature reserves in the high concentration scenario*

Species	Family	Current	High	High-change
Bidens pilosa	Compositae	0.042	0.064	0.535
Amaranthus spinosus	Amaranthaceae	0.024	0.044	0.807
Cassia mimosoides	Leguminosae	0.035	0.048	0.372
Conyza canadensis	Compositae	0.089	0.176	0.988
Daucus carota	Umbelliferae	0.078	0.174	1.244
Euphorbia hirta	Euphorbiaceae	0.027	0.047	0.721
Medicago sativa	Leguminosae	0.184	0.428	1.325
Physalis angulata	Solanaceae	0.042	0.066	0.584
Sonchus oleraceus	Compositae	0.099	0.188	0.905
Vicia sativa	Leguminosae	0.117	0.235	1.009

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DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

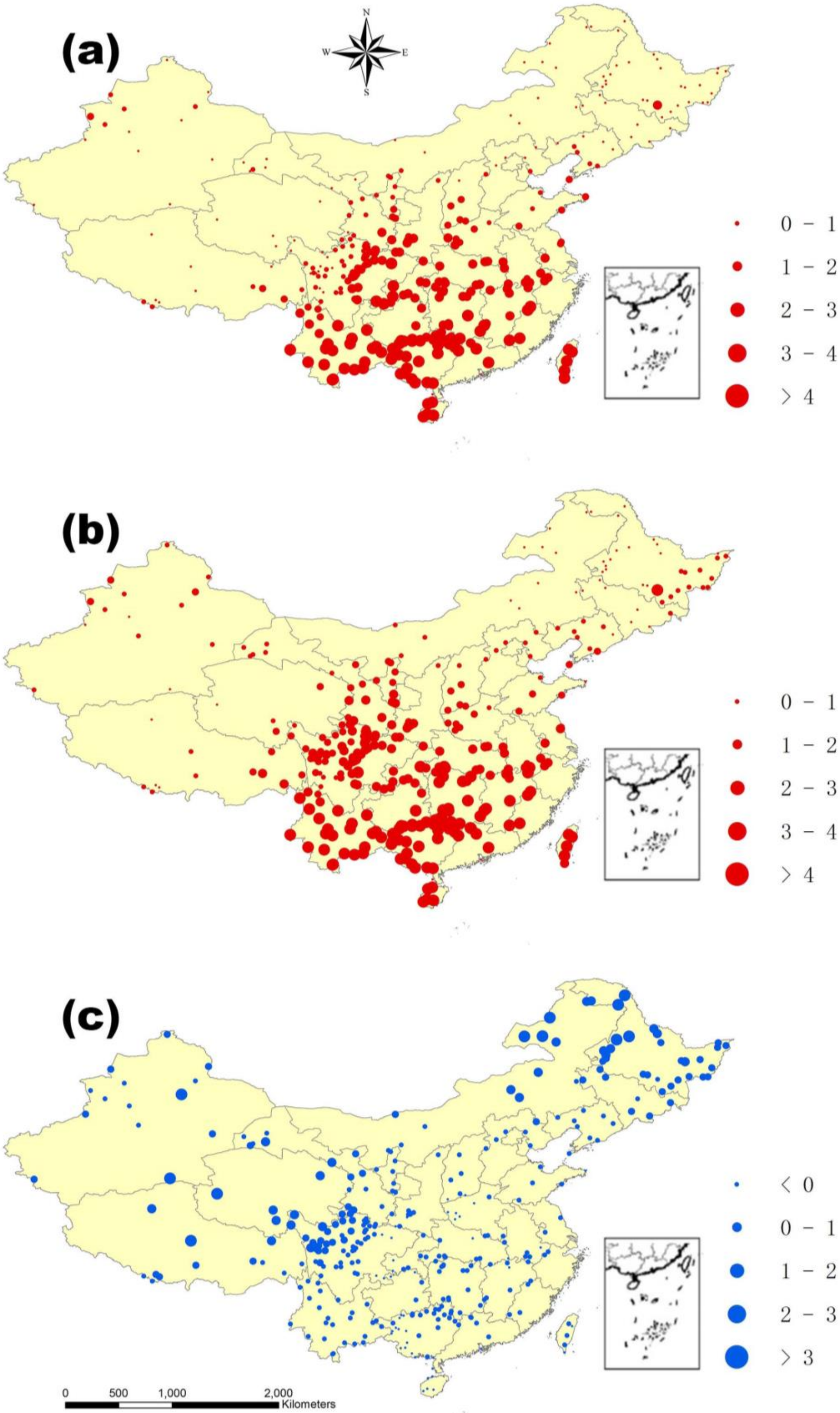

Figure 4. *The spread of invasive weeds in nature reserves (a) in the present day and (b) in high gas-concentration scenario at a spatial scale of 2.5 arc-minutes and (c) the changes in the spread of invasive weeds in the high gas-concentration scenario at a spatial scale of 2.5 arc-minutes*

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Discussion

Our study is an example of how SDMs can be applied to estimate the risk of weed spread in nature reserves with the different scales. Our results showed that using different spatial scales results in different estimates of invasive weeds distributions in current, low and high concentration future scenarios. This indicates that the spatial scale may under- or over- estimate the ability of invasive weeds to increase their distribution in nature reserves. We also found that the risk of invasive weeds in nature reserves was largest at scales of 10.0 arc-minutes. Thus, SDM prediction uncertainty caused by spatial scales could result in inaccurate estimates of invasive weed distributions and their effect on nature reserves. Previous studies have determined the appropriate scale of data to use in SDMs by comparing relationships of potential distributions between fine and coarse scales and subjectively choosing the "best" data scale based on the results (Franklin et al., 2013; Suárez-Seoane et al., 2014). Franklin et al. (2013) selected the appropriate scale by computing the extent and location of the predicted distribution area under current climate conditions depending on the differences in the estimates between fine and coarse scales. The selection of an appropriate data scale should incorporate the variance found when using different scales in SDMs (based on *Fig. 2*) and stabilize the predicted distribution of invasive weeds (Franklin et al., 2013). Hence, by comparing the SDM results of different scales, we used 2.5 arc-minutes, the second-finest scale (also, the medium scale), to evaluate invasive weed risk in nature reserves under climate change scenarios.

Millions of dollars have been invested in the global control of invasive weeds and many scientists have proposed methods to prevent and control the invasion of invasive weeds (Dewey et al., 1995; Rinella and Luschei, 2007). Some scientists have proposed designing long-term management plans at the regional or national scale to mitigate weed spread due to climate change (Chejara et al., 2010; Bohan et al., 2011; Sheppard, 2013; Qin et al., 2014). However, few studies paid attention to the spread of invasive weeds in nature reserves at the national scale. The spread of invasive weeds into nature reserves may cause serious problems (Van Wilgen et al., 2012; Lindenmayer et al., 2015). The invasive weeds can displace native species, alter community structure and ecosystem functions, and cause landscape change and habitat fragmentation (Lindenmayer et al., 2015; Thalmann et al., 2015). Consequently, nature reserves may lose their function of protecting concerned species, habitats or ecosystems (Ingwell and Bosque-Pérez, 2015; Thalmann et al., 2015). We found that nature reserves in southern China are currently dominated by invasive weeds and our estimates predict most of them will continue to be so in the future. Our data supports the need for long-term monitoring of these nature reserves to prevent the spread of invasive weeds due to climate change (Wang et al., 2017). Our finding that the ability of invasive weeds to spread within nature reserves would increase more severely in the high concentration scenario than the low concentration scenario indicated that climate change due to the increasing gas concentration may facilitate the spread of invasive weeds in nature reserves. More importantly, we found a significant relationship between increased invasive weed distributions and low and high concentration scenarios, suggesting we should prioritize the prevention and control of invasive weeds now to lessen their impact on nature reserves in the future (Rannow et al., 2014). Therefore, the prevention and control of invasive weeds in nature reserves is extremely urgent now. The challenge for biological conservationists will be in minimizing the opportunities for invasive plant species to be introduced into nature reserves under climate change. Based on the assessment of expansion risk for invasive weeds and nature reserves, we propose the following measures: (1) detailed monitoring of climate change, (2) improvement of

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effective management for human activities near or inside nature reserves, and (3) control of the introduction of invasive weeds with a high ability to naturally disperse (Foxcroft et al., 2017; Merow et al., 2017).

Our suggestion is that researchers integrate model evaluation of several different spatial scales (0.5, 2.5, 5.0 and 10.0 scales widely used in SDM studies) into their SDM analyses on invasive weeds. Although our study did not validate the Maxent estimates with ground truthing or ecological monitoring this work should be prioritized as a way to test our approach for quantifying invasive species risk (Alagador et al., 2011). Using the correct scale for SDM may lead to more accurate predictions that allow researchers and land managers to make reasonable decisions regarding the management of invasive weeds (Costa et al., 2013; Sheppard, 2013; Qin et al., 2014). Therefore, studies on the effect of data scales on SDMs must continue. We hope that future studies can expand the application of SDMs to provide practical suggestions for mitigating the impact of scale effects on SDM predictions of weeds.

Conclusion

We put forward a simple method to balance various results modeled by different spatial scales for avoiding the over- or under-estimation of SDM results due to the selection of spatial scales, and take the impact of different scales on SDM results into consideration for invasion risk of weeds. Nature reserves with a high risk of invasive weed spread were mainly distributed in southern China. We should prioritize the prevention and control of invasive weeds now to lessen their impact on nature reserves in the future. Here, we proposed the useful suggestions for the evaluation of risk of invasive species: (1) we need to compute two indicators: the ability of invasive weeds to spread in nature reserves and spread potential of invasive weeds for nature reserves; (2) we should balance the various impacts of different spatial scales on the results of SDMs; (3) we should determine the regional scales of spread risk of invasive weeds under climate change. Finally, we hope that future studies can expand the application of SDMs to provide feasible suggestions for risk evaluation of invasive species under climate change.

Acknowledgements. This work has been supported by the National Natural Science Foundation of China (No. 31800449 and 31800464) and the Basic Research Project of Qinghai Province, China (No. 2019-ZJ-936Q and 2019-ZJ-960Q).

Disclosure statement. No potential conflict of interests was reported by the authors.

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APPENDIX

Table A1. *Occurrence records and AUC values of study species. Records: the number of recorded occurrences of each study species; AUC: AUC values of study species. 0.5: 0.5 arc-minute, 2.5: 2.5 arc-minutes, 5.0: 5.0 arc-minutes, 10.0: 10.0 arc-minutes*

Species	Family	0.5 arc-minutes		2.5 arc-minutes		5.0 arc-minutes		10.0 arc-minutes	
		Records	AUC	Records	AUC	Records	AUC	Records	AUC
Bidens pilosa	Compositae	266	0.9759	245	0.9302	223	0.946	190	0.9241
Amaranthus spinosus	Amaranthaceae	159	0.9806	153	0.9816	143	0.9635	127	0.9646
Cassia mimosoides	Leguminosae	173	0.9459	168	0.9602	165	0.9447	152	0.948
Conyza canadensis	Compositae	211	0.9131	193	0.9043	186	0.9355	175	0.9076
Daucus carota	Umbelliferae	174	0.954	174	0.9525	173	0.9499	172	0.9453
Euphorbia hirta	Euphorbiaceae	249	0.9492	226	0.9227	211	0.9289	179	0.9359
Medicago sativa	Leguminosae	191	0.9471	187	0.9438	185	0.9529	180	0.9339
Physalis angulata	Solanaceae	230	0.968	219	0.9091	210	0.947	188	0.9184
Sonchus oleraceus	Compositae	225	0.9459	222	0.9458	211	0.9057	194	0.903
Vicia sativa	Leguminosae	146	0.94	146	0.9342	143	0.9241	138	0.936

Table A2. *WorldClim bioclimatic variables used in the analysis. Bioclimatic variables were used as environmental layers for the species potential habitat distribution models in Maxent; C of V represents the coefficient of variation*

Code	Bioclimatic variables	Unit
Bio1	Annual mean temperature	°C
Bio2	Mean diurnal range	°C
Bio4	Temperature seasonality	SD*100
Bio8	Mean temperature of the wettest quarter	°C
Bio10	Mean temperature of the warmest quarter	°C
Bio12	Annual precipitation	mm
Bio14	Precipitation of the driest month	mm
Bio15	Precipitation seasonality	C of V

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DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

Table A3. *The average and standard deviation values of the importance of bioclimatic variables based on Maxent jackknife test. The codes that were used in this table are defined in Table A2*

Code	0.5 arc-minutes	2.5 arc-minutes	5.0 arc-minutes	10.0 arc-minutes
Bio1	19.986±13.494	18.055±14.905	17.966±10.469	12.517±11.458
Bio2	20.470±12.681	20.359±15.160	24.238±17.660	23.131±14.188
Bio4	29.833±14.493	30.527±13.388	26.660±12.994	30.508±10.700
Bio8	4.129±3.577	4.529±3.807	3.573±3.530	5.662±4.441
Bio10	4.987±3.373	4.283±1.651	5.419±3.246	5.854±4.117
Bio12	7.912±5.462	10.075±6.505	8.288±3.559	11.475±5.658
Bio14	3.343±2.016	2.954±2.913	3.928±2.781	3.866±3.030
Bio15	9.339±10.249	9.217±7.524	9.930±9.652	6.986±9.917

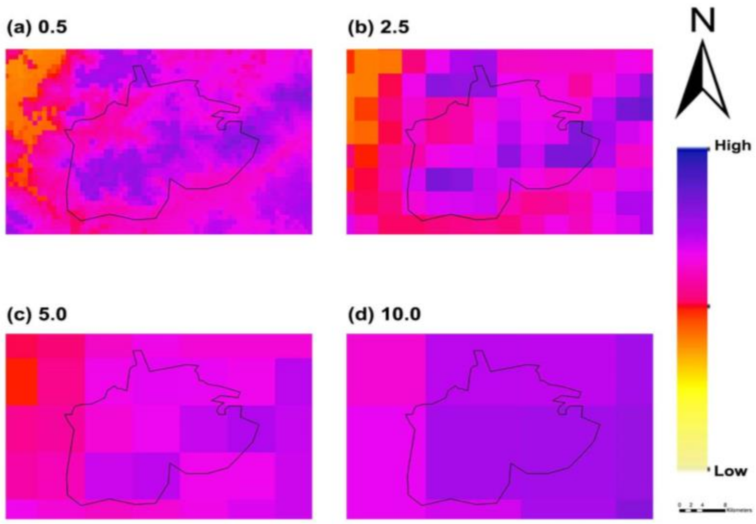

Figure A1. *The spread of invasive weeds in Jiaxi nature reserve in the present day at different spatial scales (i.e., 0.5, 2.5, 5.0 and 10.0 arc-minute resolutions)*

Table A4. *Potential risk of the spread of 10 invasive weeds in Chinese nature reserves at a spatial scale of 2.5 arc-minutes. Name refers to the names of nature reserves based on WDPA database. Current refers to the spread of 10 invasive weeds in the nature reserves in current concentration scenario. Low signifies the spread of 10 invasive weeds in the nature reserves in the low concentration scenario. High indicates the spread of 10 invasive weeds in the nature reserves in the high concentration scenario. Low-change: the changes in the ability of invasive weeds to spread in the nature reserves in the low concentration scenario; High-change: the changes in the ability of invasive weeds to spread in the nature reserves in the high concentration scenario*

Name	Current	Low	High	Low-change	High-change
Aerjinshan	0.068180366	0.1775214	0.3933055	1.603702655	4.76860353
Ailaoshan	4.218334732	4.9194589	5.5215848	0.166208756	0.308948946
An'jilongwangshan	3.610529992	4.000187	3.7872113	0.107922385	0.048935006
Anxijihanhuangmo	0.811199047	1.1095888	1.6160121	0.367837899	0.99212771
Anzihe	3.473078712	3.9443094	4.0854224	0.13568097	0.176311492
A'rengou	1.389479549	2.8543233	3.5136993	1.054239159	1.528788065
Badagongshan	3.578065628	4.0938891	3.972425	0.144162664	0.110215802
Baidongheshuiyuanlin	4.884217508	4.6070336	4.3879747	-0.056750935	-0.101601292
Baihe (Sichuan)	2.355475977	3.2660591	3.2499793	0.386581367	0.379754806

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DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

Name	Current	Low	High	Low-change	High-change
Baijitan	1.805230733	2.4081005	2.4963897	0.333957181	0.382864614
Baimaxueshan	2.38405453	3.1118643	3.1947595	0.30528235	0.340053031
Baishuijiang	3.507968046	4.0925835	3.7023217	0.166653586	0.055403485
Baiyang	2.526666136	3.227808	3.2619489	0.277496838	0.291009071
Bajie	1.184312121	2.0891855	2.7071695	0.764049749	1.285858138
Bamianshan	4.008368154	4.6635722	4.6239197	0.163459049	0.153566619
Banli	4.943201904	4.8011102	4.8733824	-0.028744872	-0.014124348
Baohuashan	3.25073457	3.4565137	3.4192301	0.063302348	0.051833063
Bayinbuluketiane	0.166414421	0.2370796	0.3055153	0.424633746	0.835870342
Bitahai	2.231175582	2.4913757	2.9686196	0.116620189	0.330518147
Buergenheli	0.72121909	1.3040109	1.5471167	0.80806487	1.145141083
Buliuheshuiyuanlin	4.69673285	4.7488565	4.5295667	0.011097853	-0.035592007
Cangshanerhai	3.959751105	4.8609742	5.2486132	0.227595895	0.325490684
Cenwanglaoshanshuiyuanlin	4.673200934	5.0158726	4.9457915	0.07332697	0.05833059
Changbaishan	0.340824228	0.5910087	0.8382921	0.734057181	1.459602432
Changjiangxinluoduanbaijitun (Hubei)	3.638086006	3.9493103	3.758875	0.085546162	0.033201248
Changjigangshidi	0.281764871	0.5188044	0.9337151	0.841267146	2.313809478
Changningzuhai	4.322759185	4.3419296	3.900583	0.004434764	-0.097663591
Changqing	3.019663695	3.4678836	3.3351798	0.148433717	0.104487167
Changshagongma	0.470046796	0.9273012	1.5938657	0.972784854	2.390866002
Chaqinsongduo	0.81403072	1.5976287	2.5128852	0.962614752	2.086966055
Chayucibagou	2.041112011	2.9757539	3.2698697	0.457908181	0.602004046
Chayuhu	0.430203311	0.7933017	0.9610103	0.844015794	1.233851473
Chengbiheshuiyuanlin	4.896073856	4.7630283	4.5597921	-0.027173928	-0.068683963
Chichengdahaituo	0.829301289	0.9031788	1.1233997	0.089084042	0.354633973
Chuandonghe	4.606035769	4.6140348	4.3998648	0.001736641	-0.044761044
Cuiyunlanggubai	3.859620279	4.0159084	3.5892596	0.040493134	-0.070048518
Dabashan	3.328029459	3.7531105	3.5995671	0.127727548	0.081591117
Dafengmilu (Jiangsu)	2.90771915	3.2236214	3.2242437	0.108642628	0.108856645
Daguisi	3.264832547	3.476791	3.4729822	0.064921692	0.063755078
Daheishan (Heilongjiang)	0.291439772	0.5273876	0.8503797	0.809593785	1.917857416
Dahongjiangshuiyuanlin	4.71794787	4.684868	4.6164774	-0.007011495	-0.021507332
Dahongshanyinxing	3.448675483	3.4305483	3.2224149	-0.005256274	-0.065607966
Dahuofangshuiyuanshuiku	0.956092565	1.2316865	1.0782893	0.288250265	0.127808477
Dalaihu	0.108859393	0.2305818	0.5355734	1.118161728	3.91986392
Dalinuoerniaolei	0.285656861	0.5563217	0.8753719	0.947517375	2.064417557
Damingshanshuiyuanlin (Guangxi)	4.889718323	4.9585741	5.01487	0.014081747	0.025594864
Danxianbaidiebei	4.985318304	4.6786053	4.8319898	-0.061523254	-0.030756011
Daozhendashahe	3.680527407	4.0983071	3.9235606	0.113510822	0.066032165
Dasuganhu	0.562605599	1.0260591	1.405613	0.823762689	1.498398527
Dawanglingshuiyuanlin	5.014520129	4.9281845	4.7289345	-0.017217127	-0.056951736
Daxiaolangou	3.590334076	3.7437164	3.5987411	0.042720906	0.002341572
Dayaoshanshuiyuanlin (Guangxi)	4.439299765	4.9065495	4.6764125	0.105253026	0.053412193
Dazhongshan	4.649500182	5.4927152	5.8394917	0.181356057	0.255939665
Dianchi	4.499969969	5.1899584	5.330356	0.153331786	0.184531461
Donganshunhuangshan	3.867737555	4.5718572	4.8515843	0.182049489	0.254372674
Dongdongtinghu (Hunan)	3.886313855	4.3183458	4.1328029	0.111167538	0.063424894
Dongzhai niaolei	3.316473124	3.4241928	3.4126855	0.03248019	0.029010449
Dugoula	0.907013901	1.5785688	2.674138	0.740401992	1.948287779
Dunhuang	0.404149414	0.7933638	1.2392571	0.963045776	2.066334027
Em Ei Shan	3.992729896	4.2901553	4.1393044	0.074491742	0.036710348
Fanjingshan (Guizhou)	3.570361712	3.9039159	3.9035081	0.09342308	0.093308862
Fenghuangshan (Heilongjiang)	0.682192057	1.1025248	1.5767282	0.616150157	1.311267309
Fenglin	0.224479499	0.3988635	0.6667784	0.776837091	1.970330934

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DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

Name	Current	Low	High	Low-change	High-change
Fengtongzhai (Sichuan)	3.081397331	3.6091109	3.8951259	0.171257878	0.264077781
Fengwugou	0.826358234	1.2335944	1.7518904	0.492808263	1.120013244
Foping	2.894701462	3.4591989	3.3299122	0.195010589	0.150347365
Funiushan	2.804172271	2.9782938	2.7362375	0.062093735	-0.024226319
Fuyushuiqing (Shuiqing)	0.256543428	0.4779255	0.8550919	0.862941895	2.333127286
Gajinxueshan	1.066909829	2.1621586	2.8080534	1.026561703	1.631950071
Ganjiahu (Xinjiang)	1.300586638	1.5906694	1.5038426	0.223039937	0.156280217
Ganligahai-zecha	0.997468313	2.1067952	2.8403099	1.112142484	1.847518927
Gaoligongshan	3.134970181	3.7814828	4.0319559	0.206226082	0.286122568
Gemu	0.909843507	1.2958836	1.6658613	0.424292848	0.830931679
Genieshenshan	1.088408407	1.7106679	2.050123	0.571715074	0.883597174
Gonggashan (Sichuan)	1.402533989	2.0132685	2.252333	0.435450774	0.605902615
Gouxiheshidi	4.079206322	4.1594578	3.728884	0.019673307	-0.08588002
Guangwushan	3.669389547	4.0787234	3.7616593	0.111553665	0.025145805
Guhaian (Tianjing)	1.329830276	1.5704211	1.5878316	0.180918444	0.194010716
Guniujiang	3.57206942	4.3096911	3.9605627	0.206497017	0.108758603
Guposhanshuiyuanlin	4.035366563	4.9541409	4.7622043	0.227680515	0.180116905
Haiyangshanshuiyuanlin	4.123817147	4.810177	4.6425934	0.166437994	0.125800014
Haizishan	0.736253343	1.118952	1.6183143	0.519792081	1.198040003
Hanasi	0.492735606	0.7246338	1.4470392	0.470634132	1.936745757
Hanma	0.096370639	0.1567511	0.3420536	0.626544159	2.549354903
Heilihe	0.666124926	0.9969191	1.3844194	0.496594799	1.078317964
Heilonggong	0.320917678	0.5791164	0.7352973	0.804563724	1.291233392
Heishantou	0.113505814	0.2222014	0.5145099	0.957621307	3.532894676
Heishuihe	3.047325448	3.6869272	3.8800535	0.209889545	0.273265218
Helanshan (Ningxia)	1.422396867	2.0784923	2.2252012	0.46126046	0.56440249
Helanshanshuiyuanhanyanglin (Neimeng)	1.299201368	1.8780089	2.0024912	0.445510254	0.541324732
Hepu rugen	5.011440922	5.0482994	5.1063827	0.007354866	0.018945006
Hesigechuor	0.184012734	0.4104674	0.6653623	1.230646712	2.615849216
Hongba	1.514917419	2.2236418	2.3874644	0.467830373	0.575969997
Honghe	0.375266436	0.6961311	1.0984756	0.855031607	1.927188511
Honghua'er'jichangzhangzisonglin	0.150298859	0.2506426	0.5995992	0.667628096	2.989379587
Honghushidi	3.714843677	3.9892489	3.8569874	0.073867233	0.038263716
Houhe	3.423165934	3.955298	4.0340362	0.155450269	0.178451842
Huagaoxi	4.045582902	4.2214755	3.884153	0.04347769	-0.039902755
Huang Long	1.468625458	2.3589348	2.7255073	0.606219467	0.855821908
Huang Shan	3.581699243	4.4166519	4.0191771	0.233116351	0.122142544
Huanghegudaoshidi	1.719022713	1.9421119	1.8753494	0.129776754	0.09093928
Huanghesanjiaozhou	1.692432243	2.1877714	1.9383856	0.29267887	0.145325379
Huangheshidi	2.604529637	2.5749698	2.3813474	-0.011349396	-0.085690035
Huangheshouqu	1.198754052	2.4242601	2.8997985	1.022316501	1.419010384
Huanglianshan	5.200654594	5.466738	5.847181	0.051163445	0.124316352
Huanglianshanshuiyuanlin	4.87267502	5.0452256	4.8449926	0.035411879	-0.005681155
Huaping	3.957146575	4.660721	4.7623428	0.177798424	0.203478999
Huashuichongshuiyuanlin	4.240136494	5.0120098	4.8664545	0.182039731	0.147711756
Huihe	0.119540898	0.236545	0.5829069	0.978778844	3.87621316
Humahe	0.159231741	0.3347174	0.7894514	1.102077123	3.957877086
Huochengsizhualugui	2.65524078	2.8764318	2.7659642	0.083303564	0.041699955
Huzhong	0.103698918	0.1655946	0.3709349	0.596878764	2.577037323
Jianfengling	6.427512043	6.4288437	6.0850099	0.000207181	-0.053286892
Jiangcun	1.321870747	1.5581834	1.9513201	0.178771377	0.476180712
Jiangxiwuyishan	4.16857114	4.5717325	4.9945192	0.096714521	0.198136971
Jiaqiaolingshuiyuanlin	4.251732296	4.7277762	4.5093215	0.111964694	0.06058453
Jiaxi	6.456783457	6.5712797	6.3103195	0.017732706	-0.022683734

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Name	Current	Low	High	Low-change	High-change
Jiejingkou	0.3406554	0.6530771	0.9676376	0.91711947	1.840517426
Jin Zhai Gou	1.58088584	2.5481558	2.8900921	0.611853137	0.828147249
Jinfoshan	3.783649179	4.0667182	3.8758498	0.07481376	0.024368174
Jingangtai	3.317290761	3.469444	3.4888029	0.045866718	0.051702474
Jingbohu	0.551775952	0.9941344	1.2141727	0.801699397	1.200481365
Jinggangshan	3.972931325	4.6766994	4.4803111	0.17714076	0.127709173
Jinpingfenshuiling	4.499785327	4.7277904	5.0365844	0.050670211	0.119294374
Jintangkongyu	1.921151073	2.5756459	3.0938951	0.34067848	0.610438213
Jinyunshan	4.457848275	4.4195618	3.9280611	-0.008588555	-0.118843698
Jinzhongshan	4.687741682	5.1219961	4.9311732	0.092636166	0.05192938
Jiudingshan	2.62135821	3.2398644	3.3155459	0.235948749	0.264819851
Jiugongshan	3.396543347	4.2514869	3.9315731	0.25170989	0.157521839
Jiuwanshanshuiyuanlin	4.195228936	4.5764742	4.7234784	0.090875914	0.125916719
Jiuyishan	4.087967301	4.9634151	4.6755687	0.214152349	0.143739261
Kalamailishan	1.216526787	1.5187629	2.0973507	0.248441807	0.724048104
Kashahu	1.311950373	2.4513161	3.0544196	0.868451849	1.328151783
Keerxin	0.43003141	0.7316846	0.8073818	0.701467807	0.877494949
Kekexili	0.116443458	0.4856354	0.664697	3.170568346	4.708324121
Kenting	4.650508798	3.7180358	3.6024545	-0.200509888	-0.225363362
Ku'erbin	0.21556771	0.3929574	0.6830063	0.822895461	2.168407272
Labahe	2.928455529	3.5021062	3.7542428	0.195888469	0.281987301
Langcun	0.829746806	1.6189519	2.6268773	0.951139659	2.16587817
Laoshan	3.502353489	4.0273222	3.6781209	0.14989027	0.050185514
Laoshan	2.47116014	2.9672197	2.5445171	0.200739544	0.029685231
Laotudingzi	0.520627921	0.8766302	0.9796121	0.683794058	0.881597318
Laoxiancheng	2.801765329	3.3794668	3.2922669	0.206191955	0.175068756
Leigongshan	3.617425677	4.1193028	4.4312704	0.138738752	0.224978976
Leiwuqi	0.717355376	1.4067066	2.5502438	0.960961954	2.555063341
Leizhou Rare Marine Life	0.279471662	0.2804547	0.2664467	0.003517487	-0.046605663
Lian Huan Hu	0.366932889	0.5407905	0.811421	0.473813104	1.211360781
Liancheng	1.26867781	2.3219657	2.8948438	0.830224886	1.281780116
Liangucheng	0.996566208	1.5130357	2.0309251	0.518249051	1.037922903
Liangyeshan	4.473078984	5.086663	5.5386353	0.137172632	0.238215404
Lingnan	3.765201678	4.5642651	4.0691275	0.212223273	0.080719666
Lingqiuqingtun	1.463160215	1.807377	1.4957921	0.235255703	0.022302332
Lishan	2.383144667	2.6577278	2.4785096	0.115218827	0.040016426
Liupanshan	2.247728102	2.9802779	3.1895395	0.325906767	0.419005927
Longbao	0.539827103	1.0910366	2.1039936	1.021085258	2.897532355
Longganhu	3.629671022	4.263526	3.5162698	0.174631523	-0.031242838
Longwan	0.38756117	0.6466648	0.8194125	0.668548993	1.114279147
Longxihongkou	3.214561441	3.6995559	3.7267688	0.150874223	0.159339732
Luanheyuancaodi	4.867275133	4.3397563	4.2873983	-0.10838073	-0.119137878
Luoshan	1.825088565	2.4842113	2.6502165	0.361145617	0.452102956
Luoxu	0.627191882	1.2530207	2.0602264	0.997826719	2.284842261
Lushan	3.933653669	4.6004531	4.2564231	0.169511474	0.082053342
Lushidani	3.064114253	3.158907	2.8135776	0.030936427	-0.081764788
Luyashan	0.950415949	1.5357447	1.7078583	0.615865876	0.796958797
Mabiandafengding	3.150848417	3.7765117	3.7137125	0.198569782	0.178638896
Mangkang	1.508791551	2.2683431	2.3152967	0.503417154	0.534537159
Mangshan	3.956484796	4.7755036	5.0121092	0.207006686	0.266808659
Manzetangshidi	1.108426009	1.9325509	2.9870393	0.74350916	1.694847717
Maoershan	3.717741689	4.5665343	4.7695168	0.228308657	0.28290699
Maoershan	0.420672612	0.7190534	0.9726003	0.709294543	1.312012411
Maojieniaolei	4.799302307	5.2228477	5.0493304	0.088251451	0.052096758

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Name	Current	Low	High	Low-change	High-change
Maolan	4.27280106	4.5967033	4.6510033	0.075805598	0.088513889
Maoshan	0.192927715	0.4251202	0.7912416	1.203520629	3.101233459
Mayangheheiyehou	3.865217812	4.1581757	3.8065134	0.075793371	-0.015187866
Meigudafengding	2.985776711	3.6786022	3.6260661	0.232041963	0.214446508
Meihuashan	4.669146936	5.2814537	5.9372162	0.131138894	0.271584784
Mengda	1.552720746	2.5167115	3.0781737	0.620839746	0.982438702
Mianshan	2.087279858	2.2833927	2.1241097	0.09395618	0.017644899
Micangshan	3.62645716	3.8435796	3.589351	0.059871779	-0.010232069
Minjiangbai	1.804775928	2.4589164	3.4289343	0.362449688	0.899922448
Momoge	0.391668358	0.580151	0.8334726	0.481230199	1.12800596
Mudanfeng	0.51741769	0.9507688	1.3357585	0.837526661	1.581586455
Mulinzi	3.422753301	3.9917032	3.9998137	0.166225798	0.168595382
Mulun	4.354657004	4.6045431	4.647664	0.057383646	0.067285895
Mupinghu	3.800973992	4.3609185	4.0455318	0.147316059	0.064340826
Namusilai	0.870281819	1.2314424	1.1692505	0.414992676	0.343530882
Nandongtihu Shidi and Shuiqin	3.802469437	4.388544	3.971799	0.154129987	0.044531472
Nangunhe	4.957876728	5.4808349	6.0436073	0.105480269	0.218991038
Nanling	3.970448966	4.7750611	5.0630911	0.202650164	0.275193597
Nansihu	2.302205243	2.6691182	2.3885047	0.159374564	0.037485562
Nanwenghe	0.146800332	0.2856544	0.7191587	0.945870259	3.898890147
Nanyangkonglongdanhuashiqaun	3.083725701	2.9745135	2.6665973	-0.035415666	-0.135267673
Nanyuehengshan	4.19229214	4.9305377	4.8986686	0.176095924	0.168494093
Nazoushuiyuanlin	4.95240428	5.2613022	5.0417035	0.062373325	0.018031488
Neixiangbaotianman	2.87574233	2.9890161	2.6838037	0.039389402	-0.066744029
Nianlong	0.673981487	1.113588	2.3452111	0.652253098	2.479637268
Nonggang	4.942850266	4.7402721	4.5984173	-0.040984079	-0.069683067
Panzhihuasutiesutie	4.376400448	4.6101161	4.6956361	0.053403626	0.072944799
Pishangou	0.743091317	1.0304883	1.0897775	0.386758634	0.466545867
Poyanghulijiyuchanluanchang	3.683235151	4.2913702	3.6359704	0.165108939	-0.012832401
Qianfoshan	3.456355012	3.8333341	3.6747835	0.109068393	0.063196196
Qiangtang	0.142320064	0.3363909	0.5623604	1.363622462	2.951378212
Qianjiadongshuiyuanlin	3.99580901	4.9413833	4.8074861	0.236641513	0.203132104
Qilianshan	0.585662053	1.2939186	1.9769024	1.20932634	2.375500239
Qinghaihuniaodao	0.620402936	1.775535	2.3756885	1.861906185	2.829267017
Qingliangfeng	3.638672235	4.2046866	3.8935602	0.155555194	0.070049718
Qingshitanshuiyuanlin	4.086825363	4.7388727	4.7408752	0.159548618	0.160038607
Qixinghe	0.496490676	0.8872769	1.208391	0.787096803	1.433864438
Qixinglazi	0.377813653	0.7623187	1.231765	1.017710832	2.260244806
Queershan	1.034280414	2.3431316	2.9973743	1.265470339	1.898028677
Quomolangma	0.402532064	0.6339384	0.9115877	0.574876778	1.264633756
Ribaxueshan	0.91896993	1.7143432	2.4622932	0.865505218	1.679405625
Riganqiaoshidi	1.395896878	2.8567253	3.0943032	1.046516003	1.216713318
Rongchengtianee	2.400741937	0.9815751	0.8954855	-0.591136771	-0.626996352
Ruoergaishidi	1.191480653	2.7467948	3.0479123	1.305362486	1.558087949
Sandagu	1.101322859	1.9048686	2.5908522	0.729618689	1.352491078
Sanjiang	0.392006461	0.7188821	1.097434	0.833852682	1.799530388
Sanjiangyuan	0.409948785	0.8813389	1.4625438	1.149875624	2.567625649
Sanpihushuiyuanlin	4.413829766	4.6808017	4.380299	0.060485326	-0.007596751
Selincuoheijinghe	0.242026402	0.6159431	1.2225865	1.54494177	4.051459221
Shankou hongshulin	4.648508413	4.694658	4.5818393	0.009927827	-0.014342044
Shapotou	1.767804306	2.3346807	2.5180899	0.320666938	0.424416657
Shedao-laotieshan	2.35352009	3.0046636	2.5923355	0.276667921	0.101471583
Shei-Pa	5.529632737	5.8106414	5.9472897	0.050818685	0.075530688
Shenmuchoubai	1.572859382	2.0394837	1.9446701	0.296672623	0.236391582

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Shennongjia	3.03960801	3.3772771	3.455241	0.111089683	0.13673901
Shimenhupingshan	3.522257613	4.0369354	4.0200765	0.146121563	0.141335172
Shimentai	4.34055815	4.9686015	5.2527353	0.144691841	0.21015204
Shiwandashanshuiyuanlian	5.361982368	5.2986107	5.2353774	-0.011818701	-0.023611597
Shouchengshuiyuanlin	4.136012421	4.6743513	4.5535564	0.130158913	0.10095327
Shoulushan	1.329549648	2.2403738	2.7076785	0.685062159	1.036538089
Shuangtaihekou	1.280107139	1.3249515	1.5153521	0.035031725	0.183769744
Shuguang	0.495156359	0.8889053	1.3803384	0.79520122	1.78768186
Simianshan	4.342941694	4.2967751	3.8913278	-0.010630259	-0.103988017
Songfengshan	0.311807626	0.5730393	0.739885	0.837797578	1.372889366
Songhuaba	4.136122479	4.6244788	5.0062412	0.118071049	0.210370637
Songhuajiangsanhu	0.487841987	0.7779641	0.8678869	0.59470509	0.779032808
Suoxiyu	3.744563228	4.0814605	3.9500149	0.089969711	0.054866659
Susu	0.427362245	0.7864597	1.1640926	0.840264809	1.723901359
Tachengyebadanxing	1.113086442	1.5539888	2.244644	0.396107922	1.016594503
Taibaishan	2.190025012	2.8556487	2.9680267	0.303934286	0.355247855
Taitong - kongdongshan	2.479497096	3.1889538	3.2369633	0.286129274	0.30549187
Talimuhuyanglin (Tarim)	0.731850107	0.7827091	1.4347354	0.069493729	0.960422478
Tangjiahe	3.26731949	3.9000298	3.6454454	0.19364813	0.115729702
Taoyuandong	4.010578083	4.65248	4.5882173	0.160052218	0.144028917
Taroko	6.40065087	6.4569357	6.3754622	0.00879361	-0.003935329
Tashikuerganyeshengdongwu	0.374775574	0.4841934	1.0601476	0.291955596	1.828753189
Tawushan	6.449766139	6.5764015	6.6226019	0.019634101	0.026797214
Tianchi (Xinjiang)	0.413640792	0.8270696	1.8216088	0.999487517	3.403842259
Tianma	3.260532899	3.7689337	3.9094255	0.155925677	0.199014278
Tiebu	1.49233669	2.6581784	3.1729697	0.781218955	1.126175495
Tiexi	0.757873908	1.1927998	1.6746278	0.573876324	1.209639074
Tongbiguan	4.695141012	5.708225	5.9362822	0.215772857	0.264345881
Touersantan	3.485723924	3.9518337	3.5432415	0.133719648	0.016500898
Tunhuangxihu	0.78660997	0.8856022	1.2197254	0.125846651	0.550610145
Tuomuerfeng	0.247492323	0.4853855	0.6563681	0.961214369	1.652074586
Wahuishan	1.008375306	1.3062069	1.6669644	0.295357881	0.653119022
Wanglang	1.966656941	2.913366	3.0888863	0.481379868	0.060246567
Wawushan	3.393578096	3.9592295	3.9729427	0.16668289	0.170723816
Weiyuanjiang	5.296218996	5.3039563	5.541466	0.001460911	0.046306054
WenchuanCaopo	2.482182373	3.0895488	3.3849365	0.244690492	0.363693714
Wenlanjiang	5.512020513	5.3077275	5.3905429	-0.037063181	-0.022038672
Wenshanlaojunshan	4.625134114	5.0914454	5.5123206	0.100821138	0.191818543
Wolong	2.226933152	2.9311619	3.341406	0.316232549	0.500451864
Wu Ling Yuan	3.706937481	4.1440955	4.016746	0.117929698	0.083575329
Wudalianchihuoshan	0.192568797	0.4054511	0.8224395	1.105487007	3.270886628
Wujiao	2.506918055	3.3708793	3.3205089	0.344630828	0.324538269
Wulatelenglenglin--mengguyelv	0.552223979	0.8240096	1.1897453	0.492165555	1.154461496
Wuliangshan	4.276610942	4.9705823	5.6408029	0.162271333	0.318989026
Wuliangsuhainiaolei	0.642323075	1.0380845	1.0480873	0.616140756	0.631713605
Wulingshan	0.785673619	1.0396885	1.2165839	0.3233084	0.548459654
Wulushan	2.115315374	2.6604441	2.54036	0.257705651	0.200936764
Wuyishan	4.249849161	4.6599941	5.1109583	0.096508117	0.202621106
Wuzhishan	6.686374936	6.7594269	6.6454659	0.010925496	-0.006118268
Xianghai	0.405432707	0.7253346	0.817071	0.789038199	1.015306081
Xiangshan	3.268353297	3.8189908	3.7705003	0.168475514	0.153639144
Xiangtoushan	5.40113347	5.9018235	5.6097032	0.092700918	0.038615919
Xianrendong	1.406685222	2.025562	1.9842726	0.439953991	0.410601725
Xiaohe	3.564841683	3.9950627	4.0655956	0.120684467	0.14047017

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Xiaohegou	2.628934089	3.4445591	3.3231073	0.310249319	0.264051204
Xiaosuganhu	1.02197917	1.3578324	1.6552772	0.328630211	0.619678021
Xiaowutaishan	0.859756071	1.2860287	1.2326404	0.495806477	0.433709446
Xiaoxi	3.597079523	3.9896132	4.0923405	0.10912566	0.137684189
Xiaozhaizigou	2.561513663	3.1436491	3.2286073	0.227262281	0.260429467
Xidamingshanshuiyuanlin	4.953416169	4.7663636	4.8259502	-0.037762337	-0.025732942
Xi'e'erduosi	1.280985413	1.8391057	1.8368926	0.435696052	0.433968398
Xilinguolecaoyuan	0.24001529	0.5176482	0.7557911	1.156730098	2.14892897
Xingdoushan	3.624776709	4.1074179	3.969299	0.133150599	0.095046487
Xingkaihu	0.801902821	1.2059164	1.7160298	0.503818628	1.139947329
Xinglongshan	1.592556376	2.5735305	3.1120161	0.615974504	0.954101058
Xinjiangluobupoyeshuangfengtuo	0.377652057	0.6351586	1.0385473	0.681861884	1.750010971
Xinningshunhuangshan	3.985194504	4.6291497	4.7515863	0.161586893	0.192309759
Xionglongxi	1.126909278	1.9786615	2.5893394	0.755830339	1.297735453
Xishuangbanna	5.576098026	5.8063043	5.7907511	0.041284474	0.038495212
Xishuangbanna	5.624244814	5.88671	5.8572166	0.046666743	0.041422768
Xishuizhongyaredaisenlin	3.929326843	4.1611994	3.882315	0.059010758	-0.01196435
Xitianshan	1.282375399	1.5494784	1.6335789	0.208287683	0.273869493
Xuebaoding	2.815380854	3.4853574	3.4141119	0.237970129	0.212664317
Xunbielahe	0.229326621	0.4875641	0.8174838	1.126068478	2.564713928
Yading	1.381329549	1.6796899	1.9996408	0.215995054	0.447620375
Yalujiangbinhaishidi	1.779385149	2.3025369	2.1080035	0.294007035	0.184680844
Yaluzangbudaxiagu	2.811536007	3.53505	3.8080543	0.257337623	0.3544391
Yaluzangbujiangzhongyouheguoheijinghe	0.536832254	1.0595916	1.5541322	0.973785279	1.895005262
Yanboyezeshan	1.158267795	2.2741909	2.973453	0.963441365	1.567155033
Yancheng	2.570085802	2.3541128	2.3982959	-0.084033382	-0.066842088
Yangchengmanghemihou	2.564979596	2.6777729	2.3767916	0.043974347	-0.073368223
Yangxianzhuhuan	3.636850474	3.7548798	3.3665618	0.03245372	-0.074319435
Yangzie	3.604495881	4.0008207	3.5960218	0.109952912	-0.002350975
Yihuanghuananhu	3.873180847	4.3230026	4.2315213	0.116137555	0.092518389
Yindingshanshuiyuanlin	4.077412271	4.8908244	4.6513785	0.199492245	0.140767279
Yiwulushan	1.141283391	1.3711778	1.5506742	0.20143499	0.358710915
Yongzhoudupangling	4.042135196	4.9242673	4.744988	0.218234191	0.173881568
Youyi	0.94899932	1.8821107	2.7550495	0.983258218	1.903110089
Yuanbaoshanshuiyuanlin	3.949066789	4.60341	4.7112504	0.165695656	0.193003474
Yuanshan	1.966113109	2.4518978	2.0421601	0.24707871	0.038678848
Yueyahu	0.592437574	0.9492332	1.4326278	0.602250164	1.418191997
Yuke	0.952112713	1.8103647	2.7074516	0.901418472	1.843625091
Yulongxueshan	2.427936926	2.8823653	3.1887478	0.187166466	0.313356935
Yunchengtianee	2.721900784	2.9060026	2.5767111	0.067637225	-0.053341284
Yunling	3.607845269	4.1090513	4.4952158	0.138921155	0.245955817
Yunnandaweishan	5.732517823	5.7859962	5.9304935	0.009328951	0.034535554
Yunwushancaoyuan	2.126109799	2.7576579	3.0100536	0.297043973	0.415756421
Yushan	5.693405357	5.9928932	6.0700757	0.052602586	0.066159059
Zhagashenshan	1.11401156	1.8823403	2.5351139	0.689695482	1.275662113
Zhalong	0.288557936	0.519417	0.8907593	0.800044065	2.086933987
Zhangjiajiedani	3.746332725	4.1066225	3.9822749	0.096171323	0.062979504
Zhangmukouan	1.299713281	1.4389909	1.970977	0.107160265	0.516470616
Zhouzhijinsihou	3.039861443	3.4981733	3.3203029	0.150767351	0.092254684
Zhuchanggou	1.490779594	2.1443633	3.1438463	0.438417395	1.108860567
Zhujiangkouzhonghuabaijitun	0.274393703	0.0371043	0.0371043	-0.864777145	-0.864777145
Zhujiangyuantou	4.046219123	4.5566399	4.8763739	0.126147587	0.205168023
Zhumulangmafeng	0.346179922	0.55216	0.9369674	0.595008737	1.706590823
Ziyunwanfengshan	3.674821498	4.3552625	4.7189632	0.185163008	0.284133992

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35133533

THE ROLE OF ONTOGENETIC DEVELOPMENT IN FISH SCALE SHAPE CHANGES

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(Received 12th Nov 2018; accepted 4th Feb 2019)

Abstract. Fish scale investigation has been used in many ways. Recently several species' scale shape was used to differentiate species, populations or stocks. Effects of allometric growth on scale shape proved to be a common phenomenon in case of numerous species, however there is no information regarding the impact of temporal (ontogenetic timescale) changes. In this study the effect of intrapopulation age distribution on the scale shape was tested. Seven age groups of a gibel carp (*Carassius gibelio*) population were identified and analyzed using landmark-based geometric morphometric methods. The results indicated a clear trend of ontogenetic-driven shape change of gibel carp scales: the adult fish specific scale shape occurs at the age of 3+, along with sexual maturation, the alterations among the older age classes show no significant differences. These results suggest that the asymmetric age distribution of fish populations should be taken into consideration during scale morphometric analyses.
Keywords: *geometric morphometrics, shape analysis, allometry, Carassius gibelio, age*

Introduction

Increasing number of papers reported the usefulness of morphometrical studies of fish scale shape (Avigliano et al., 2017; Ibanez and Jawad, 2018; Albertson et al., 2018). The methodology has numerous advantages in fish population studies (e.g. cheap, fast), however more precise assessment of application conditions are necessary. Previous studies described that allometric growth has to be taken into consideration during scale shape analyses (Ibáñez et al., 2012; Staszny et al., 2012.), however the correlations of the specimens' age and scale shape were neither considered, nor studied yet. The correlation between the age and body shape was proved in the case of fish (Wimberger, 1992), and similar patterns were observed in wide taxonomical range, e.g. in case of the human ribcage (Gayzik et al., 2008) or the wing shape of Blackcaps (*Sylvia atricapilla* Linnaeus, 1758) (Pérez-Tris and Tellería, 2001).

As an easily conductible, well-repeatable, non-destructive method, fish scale shape related analyses might emerge importance in various ecological studies (Takács et al., 2016); therefore, assessment of the source of shape variability within a studied group is crucial. The aim of our study was to test the effect of within-population age distribution on scale morphometric analyses, in order to assess the presence of ontogenetic changes in scale shape.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35353544

Review of literature

Answers can be found for many population level questions, encoded in fish scales. Generally, fish scales are used in taxonomy in species identification (e.g. in case of gut content analyses of Piscivores (Bräger et al., 2016)), also used in population level separations (as a cheaper alternative of population genetic methods) (Ibáñez et al., 2007; Garduño-Paz et al., 2010; Staszny et al., 2012; Ibáñez, 2015) and species separation (Şerban and Grigoraş, 2018). It is suitable for age determination and for the back-calculation of individual- or population-level growth in temperate climate zone (Pompei et al., 2011; Soriguer et al., 2000). Similarly to other body parts, the phenotype of scales is affected by genetic, environmental and their covariate effects, during the lifespan of the fish (Ibáñez et al., 2012; Staszny et al., 2013). This phenomenon is partially confirmed by the study of Garduño-Paz et al. (2010), which successfully discriminated sympatric ecotypes of Arctic charr (*Salvelinus alpinus* Linnaeus, 1758) within Loch Tay lake and Loch Awe lake, based on their scale shape.

Materials and methods

Fish samples were collected during the summer of 2011 from the littoral zone of the Kis-Balaton Water Quality Protection System (Hungary, Coordinates N46.657537 E 17.194324) (*Fig. 1*), using a battery-powered electric fishing device (SAMUS 725MP).

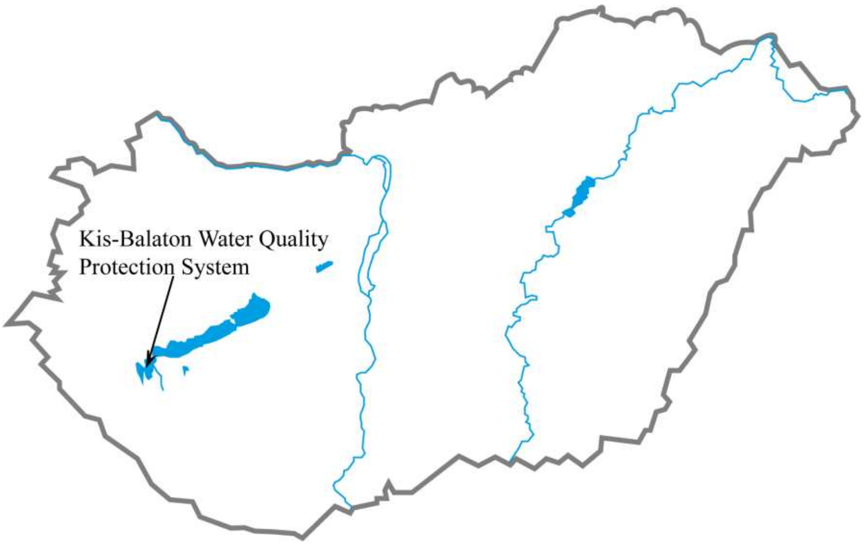

Figure 1. *The sampling area of the study*

The non-indigenous Cyprinid gibel carp (*Carassius gibelio* Bloch, 1782) was selected as model species. Scale samples (one per specimen) were collected from the flank, anterior to the dorsal fin (Garduño-Paz et al., 2010), from the left side of the body in order to avoid the variance caused by the fluctuating asymmetry (Sheridan and Pomiankowski, 1997). Scales were then placed between two glass slides and digitalized by using a HP ScanJet 5300C XPA scanner at 2400 dpi (upper lighting). Altogether 273 gibel carp specimens were collected. The age of each fish was determined based on the number of annual rings, determined based on the digital images by the same expert. Seven experimental groups were created using the scales of randomly selected 20-20

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specimens from age groups of 0+ - 6+, where 0+ stands for young-of-the-year individuals and 6+ represents seven year-old fish. The average standard length and weights of the age groups are shown in *Table 1*.

Table 1. *Average standard length (mm) and weights (g) of the age groups*

Age group	Weight (g)	Standard length (mm)
0+	30.18±19.41	89.27±29.02
1+	35±11.98	101.06±12.52
2+	119±51.98	146.67±23.23
3+	289.67±66.23	205.5±17.73
4+	411.92±57.07	234.54±12.55
5+	586.67±111.35	257.89±17.93
6+	790.67±60.74	287.17±10.74

Shape was analysed by using landmark-based geometric morphometric methods (Zelditch et al., 2004). Seven easily definable landmarks (Ibáñez et al., 2007) were recorded on each scale by using tpsUtil (Rohlf, 2015) and tpsDig2 (Rohlf, 2005) software (*Fig. 2*). Landmarks 1 and 3 are the ventro- and dorso-lateral tips of the anterior portion of the scale. Landmarks 4 and 6 are at the boundary between the area covered by other scales and the exposed area, landmark 7 is positioned at the tip of the posterior portion of the scale. Landmark 2 is in the center of the anterior edge of the scale, and landmark 5 is the focus of the scale.

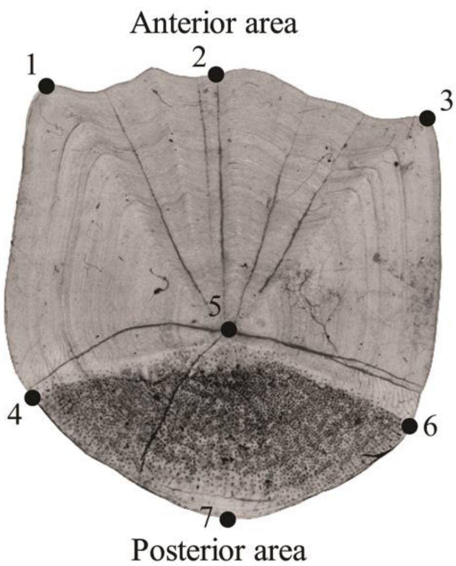

Figure 2. *Landmarks used to define the shape of the scales. The areas of the scales are described with respect to the fish position*

MorphoJ software package was used for further multivariate analysis (Klingenberg, 2011). Generalized least-squares Procrustes superimposition (GLS) was applied to the coordinates of raw landmarks to scale, translate and rotate and get new shape variables independent of the scale size (Rohlf, 1990). A regression analysis was performed

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between the logarithm of centroid sizes and Procrustes coordinates within each group to eliminate the within-group size-variances. Canonical Variates Analysis (CVA) and Discriminant Function Analysis (DFA) were performed to examine the separation of different age groups. Permutation test was performed (10 000 iterations) to assess the reliability of the results.

Results

The result of the regression analysis shows significant allometry in all age groups ($p = 0.001$). The average variance of size accounted for 3.47% of the whole shape variance. All experimental groups could be discriminated significantly from each other, except two pairs (3+-5+ and 4+-5+) (*Table 2*).

Table 2. *Analysis of the seven age groups of gibel carp (Carassius gibelio) scale shape using geometric morphometrics. Left half-matrix: T^2 statistics; right half-matrix: p-values; diagonal in italics: average similarities in groups. The non-significant results are in bold.*

	0+	1+	2+	3+	4+	5+	6+
0+	*0.645*	0.027	<0.001	<0.001	<0.001	<0.001	<0.001
1+	32.72	*0.636*	0.006	<0.001	<0.001	<0.001	<0.001
2+	147.29	42.81	*0.498*	0.002	0.02	0.022	<0.001
3+	616.94	135.57	49.56	*0.498*	0.049	**0.207**	0.008
4+	357.96	147.64	34.81	28.57	*0.373*	**0.145**	<0.001
5+	340.58	97.12	33.92	**19.06**	**21.47**	*0.458*	0.004
6+	761.58	214.29	115.26	40.92	75.64	46.15	*0.484*

CVA analysis revealed that scale shape of younger age groups (0+ – 2+) could be discriminated along with the first canonical axis, while older age-classes (3+ - 6+) form an integrated group, which is only dissolved along the second canonical axis, accounted for considerably less variance (7.3%; *Fig. 3*).

The classification rates proved to be high: 91.6 ± 7.37 % (mean ± SD) (not including the non-significant groups) (*Table 3*).

The comparison of average scale shape of the different age groups shows that up until the 3+ group the change in direction of shape is very similar (*Fig. 4*). Between the 3+ and 4+ groups the scales begin to widen, then the caudal edge starts to narrow and the proportion of the exposed area decreases.

Table 3. *Classification rates of seven age groups of gibel carp (Carassius gibelio) scale shape: results of discriminant function analysis. Left half-matrix: cross-validation rates; right half-matrix: classification rates. The non-significant results are in bold.*

	0+	1+	2+	3+	4+	5+	6+
0+		85%	100%	100%	100%	100%	100%
1+	57.5%		87.5%	95%	95%	95%	97.5%
2+	92.5%	72.5%		87.5%	80%	85%	90%
3+	97.5%	85%	77.5%		77.5%	**77.5%**	85%
4+	95%	95%	67.5%	60%		**80%**	95%
5+	97.5%	85%	62.5%	**55%**	**55%**		85%
6+	97.5%	97.5%	87.5%	67.5%	87.5%	70%	

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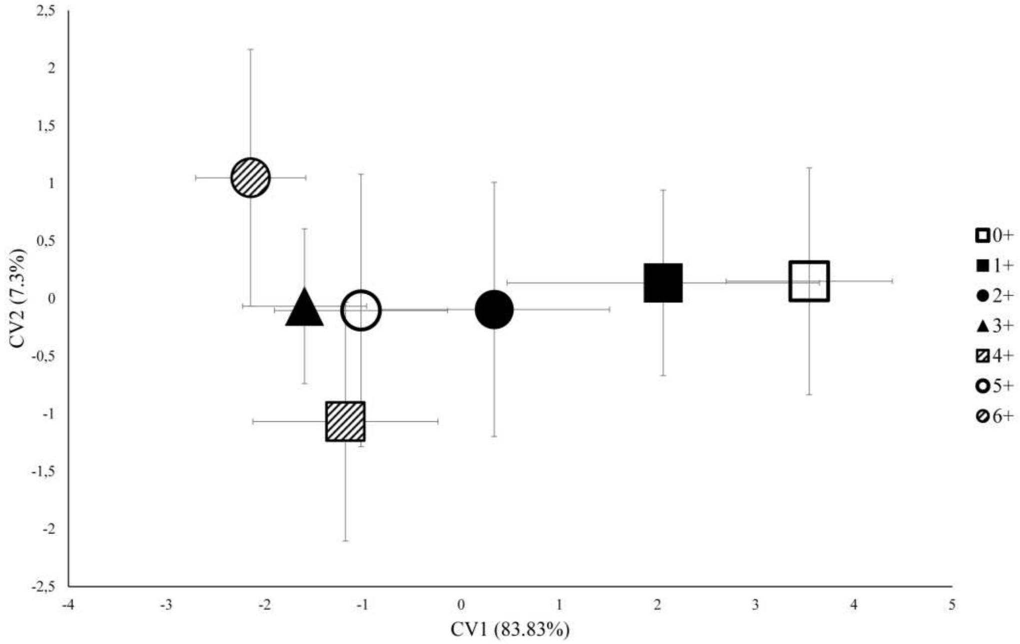

Figure 3. *Differences between the seven gibel carp (Carassius gibelio) age groups based on their scales shape. Symbols shows the groups average, crosshairs shows the standard deviation, percentage values shows the carried variance proportions of the axes.*

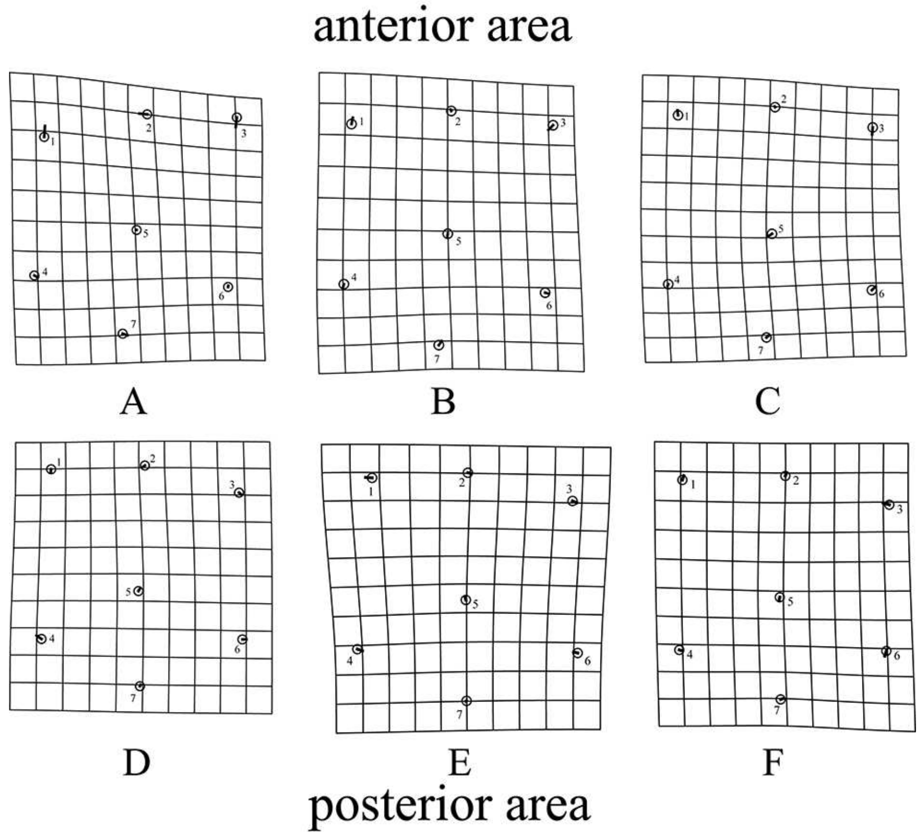

Figure 4. *Differences between the gibel carp (Carassius gibelio) age groups' scales shape illustrated with thin plate spline (A – 0+-1+, B – 1+-2+, C – 2+-3+, D – 3+-4+, E – 4+-5+, F – 5+-6+)*

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The experimental groups were further categorized, based on the CVA results: the former age groups were ranked into three categories, referring as "life stages" (juvenile: 0+, 1+; subadult: 2+; adult: 3+, 4+, 5+, 6+) (Harka and Sallai, 2004). Another CVA was performed on the three new groups ("life stages"). Based on the results of regression analysis the size affected the shape significantly ($p < 0.001$), in similar degree (4.59%) as in age groups. All the three groups separated from each other significantly ($p < 0.001$) (*Table 4*).

Table 4. *Examination of three life stages of gibel carp (Carassius gibelio) scale shape with geometric morphometrics. Left half-matrix: T^2 statistics; right half-matrix: p-values; diagonal in italics: average similarities in groups*

	Juvenile	**Subadult**	**Adult**
Juvenile	*0.79*	<0.001	<0.001
Subadult	72.2	*0.368*	<0.001
Adult	509.37	76.92	0.241

The three groups separated from each other along the first canonical axis (*Fig. 5*), which is accounted for the 98% of the total variance.

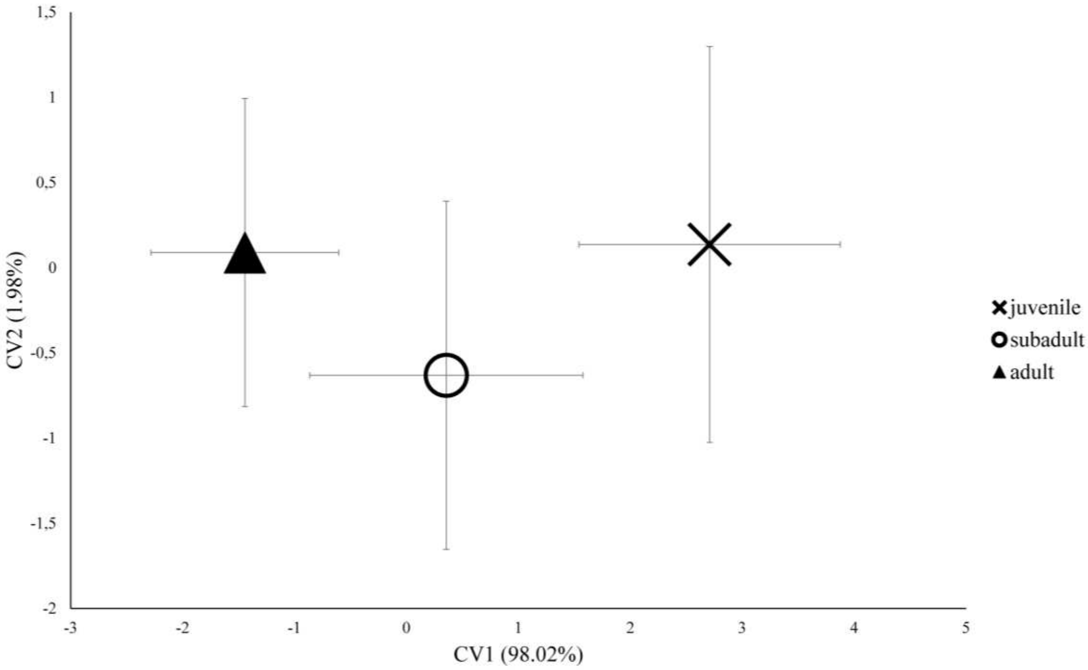

Figure 5. *Separation of the gibel carp (Carassius gibelio) three life stages based on scales shape. Symbols show the groups average, crosshairs show the standard deviation, percentage values shows the carried variance proportions of the axes.*

The classification rates were very similar to the analysis of the seven age groups, (mean ± SD): 89.9 ± 3.54% (*Table 5*).

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Table 5. Classification rates of three life stages of gibel carp (Carassius gibelio), scale shape analyzed with discriminant function analysis. Left half-matrix: cross-validation rates; right half-matrix: classification rates.

	Juvenile	Subadult	Adult
Juvenile		88.75%	93.75%
Subadult	81.25%		86.90%
Adult	96.90%	76.25%	

Discussion

Our analyses revealed a clear trend of scale shape development in case of gibel carp, which was separated into three stages: ‘0+-1+’: juvenile; ‘2+’: subadult; ‘3+ -6+’: adult. Microstructural, histological changes, and genetic background processes of scale development are well known during the ontogenesis of fishes (Breitler-Hahn and Zylberberg, 1993; Sire and Akimenko, 2004; Levin, 2011), however according to our knowledge no comparable data is available regarding the shape changes of other fish species. Our results suggest that however the scale itself appears in a very early life stage (e.g.: at 8 mm standard length in case of zebrafish (Le Guellec et al., 2004)), the shape might have its own ontogenetic development.

In the case of gibel carp, the background of scale shape changes might be related to the sexual maturity, since the time of maturation could be dated to the 3+-4+ age in Central Europe (Szczerbowski, 2001). *Figure 3* shows a clear pattern with opposite shape changes amongst the groups, which is possibly in connection with maturation. In the case of young groups (0+-3+), the groups follow each other step by step in a negative direction along the first canonical axis. Later the groups were separated along the second canonical axis. This phenomenon is likely in connection with the ontogenetic development of body shape, which was described in detail in several studies. In case of red-bellied piranha (*Pygocentrus natterei* KNER 1858) the study of Zelditch and Fink (1995) demonstrated that during the ontogenetic development the middle section of the body, the postorbital section of the head, as well as the nape area are elongating, and the whole head becomes higher. It was also reported in case of cascarudo (*Callichthys callichthys* L. 1758) that the body and the postorbital region elongated, the caudal peduncle became thinner and the height of abdominal region increased (Reis et al., 1998). Hood and Heins (2000) had similar findings in Blacktail shiner (*Cyprinella venusta*), which suggests that ontogenetic changes in reproductive status is in connection with body shape change. The results of our study coincide with these findings. As the number of scale lines are fixed along the body of the fish, after the age 3+, the body height increased relative to body length, which cause the widening of the scales. After that, the middle section of the fish elongated (the body and the scales were growing in higher proportion to cranio-caudal direction), that is the reason of the scales narrowed in dorso-ventral direction.

Conclusion

In conclusion, the age structure of the sample may have an important role in scale shape morphometric studies. In our opinion this finding has to be taken into consideration in further comparative scale morphometric analyses, while the

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asymmetric age distributions might affect the results significantly, false population level separation could be observed without the presence of genetic and/or environmental differences. Furthermore, involvement of specimens' size as a supplementary variable in the scale morphological analyses (Ibáñez and O'Higgins, 2008) became questionable, since length is strongly correlated with the age of the fish (Le Cren, 1951). Further studies needed to test our findings on different other species and the quantified effect of the asymmetric age distribution of the compared groups.

Acknowledgements. This study was supported by the NVKP_16-2016-1-0003: Risks of some endocrine disruptors and developments for risk mitigation in Budapest Metropolitan Region project, financed by the NRDI Fund. Árpád Ferincz, Péter Takács and Ádám Staszny were supported by the Bolyai Fellowship of the Hungarian Academy of Sciences and the Ministry of Human Capacities ÚNKP-18-4-SZIE New National Excellence Program. Gábor Paulovits and Péter Takács were supported by the GINOP 2.3.2 – 15 – 2016 – 00004 project: Establishing the sustainable angling-aimed management of Lake Balaton. The publication is supported by the EFOP-3.6.3-VEKOP-16-2017-00008 project. The project is co-financed by the European Union and the European Social Fund.

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DOI: http://dx.doi.org/10.15666/aeer/1702_35353544

AN AUTOMATED CALCULATION OF PLANT ROOT DISTRIBUTION PARAMETERS BASED ON ROOT LENGTH DENSITY DATA

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(Received 11th Oct 2018; accepted 22nd Nov 2018)

Abstract. Pattern of vertical distribution of plant roots determines plant water and nutrient uptake and influences various soil processes under field conditions. However, information of root distribution in the whole rooting profile is rarely available in practice. This paper documents a method to quickly quantify root vertical distribution pattern using limited root length density data obtained from soil cores under field conditions. It was implemented through the development of a Minitab macro and an R script, with sample calculations conducted on field collected root length density data from the winter wheat crop in Texas, USA. The method is potentially useful for quantifying plant root activities in natural soils where deep roots are difficult to access by physical means and data of root length density distribution are only available in the upper root zone.
Keywords: *computer code, data processing, field measurement, rooting depth, soil core*

Introduction

Vertical distribution of roots of land plants generally follows an exponential decay pattern (Weaver, 1926) and this has been frequently recognized in models describing plant water uptake from the soil profile (Ojha and Rai, 1996; Wu et al., 1999; Yadav et al., 2009; Dong et al., 2010; Dong, 2016). This fashion of root distribution is also an important trait dictating plant resource acquisition from the soil environment (Xue et al., 2003; Acuña and Wade, 2013; Sharma et al., 2014; Wasson et al., 2014; Hodgkinson et al., 2017). For example, the depth at which the densest roots locate in the soil profile influences plants' capacity to tolerate drought stress (Wan et al., 2000; Yu et al., 2007). The roots of numerous plants can easily reach 2 meters deep in the soil (Coupland and Johnson, 1965), but it is difficult to measure the roots from deeper soil profile. As a result, field studies usually only report root data collected from upper soil layers (Xue et al., 2003), and a major challenge for researchers is to extract the most information from the limited survey data of plant roots. The need for obtaining simple indices to summarize root distribution becomes more acute for large field experiment involving a complex design structure. The pattern of vertical distribution of plant roots in the soil profile is described elsewhere (Wu et al., 1999; Zuo et al., 2004; Yadav et al., 2009; Zuo et al., 2013). Despite complexities due to variations in plant growth and soil micro-environment, a salient feature pervading the literature is the exponential decay of the roots with the increase of soil depth. This fashion of root distribution allows calculation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35453552

of the normalized root length density (Wu et al., 1999) that has important applications in describing water uptake by plant roots. The main purpose of this paper is to describe a simple procedure for estimating root distribution pattern using limited field data. This will include an automated calculation to extract root information from large survey data sets, so that variables such as normalized root length density can easily be obtained based on field survey data of root length density distribution.

Method

The relative importance of roots located in a particular soil layer to plant water uptake is conveniently described by the normalized root length density L_{nrd} (dimensionless), which can be calculated according to Wu et al. (1999):

$$L_{nrd} = \frac{L_d(z_r)}{\int_0^1 L_d(z_r)\,dz_r} \quad \text{(Eq.1)}$$

where $L_d(z_r)$ is root length density (cm cm^{-3}), $z_r = z/L_m$ is the relative soil depth containing plant roots and z (cm) and L_m are soil depth and the maximum rooting depth (cm), respectively. It has been shown that *Equation 1* can be used as a weighting factor to partition potential transpiration into components of root water uptake occurring from different soil layers (Wu et al., 1999; Zuo et al., 2013).

Under field conditions, values of L_m at different growth stages of a crop may be estimated using a sigmoidal growth curve (Wu et al., 1999; Dong et al., 2010), or based on the ratio of potential transpiration and measured maximum rooting depth at the peak growth stage (Ojha and Rai, 1996). While $L_d(z_r)$ can easily be measured for a particular soil layer by destructive sampling, the denominator of *Equation 1* normally needs to be estimated based on measured root length density data, as well as a model describing root length density as a function of soil depth. Assuming that root length density $L_d(z)$ is measured for the first L' cm of soil for n intervals, each of L'/n cm in depth, root length density as a function of soil depth may be described using an exponential decay function

$$L_d(z) = Ke^{-\alpha z} \quad \text{(Eq.2)}$$

in which K and α are parameters that can be estimated using a nonlinear least-squares regression procedure. Then, with the estimated values of K and α, the total lengths of roots (per unit of soil surface area) contained in the soil depth from L' to L_m can be estimated by integrating *Equation 2:*

$$L_{bottom} = \int_{L'}^{L_m} Ke^{-\alpha z}dz = -\frac{K}{\alpha}\left[e^{\alpha L_m} - e^{\alpha L'}\right], \quad \text{(Eq.3)}$$

where K is maximum root length density near soil surface (z = 0 cm), α is a parameter determining the rate of decay of root length density with soil depth z (cm).

The normalized root length density (*Equation 1*) for the ith soil layer can be calculated as

$$L_{nrd,i} = \frac{L_m L_{d,i}}{\sum_{k=1}^{n} \Delta z_k L_{d,k} + L_{bottom}}, \quad \text{(Eq.4)}$$

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where the 2nd term of the denominator (L_{bottom}) is calculated according to *Equation 3*. Also, we used the relation $\int_0^{L_m} L_d(z)dz/L_m = \int_0^1 L_d(z_r)dz_r$. *Figure 1* shows the distribution of normalized root length density at different relative depths of soil.

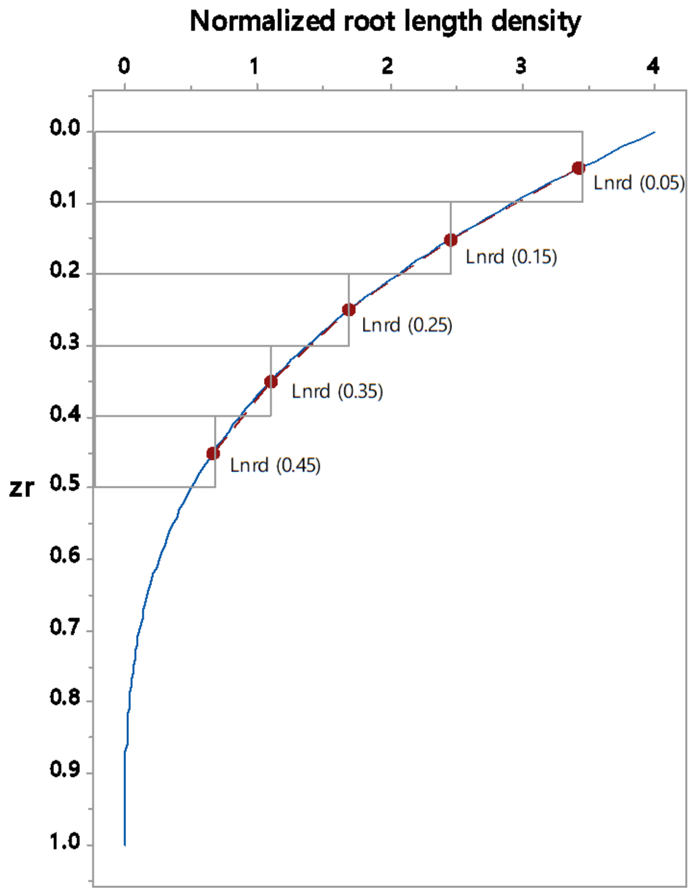

***Figure 1.** Normalized root length density (L_{nrd}) as a function of relative soil depth (z_r), based on measured root length densities in the upper root zone (rectangles). Numerals enclosed in parentheses indicate the relative depths of centers of the soil depth intervals with root measurement. Also shown is the predicted distribution of L_{nrd} in the root zone, assuming $\beta = 3.0$*

The normalized root length densities calculated in *Equation 4* will be fitted to a one-parameter nonlinear model of Ojha and Rai (1996), assuming maximum rooting depth (L_m) is known:

$$L_{nrd} = (\beta + 1)(1 - z_r)^{\beta}, \quad \text{(Eq.5)}$$

where β is the unknown parameter to be estimated using nonlinear least-squares. Compared with the four-parameter polynomial equation of Wu et al. (1999), *Equation 5* is simpler, containing only one parameter. It attains the maximum value near soil surface when $z_r = 0$; it gives zero value at bottom of the root zone ($z_r = 1$). Also, the integral of *Equation 5* over the root zone is unity: $\int_0^1 L_{nrd}(z_r)dz_r$. It should be noted that *Equation 5* was also obtained independently from a different derivation by Zuo et

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35453552

al. (2004, 2013) as a generalized root length density function. It was used by Dong et al. (2010) to characterize plant root distribution pattern in a mixed-grass prairie. The best parameter can be estimated using nonlinear curve-fitting method based on measured root length density data.

Once the values of β are estimated based on experimental data, *Equation 5* can be employed to calculate the relative soil depth $z_{r,95}$ (or absolute soil depth z_{95}) above which 95% of roots are found. Knowing how much deep into the soil profile should we dig in order to find 95% of roots will surely help our future root survey by concentrating our efforts in the depth of soil that is most important for root growth and function. We can estimate $z_{r,95}$ by evaluating the following definite integral:

$$\int_0^{z_{z,95}} (\beta + 1)(1 - z_r)^{\beta} dz_r = 0.95, \quad \text{(Eq.6)}$$

which yields

$$z_{r,95} = 1 - e^{\frac{ln(0.05)}{\beta+1}}. \quad \text{(Eq.7)}$$

Using *Equations 6* and *7,* the absolute depth above which 95% of plant roots are found (i.e., z_{95}) can be calculated as $z_{95} = L_m z_{r,95}$.

Computer code with sample data testing

The root distribution parameters can be calculated using the Minitab macro **len.mac**, or the R script **len_R.txt**, as can be found in the *Appendix*. As a test of the computer code, a sample input data file, **soil.csv**, is provided and the output results are shown in **out.csv**. The main output results include (a) distribution pattern of root length densities as measured from soil coring (not normalized within the whole root zone; see the documentation of **len.mac** in the *Appendix*); (b) the ratio of total roots contained in the upper and lower profile (the dividing line between the upper and lower profile is set at 40 cm in the illustration, but this can be changed easily); (c) the distribution pattern of normalized root length density as measured from soil coring; and (d) the estimated depth of soil containing 95% of total root lengths. Parameter values estimated from the nonlinear regression procedure are accompanied by estimated standard errors. As can be seen from the sample output file **out.csv**, standard errors of the model parameters for some soil depths are quite high, primarily due to the small sample size (5 for each soil core in the testing data set). The errors could be reduced for larger sample sizes with soil layers divided into thinner segments. It has been found that root vertical distribution is strongly influenced by soil bulk density (Zhang et al., 2012). Thus, under some situations, such as one in which there exists a hard plow pan, root distribution can become more complex. In that case, different nonlinear models may be used in place of current ones (modifications may be made to lines marked with *** in **len.mac**), as well as some other minor changes to properly collect the outputs from new nonlinear models.

The current setup of the programs gives reasonable estimates of the average root distribution patterns of winter wheat varieties from a relatively large data set obtained in field conditions, as seen in *Figure 2,* in which fitted values for normalized root length densities for 240 soil core samples are compared against the measured ones. The root samples were collected in two seasons in 2016 and 2017 during the flowering stage

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35453552

from the same set of ten winter wheat varieties planted in two locations in Texas: Amarillo and Uvalde. The soil in Amarillo is clay loam (Xue et al., 2003) while that in Uvalde is clay (Sharma et al., 2014). Amarillo (35°52'N, 101°58'W; elevation 1114 m) is located in Texas High Plains, with an annual rainfall of 516 mm, average temperature of 14.1 °C and average wind speed of 6.4 m/s. Uvalde (29°13'N, 99°45'W; elevation 283 m) is located in the southwest of Texas (Wintergarden), with an annual rainfall of 594 mm, average temperature 27.6 °C and average wind speed 3.1 m/s. Root samples were taken using a hydraulic soil corer from 1 m profile at 20 cm intervals. The samples were promptly stored in a -20 °C freezer and later washed to obtain the fine roots, which were scanned into digital images and measured for root length using the WinRhizo software version 2013b (Reagent Instruments Inc., Quebec, Canada). Root length density was expressed as the total length of roots contained in unit soil volume (cm/cm^3). The winter wheat plots were planted with a seeding rate of 5.8 g/m^2 and row spacing of 19 cm (see Liu et al., 2018 for detail).

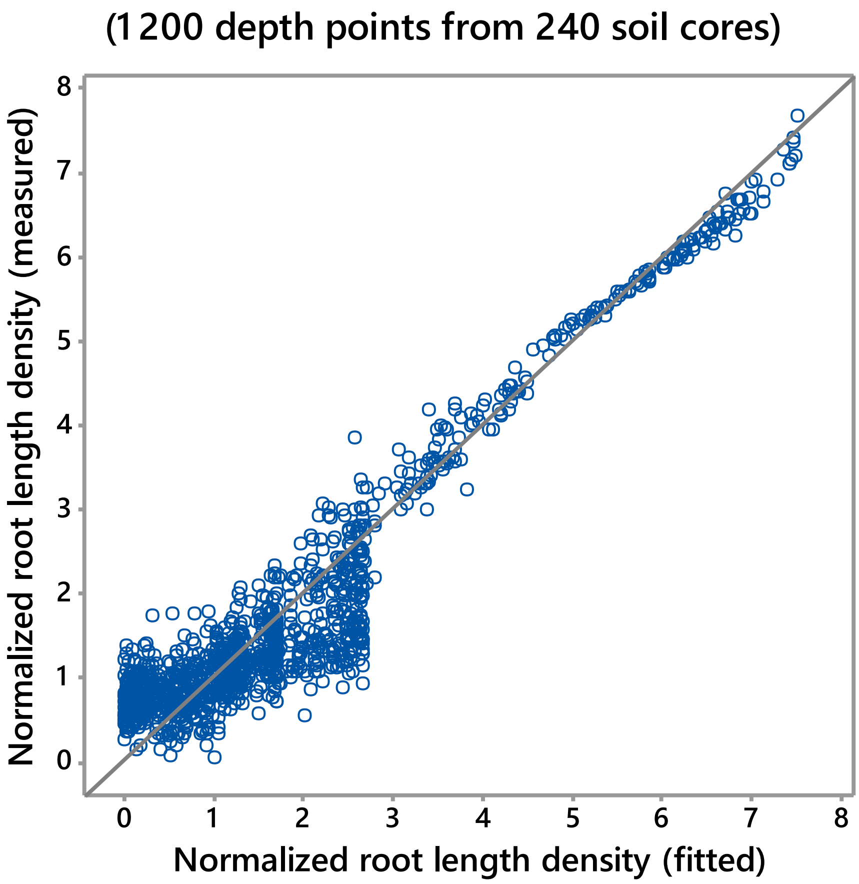

Figure 2. *Measured normalized root length density (L_{nrd}) as compared against the values fitted using the nonlinear regression based on 1200 depth points from 240 soil cores collected in winter wheat fields in Uvalde and Amarillo, Texas, USA. Also shown is the 1:1 line*

The proposed root data processing method allowed us to estimate average soil depths (z_{95}) containing 95% of roots in two Texas locations. As seen in *Figure 3,* the average depth of z_{95} in Amarillo was 117 cm, while the value for Uvalde was only 63 cm. A further discussion of the agronomic significance of these data is out of the scope of this

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DOI: http://dx.doi.org/10.15666/aeer/1702_35453552

method paper, but we do see clearly that the shallow rooting depths in Uvalde are associated with the heavy clay soil prone to compaction, while the soils in Amarillo are of clay loam type that promote deep root growth, especially for winter wheat (the dry, windy weather conditions in Amarillo may also be a factor influencing root distribution). Full data of root length density used to generate the results in *Figures 2* and *3,* as well as other related results, are collected in the *Appendix*. Finally, the computer code (both R script and Minitab macro), testing data and full data set are freely available at Zenodo (https://zenodo.org/record/1484655#.W-o6BzFReUk).

Plant root distribution is frequently described by an empirical equation relating the cumulative proportion of roots to soil depth according to $Y = 1 - \beta^D$, where Y is the cumulative proportion of roots (from 0 to 1), D is soil depth (cm) and β is a fitted extinction coefficient (Gale and Grigal, 1987; Jackson et al., 1996). While this equation can also be used to calculate the soil depth at which 95% of roots can be found, the parameter β is defined differently from that in *Equation 5,* in which β is associated with normalized root length density, a quantity that is directly useful for modeling root water uptake (Wu et al., 1999).

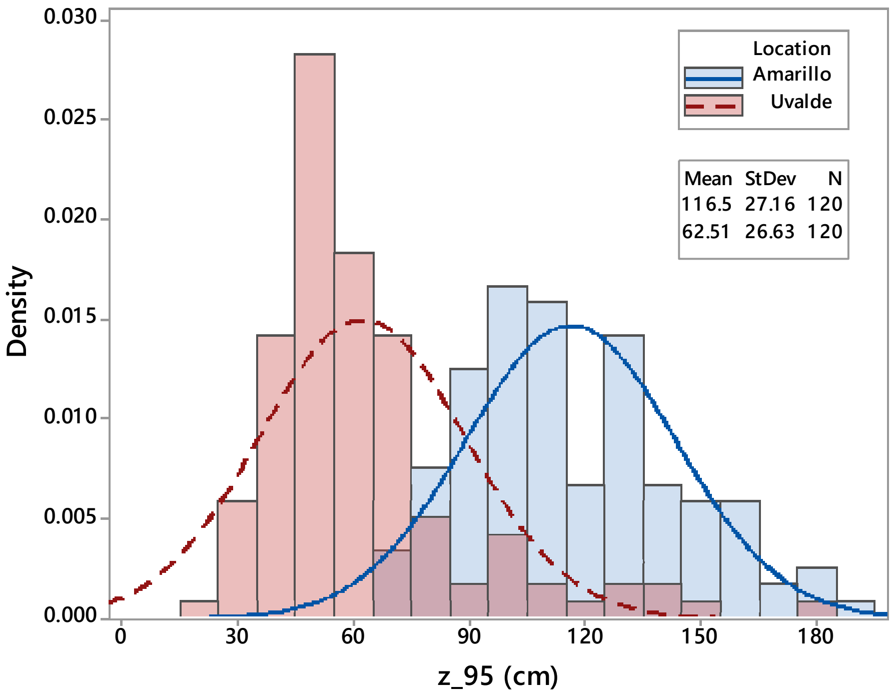

Figure 3. *Histograms of soil depths that contain 95% of total root lengths (z_{95}) for 240 plots of winter wheat growing in Amarillo and Uvalde of Texas, USA. The values of z_{95} are calculated according to Equation 7 of the main text, which uses the estimated values of corresponding to each of the 240 soil core samples. "Density" in the vertical axis indicates probability density of the fitted normal distribution curves*

Implications and potential uses of the computer code

Although root densities of plants generally tend to decrease exponentially from top to lower soil layers, the rate at which this decrease occurs may change under different situations. The computer code documented in this paper can facilitate a rapid extraction of parameters dictating root distribution patterns, with the potential of uncovering root

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traits associated with different genotypes, environment or management interaction scenarios. With the accumulation of field data of root distribution patterns in regional and global scales, rapid methods, such as the one proposed in this paper, are much needed in order to conduct meta-analysis using large data sets. In particular, the method proposed in this application note can handle variable sample sizes (one sample is defined as one set of roots obtained from one soil core divided into different segments), as well as flexible depth intervals (i.e., variable and/or uneven depth intervals for samples collected from the same ecosystem by different researchers under different circumstances). Finally, as the current method relies on the nonlinear regression procedure, the precision of parameter estimation is heavily dependent on the quality of field data (i.e., with soil cores sampled at fine, instead of coarse, segments).

Acknowledgements. We thank Shane Sieckenius and Dalton Thompson at the Uvalde Research Center and Kirk Jessup at the Amarillo Research Center for field assistance. We also thank Cathy Akritas at Minitab, Inc. for helpful suggestions for the development of the Minitab macro. The work was supported by the Texas A&M AgriLife Research cropping system seed grant entitled "Root-shoot phenotyping and water balance characterization to improve water use efficiency and productivity of cropping systems in Texas", and by the USDA National Institute of Food and Agriculture NIFA Hatch project 1018758.

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ELECTRONIC APPENDICES

1. List of Minitab macro **len.mac**.
2. List of R script **len_R.txt**.
3. Sample input data **soil.csv**.
4. Sample output results **out.csv**.
5. Full data set for generating results in *Figures 2* and *3* (**full.csv**).
6. Output results from the full data set (**full_out.csv**).

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DOI: http://dx.doi.org/10.15666/aeer/1702_35453552

IMPACT OF STAND DENSITY ON SOIL QUALITY IN CHINESE FIR (*CUNNINGHAMIA LANCEOLATA*) MONOCULTURE

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(Received 30th Nov 2018; accepted 18th Feb 2019)

Abstract. It is known that forest management practices and land use affect soil quality worldwide. This study was conducted to assess the potential effects of stand density on soil quality in Chinese fir plantations. Low- (1,450 trees/ha with 2.36 × 2.36 m spacing), intermediate- (2,460 trees/ha with 1.83 × 1.83 m spacing), and high-density (3,950 trees/ha with 1.44 × 1.44 m spacing) stands in a 10-year-old Chinese fir monoculture plantation were examined, and different soil quality indicators were measured. The results indicated that stand density affected nitrogen (N), phosphorous (P), and magnesium (Mg) content, whereas potassium (K) and calcium (Ca) were not affected. Total N and total P contents were higher in the low-density stands, whereas total Mg was higher in the intermediate-density stands. Available N was higher in the low-density stands, whereas available P was higher in the intermediate-density stands. No significant difference was observed in the contents of available K, total K, and total Ca among all densities. Soil organic matter was significantly higher in the intermediate-density stand than in the high- and low-density stands. Soil bulk density increased from the surface layer to the 40-60 cm soil layer. Soil pH was lowest in the surface layer of soils of all three densities and increased from the 0-20 cm layer to the 20-40 cm layer; however, it decreased from the 20-40 cm layer to the 40-60 cm layer. Soil pH differed significantly between soils of different densities but remained within an optimum range (4.1-4.5) for Chinese fir plantations. Soil moisture content was significantly higher in the high-density stand than the other stands. The observed effects of stand density on soil quality may be useful for policy makers and forest managers to implement improved forest conservation practices for preserving soil quality and stand production.
Keywords: *forest conservation, soil health, sustainable forestry, nutrient status, competition*

Abbreviations: SOM−soil organic matter, SMC−soil moisture content, BD−soil bulk density, DBH−diameter at breast height, TN−total nitrogen, TP−total phosphorus, TK−total potassium, TCa−total calcium, TMg−total magnesium, AN−available nitrogen, AP−available phosphorus, AK−available potassium, EC−soil electrical conductivity, SO−silty oxisol

Introduction

Chinese fir (*Cunninghamia lanceolata* (Lamb) Hook) is an indigenous, typically evergreen, sub-tropical conifer species, and it is a widely cultivated timber specie in China due to its high yield, excellent timber quality, and fast growth. According to the Eighth National Forest Inventory, the planting area of Chinese fir covers more than 11 million ha, which accounts for about 18.2% of all forest plantations in China, and for 6% of the global forest plantation area (State Forestry Administration, 2014). The yield of Chinese fir plantations has declined continuously over the past years (Yang et al., 2005a), and standing wood volumes in the second and third rotations were 30% and

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

47% less those in the first rotation, respectively (Bi et al., 2007). Serious concerns have been raised regarding the decrease in yield and the long-term decline in the productivity of Chinese fir (Ma et al., 2007; Zhao et al., 2013; Zhou et al., 2015). In other countries such as Brazil, deforestation rates are either decreasing or stabilizing; however, in China, Malaysia, and Indonesia, deforestation rates are increasing due to the increasing demand for wood-derived products (Margono et al., 2014; Abood et al., 2015).

In forest research, soil quality monitoring has been recognized as a primary aspect for sustainable management. Soil quality can vary due to management and land use practices, which is strongly correlated with physio-chemical, bio-chemical, and biological soil properties (Dudley et al., 2014). Land over-use and intensive cultivation resulted in millions of ha of degraded land, and soil erosion has led to the loss of topsoil and exposure of soils with low nutrient content (Liu and Zhao, 1993; Wei et al., 2006; Zhou et al., 2006). For commercial purposes such as timber production, deforestation continues to be carried out globally on a large scale, leading to significant decreases in soil quality and ecosystem productivity, and Chinese fir plantations are no exception (Wang et al., 2014).

In Chinese fir plantations, surface soil hydrolysable nitrogen (N) and available phosphorous (P) declined under continuous cropping (Horner et al., 2010). Various studies demonstrated a reduction in soil nutrient availability, microbiome structure, microbial enzyme activity (Yang et al., 2010; Vitali et al., 2016; Wang et al., 2017; Zhang et al., 2017b), N stocks, and soil organic carbon after conversion of native forests to plantations (Chen et al., 2013; Marin-Spiotta and Sharma, 2013; Guan et al., 2015; van Straaten et al., 2015). Therefore, a decline in timber yield and productivity under the current management practices of Chinese fir plantations is likely a response to impoverished soils.

Soil quality indicators have been assessed in previous studies in different regions of China with the main research focus on different provenances or age of the plantations. These studies are useful, however, there is no comprehensive understanding of soil quality in Chinese fir stands of different planting densities. The current study was conducted to fill this gap by studying different soil quality indicators in Chinese fir monoculture plantations of the same age (ten years) but with different stand densities. The soil quality parameters total soil nutrient and available nutrient content, soil organic matter content (SOM), soil pH, soil bulk density (BD), and soil moisture content (SMC) were analyzed at low-, intermediate- and high-density stands. We hypothesized (1) that due to competition by higher under-story vegetation and higher number of trees in low- and high-density stands respectively, total and available nutrient content should be higher in intermediate-density stands; (2) that stand density should have no effect on soil pH and electrical conductivity (EC), while SOM and SMC should be higher in high-density stands than in low- and intermediate-density stands due to maximum shade and less sun light availability.

Materials and methods

Study site

The Xinkou Experimental Forest Farm was established in 2007 as Chinese fir monoculture plantations, in Sanming City, Fujian Province, China (*Fig. 1*). The study site was located at 26°10′ N and 117°27′ E. Soils were acidic and the climate of the region was a typical mid-subtropical monsoon climate (Zhou et al., 2015), with mean

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

annual rainfall of 1,612 mm and a mean annual temperature of 19 °C, according to an on-site meteorological station. The average monthly temperature and precipitation is shown in *Table 1*. Before the plantation was established, the area was cut clear, the debris was piled and burned, and complete tillage was carried out. After this, the surface biomass was burned before establishing stands, and one-year-old Chinese fir seedlings were planted. Three plantation stands with different densities were selected and categorized as low-, intermediate-, or high-density stands. The understory vegetation in the selected Chinese fir stands were *Maesa japonica*, *Woodwardia japonica*, *Callicarpa kochiana*, *Selaginella moellendorffii*, *Ilex pubescens*, and *Alpinia japonica.* Further details on the plantation stands are shown in *Table 2*.

Soil sampling

Soil was sampled in the second week of November 2017. Four plots of 20 × 20 m were established in each stand, and in each plot, four pits were dug diagonally. A soil corer was used to obtain soil samples from 0-20, 20-40, and 40-60 cm depths from each pit. After collection, the soil samples from the four sampling pits were mixed to produce one sample for each soil layer and measure pH. Eight composite samples per density from a 0-60 cm depth were prepared to measure the total and available nutrient content, SOM, and SMC. All samples were sealed in clean, air-tight polythene bags. Three soil samples were collected from each stand to determine soil BD at 0-20, 20-40, and 40-60 cm depths using 200 cm^3 volumetric rings.

Table 1. *Average monthly temperature and precipitation in Sanming City, Fujian, China*

	Average temperature (°C)	**Minimum temperature (°C)**	**Maximum temperature (°C)**	**Average precipitation (mm)**
January	10.4	5.9	14.9	57
February	11.4	7.3	15.5	101
March	15.2	10.9	19.6	180
April	19.9	15.4	24.4	203
May	23.6	19.4	27.9	279
June	26.5	22.1	31.0	276
July	28.8	23.7	34.0	122
August	28.4	23.4	33.4	134
September	26.1	21.5	30.8	108
October	21.3	16.5	26.1	63
November	16.7	12.1	21.3	49
December	11.3	6.5	16.1	40

Table 2. *Details of plantation stands. Shown are the means ± SE values of average height and diameters at breast height (DBH). "SO" indicates the soil type silty oxisol*

Stand density	**Number of trees/ha**	**Spacing (m)**	**Altitude (a.s.l)**	**Avg. DBH (cm)**	**Avg. height (m)**	**Soil type**
Low	1,450	2.36 × 2.36	206	13.05 ± 0.12	12.41 ± 0.14	SO
Intermediate	2,460	1.83 × 1.83	204	12.46 ± 0.43	12.01 ± 0.30	SO
High	3,950	1.44 × 1.44	210	11.04 ± 0.19	11.63 ± 0.23	SO

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

Figure 1. *Location of the study area, Xinkou Experimental Forest Farm, in Sanming City, Fujian province, China*

Sample preparation

All samples were transferred to a laboratory for further analyses. Debris and roots were removed carefully by hand. The soil samples were air-dried at room temperature and then crushed and passed through sieves of 2 mm and 0.15 mm mesh size for subsequent analyses. To determine soil BD, the samples were weighed and then dried to constant weight before re-weighing.

Laboratory analyses

All measurements were conducted in the key laboratory of soil and water conservation, College of Forestry, Fujian Agriculture and Forestry University, China. The respective analysis methods are shown in *Table 3*.

Table 3. *Methods and equipment used for soil quality analyses*

Analysis	Method/Equipment
pH	Potentiometric method (1:2.5 soil:water)
Electrical conductivity (EC)	Conductivity meter
Bulk density (BD)	Core method of the Nanjing Institute of Soil Science (1978)
Total nitrogen	CN elemental analyzer
Total phosphorus	Molybdenum-antimony colorimetric method
Total potassium	Flame photometry
Total calcium	CN elemental analyzer
Total magnesium	CN elemental analyzer
Available nitrogen	Kjeldahl method (Ryan et al., 2007)
Available phosphorus	Bray and Kurtz (1945)
Available potassium	Schollenberger and Simon (1945)
C:N ratio	CN elemental analyzer
Moisture content (SMC)	Calculated based on wet and dry weight
Soil organic matter (SOM)	Soil organic carbon was first measured by wet oxidation (CN elemental analyzer) and then calculated as SOM

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

Statistical analyses

All measurements are reported as mean ± standard error. A one-way analysis of variance was used to determine correlations between soil quality indicators and stand densities using the SPSS software (SPSS 13.0, SPSS Inc., IL, USA). Means of variables that showed significant differences were compared using Tukey's test at a 5% level of significance. Origin 9.1 and ArcGis software were used to produce graphs and figures.

Results

Variation in total soil nutrient contents

A significant difference in total N and P contents between stands was observed at $P < 0.05$. Total N content was 0.99 ± 0.2 g/kg, 0.74 ± 0.1 g/kg, and 0.92 ± 0.08 g/kg, and total P content was 0.59 ± 0.03 g/kg, 0.48 ± 0.1 g/kg, and 0.59 ± 0.2 g/kg in low-, intermediate- and high-density stands, respectively. Both total N and total P contents were highest in the low-density stand and lowest in the intermediate-density stand.

Total Mg contents were highest in intermediate-density stand and lowest in the low-density stand. No significant differences between stand densities were found in total K and Ca contents. Total K contents were 22.5 ± 7.5 g/kg, 21.2 ± 8.2 g/kg, and 21.8 ± 2.5 g/kg and total Mg contents were 7.41 ± 3.9 g/kg, 8.51 ± 3.7 g/kg, and 7.91 ± 0.5 g/kg in low-, intermediate-, and high-density stands, respectively. Total Ca contents were 2.23 ± 0.4 g/kg, 2.25 ± 1.09 g/kg, and 2.14 ± 0.9 g/kg in low-, intermediate-, and high-density stands, respectively (*Fig. 2*).

Variability in available soil nutrient contents

Available N and available P were significantly different between soils of different stand densities, whereas available K contents did not differ significantly. Available N contents in soils of low-, intermediate- and high-density stands were 67.69 ± 2.2 mg/kg, 59.04 ± 3.0 mg/kg, and 54.07 ± 1.9 mg/kg, respectively, and available P contents were 3.92 ± 0.2 mg/kg, 5.16 ± 0.4 mg/kg, and 4.79 ± 0.5 mg/kg, respectively. Available K contents in soils of low-, intermediate- and high-density stands were 87.23 ± 2.1 mg/kg, 81.44 ± 3.9 mg/kg, and 87.30 ± 5.4 mg/kg, respectively.

Available N was highest in the low-density stand and lowest in high-density stand, whereas available P was highest in the intermediate-density stand lowest in the low-density stand. Available K did not differ significantly between the low- and high-density stand, both of which differed significantly from the intermediate-density stand (*Fig. 3*).

Soil pH, BD, SOM, EC, and SMC

Modest variations in the pH values were observed between stands in each soil layer (*Fig. 4a*). Soil pH in the 0-20 cm soil layer was 4.21 ± 0.1, 4.28 ± 0.04, and 4.27 ± 0.2 in the low-, intermediate-, and high-density stands, respectively. The soil pH values in the 20-40 cm soil layer were 4.27 ± 0.03, 4.37 ± 0.02, and 4.29 ± 0.1, respectively. In the 40-60 cm layer, the pH values were 4.15 ± 0.04, 4.28 ± 0.02, 4.27 ± 0.1 in the low-, intermediate-, and high-density stands, respectively. The soil pH values in the 0-20 cm layer was lower in intermediate- and high-density stands compared to the 20-40 cm and 40-60 cm layers (*Fig. 4a*). Soil pH was higher in the 20-40 cm layer and lower in the

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):3553-3566.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

40-60 cm layer. There was no consistent decline from the 0-20 and 40-60 cm layers in the intermediate- and high-density stands, however, these layers differed significantly different in the low-density stand.

Soil BD generally increased with increasing stand density, which was consistent in the 0-20 cm, 20-40 cm, and 40-60 cm soil layers (*Fig. 4b*). In the surface layer, soil BD was 1.06 ± 0.1 g/m^3, 1.24 ± 0.04 g/m^3, and 1.36 ± 0.2 g/m^3, and in the 20-40 cm layer soil BD was 1.19 ± 0.2 g/m^3, 1.24 ± 0.1 g/m^3 and 1.34 ± 0.3 g/m^3 in low-, intermediate-, and high-density, respectively. Furthermore, in the 40-60 cm layer, soil BD was 1.04 ± 0.1 g/m^3, 1.15 ± 0.3 g/m^3, and 1.29 ± 0.1 g/m^3 in the low-, intermediate-, and high-density stand, respectively. In the surface layer, soil BD differed significantly between stands and increased from the low- to the high-density stand. Soil BD in the 20-40 cm and 40-60 cm layers was higher in the high-density stand and lowest in low-density stand (*Fig. 4b*).

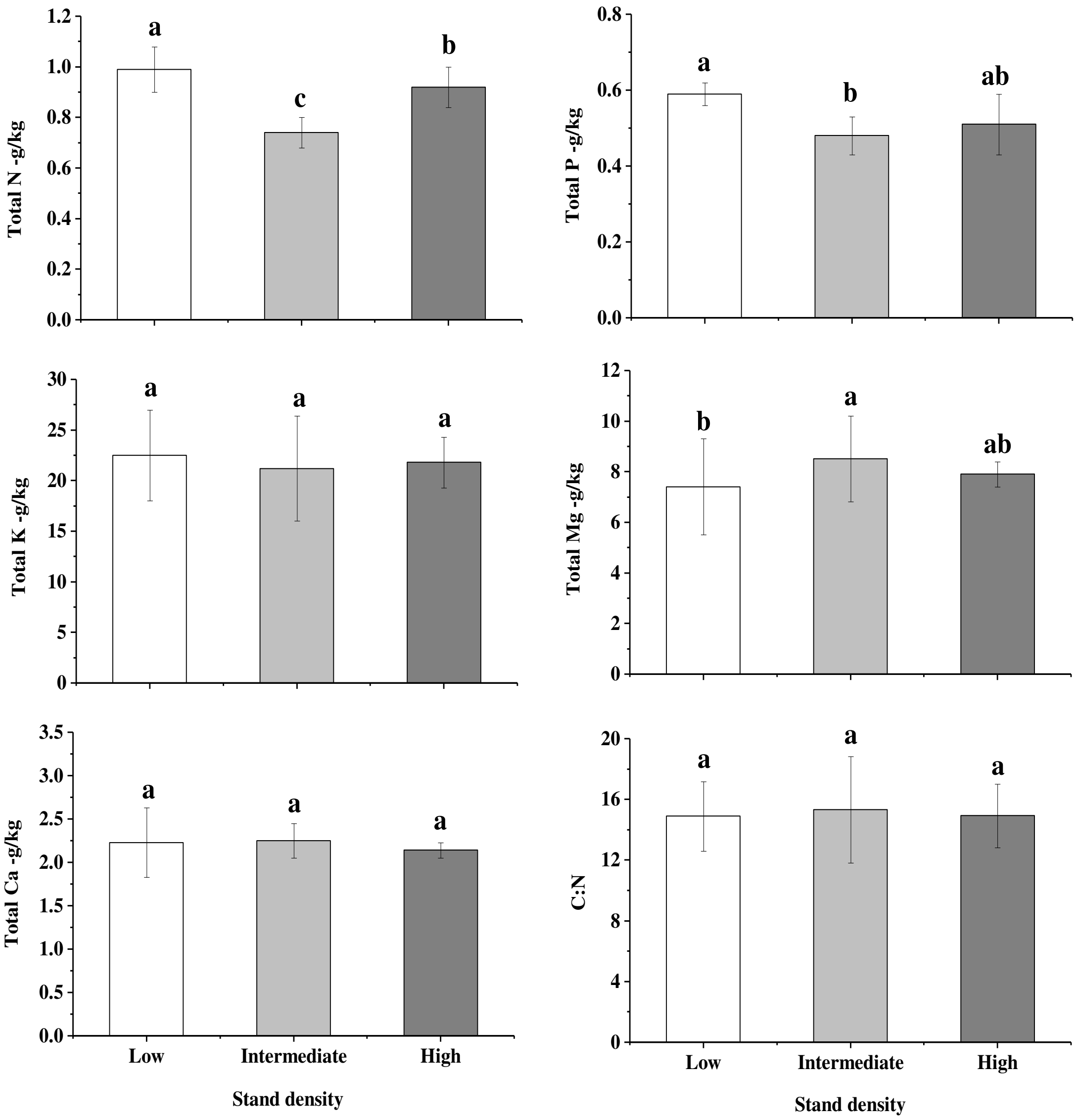

Figure 2. *Variation among total soil nutrient contents in low-, intermediate-, and high-density stands (N = 24). Columns indicate mean ± SE, different letters indicate significant differences at P < 0.05*

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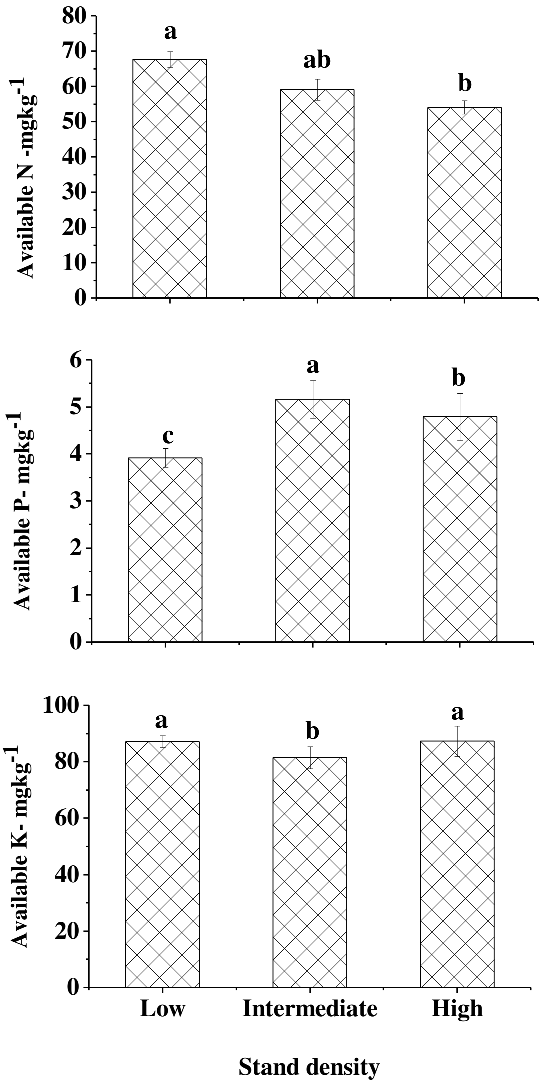

Figure 3. *Available N, available P, and available K at low-, intermediate-, and high-density stands (N = 24). Columns indicate mean ± SE, different letters indicate significant differences at P < 0.05*

At a 0-60 cm soil depth, the soil pH was highest in the intermediate-density stand and lowest in the low-density stand. In both the intermediate- and the high-density stands the soil pH values were significantly different from the low-density stand. Soil pH values were 4.21 ± 0.04, 4.31 ± 0.03, and 4.27 ± 0.03 in the low-, medium- and high-density stands, respectively. Soil BD was highest in the intermediate-density stand and lowest in the high-density stand. Soil BD was 1.22 ± 0.07, 1.28 ± 0.01, and 1.16 ± 0.09 in low-, medium-, and high-density stands, respectively, and differed significantly between stands (*Table 4*).

No significant difference in soil EC was observed among the stands. SOM was higher in the intermediate-density stand and lower in low-density stand; the SOM values were 31.90 ± 1.34 g/kg, 36.56 ± 1.61 g/kg, and 35.47 ± 0.8 g/kg in the low-, intermediate-, and high-density stands, respectively. No significant difference was observed between the intermediate- and high-density stands; however, both were significantly different from the low-density stand. SMC was higher (17.2%) in the high-

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

density stand than in the intermediate- (9.53%) and low-density (11.49%) stands. SMC in the high-density stand differed significantly from the intermediate- and the low-density stands (*Table 4*).

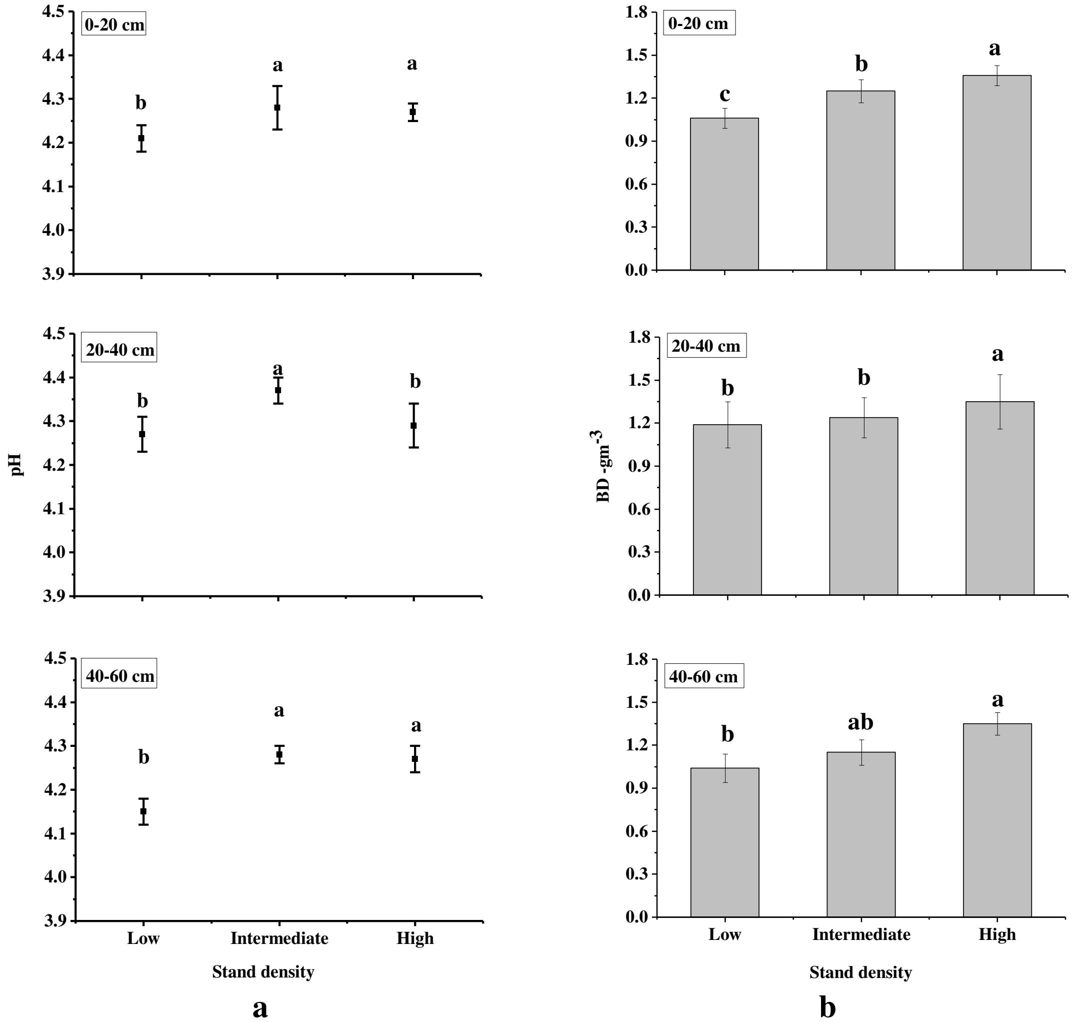

Figure 4. *Soil pH and soil BD in low-, intermediate-, and high-density stands. Columns indicate mean ± SE, different letters indicate significant differences among densities at P < 0.05*

Table 4. *Soil pH, soil electrical conductivity (EC), soil bulk density (BD), soil organic matter (SOM) and soil moisture content (SMC) in low-, intermediate-, and high-density stands at a 0-60 cm soil depth (N = 24)*

Soil parameters	Stand density		
	Low	**Intermediate**	**High**
pH	4.21 ± 0.04 b	4.31 ± 0.03 a	4.27 ± 0.03 a
BD (g/m^3)	1.22 ± 0.07 ab	1.28 ± 0.01 a	1.16 ± 0.09 b
EC	0.02 ± 0.01 a	0.02 ± 0.01 a	0.02 ± 0.01 a
SOM (g/kg)	31.90 ± 1.34 b	36.56 ± 1.61 a	35.47 ± 0.74 a
SMC (%)	11.49 b	9.53 b	17.2 a

Note: Shown are the mean ± SE, different letters indicate significant differences between the stands at *P* < 0.05

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DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

Discussion

In forest conservation and management, stand density is an important factor influencing the trees' competition for growth space, light, and below-ground resources (nutrients and water) (Farinelli and Lemos, 2012; Fan et al., 2014; Sitienei et al., 2016). The soil quality indicators used in the current study were successfully used in previous studies on different forest ecosystems (Kay and Grant, 1996; Wei et al., 2009; Bautista-Cruz et al., 2012; Zhijun et al., 2018), suggesting that these parameters can be used for effective soil quality evaluation.

Watt et al. (2005, 2008) identified total P as important soil quality indicator due to its pivotal role in biochemical reactions and in the nutrient cycling of plantation systems (Reganold and Palmer, 1995; Turrión et al., 2007). In the present study, stand density had a significant effect on total N, available N, total P, available P, and total Mg but had no significant effect on total Ca, total K, and available K. In these plantations of ten years of age, both total N and total P were higher in the low-density stand and lower in the intermediate-density stand. Available N content was higher in the low-density stand and lower in the high-density stand, whereas available P content was higher in the intermediate-density stand and lower in the low-density stand. Our results contradicted those of Fang et al. (2017) who found that stand density had no significant effect on total soil N and on C:N ratio. Cancellier et al. (2011) reported that varying planting density influenced the soil chemical properties, and in low planting density, available P and exchangeable Mg, N, and Ca concentrations were significantly higher than at other planting densities, but little difference was observed in soil organic matter content. Furthermore, our results support the assumption that total P is an important indicator of soil quality for conifer plantations (Yang et al., 2004; Ma et al., 2007; Ross et al., 2009).

SOM acts as a storage reservoir for soil nutrients and is an important parameter for assessing sustainable forest management (Haynes and Beare, 1996; Tanaka et al., 2005). Availability of C and N in soil is crucial regarding SOM and abundance of the microbial biomass (Xing et al., 2010; Zhang et al., 2017a). According to our results, SOM was affected by stand density and was significantly higher in the intermediate-density stand than in the other stands. Moreover, previous studies showed that in Chinese fir plantations SOM increased due to a large quantity of debris input following artificial thinning (Lin et al., 2001; Ma et al., 2014). Besides stand population density, continuous rotations can also have a significant impact on soil fertility (Black, 2013). Huang et al. (2004) stated that in Chinese fir plantations SOM decreased significantly with continuous cultivation (by 10% and 15% in second and third rotation, respectively). Soil enzyme activity and microbial diversity decreased with successive rotations (Chen and Wang, 2003; Liu et al., 2010). According to a different study, continuous planting does not necessarily affect soil degradation, SOM, total P, total K, and available N content; it was therefore hypothesized that sustainability and soil fertility could be consolidated under a continuous cropping regime if plantation stand density was optimized (Wei et al., 2012). In contrast to our results, Wright (1992) reported that plant density has no substantial effect on soil fertility. Taken together, the relationship of plant density and soil fertility seems not to be entirely consistent.

Soil pH is an important indicator of soil quality, influencing plant nutrient availability and soil chemical reactions (Schoenholtz et al., 2000; Cheng et al., 2016). Previous studies have emphasized the importance of pH as a soil quality indicator for assessing different land use and forest conservation practices (Doran and Parkin, 1994; Bautista-Cruz et al., 2012; Yu et al., 2017). Soil pH was lowest in the surface layer of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

soils of all three densities and increased from the 0-20 cm layer to the 20-40 cm layer; however, it decreased from the 20-40 cm to 40-60 cm layer. No significant difference in pH was observed between intermediate-density and high-density stands, both of which differed significantly from the low-density stand; however, the pH values generally remained within the optimum range for Chinese fir plantations (Zhou, 1985). Soil BD increased from the surface to the 40-60 cm soil layer, and significant differences between the stands were observed in each layer. Our results differed from those of Fang et al. (2017) who found that stand density had no significant effect on soil pH and BD.

The understanding of stand density and soil heath under continuous cropping conditions have become important for understanding forest productivity, plant population dynamics, and land use for sustainable production (de Bello et al., 2010; Van der Putten et al., 2013). Nutrients are typically removed from growing sites due to stem harvesting and clear cutting (Chen et al., 2013). Yang et al. (2005b) found that nutrient removal due to clear-cutting was higher than that due to pre-burning; furthermore, the total N and organic C contents of topsoil were reduced by 19% and 17%, respectively. Therefore, besides fertilization, plant residues should be buried on site to replenish the soil nutrient contents. A lack of sufficient soil remediation may lead to a reduction in soil nutrient availability, which may, in turn, limit harvesting potential to only a few rotations (Zhou et al., 2016).

Conclusions

Assessing the effect of stand density may help maintain and enhance sustainable crop production and soil health. Our results indicated that stand density had a significant impact on total and available N and P but not on total and available K. Moreover, total Ca was not affected by stand density. Total P may be a useful indicator for assessing soil quality in Chinese fir plantations. A significant difference in soil pH between stand densities was observed; however, soil pH was within the optimum range for Chinese fir plantations. Soil BD was higher in the intermediate-density stand, and SMC was higher in the high-density stand; SOM was higher in the intermediate-density stand and lower in the low-density stand. No significant difference in the SOM was found between intermediate- and high-density stands, both of which were significantly different from the low-density stand. Soil nutrients typically decline due to clear cutting; therefore, the residues should be buried on site to increase soil fertility. A long-term strategy for maintaining and improving yield and sustainable land use requires that policy makers and managers have access to information on the quantitative relationship between stand density and soil quality. To preserve soil quality and ensure high timber production, variations in soil quality parameters, which are affected by stand density, may help policy makers develop better management practices. Moreover, the influence of aspects, such as enzymatic activity, biological variables and microbiological communities, on soil functioning should also be investigated.

Acknowledgements. This study was financially supported by the *National Natural Science Foundation of China* (31870614 and 31370531), the *Science and Technology Major Project of Fujian Province, China* (2018NZ0001-1) and the Special Technology Innovation Foundation of Fujian Agriculture and Forestry University (CXZX2017112).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35533566

REDUCTION OF GLYCOALKALOIDS IN POTATO UNDER THE INFLUENCE OF BIOSTIMULATORS

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(Received 30th Nov 2018; accepted 14th Feb 2019)

Abstract. The aim of the conducted research was to determine the effect of biostimulators on the content of glycoalkaloids in leaves and tubers of three edible potato varieties. The experiment was established in a split-plot system in three replications. The examined factors were: I – three varieties of edible potato: Honorata, Jelly, Tajfun, and II – five types of using biostimulators: biostimulator BrunatneBio Złoto Cytokinin®, GreenOk®, Kelpak SL®, Titanit®, control variant. In the biostimulators group there were products containing functional elements, eg titanium, products based on algae extracts, and products containing amino acids. The lowest concentration of glycoalkaloids in leaves and tubers of edible potato was found in the Jelly variety –251 mg·kg^{-1} on average in leaves, and – 80.5 mg·kg^{-1} in tubers, while the largest in the Tajfun variety – on average in leaves 370 mg·kg^{-1}, in tubers 110 mg·kg^{-1}. Under the influence of all bioregulators, there was a significant reduction in the content of harmful glycosides as compared to the control variant. The lowest concentration was recorded under the influence of the BrunatneBio Złoto biostimulator – on average in the leaves 293 mg·kg^{-1}, in tubers 91 mg·kg^{-1}.
Keywords: *Solanum tuberosum L., glycosides, BrunatneBio Złoto Cytokinin®, GreenOk®, Kelpak SL®, Titanit®*

Introduction

Glycoalkaloids (TGA - total glycoalkaloids) are toxic steroid glycosides naturally occurring in a potato plant (*Solanum tuberosum*) (Friedman, 2006). In leaves, stems, flowers and sprouts, the content of these compounds is several times higher than in tubers. The maximum content of glycoalkaloids, regardless of the variety, is up to 200 mg·kg^{-1} of fresh tuber weight (Mazurczyk and Lis, 2000; Barceloux, 2008). In the studies of many authors (Mazurczyk and Lis, 2000; Percival and Dixon, 1997; Lachman et al., 2001), the total content of glycoalkaloids (cTGA; content of total glycoalkaloids) in potato tubers usually ranged from 10 to 150 mg·kg^{-1} of fresh weight. Wroniak and Mazurczyk (2006), on the basis of many years of research between 1988–2005, including studying 145 varieties showed that fluctuations in the content of glycoalkaloids constituted from 3 to 350 mg/kg of fresh tuber mass. TGA accumulation in tubers above 100 mg·kg^{-1}of fresh mass causes significant deterioration if taste (Mazurczyk and Lis, 2000), reducing their nutritional value and may also adversely affect human health. Consumption of potatoes with excessive content of glycoalkaloids may be toxic, damaging the nervous system and cause gastrointestinal disorders (Friedman and Dao, 1992; Percival et al., 1996). According to Grenda (2003), Pusz and Pląskowska (2008), Von Bennewitz Alvarez et al. (2008), Matysiak et al. (2011) and Bulgari et al. (2015) biostimulators used in the cultivation of plants are a natural method of better utilization of plant growth conditions (temperature, air and substance humidity, amount and intensity of light) and nutrients, which in turn contributes to the increase in the quantity and quality of the crop.

Our hypothesis was that the use of biostimulators reduce the concentration of glycoacaloids in potatoes. Here we present the impact of the types of biostimulators on

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

the content of anti-nutritional compounds called glycoalkaloids in leaves and tubers of three edible potato varieties.

Materials and methods

The experimental site

Field research was carried out from 2015 to 2017 in an individual farm in Międzyrzec Podlaski (51°59' N i 22°47' E), Biała Podlaska county, Poland (*Figure 1*).

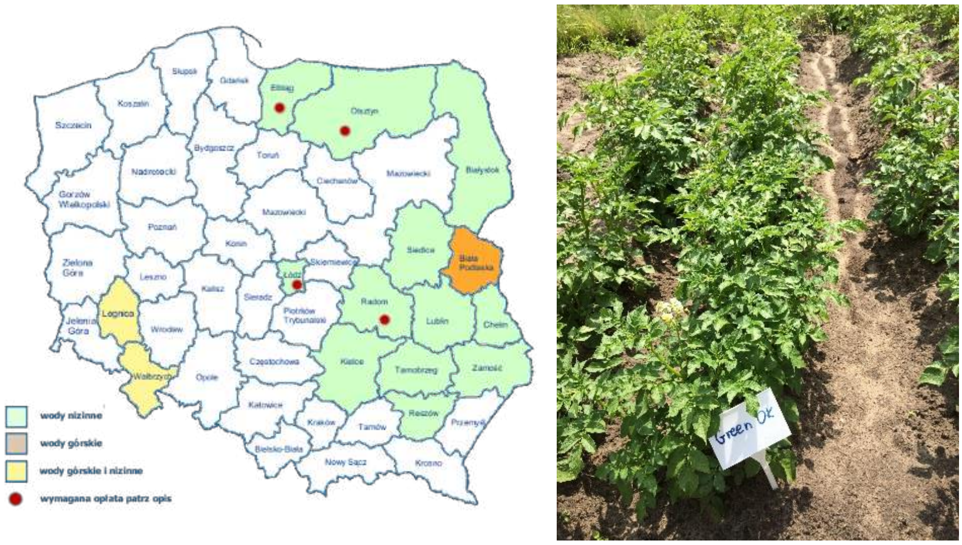

***Figure 1.** Location of the experiment*

The experiment was established in three replications using the split-plot method, on the soil included in the very good rye complex, of the IV a soil class. In individual years of research, soils differed in the content of organic matter and available macro-elements. In 2015 and 2016, the soil was characterized by slightly acidic reaction, and in the last year of research, alkaline. The content of organic matter ranged from 15.0 to 18.7 $g \cdot kg^{-1}$. The content of available phosphorus (P) was from high to very high, potassium (K) from medium to very high, and magnesium (Mg) was high. The first factor were three moderately early varieties of edible potato: Honorata, Jelly and Tajfun, and the second one, four types of biostimulators used in three dates (beginning of flowering, fully flowering and after flowering of plants):

- Control variant – without the use of biostimulators spraying with distilled water.
- Biostimulator BrunatneBio Złoto (active substances – plant hormones: auxin – 0.06 $mg \cdot l^{-1}$ and cytokinin - 12 $mg \cdot l^{-1}$) at a dose of 0.20 $l \cdot ha^{-1}$.
- Biostimulator GreenOk® (active substance – humus substances 20 $g \cdot l^{-1}$) at a dose of 0.20 $l \cdot ha^{-1}$.
- Biostimulator Kelpak®SL (active substance - *Ecklonia maxima* algae extract), containing plant hormones: auxin - 11 $mg \cdot l^{-1}$ and cytokinin – 0.031 $mg \cdot l^{-1}$, at a dose of 0.20 $l \cdot ha^{-1}$.
- Biostimulator Tytanit® (active substance – titanium) at a dose of 0.20 $l \cdot ha^{-1}$.

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DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

The forecrop for potato in particular years of research was winter wheat. After harvesting the forecrop, a team of post-harvest crops was made. In autumn, each year preceding planting, organic fertilization in the form of manure in the amount of 25.0 $t \cdot ha^{-1}$ and mineral fertilization with phosphorus-potassium in the amount of P – 44.0 (100 $P_2O_5 \cdot 0.44$) $kg \cdot ha^{-1}$ (lubofos for potatoes 7%) and K – 124.5 (150 $K_2O \cdot 0.83$) $kg \cdot ha^{-1}$ (lubofos for potatoes 25%) was applied. These fertilizers were plowed pre-season plowing. Nitrogen fertilizers were applied in the spring in an amount of N 100 kg/ha (nitro-chalk 27%) and mixed with the soil using a cultivator. Potatoes were planted manually under the marker at a spacing of 67.5 x 37 cm, in the third decade of April (2015, 2016, 2017). Each plot with an area of 15 m^2 accounted of five ridges. Cultivation and care treatments were carried out in accordance with the requirements of correct. During the growing season, the potato plantation was protected with the following incidences: Actara 25 WG (thiametoksam) at the rate of 0.08 $kg \cdot ha^{-1}$ and Caliypso 480 SC (thiacloprid) at the rate of 0.1 $l \cdot ha^{-1}$, and fungicides: Copper Max New 50 WP at the rate of 2.0 $kg \cdot ha^{-1}$ and Dithane Neo Tec 75 WG the rate of 2.0 $kg \cdot ha^{-1}$. Samples of potato leaves (10 leaves from central part of a stalk) for chemical analyses were taken from each treatment at the flowering stage in July (after chemical treatments). Samples of potato tubers (50 tubers) were taken from each of the plots during harvest and stored at 10-12°C, for 8-10 days. Chemical analyses were performed using fresh material from 10 representative tubers in three replications. The content of glycoalkaloids in leaves and tubers of potatoes was determined by the Bergers colorimetric method (Bergers, 1980). Fresh potatoes were homogenized; then, 150 ml of ethanol were added to a 50 g sample and extraction in a water bath was performed at 90°C. , the filtered extract was evaporated at 60°C using a rotary evaporator to the volume of 5 ml. After addition of 50 ml of 10% acetic acid and centrifuging, the liquid part was poured into a flask, the sediment was poured to the supernatant (solution above the sediment) and 4 ml of ammonia were added (NH_3) to adjust to pH = 10 The flask was heated in a water bath (70°C) for 20 min, then cooled at 4°C for 3 h and centrifuged. The sediment was dissolved in 5 ml 7% phosphoric acid ($H_3P\ O_4$). Next, 0.2 ml of the solution were mixed with 2 ml of 85% phosphoric acid with paraformaldehyde (30 $mg \cdot l^{-1}$) and mixed again. After 40 min, absorption was recorded at the wavelength of 600 nm (solution colour changes to blue and then gets lighter). The amount of potato total glycoalkaloids was calculated based on the L-solanine standard curve. The results of the analyses are given as mg per 1 kg of fresh matter.

Table 1. *Weather conditions during of potato vegetation [Zawady Meteorological Station, (52°03'N and 22°33'E), Poland]*

Months	Air temperature (°C)				Rainfall (mm)			
	Multi-year mean	Monthly means			Multi-year mean	Monthly sums		
	1996-2010	2015	2016	2017	1996-2010	2015	2016	2017
April	8.0	8.2	9.1	6.9	33.6	30.0	28.7	59.6
May	13.5	12.3	15.1	13.9	58.3	100.2	54.8	49.5
June	17.0	16.5	18.4	17.8	59.6	43.3	36.9	57.9
July	19.7	18.7	19.1	16.9	57.5	62.6	35.2	23.6
August	18.5	21.0	18.0	18.4	59.9	11.9	31.7	54.7
September	13.5	14.5	14.9	13.9	42.3	47.1	13.6	80.1
April – September	15.0	15.2	15.8	14.6	335.4	295.1	200.9	335.4

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DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

Meteorological conditions

In the years of research, varied weather conditions prevailed (*Table 1*). The growing season of 2015 proved to have an average of air temperature of 15.2°C, higher by 0.2°C than the long-term mean and precipitation of 295.1 mm. The highest average air temperature was recorded in 2016 and it amounted to 15.8°C, it was higher than the long-term average by 0.8°C, while this year was characterized by the lowest precipitation – 200.9 mm, lower by 134.5 mm from the long-term sum. The highest number of rainfall was recorded in the growing season 2017–325.4 mm and the lowest average air temperature –14.6°C.

Statistical analysis

Results of the study were analysed by ANOVA. Significance of sources of variation was checked with the Fisher-Snedecor test and the significance of differences between means was tested using the multiple comparison Tukey's test at the significance level of P = 0.05. Statistical calculations were performed in Excel using the authors' own algorithm based on the split-plot mathematical model.

Results and discussion

The content of glycoalkaloids in potato tubers depending on the types of biostimulators used (*Table 2*). Statistical evaluations showed a significant effect of cultivars on the content of glycoalkaloids in potato tubers. The TGA content was the highest in the Tajfun variety, an average of 109.7 mg·kg^{-1}, and the lowest in the Jelly variety, an average of 80.5 mg·kg^{-1}. The results are similar to the results presented by Eltayeb et al. (2003), Hamouz et al. (2014) and Valcarcel et al. (2014), who stated that the genotype had a dominant effect on the content of glycoalkaloids in potato tubers. The biostimulators used in the experiment reduced the content of glycoalkaloids in tubers compared to the control. The lowest TGA concentration was noted in tubers from variants sprayed with the BrunatneBio Złoto biostimulator (variant 5), an average of 91.3 mg·kg^{-1}. In the studies by Zarzecka et al. (2015), there was an increase in glycoalkaloids after application with herbicide and biostimulator. While Hamouz et al. (2004; 2005) and Wierzbicka (2014) observed a trend towards increased TGA accumulation in edible potato tubers.

Table 2. *Total glycoalkaloid content in potato tubers mg·kg^{-1} fresh matter*

Types of biostimulators (variants)	Cultivars			Years			Mean
	Honorata	Jelly	Tajfun	2015	2016	2017	
1. Control variant	85.5a	81.5a	110.3a	92.6a	91.2a	93.5b	92.4a
2. Kelpak SL	84.8b	80.5b	109.8b	91.8b	90.4b	93.3b	91.8b
3. Tytanit	84.9b	80.7b	109.7b	91.6bc	90.3bc	93.5b	91.7b
4. GreenOk	84.9b	80.2c	109.4c	91.3c	90.0c	93.5b	91.5c
5. BrunatneBio Złoto	84.8b	79.8d	109.3c	90.7d	89.4d	93.9a	91.3d
Mean	85.0c	80.5b	109.7a	91.6b	90.2c	93.5a	91.8

Explanation: cultivars – a; variants – b; years – c

There are many changes in the literature confirming the growth of TGA in tubers during the growing season due to stress conditions such as too low or too high

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DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

temperature, prolonged cold or heat conditions, lack of water or its excess, and strong sunlight (Sinden et al., 1984; Bejarano et al., 2000; Hamouz et al., 2014). The cooperation of years and used biostimulators has been proved, which indicates a different effect of the biostimulators in changing climatic conditions during the tests (*Table 2*). Under the influence of the BrunatneBio Złoto biostimulator in the growing season 2015-2016, the lowest TGA concentration in tubers was obtained – on average from 90.2 to 91.6 mg·kg^{-1}, while in 2017 under the influence of Kelpak SL biostimulator, the lowest concentration of glycoalkaloids was obtained, on average 93 mg·kg^{-1}. Weather conditions during the study years differentiated the level of glycoalkaloids in potato tubers. The least glycoalkaloids were found in 2016 with the highest average air temperature of 15.8°C and were higher than the long-term average of 0.8°C, this year characterized by the lowest precipitation – 200.9 mm, lower by 134.5 mm from the multiannual sum. The interaction of years with varieties confirmed the different accumulation of TGA in the years of research. Most TGA was found in three studied varieties in 2017, and the least in 2016 (*Table 4*). Similar results were described by Morris and Petermann (1985).

The content of glycoalkaloids in potato leaves depending on the types of biostimulators used (*Table 3*). The lowest concentration of glycoalkaloids in potato leaves was found in the Jelly variety – an average of 251 mg·kg^{-1} and the largest in the Tajfun variety – an average of 370 mg·kg^{-1}. The content of glycoalkaloids in potato leaves was three times higher than in tubers. Zaczecka et al. (2015) in her studies found that the content was ten times higher in leaves than tubers, while Uppal (1987) and Żołnowski (2001) showed a 40-50 times higher concentrations in leaves compared to potato tubers. Biostimulators used in the experiment reduced the glycoalkaloids content in the potato leaves as compared to the control object. The smallest concentration of these compounds in potato leaves was obtained on plots sprayed with the BrunatneBio Złoto biostimulator (average of 293.5 mg·kg^{-1}). Other authors (Gugała et al., 2016) in relation to potato, using herbicides in combination with biostimulators (Harrier 295 ZC 2.0 l·ha^{-1} + Kelpak SL 2.0 l·ha^{-1} and Sencor 70WG 1.0 kg·ha^{-1} + Asahi SL 1.0 l·ha^{-1}) received an increased concentration of glycoalkaloids in leaves and tubers in relation to the control plot.

Table 3. *Total glycoalkaloid contents in potato leaves mg·kg^{-1} fresh matter*

Types of biostimulators (variants)	Cultivars			Years			Mean
	Honorata	Jelly	Tajfun	2015	2016	2017	
1. Control variant	266.1a	252.6a	371.6	296.7a	293.7a	299.9a	296.8a
2. Kelpak SL	262.6b	251.2b	369.8	292.0b	292.4b	299.2ab	294.5b
3. Tytanit	263.7c	250.9b	369.3	292.0b	292.1bc	298.9bc	294.2b
4. GreenOk	261.5d	250.2c	369.0	290.8c	291.6cd	298.3c	293.6c
5.BrunatneBio Złoto	261.2d	250.4c	369.1	291.0c	291.3d	298.2c	293.5c
Mean	263.0b	251.1c	369.8a	292.2b	292.2b	298.9a	294.5

Explanation: cultivars – a; variants – b; years – c

Statistical evaluations showed the cooperation of varieties with the types of biostimulators used (*Table 2*). As a result of the use of biostimulators in the tested varieties, a significant reduction in the content of glycoalkaloids was observed. Regardless of the biostimulators used, the Jelly variety accumulated less of these

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

compounds than the Honorata and Tajfun varieties. The cooperation of years and biostimulators used has been proven, which indicates different effects of the biostimulator in changing climatic conditions during the tests (*Table 2*). Under the influence of the BrunatneBio Złoto biostimulator in all growing seasons, the smallest TGA concentration was obtained – on average from 291 to 298.2 $mg \cdot kg^{-1}$. Weather conditions in the study years differentiated the level of glycoalkaloids in potato leaves. The least glycoalkaloids were found in 2015, while the most of this component was accumulated by tubers in 2017, where the highest rainfall was recorded –325.4 mm and the lowest average air temperature –14.6°C (*Table 4*).

Table 4. *Content of glycoalkaloids in fresh mass of edible potato tubers and leaves depending on the cultivars and years of conduct of tests (mg kg^{-1})*

Cultivars	**Potato tubers**			**Potato leaves**		
	2015	**2016**	**2017**	**2015**	**2016**	**2017**
Honorata	85.0b	84.0b	86.2b	261.2b	257.8b	269.5b
Jelly	80.8c	79.1c	81.6c	250.7c	249.2c	253.3c
Tajfun	108.9a	107.5a	112.7a	365.6a	369.7a	374.1a

Explanation: cultivars – a; variants – b; years – c

In the present study, an interaction was observed between the varieties studied. The content of glycoalkaloids in potato leaves and tubers has a significant impact on the genetic characteristics of the variety, and weather conditions during the growing season. The use of four biostimulators contributed to the reduction in TGA in leaves and tubers of edible potato.

Summarizing, three varieties of edible potato grown in the study differed in terms of glycoalkaloid content in leaves and tubers. The lowest concentration of glycoalkaloids was found in the Jelly variety. Biostimulant BrunatneBio Gold reduced the content of glycoalkaloids in leaves and tubers of edible potato compared to the control. The weather conditions during the growing season of the potato crop can largely affect the content of glycoalkaloids in leaves and tubers of potato. The lowest content of glycoalkaloids in the leaves was found in the year 2015, and in tubers in the year 2016.

Conclusions

The use of four biostimulators contributed to the reduction in TGA in leaves and tubers of edible potato. Summarizing, three varieties of edible potato grown in the study differed in terms of glycoalkaloid content in leaves and tubers. The lowest concentration of glycoalkaloids was found in the Jelly variety. Biostimulant BrunatneBio Gold reduced the content of glycoalkaloids in leaves and tubers of edible potato compared to the control. The weather conditions during the growing season of the potato crop can largely affect the content of glycoalkaloids in leaves and tubers of potato. The lowest content of glycoalkaloids in the leaves was found in the year 2015, and in tubers in the year 2016. In the experiment discussed in this paper, TGA levels did not exceed 200 $mg \cdot kg^{-1}$ fresh weight of tubers. The three cultivars examined are thus safe for human consumption.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35673574

ASSESSMENT OF CHROMOPHORIC DISSOLVED ORGANIC MATTER AND NUTRIENT DISTRIBUTION IN NEW ZEALAND RIVERS USING SATELLITE IMAGES

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(Received 2nd Dec 2018; accepted 20th Feb 2019)

Abstract. The importance of chromophoric dissolved organic matter (CDOM) and water parameters for the structure and function of rivers and lake ecosystems has led to the development of a way to estimate the amount of CDOM, TP (total phosphorus) and TN (total nitrogen) in rivers and lakes over large geographic areas. Operational Land Imager (OLI) images have been obtained of the North and South Islands of New Zealand for the March 2014 and 2015, with in-situ measurements of bio-optical properties and the water parameters of 18 stations. Satellite images of the date closest to the water sampling were used to statistically correlate the in-situ measurements with various combinations of Landsat 8 bands in order to develop algorithms that best describe those relationships and calculate accurately the aforementioned water quality components. Optimal models were selected based on statistical criteria and indices; finally, the predictive models of CDOM, TP and TN concentrations involving the combination of ultra-blue (B1), blue (B2) and green (B3) OLI bands of Landsat 8 satellite sensor were achieved. As a result of the validation process, CDOM absorption at 420 nm (R = 0.35), TP (R = 0.57) and TN (R = 0.71) concentrations were shown to be the most accurately estimated components, respectively.
Keywords: *CDOM, algorithm, TN (total nitrogen), TP (total phosphorus), water quality*

Introduction

Dissolved organic matter (DOM) is the main structuring constituent of aquatic ecosystems. Imported terrestrial dissolved organic matter constitutes a carbon source for heterotrophic bacteria within the aquatic ecosystems, thereby making them partly independent of the primary production of organic substrates within the aquatic ecosystems (Kutser et al., 2005a). Coloured (chromophoric) dissolved organic matter (CDOM) is the part of DOM which absorbs light. According to Slonecker et al. (2016), “CDOM is the humic-rich, optically active fraction of dissolved organic matter that is present in natural waters from the decomposition of detritus and other organic substances”. They are also known as chromophoric dissolved organic matter, yellow substance and humic colour (Slonecker et al., 2016; Hoge et al., 1995).

The importance of measuring water quality and CDOM has important implications for drinking water, aquatic ecosystems and element transport (Sloneker et al., 2016; Häder et al., 2007). Generally, there is a strong correlation between the concentrations of DOM and CDOM (Tranvik, 1990) in aquatic ecosystems. The absorption of light by CDOM affects the availability of light for the primary producers (Jones, 1998). For instance, CDOM reduces the light availability in the water column and tends to prevent biological activity, such as photosynthesis, thereby controlling growth in phytoplankton populations, which are essential to aquatic food chains (Slonecker et al., 2016; Häder et al., 2007).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

The CDOM efficiently absorbs short wavelength solar radiation, which effects photochemical reactions, and protects aquatic biota from UV-B induced damage, an effect that is perhaps particularly significant in high latitude waters (Pienitz and Vincent, 2000; Kutser et al., 2005b). The importance of DOM and CDOM to the structure and function of the aquatic ecosystems is a procedure that is necessary to estimate their amounts in freshwater over extensive regions would be highly beneficial. CDOM reflectance happens in the ultraviolet-blue part of the electromagnetic spectrum, but has no unique identifying spectral absorbance or reflectance features. CDOM displays a gently decreasing slope from the ultraviolet through the blue regions (*Fig. 1*).

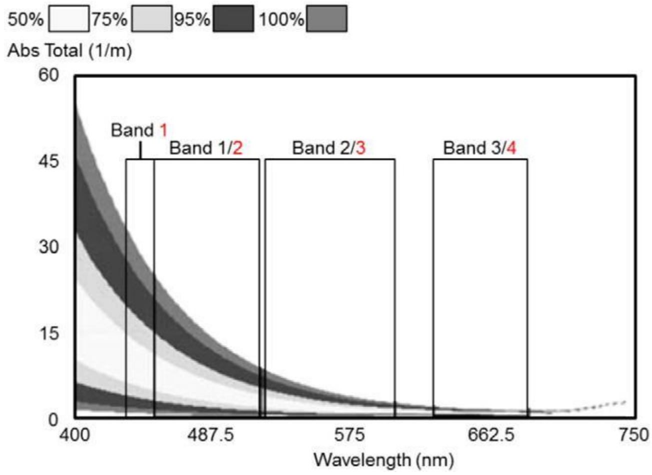

***Figure 1.** The gradually decreasing absorbance of CDOM in the electromagnetic spectrum and the band location of typical multispectral satellite sensors. Dark numbers represent earlier Landsat sensors and red numbers indicate Landsat 8 bands and the improvement of Band 1 relative to the available energy. The shaded intervals represent typical ranges of 500 simulations (Slonecker et al., 2016)*

CDOM is typically derived using absorbance or fluorescence techniques. Spectral absorbance measures how much light at a specific wavelength (typically 254 nm or 440 nm) is absorbed over a range of wavelengths. The main problem in regards to CDOM is that it is neither measured nor reported uniformly. CDOM is sometimes explained as a function of colour at 440 nm (C440) (Brezonik et al., 2005).

The remote assessment of CDOM helps to get a comprehensive view of the distribution and accessibility of DOM in water and to investigate the response of water DOM concentrations to climate change. Optical remote sensing potentially provides the means to make synoptic measurements of the rivers' and lakes' CDOM over large geographic areas. In summary, remote sensing easily solves the considerable problems of CDOM, since in rivers, CDOM is the dominating absorbing composition with a predictable effect on the reflectance spectrum (high absorption of UV, decreasing exponentially with increasing wavelength) (Kutser et al., 2005a).

A new generation of land observation sensors, such as the Advanced Land Imager (ALI) and Operational Land Imager (OLI), have modified spatial, spectral and radiometric resolution (Kutser et al., 2005a; Slonecker et al., 2016). In practice, such measurements have been limited by main three factors:

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

- River and lake size is often small with limited width compared to the most aquatic remote sensing satellite sensors.
- High absorption of CDOM associated with shallow rivers or lakes results in low reflectance and therefore requires satellite measurements of high radiometry sensitivity.
- Limitation in finding suitable images, particularly those areas with high cloud coverage.

The absorption by CDOM decreases with increasing wavelength, thus the influence of CDOM on the remotely sensed signal in OLI band 1 (430-450 nm) is stronger than band 2 (450-510 nm). Band 1 on Landsat 8 is a new addition to the Landsat suite of bands and could be potentially important to CDOM research as it is relatively narrow and centred in a part of the spectrum that is sensitive to coastal phenomena (Markogianni et al., 2017). Atmospheric correction of the blue wavelength (band 1) always creates a problem, particularly in the case of high turbidity or strongly absorbing waters with inappropriate reflectance, such as in rivers. As such, these cases' sensitivity of blue sensors is often insufficient, especially in cases where blue wavelengths are close to zero in the case of brown, CDOM-rich rivers or lakes. On the other hand, phytoplankton absorption also influences band 1 more than band 2. Therefore, the ratio band 2/band 3 is more suitable than the band 1/band 3 ratio for CDOM. This is similar to the band 2/band 3 relationship reported by Kutser et al. (2005a) and Slonecker et al. (2016). In addition, the band 2/band 3 relationship on the Landsat 8 OLI sensor shows a potentially important relationship with respect to a_{CDOM} remote sensing.

Kutser et al. (2005a) and Slonecker et al. (2016) mapped CDOM values and Landsat potential respectively for ALI and OLI water pixels utilising a simple power function (*Eq. 1*):

$$a_{\text{CDOM}}(420) = 5.20x^{-2.76} \qquad \text{(Eq.1)}$$

where x is the ratio of ALI bands 2 (565 nm) and 3 (660 nm), and a_{CDOM} (420) is the absorption coefficient of CDOM at 420 nm. According to Kuster et al. (2005a), there was a strong correlation between in-situ CDOM measurements and the ALI band ratio ($R^2 = 0.83$).

According to Proffitt (2010) investigates on the state of New Zealand's freshwater, pollution and water-related issues as the number one environmental issue facing New Zealand. Many rivers show excessive nutrients, reduced visual clarity due to suspended sediments, and pollution by faecal bacteria. Water quality is appreciably worse at several hundred sites in lowland rivers monitored by regional councils and it was showed among 134 lakes monitored, 56 percent are eutrophic or worse.

The monitoring of water quality is the first step toward understanding the characteristics of water pollution and designing effective mitigation strategies; however, basically water quality parameters (WQPs), such as total nitrogen (TN) and total phosphorus (TP), are obtained by routine monitoring methods (colorimetry) of field sampling and laboratory analyses (Zhang et al., 2018; Song et al., 2012). In addition to cost and labour intensiveness, these traditional approaches are not suitable for monitoring a large number of water bodies at a regional or national scale because of spatial heterogeneity and temporal changes of water quality across aquatic ecosystems. This condition is especially true for urban lake and river systems, where numerous point

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

and non-point inputs occur over relatively short distances (Li et al., 2013). With the permanent development of environmental information technology, remote sensing plays an important and effective role in monitoring water quality, because of its large coverage, better efficiency, and lower cost than traditional sampling methods (Liu et al., 2014).

A number of satellite sensors have been applied to the study of surface water quality (Mathews, 2011). Kutser (2012) has recently provided a significant review of remote sensing instruments which can be applied to assess water quality in inland and inshore coastal waters. Although Landsat sensors were not designed for aquatic applications (McCullough et al., 2012), many examples of applications of Landsat images for estimating and/or monitoring inland water quality are founded. Several studies proposed reliable algorithms between Landsat data and water quality parameters, including chlorophyll, phytoplankton concentrations (Brezonik et al., 2005), water quality (Guan et al., 2011), CDOM (Brezonik et al., 2005), blooms of cyanobacteria (Vincent et al., 2004) and total suspended sediments (Zhou et al., 2006).

Few studies have attempted to monitor and model nutrient data, since those data do not have optical properties, and regression models were achieved from statistically insignificant results (Chen and Quan, 2012). Chen and Quan (2012) particularly used Landsat TM imagery to attempt to predict nutrients (nitrogen and phosphorus) concentrations in Tiahu Lake, China with some acceptable results for phosphorus and less successful results for nitrogen. In general, the previous research considerably increases knowledge of water quality, and most of their developed algorithms are commonly based on empirical relationships using classical, simple linear regression models between remotely sensed reflectance values and measurements collected at the same times in the field.

Because CDOM is one of the most important water color parameters and is closely linked to nutrients, some studies have proposed defining the lake trophic state using the nutrient-color paradigm, which includes CDOM absorption (Liu et al., 2014) and this study was one of the first study in this area which has done. Therefore the aims of this study were to explore whether mapping of CDOM is possible for a larger variety of rivers in the case of satellite images collected in variable illumination conditions and the assessment of various water quality parameters (e.g. TP and TN) in the area to develop predictive algorithms for these parameters in order to discuss the potential application of assessing and monitoring the trophic state of the rivers. Finally, developed model and then applied by using operational land imager (OLI)/Landsat-8 images to show the a_{CDOM} (440), TP and TN distributions. Selected optimal algorithms were applied to another Landsat 8 images of different dates with available in-situ CDOM, TP and TN absorption data, in order to validate the results.

Methods

Source of data and dataset reduction

During this study background information and the data were obtained through this web portal: https://hydrowebportal.niwa.co.nz/, and Larned et al. (2015). We had no control over the quality of data provided to us, but most analytical laboratories have quality assurance programmes in place, and are accredited by International Accreditation New Zealand (the national authority for testing laboratories).

In the present study, surface water samples were collected for laboratory analysis. The amount of CDOM is demonstrated by the absorption coefficient at wavelength 440 nm, $a_{CDOM}(440)$, obtained from spectrophotometric measurements of filtered water (pore size 0.45 µm) in a 4 cm cuvette, relative to a reference of distilled water (Davies-Colley and Vant, 1987; Verburg, 2011). Absorption units were converted to absorption coefficients using the following relationship (Bricaud et al., 1981):

$$a(\lambda) = 2.303 * A(\lambda)/l \qquad \text{(Eq.2)}$$

where $a(\lambda)$ = absorption coefficient (m^{-1}), $A(\lambda)$ = absorbance, and l = cell's light path length (m).

The $a_{CDOM}(420)$ values are calculated from the $a_{CDOM}(440)$ using the common formula of Bricaud et al. (1981):

$$a_{CDOM}(\lambda) = aCDOM(440)exp[-S(\lambda - 440)] \qquad \text{(Eq.3)}$$

According to Kirk (1994), *S* varies between 0.01 and 0.02 nm, and even over a wider range according to Kowalczuk (1999). In addition, according to estimations by Davies-Colley and Vant (1987), the slope factor S = 0.0187 nm^{-1} gives the best results for New Zealand freshwater. Based on Kutser et al. (2005a), using the *Equation 2* to process the data for a time period of ten days before and after the OLI image acquisition allowed us to drive $a_{CDOM}(420)$ values from 18 stations (*Table 1; Fig. 2*) in this research.

Table 1. *Bands and geographical coordinates of the sites*

Site code	Type	Longitude (°E)	Latitude (°S)	b_1	b_2	b_3	g_{420}
RO1	Baseline	176.499019	38.184519	0.0155	0.0043	0.0041	4.360611
RO3	Impact	176.697483	38.458011	0.0315	0.0195	0.0358	8.721223
RO4	Baseline	176.746392	38.483561	0.02	0.0076	0.0283	13.08183
RO5	Impact	176.800183	38.039678	0.022	0.0071	0.0045	2.907074
TU2	Baseline	175.814097	38.995106	0.0101	0.0017	0.0158	18.89598
WA4	Impact	175.142414	39.786872	0.0147	0.003	0.0059	15.98891
WA5	Baseline	175.808428	39.809578	0.0774	0.079	0.1177	20.34952
GS3	Impact	177.621308	38.199558	0.0134	0.0006	0.0083	29.07074
GS4	Impact	177.632225	37.863189	0.1011	0.0977	0.1134	5.814149
HV1	Baseline	176.304164	39.818406	0.0078	0.0079	0.0134	2.907074
HV2	Impact	176.926947	39.716106	0.0346	0.0257	0.0458	11.6283
HV3	Impact	176.877156	39.594253	0.0235	0.0136	0.027	7.267686
HV6	Baseline	176.636025	39.177683	0.0631	0.0515	0.0734	7.267686
GY2	Impact	171.297483	42.452764	0.0125	0.0022	0.0099	26.16367
GY4	Baseline	169.299189	43.942036	0.0412	0.0417	0.0509	1.453537
AX2	Baseline	168.864672	45.006886	0.1204	0.1302	0.1468	1.453537
DN4	Impact	169.747208	46.236483	0.0084	0.0065	0.0255	13.08183
DN8	Impact	168.266664	46.326156	0.0378	0.0207	0.0311	10.17476

RO: Rotorua, TU: Turangi, WA: Wanganui, GS: Gisborne, HV: Havelock North, GY: Greymouth, AX: Alexandra, DN: Dunedin

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DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

Figure 2. *Map showing sampling station from the New Zealand*

During present study, satellite images were downloaded from the USGS Hazard Data Distribution System Explorer (http://earthexplorer.usgs.gov) for March 2014 and 2015. Scenes were then atmospherically corrected and processed for CDOM following methods detailed in Kutser et al. (2005a), Griffin et al. (2011) and Slonecker et al. (2016). For this study, and at all stations, the in-situ data were available for nearest dates coinciding with the OLI scenes, therefore making absolute CDOM levels possible to estimate. Unfortunately, relatively limited images were applicable due to the fact that the sky was often cloudy.

Atmospheric correction of satellite measurements is critically important in aquatic remote sensing as a considerable amount (more than 90% in many cases) of radiation detected by a satellite sensor is backscattered from the atmosphere without ever penetrating the water. Therefore, removing atmospheric effects is especially important when using multiple images.

In this study, and to validate the coloured dissolved organic matter estimation algorithm, and based on two months of field data (2015) and nine OLI images, we used three images from the North and South Islands of New Zealand that were available in the OLI data archive. The rivers in these images are included in the National Institute of Water and Atmospheric Research (NIWA) monitoring programs; thus, water colour determinations are included in these programs and are available.

The water colour measurements enabled us to compute a_{CDOM} (420), as it was described in the methods section (*Eq. 3*) for comparison with a remote estimate of a_{CDOM} (420). The estimated values of a_{CDOM} (420) from the OLI image were correlated with the a_{CDOM} (420) values calculated from the water colour monitoring data.

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In order to classify the water quality of the areas, the EPA classification system was used (EPA, 2000). Based on this scheme, the classification of water quality in rivers and lakes is according to the TP, TN and trophic index (Trophic State Index-TSI). Based on Zhang et al. (2018), the trophic index TSI is calculated for each classification quality parameter as follows (*Eqs. 4–6*):

$$TSI\ (TP) = 10(9.436 + 1.624\ln TP) \quad (Eq.4)$$

$$TSI\ (TN) = 10(5.453 + 1.694\ln TN) \quad (Eq.5)$$

$$TSI = 0.219TSI(TN) + 0.230TSI(TP) \quad (Eq.6)$$

where TSI (TP) and TSI (TN) were trophic state indexes in relation to TP and TN, respectively; TP and TN were the total phosphorus (mg/L and total nitrogen (mg/L).

Predictive algorithms

Numerous researchs have investigated one bands, band combinations and band ratios to estimate mainly chl-*a* and CDOM absorption and to a lesser extent nutrient concentrations in freshwater bodies (Tebbs, et al., 2013; Brezonic, et al., 2015). Firstly, attempts were made to find combinations, transformations, or logarithmic transformations of Landsat 8 OLI bands which would provide more information about the under study parameters in the rivers than only one bond. Such combinations are: ratios of B2/B3, B2/(B1+B2+B3), B1/(B1+B2). Subsequently, pixel values of each transformed image were retrieved from those regions where the 18 sampling stations are located among all stations (*Table 1; Fig. 2*).

To develop predictive $a_{CDOM\ (440)}$ and nutrients (TN and TP), different regression models and MLR (Multi Liner Regression) relating to in-situ data and reflectance values of the selected bands and band combinations were established. Several regressions resulted in unsuccessful results accompanied by statistically insignificant correlations. Taking into account certain previously mentioned statistical indices, optimal models were recorded for each studied water quality parameter.

Data analysis

In this study, statistical analysis including the mean values as well as linear and non-linear fitting, were performed using Statistical Program for Social Sciences (SPSS) 15.0 software. The data from March 2014 were used to establish the relationships and March 2015 data were used to validate between parameters. Distribution maps of rivers and sampling sites were made using ArcGis 11.2 and other data graphs were made using Excel 10 software. In this study, atmospheric correction of the images was performed using the FLAASH (Fast Line-of-sight Atmospheric Analysis of Spectral Hypercubes) software package in ENVI 5.1.

Results

In this study, it was not possible to use existing data at all stations at exactly the same time as the satellite overpassed. For instance, in both the North and South Islands, field data was gathered from the stations during two weeks, and the satellite overpass took

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DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

place at the end of the first week. Images acquired during fieldwork, particularly in the South Island, were too cloudy for analysis, and that hindered the way first suitable OLI images were acquired ten days before and after the field data were collected. Since, there was not significant differences between the amounts of CDOM in before and after sampling dates so we assumed that the CDOM concentration did not vary dramatically within the time frame that separated the in-situ measurements from the satellite overpasses. Nevertheless, the optical remote sensing make synoptic measurements of inland water CDOM over large geographic regions. In total, measurement of CDOM should be problem that is easily solved by remote sensing.

In this research, the relationship between the B2/B3 ratio calculated from the atmospherically corrected OLI images and CDOM absorption coefficient at 420 nm measured is shown in *Figure 3*. The logarithmic model showed the strongest relationship ($R^2 = 0.65$) and highly significant ($p = 0.0001$) (*Fig. 3*). From the results of this study, the reflectance modelling results suggest that the B2/B3 ratio is the best for estimating CDOM concentration in rivers as it was suggested by Kutser et al. (2005a) and Slonecker et al. (2016).

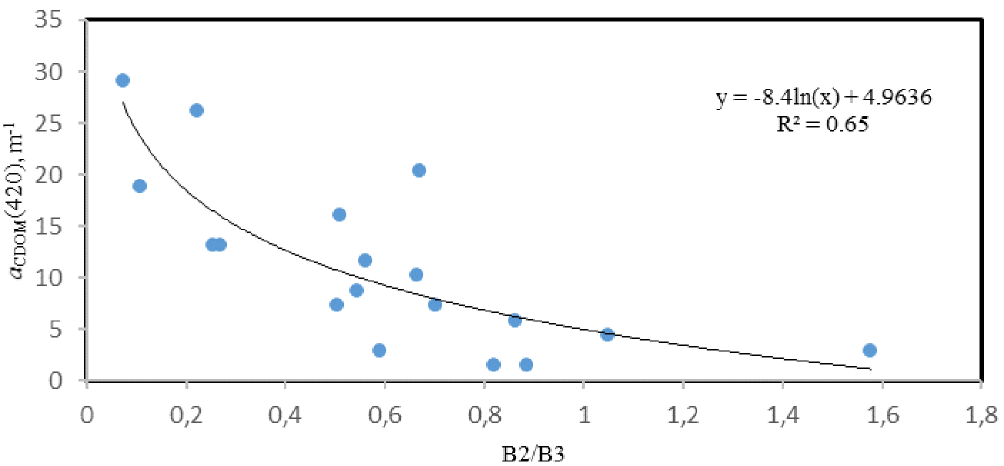

Figure 3. *Correlation between OLI band 2 (B2: 520-600 nm) and band 3 (B3: 630-690 nm) ratio calculated from atmospherically corrected OLI images acquired and CDOM absorption coefficient at 420 nm measured from water samples and the important regression algorithm (logarithmic model)*

During present research, the sampling sites covered a wide range of nutrient compositions and trophic states (*Table 2*). The TN and TP values ranged from 0.019 to 0.719 mg/L and 0.001 to 0.040 mg/L, respectively. The maximum values of TN, TP and TSI were 0.719, 0.040 and 19.064 and showed about 38, 40 and 35 fold greater than the minimum values, respectively. However, the TSI values showed smaller variations from 0.541 to 19.064, revealing oligotrophic rivers, which are according to the standard trophic categories (Jing et al., 2008): TSI < 30 indicates oligotrophic (*Table 3*).

During this study, the multi-liner regression model, including the spatial correlation structure and relating it to Landsat 8, bands 1 (ultra-blue), bands 2 (blue) and 3 (green), was shown to be the most appropriate for forecasting CDOM absorption at 420 nm and total phosphorus built on the logarithmic models. The correlation coefficient is equivalent to 0.81, while the Durbin-Watson value shows independence of residuals to CDOM (*Table 4*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

***Table 2.** Descriptive statistics of the CDOM, total nitrogen (TN), total phosphorus (TP) and trophic state index (TSI)*

	a(440)(m^{-1})	TN(mg/L)	TP(mg/L)	TSI
Min	0.020	0.019	0.001	0.541
Max	2.820	0.719	0.040	19.064
Mean	0.488	0.191	0.016	10.052
Standard deviation	0.648	0.198	0.012	5.438
Median	0.305	0.121	0.010	11.290

***Table 3.** Trophic state classification based on the estimated TSI*

<table>
<tr><th></th><th>Oligotrophic</th><th>Mesotrophic</th><th colspan="3">Eutrophic</th></tr>
<tr><td rowspan="2">TSI</td><td rowspan="2">TSI < 30</td><td rowspan="2">30 ≤ TSI ≤ 50</td><td colspan="3">TSI > 50</td></tr>
<tr><td>Light eutrophic
50 < TSI ≤ 60</td><td>Moderate eutrophic
60 < TSI ≤ 70</td><td>Hyper eutrophic
TSI > 70</td></tr>
<tr><td>This study</td><td colspan="5">TSI = 10 (strictly oligotrophic)</td></tr>
</table>

***Table 4.** Water quality parameters and best predictive models summary*

Model	R	R^2	Std. error of the estimate	Adjusted R square	Durbin-Watson
$a_{CDOM420}$ = -8.4 Ln[B2/B3] + 4.964	0.81	0.65	5.04	0.63	1.637
TP = -0.01Ln [B2/(B1+B2+B3)] -0.001	0.53	0.29	0.01	0.24	1.393
TN = 0.209 * $[B1/(B1+B2)]^{1.308}$	0.28	0.08	1.04	0.02	2.037

In the present work, the ideal model for calculating the total phosphorus was confirmed to be the one established on the logarithmic model. While the amount of the correlation coefficient is equivalent to 0.53, the Durbin-Watson value of 1.393 for TP indicates independence of the residual value as well. The best estimating model of total nitrogen concentrations involves band 1 (ultra-blue) and 2 (blue), which is the power model, while the correlation coefficient is 0.28.

To investigate the consistency of the final predictive models, regression between Landsat 8-estimates of the a_{CDOM} (420), total phosphorus and total nitrogen in the stations versus corresponding in-situ data from 2014 were determined (*Fig. 4*). Several models (linear, logarithmic, quadratic, cubic, power and exponential) have been utilised to discover the best possible concurrence between the monitored and satellite approximated values with the cubic model showing the highest correlation coefficients for all parameters (*Table 5*). However, the reasonable fit between in-situ and anticipated water quality parameters by each particular MLR showed these models to have adequate and low predictive power, especially the topmost correlation coefficient regarding the a_{CDOM}420 estimation was 0.35 with a standard error of 0.665 m^{-1}. The resulting correlation coefficients of total phosphorus and total nitrogen concentrations were determined to be 0.57 and 0.71 with standard errors of 0.017 and 0.037 mg/l, respectively.

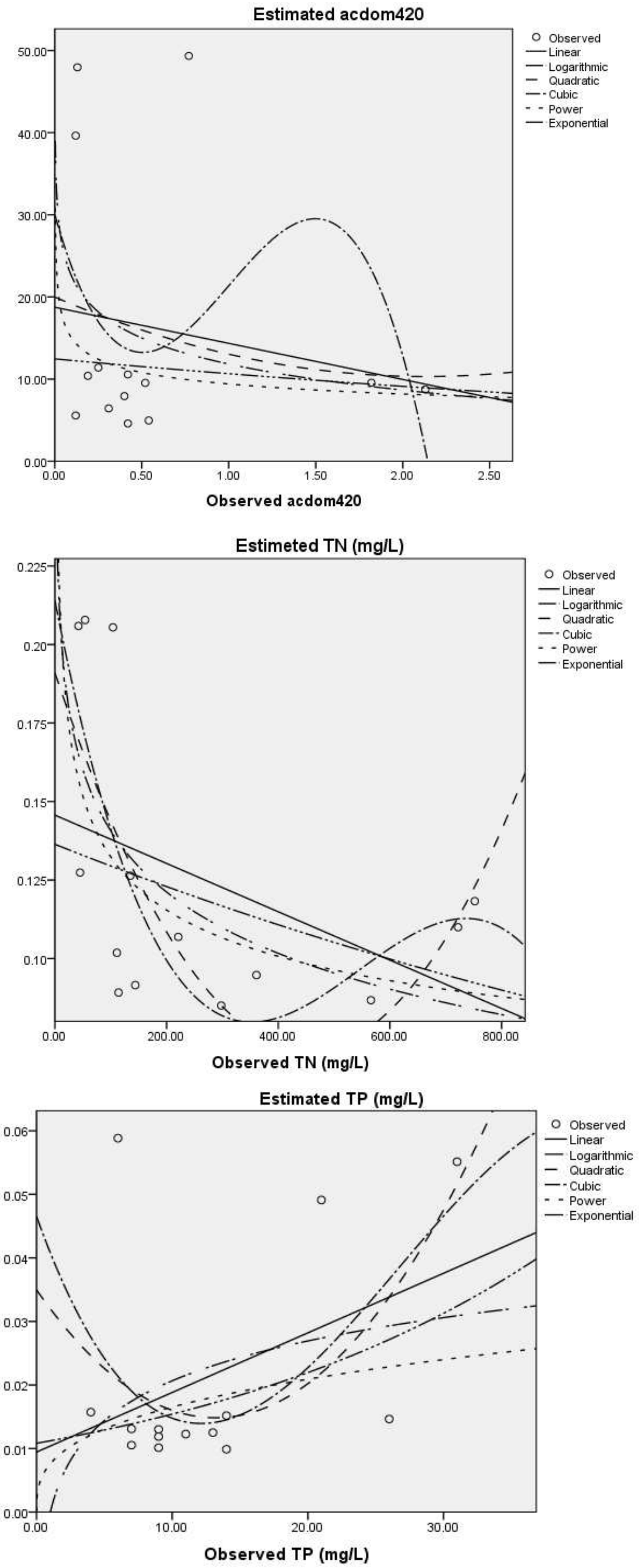

Figure 4. *Scatterplots among observed and satellite-derived data*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

Table 5. *Models summery for water quality parameters predictive models validation*

a_{CDOM}420	R	R^2	Adjusted R square	Std. error of the estimate
Linear	0.168	0.028	-0.053	0.638
Logarithmic	0.255	0.015	-0.068	0.642
Quadratic	0.172	0.030	-0.147	0.666
Cubic	0.347	0.120	-0.144	0.665
Power	0.225	0.051	-0.028	0.815
Exponential	0.121	0.015	-0.068	0.830
Total phosphorus				
Linear	0.413	0.171	0.101	0.017
Logarithmic	0.289	0.084	0.007	0.018
Quadratic	0.558	0.312	0.187	0.016
Cubic	0.566	0.321	0.117	0.017
Power	0.304	0.092	0.017	0.636
Exponential	0.436	0.190	0.123	0.601
Total nitrogenous				
Linear	0.415	0.172	0.103	0.043
Logarithmic	0.599	0.359	0.305	0.038
Quadratic	0.688	0.473	0.377	0.036
Cubic	0.713	0.509	0.361	0.037
Power	0.592	0.350	0.296	0.273
Exponential	0.395	0.156	0.086	0.311

Discussion

Remote sensing offers appropriate data regarding water quality and aquatic systems management. During this study, we demonstrated the applicability probability of Landsat 8 OLI imagery combined with in-situ water parameter concentrations to distinguish applicable algorithms for water quality representation in an oligotrophic water body, New Zealand's rivers. Water specimens from these rivers were evaluated in March 2014 concerning the concentrations of total nitrogen, total phosphorus and CDOM concentration, which then was established as absorption at 440 nm, $a_{CDOM(440)}$, inferred from the absorption spectra.

Multiple linear regressions were conducted among available data, and insignificant statistical correlations characterised the majority of models. Optimal models were selected based on statistical criteria and indices but they presented low coefficients and unsuccessful results. The selected predictive models of TN, TP and CDOM concentrations involved the combination of ultra-blue (B1), blue (B2) and green (B3) OLI bands of Landsat 8 satellite sensor. These results are in accordance with those of Pahlevan et al. (2014) and Markogianni et al. (2017).

Pahlevan et al. (2014) found out that in waters with relatively low CDOM concentrations ($a_{CDOM(440)}$ < 0.5 m^{-1}), the blue and green bands exhibit the highest sensitivity, whereas the red band was found insensitive to the changes in CDOM absorption. In general, it was found that the OLI is, on average, sensitive to changes in CDOM absorption larger than 0.1 m^{-1}. Although actual retrievals can be improved by the use of multiple bands, in our case detected changes to a lesser extent in CDOM absorption are slightly equal to the aforesaid threshold values which could be the main

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

reason for lack of precision assessment results. Furthermore, Pahlevan and Schott (2013) used a physics-based approach to fully examine the potential of OLI in terms of its enhanced features in a water constituent retrieval framework. Based on the results of Pahlavan and Schott (2013), it was concluded that the disparity between the response functions of OLI is more noticeable in turbid waters than in clearer waters when mapping CDOM absorption. Development of reliable methods to retrieve CDOM information from spectral reflectance data is difficult. Indeed, among the major water quality variables measurable by remote sensing (e.g. suspended solids, chlorophyll, Secchi depth), for many reasons, CDOM may be the most difficult to measure accurately in inland waters. CDOM absorbs but does not scatter or reflect light while it has no absorbance peaks, such as those found in plant pigments; instead, light absorption by CDOM follows a simple quasi-exponential decrease with increasing wavelength. There are no wavelength bands in the visible spectrum unlikely associated with CDOM that can be used for measurement purposes. Thus, measurement of low to moderate levels of CDOM in optically complex Case-2 waters is particularly difficult because light scattering by these particles dominates their reflectance spectra (Markogianni et al., 2017).

Predicting the concentration of total nitrogen and phosphorus in inland waters can be a hard task since very few studies have attempted to monitor data with non-optical properties, such as nutrient concentrations. Furthermore, not many previous studies have been able to provide total nitrogen and phosphorus models with statistically significant results or reasonable adjusted R^2 values (Isenstein and Park, 2014). Our research resulted in the total nitrogenous predictive model incorporating ultra-blue, and blue bands yielding a regression coefficient equal to 0.71, regarding the validation process. Similar results, regarding the utilised wavelengths, presented by Dewidar and Khedr (2001) and Isenstein and Park (2014), who detected the strongest correlation among total nitrogen and phosphorus with Landsat TM bands 1 (blue), 2 (green). Meanwhile the latest researchers have predicted total nitrogen concentrations with Landsat TM bands 1 (blue), 2 (green), 3 (red), and 4 (NIR), although all of these results were not very successful ($R^2 = 0.24$).

The outcome of this study initiates new perceptions in limnology over various scales. For instance, it is conceivable to investigate the unpredictability and dynamics of CDOM concentrations in a limited area, such as lakes, rivers and estuaries. This research revealed that the spatial variability of CDOM concentrations within bigger lakes or rivers could be substantial since those wider and deeper sites showed acceptable results by comparing the results of in situ with the satellite images. A single OLI image comprises a wide area and permits research into river and lake CDOM distribution on a regional scale. The results of this study showed that more measurement accuracy was particularly impeded from the very low concentrations which effectively describe rivers and the absence of any value differentiation among the specimen locations. However, some model predictions applied to oligotrophic water bodies are not so exact (McCullough et al., 2012), and the models resulting from this research increase understanding of water quality deterioration.

Conclusion

This paper was intended to provide a framework that would lead to method of mapping CDOM for New Zealand inland waters using satellite images and open a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

discussion about the possibility of developing an algorithm to assess and monitor rivers water quality.

The results of this study showed that extremely low concentrations and inland waters are significant factors that hiders the assessment accuracy which strongly characterize inland waters of any value differentiation among the sampling stations. Although, different models predictions applied to oligotrophic lakes are less accurate and normally these models increase knowledge of their water quality and can be useful indicators of water deterioration.

From the results of this study, it is recommended that future work is still needed to establish the fundamental equations linking the remotely sensed data to the in-situ measurements. The specific research considerations include:

1. More direct considerations between in-situ sensors and Landsat 8 overpasses, especially for lakes and estuaries.
2. More corrections for Landsat 8 surface reflectance product, since there is still a high degree of variability in Landsat 8 reflectance values that could potentially be adjusted by additional spectral and atmospheric corrections.
3. Comparative research of CDOM in lakes, rivers, estuaries and coastal environments are needed to understand significant differences, especially in terms of remote sensing capabilities.

Acknowledgements. Special thanks to NIWA (National Institute of Water and Atmospheric) for providing the data, my Supervisor Dr. Luitgard Schwendenmann in University of Auckland to her guidance during my sabbatical for generous help, particularly in data analysis.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35753589

LANDSCAPE DYNAMICS CHANGES OF THE PROTECTED MARY VALLEY, TURKEY

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(Received 4th Dec 2018; accepted 20th Feb 2019)

Abstract. This paper presents the case of Mary Valley's protected catchment, which is located in the northeast Black Sea Region of Turkey, and was declared a national park in 1987. The study area has sensitive ecosystems, with rare and endangered endemic plants. Nevertheless, current unsustainable economic activities, uncontrolled tourism and population migration have adversely affected its sensitive ecological integrity. This research aims to monitor and analyze the landscape dynamics changes of land use-land cover and landscape structure by change detection and landscape metrics in 1987, 1998, 2009 and 2016. The relationship between LULC and habitat changes linked to those of endemic plants were aimed to be determined. The findings indicated that forest areas with the highest loss transformed into grassland, shrubland and bare land areas, while bare land experienced the highest gain. However, forest area has the highest and bare land has the lowest habitat function. Additionally, endemic plant habitats have an inverse relationship with the habitat value of forest and bare land areas and a direct relationship with shrublands. These unsuitable changes can adversely pressure the sensitive landscape dynamics of Mary Valley protected area. Thus, landscape dynamics and their changes should be monitored to protect and manage for the sustainable development of Mary Valley resources.
Keywords: *environmental monitoring, landscape dynamics change, habitat function, landscape metrics, protected area*

Introduction

Landscape is an area where there is action, interaction and various relationships between natural and/or cultural resources (CE, 2000). These effects and relationships inevitably cause a change in landscape dynamics. Since the dawn of time, the change of landscape patterns, landscape sensitivity and landscape dynamics have been generated by natural disasters and unsustainable, unplanned decision making (Antrop, 2000). The idea to analyze landscape changes has been raised for the determination of the complex and significant interactions among natural, cultural and social processes (Esbah et al., 2010; Martinuzzi et al., 2015). Thus, the need for monitoring and analyzing the temporal and spatial changes of natural and cultural areas and providing for their sustainable development have become essential concerns in the last few decades (Lausch and Herzog, 2002).

Protected areas are valuable components for biodiversity, culture and history. Nevertheless, they have been under pressure due to uncontrolled and unmanaged land use and land cover change (LULCC) caused by anthropogenic activities that are mainly influenced by socioeconomic, political and technological factors (Braimoh, 2006; Esbah et al., 2010; Mariota et al., 2013; Scullion et al., 2014). Thus, monitoring and assessment are important for sustainable land management because they can define environmental trends that can be used in the planning of future endeavors in protected areas, solving problems of the landscape, and finally sustainably using resources (Estes et al., 2012; Angonose and Grau, 2014; Tian et al., 2014; Oinam et al., 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

LULCC is a key driver of global landscape change and can describe the relationship between humans and the use of the surrounding land (Fichera et al., 2012; Scullion et al., 2014). LULCC in landscape patterns is related to urban and population growth, negative impacts of anthropogenic uses, socioeconomic, political and technological variables. It increases impervious land use and deforestation and decreases agricultural and grassland areas (Seto et al., 2005; Bozkaya et al., 2015). Effective monitoring and assessment of LULC is a suitable way to assess landscape mechanisms and ecological processes, provide ecological and cultural sustainability, understand sensitivities of landscapes based on erosion and flood risk, biodiversity and habitat potential or loss, soil, water and air quality, predict future change in landscape patterns, mitigate undesirable effects on landscape resources and solve problems of landscape dynamics (Estes et al., 2012; Fichera et al., 2012; Tian et al., 2014; Demir, 2017).

Landscape ecology is focused on characterizing habitat function using landscape metrics at the patch, class and landscape level, i.e. number, size, density, edge and shape of patches (McGarigal and Marks, 1995; Lausch and Herzog, 2002; Seto and Fragkias, 2005). Overall landscape is defined by analyzing the landscape structure to understand its habitat function using landscape metrics (Turner et al., 2001; Malaviya et al., 2010; Mairota et al., 2013; Demir and Demirel, 2018). Therefore, these metrics can describe the distribution of landscape disturbance and explain sustainable landscape development and landscape patterns and dynamics (Neel, 2008; Esbah et al., 2010, Fan et al., 2018). Understanding landscape dynamics is associated with landscape functions and changes (Seto and Fragkias, 2005; Ghosh et al., 2012; Bruton et al., 2016). LULCC integrated with landscape metrics generates useful information for land use researchers, landscape and urban planners, decision and policy makers that can be effectively used for landscape planning and management (Esbah et al., 2009; Malaviya et al., 2010; Fichera et al., 2012). Due to this, monitoring and analyzing LULCC and landscape structure have gained attention internationally in recent years (Lausch and Herzog, 2002; Kara et al., 2013).

Unsustainable land use can damage and reduce biological diversity and cultural-historical resources of protection areas (Martinuzzi et al., 2015). Therefore, they cannot be assessed in isolation, separately from their surroundings (Demir et al., 2016) because ecological processes form an integrated system that is not limited by administrative boundaries. This study monitored and analyzed Altindere Valley National Park and its surrounding Mary Valley catchment, based on ecological boundaries, located in the Maçka District of Trabzon, Turkey. With its protected ecological and cultural resources, Mary Valley has gained national and international tourism potential. Especially the historical Sumela Monastery is visited each year by Orthodox Christians from around the world. It is host to 31 endemic plants species (Salapaş, 2002; Uzun, 2002). Although there are many ecological, cultural and touristic attractions in the park, the human population of the study area diminished by about 25% from 1987 to 2016 due to migration to urban areas (TURKSTAT, 2016). This study monitored and analyzed temporal and spatial LULCC and habitat function in the protected Mary Valley. In this context, this study's objectives are to determine temporal habitat value based on landscape structure analysis using landscape metrics (1) and to identify the relationships among LULCC, habitat function and endemic plant distribution areas (2). To reach this aim, temporal landscape dynamics were evaluated to understand the changes of LULC types, endemic plant habitats and their value. The use of landscape structure analysis integrated with the normalization method and determining the effects of LULC changes

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on sensitive endemic plant habitats were the primary objectives of this research. Therefore, the findings of this research can be an example for Turkey and other developing counties to support ecological and cultural integrity of protected areas.

Methodology

The research process included the characterizing the study area, data collection, image pre-processing, image classification, determining and comparing LULCC and determining habitat function using landscape structure metrics analysis (*Figure 1*).

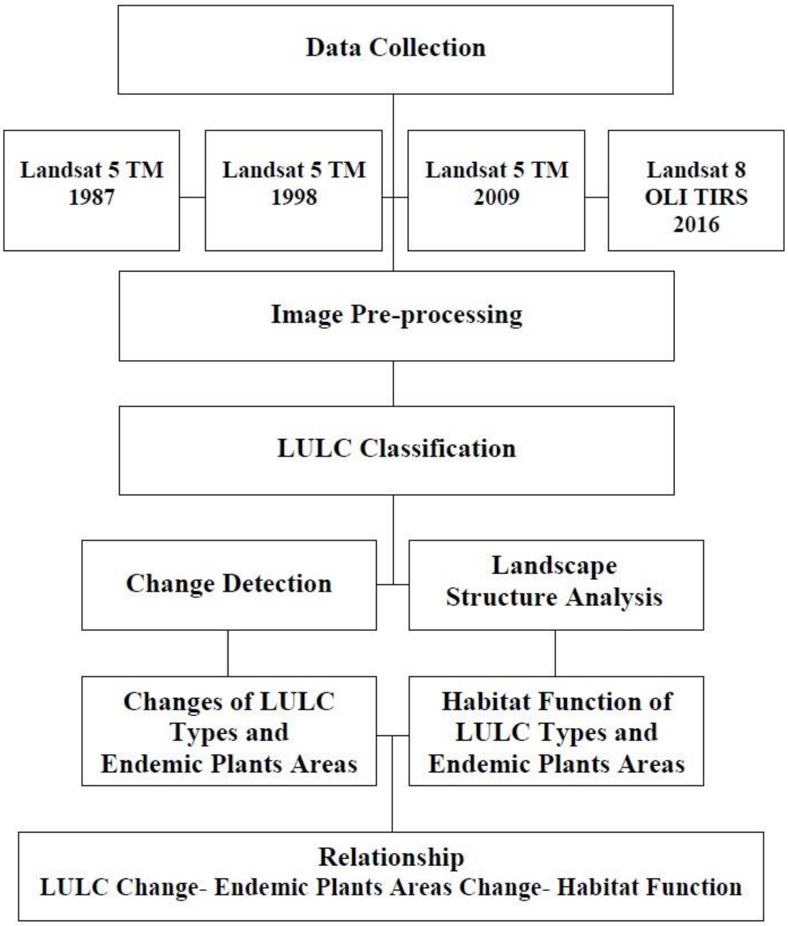

Figure 1. *Flowchart of the research process*

Study area

Mary Valley, located in the Maçka District of Trabzon Province in Turkey's Northeast Black Sea Region, lies in a catchment area of approximately 7,802 hectares (40° 45' 48"-40° 37' 17" N, 39° 35' 55"-39° 42' 26" E) (*Figure 2*). It is a rural area with six traditional villages and plateau settlements on the steep and deep valley, with a rich natural, cultural and historical landscape. Therefore, Mary Valley is one of the most internationally and nationally important protected areas and tourism destinations of Turkey. 4,468 hectares of the study area were declared as the Altindere National Park in 1987 by the Ministry of Environment and Urbanism.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

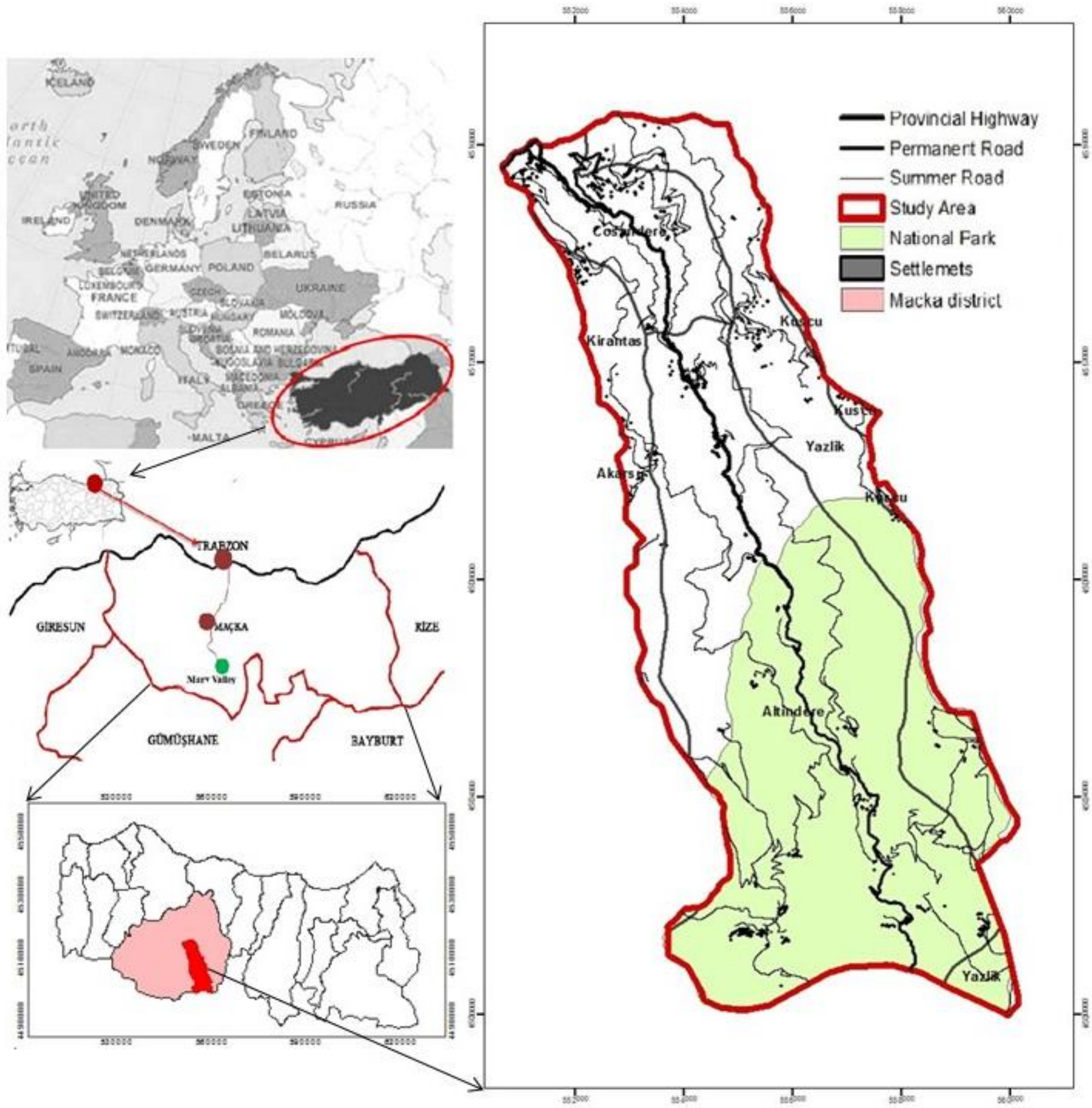

***Figure 2.** The study area of Mary Valley, Turkey*

A difference in elevation from 480 meters to 2,718 meters shapes the area's topography, climate and vegetation (Doganay, 2003; Demir, 2017). The study area includes different kinds of ecosystems including riparian buffers, dense broadleaf forests (1,350 m-1,750 m), coniferous forests (1,750 m-2,000 m), and alpine meadows (2,000 m and higher). It hosts 31 endemic plants species (*App. 1*) that are declared on the red list of IUCN as threatened species (Salapaş, 2002; Uzun, 2002; Demir, 2017). It has been designated as an Important Bird Area concerning Eastern Europe's bird migration (BirdLife International, 2015; Development Plan, 2005). The historical Sumela Monastery has an archaeologically and historically important cultural landscape value that manifests in the religious ceremonies of monks and pilgrims (Zaman, 2010; Demir et al., 2015).

The current land cover types of the study area include forests, shrublands, grasslands, croplands, bare land and settlements. The main economic activities are tourism, agriculture, forestry and livestock breeding. Forestry and agricultural activities are restricted within the national park's boundaries. These reasons lead to the migration of locals to big cities, and the population of study area diminished from 4,142 to 1,602 people between 1987 to 2016 (TURKSTAT, 2016). Additionally, the conservation status Mary Valley was under changed from 1972 to 1998. Mary Valley has gained national and international conservation value and tourism potential due to its wide range of natural and cultural resources. Thus, ecology-based tourism has been proposed for this protected catchment area by the KAMAG (2011) and in DOKA (2012) projects and by several studies (Demir et al., 2015; Pirselimoğlu and Demirel, 2015a, 2015b). The changes of LULC and habitat can be a determining factor in promoting sustainable development of this area.

Data collection

Three different Landsat 5 TM images taken on September 8, 1987, September 22, 1998, and September 4, 2009, in addition a Landsat 8 OLI TIRS image taken on September 3, 2016 were downloaded from the USGS website and used in this research to examine LULCC in Mary Valley. The spatial resolution of Landsat 5 TM images and the Landsat 8 OLI TIRS image is 30 meters. All these images had cloud cover of less than 5%. Moreover, one rectified digital aerial photo with a spatial resolution of 3 m taken on August, 12 2013 and a map with a spatial resolution of 30 m called Globeland 30-N37 from September 16, 2010 was used for visual interpretation and accuracy assessment (GLC30, 2015). Demographic data (TURKSTAT, 2016) and endemic plant species data (Salapaş, 2002; Uzun, 2002) were examined for habitat and local population change. ERDAS 2014 software was used for image pre-processing, classification and haze reduction. ENVI+IDL programs were used to reduce the black line gaps of the images based on the Landsat gap fill process. ARCGIS 10.2 was used for geometric corrections, converting raster data to vector data and determining the LULCC. FRAGSTAT 4.2 (McGarigal et al., 2012) was used to determine habitat value of land cover types using landscape structure metrics.

Image pre-processing

The 5 TM images taken in the year 1987, 1998 and 2009, in addition to the Landsat 8 OLI TIRS image taken in the year 2016 were evaluated directly for image pre-processing. For this step, Image pre-processing was used to eliminate band combination based on stack layers, top of atmospheric radiances (TOA), atmospheric correction, and for haze reduction. Firstly, the digital number (DN) layers were combined with the stack layer modeler. For next step, a top of atmospheric radiance and reflectance computation (TOA) modeler was created for each image to minimize atmospheric effects (Özyavuz et al., 2011; Tian et al., 2014). The radiative transfer model was used for the atmospheric correction of these four satellite images from 1987, 1998, 2009 and 2016 using the simulation of the satellite signal in the solar spectrum (5S) to compute the atmosphere's attenuation of the solar radiation that reflected radiation or radiance from a surface. Then all the values were evaluated for the satellite images, separately. The Landsat images were geometrically corrected and registered in the Universal Transfer Mercator projection system (datum WGS 84, zone 37N) for geometric correction. The Landsat fill gap was used to eliminate and reduce the black line gap and haze effects in the visible bands of Landsat images (Yale, 2015) to enhance the accuracy of classification. Images of four different times were used to determine landscape dynamics changes through LULC and landscape structure analysis.

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Image classification

The classification process groups pixels that have similar spectral values (Kara et al., 2013; Bozkaya et al., 2015). This research was carried out using the maximum likelihood supervised classification algorithm to detect land use and land cover types. In this process, 342, 329, 303 and 325 pixels were selected for 1987, 1998, 2009 and 2016, respectively. After that, forest areas (deciduous and coniferous), shrublands, grasslands, bare land (gravel and bare ground) and croplands were identified as land use and land cover classes. These classified data were compared with the GlobeLand 30-N27 map and the digital aerial photo (2013) using on-screen digitizing to improve the accuracy rates of the classification maps and solve the mixed pixel problem. Overall accuracy was calculated using 800 independent test pixels. The statistical results were 94% 94% 92% and 93% respectively for the 1987, 1998, 2009 and 2015 classified images.

Change detection

Change detection was used to compare two years using from-to analysis (Francisco and Hochschil, 2012; Tian et al., 2014). It is the most effective technique for detecting the differences between two images and demonstrates the transformation of LULCC by post-classification comparison (Fichera et al., 2012; Kara et al., 2013; Hussain et al., 2018). This comparison indicates the percentage of each class on diagonal and off-diagonal axes (Congalton et al., 2014; Tian et al., 2014; Rawat and Kumar, 2015). The changes in LULCC were determined and described for three intervals: 1987-1998, 1998-2009 and 2009-2016. According to Leitao et al. (2006), Esbah et al. (2009, 2011), Erdogan et al. (2013) an at least 100 meter buffer zone area is a viable way of protecting the sensitive core zone of a habitat. Therefore, this zone was created for the point data of endemic plant species in order to detect and monitor the LULCC (Leitao et al., 2006; Esbah et al., 2011; Erdogan et al., 2013) of these areas from 1987 to 2016. This process was summarized in terms of net change, total change, persistence, gross gains, losses and swaps in LULCC (Braimoh, 2006; Angonese and Grau, 2014). After detecting the different areas of persistence, gain and loss for LULCC, these areas were associated with the endemic plant habitats. This post- classification method (from-to) was used by comparing images taken at different times to monitor the transitions of LULC and endemic plant habitats.

Landscape structure

Landscape metrics analysis was used to assess the habitat function and landscape changes for the quantification of landscape pattern to compute the complexity of landscape structures (Leitao et al., 2006; Esbah et al., 2010; Gökyer, 2013). In this research, 16 landscape metrics were used in FRAGSTAT 4.2 software at the class level for four LULCC classified map-years (*Table 1*). The metrics were categorized in four groups according to their characteristics (McGarical et al., 2002): 1) area/edge metrics; including percentage of landscape (PLAND), total edge (TE), edge density (ED), patch number (PN), patch density (PD), mean patch size (MPS), area weighted patch size (AWP); 2) shape metrics, including mean size index (MSI), area weighted mean size index (AWMSI), area weighted mean perimeter-area ratio (MPAR), area weighted mean fractal (FRAC_AM); 3) core metrics, including total core area (TCA), mean core area index (CAI_MN), and 4) the isolation/contiquity metrics, including mean euclidean nearest neighbors distance (ENN_MN), mean contiquity (CONTIG_MN).

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***Table 1.** Explanation of relationship between landscape metrics and habitat function (Turner et al., 2001; Esbah et al., 2009; Demir and Demirel, 2018)*

Landscape Metrics	**Symbol**	**Explanation**
Percentage of Landscape	PLAND	It measures the percentage of the each land cover class according to all landscape cover classes.
Mean Patch Size Area Weighted Mean Patch Size Patch Number Patch Density	MPS (AREA_MN) AWMPS (AREA_AM) PN PD	MPS measures the size of discrete patches summarized across all patches of a particular land cover class. If the patch size is bigger, it has more habitat potential. If it is used by Patch Number (PN) and Patch Density (PD), it can serve as a fragment. Displays the magnitude of the presence of similar patches in the landscape, indicates isolation if PD decreases. More patches (PN) mean more boundaries between land cover classes, which can mean barriers to movement between habitats. MPS and PD are directly proportional and PN is inversely proportional to habitat function. MPS is interpreted better by PLAND, PN and PD.
Total Edge Edge Density	TE ED	Total Edge (TE) is the total edge numbers for each landscape class. Edge Density (ED) is the total length of per hectare for each landscape class area. TE and ED are inversely proportional to habitat function, because more edges mean higher possibility of edge effects. Edge effects alter vegetation structure and animation abundance.
Mean Size Index Area Weighted Mean size Index Area Weighted Mean Perimeter-area Ratio	MSI (SHAPE_MN) AWMSI (SHAPE_AM) MPAR (PARA_AM)	It measures the average of the mean patch shape for each land cover class. AWMSI and MSI are related to the complex geometric shapes of a patch because these affect the edge effects and cross boundaries. Linear, corridor-like, lobed, complex and convoluted patch shapes have a higher amount of boundaries than round, compact and simple patch shapes. Rounded, compact and simple patch shapes garner a higher habitat potential than others because more boundaries mean higher possibility of edge effects. If the number is close to 1, it means the class can have a higher habitat value. Most of shape metrics have a relationship with perimeter-area metrics. MPAR is inversely proportional to habitat function.
Area Weighted Mean Fractal	FRAC-AM	It measures the total edge length of the patches of each land cover class. It displays the fragmentation of habitat. If the number is smaller, it means the fragmentation is not so severe. FRAC-AM is inversely proportional to habitat function.
Mean Contiquity	CONTIG_MN	It measures the average possibility of neighbors (contiguity) of each land cover class. CONTIG-MN is inversely proportional to habitat function.
Mean Euclidean Nearest Neighbors Distance	ENN_MN	It measures the average distance of the nearest neighbors (contiguity) of each land cover class. ENN_MN is inversely proportional to habitat function.
Total Core Area Mean Core Area Index	TCA CAI_MN	It measures the total core area of each landscape classes.100 m is based on the core area. It can be assessed with CAI-MN to display the detection of habitat function of each land cover class.

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The directly and inversely proportional metrics that affect habitat function were evaluated separately for each category. Finally, one of the well-known linear normalization methods called Min-Max method was used to normalize the data linearly. Minimum; refers to the lowest value, maximum; refers to the highest value that data can receive. The Min-Max normalization method (*Equation 1*) sorts data in the range of 0 to 1 (Wang and Cumming, 2011; Yavuz and Deveci, 2013). Data is calculated according to the following *Equation 1*:

$$x' = \frac{x_i - x_{\min}}{x_{\max} - x_{\min}} \qquad \text{(Eq.1)}$$

In this formula x' is normalized data, x_i is input value, x_{min} is the smallest and x_{max} is the largest number in the input set (*Equation 1*). In this context, Min-Max normalization method was preferred to generalize objectively the range of landscape structure metrics values of each LULC type.

Results and discussion

Land use and land cover change

The LULC types in the study area consist of forests, grasslands, bare land, shrublands and croplands. According to the LULC distributions from 1987 to 2016, the dominant LULC types were forests and grasslands (*Figure 3*). The LULC types gained bare land at the cost of losing forest, grassland, shrubland and cropland, respectively. Forests decreased from 49.87% in 1987 to 43.92% in 2016, while bare land increased significantly. In 2016, the forests were still dominant followed by croplands. The bare land area increased significantly in 2016 compared to previous years, while forests declined sharply in 2016 (*Figure 4*).

The change detection indicated the amounts of net change, total change, persistence, gains, losses and swaps of LULCC types from 1987 to 2016. *Table 2* shows that grasslands had the highest total change (13.06%) and highest swap (12.10%) in terms of gain and loss amounts. Cropland had the lowest total change (gain+loss) and the lowest swap amounts. The highest net change (gain-loss) of LULCC belonged to bare land, and the lowest change was that of croplands. Forests experienced the highest loss with 580.23 ha, whereas bare land experienced the highest gain with 615 ha (*Table 2*).

Eventually, the LULC types of the research area include forest, grassland, bare land and shrubland. Forest and grasslands are the main LULC types of this research. Loss of forest is most likely due to lumber production and the expansion of the road network for villages, summer homes and ski centers, whereas gain in bare land is due to the transformation of grasslands. Cropland areas decreased constantly from 1987 to 2016 (*Table 2*). The main causes of this decline were the limitations on agricultural and forestry activities in the national park, and local migration. Although forestry activities were limited, the forestry area had a similar decreasing trend due to hydroelectric power plants, road networks, deforestation and floods. In these cases, forest areas were transformed to grasslands, shrublands and bare land within 29 years, showing that, besides anthropocentric activities, lack of conservation and management strategies negatively affected LULCC.

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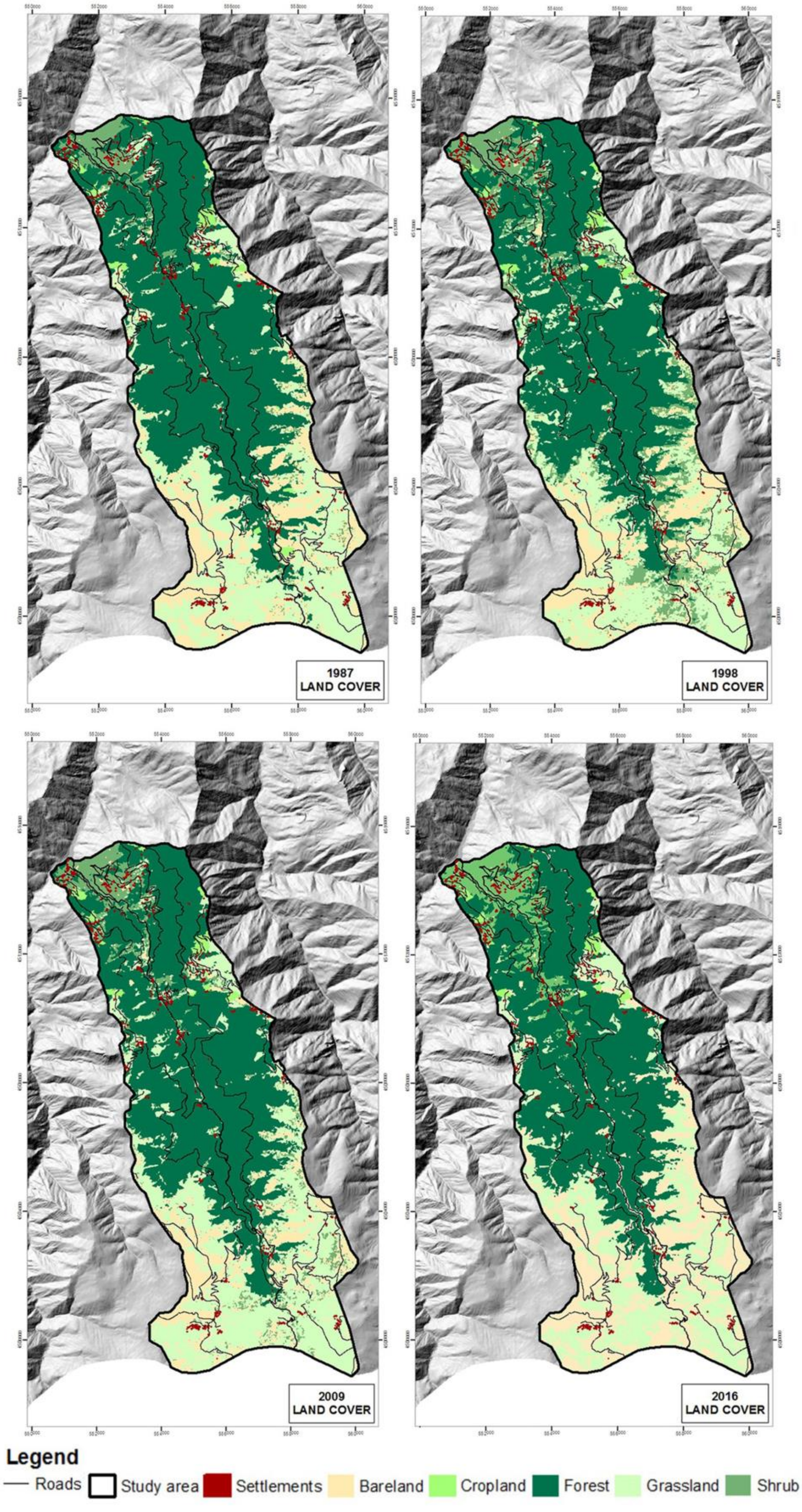

Figure 3. *LULC (land use-land cover) classification of 1987-1998-2009-2016*

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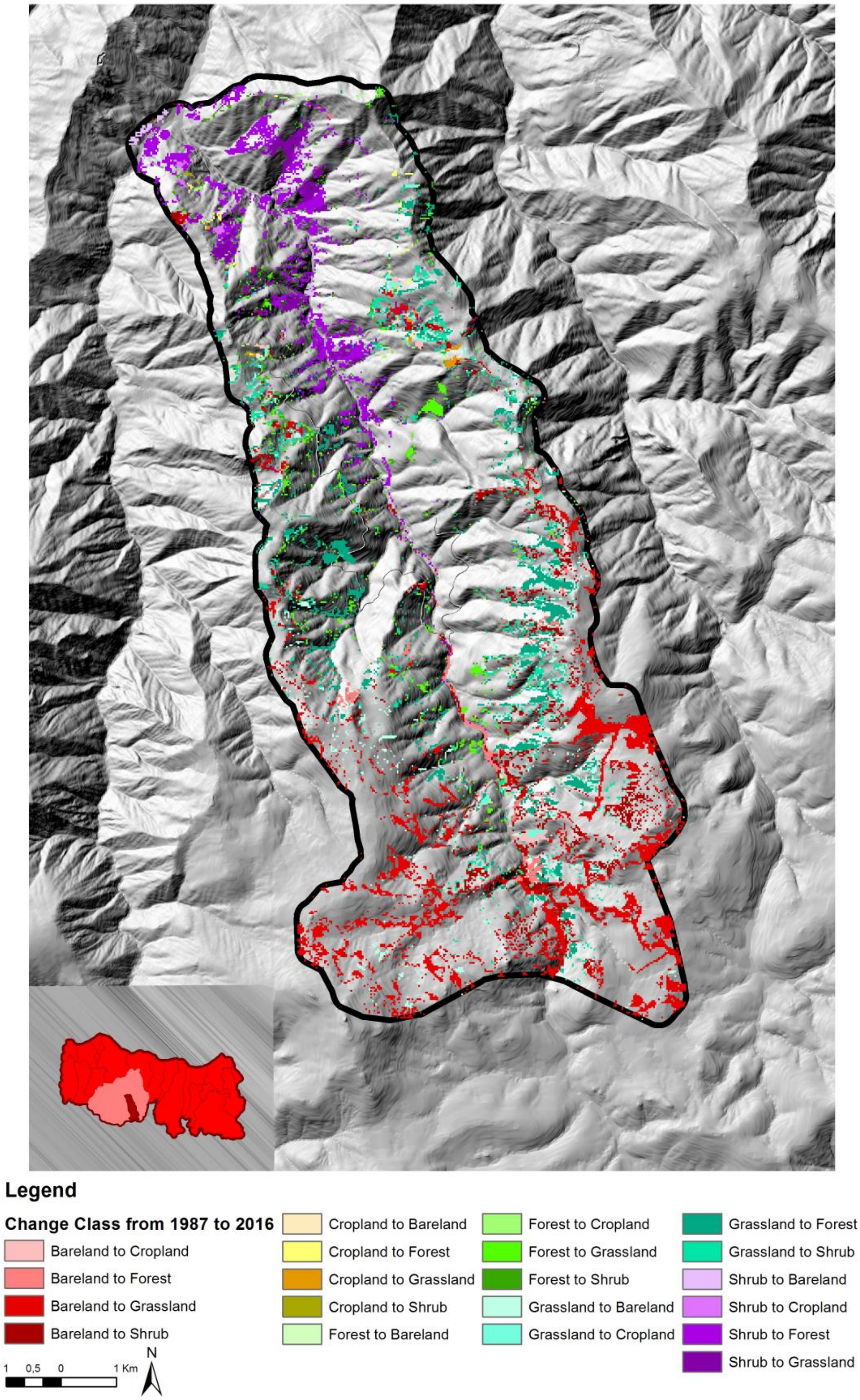

Figure 4. *Transition of LULC (land use-land cover) types from 1987 to 2016*

***Table 2.** Change detection and summary of LULC (land use-land cover) changes*

Change detection of LULC (ha)

	2016 (ha)							
	LAND COVER	**FOREST**	**GRASS-LAND**	**SHRUB-LAND**	**BARE LAND**	**CROP-LAND**	**Total 1987**	**Loss**
1987 (ha)	**FOREST**	**3310.56**	260.82	235.62	76.95	6.84	3890.79	580.23
	GRASSLAND	73.35	**1682.28**	99.36	367.56	6.57	2229.12	546.84
	SHRUB	36.63	131.49	**375.75**	159.39	3.24	706.50	330.75
	BARELAND	1.80	63.18	3.51	**811.80**	0.63	880.92	69.12
	CROPLAND	4.41	16.47	6.12	11.16	**55.62**	93.78	38.16
	Total 2016	3426.75	2154.24	720.36	1426.86	72.90	**7801.11**	**1565.10**
	Gain	116.19	471.96	344.61	615.06	17.28	**1565.10**	**20.06 %**

Summary of LULC changes (%)

LAND COVER	**Total 1987**	**Total 2016**	**Gain**	**Loss**	**Total Change**	**Swap**	**Absolute value of net change**
FOREST	49.87	43.92	1.48	7.45	8.93	2.98	5.95
GRASSLAND	28.57	27.61	6.05	7.01	13.06	12.10	0.96
SHRUB	9.06	9.23	4.42	4.24	8.66	8.48	0.18
BARELAND	11.29	18.29	7.88	0.89	8.77	1.77	7.00
CROPLAND	1.20	0.93	0.22	0.49	0.71	0.44	0.27
Total	100.00	100.00	20.06	20.06	40.13	25.77	14.35

Endemic plant areas change

The research area has 31 endemic plants species in the highlands (*Figure* 5). According to Leitao et al. (2006), Erdogan et al. (2013), Esbah et al. (2009, 2011), a 100 m wide buffer zone was created to protect the core zone for each sensitive and endangered endemic plant habitat. The species, listed in the IUCN red list category of threatened species, are most often located in grasslands, forests and shrublands. The LULCC affected endemic plants significantly through the increase of bare land and the loss of grasslands and forests. Even though these plants are protected by the IUCN categories, some changes were found in each endemic plant habitat. LULCC adversely affected their habitats and diminished the environmental properties of the buffer area.

The LULCC distributions from 1987 to 2016 showed that grasslands have the most endemic plants (*Figure 5*), although the highest loss, swap and especially net change amounts were observed in grasslands. *Table 3* included that endemic plant occurrence in shrublands, which is the subdominant LULC type, decreased significantly from 8.70 % in 1987 to 1.81 % in 2016 with the highest loss area. It indicated that the endemic plant habitat in shrublands was under threat with this sharp, constant decline trend. The bare land area experienced more gain, on the contrary, grassland, forest and shrubland experienced more loss from 1987 to 2016.

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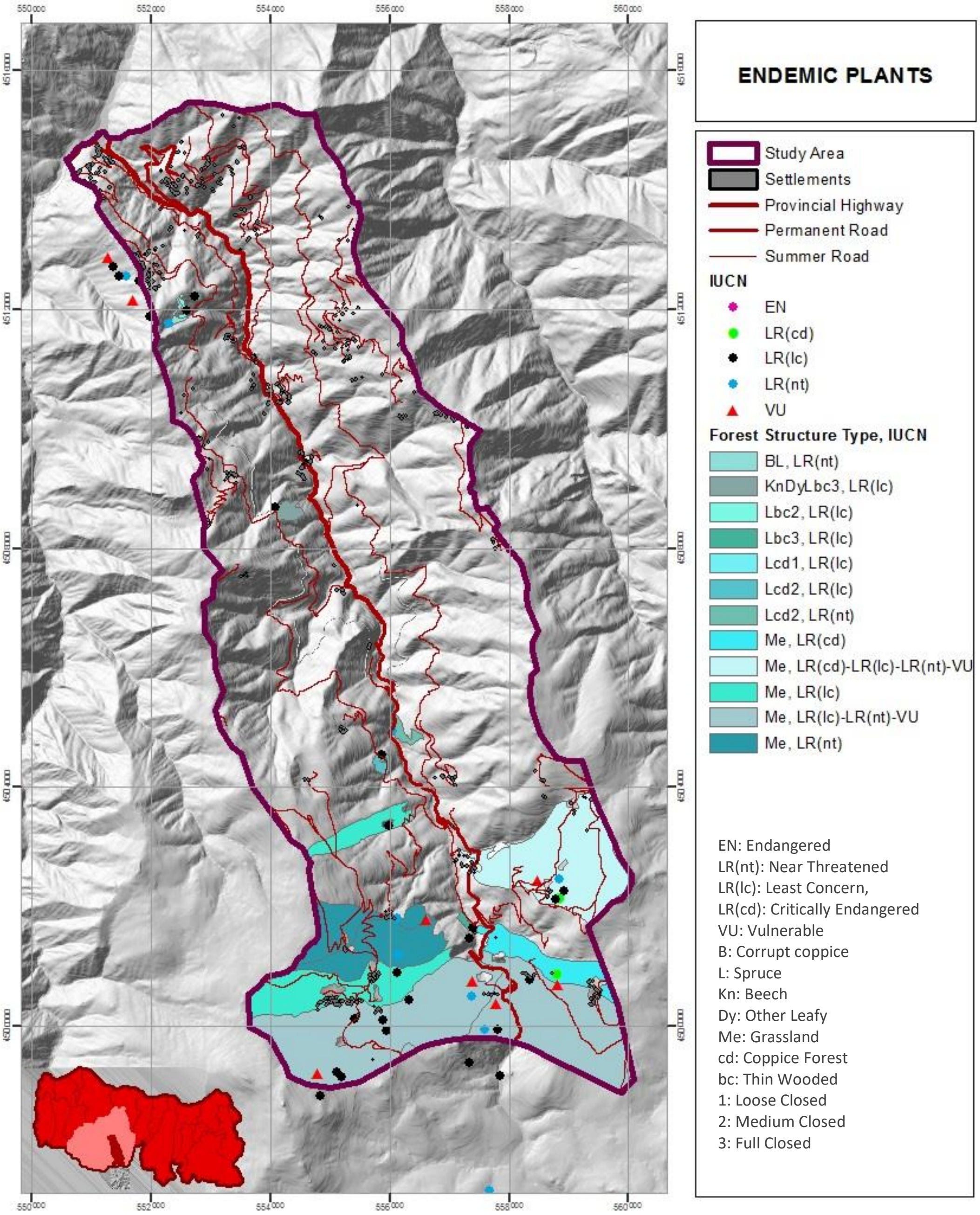

Figure 5. *Endemic plants in LULC (land use-land cover) types*

Each endemic plant habitat has its own specific ecosystem characteristics (Salapaş, 2002; Uzun, 2002; Scullion et al., 2014). Therefore, the LULCC of the surrounding endemic plants had negative effects on their ecological integrity (Neel, 2008; Esbah et al., 2009). The grassland, forest and shrubland areas, habitats for endemic plants changed dramatically from 1987 to 2016. These changes can put pressure on sensitive, rare and endangered endemic plants (Ghosh et al., 2012; Demir, 2017). These negative effects on endemic plant areas from 1987 to 2016 can affect the sustainability of local resources in the protected zone.

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LULCC may currently be the most important threat to endemic plants (Neel, 2008; Bozkaya et al., 2015). Conservation plans should be created for tourism and land management and should be supported by local participation to ensure sustainable protection (Malaviya et al., 2010; Francisco et al., 2012; Mairota et al., 2013). In this research, continuous annual data on the population density of endemic plants was missing. Utilizing continuous annual data would enhance understanding of the seasonal distributions of endemic plants.

Table 3. *Change detection and summary of endemic plant habitat changes*

Change detection of endemic plants areas								
	2016 (ha)							
	LAND COVER	**FOREST**	**GRASS-LAND**	**SHRUB-LAND**	**BARE LAND**	**CROP-LAND**	**Total 1987**	**Loss**
1987 (ha)	**FOREST**	**16.92**	1.08	0.36	1.62	0	19.98	3.06
	GRASSLAND	0.9	**48.69**	0.72	9.81	0	60.12	11.43
	SHRUB	1.44	2.52	**0.72**	3.96	0	8.64	7.92
	BARELAND	0.09	0.9	0	**9.63**	0	10.62	0.99
	CROPLAND	0	0	0	0	**0**	0	0
	Total 2016	19.35	53.19	1.8	25.02	0	**99.36**	**23.4**
	Gain	2.43	4.5	1.08	15.39	0	**23.4**	**23.55 %**

Summary of endemic plants areas changes (%)							
LAND COVER	**Total 1987**	**Total 2016**	**Gain**	**Loss**	**Total Change**	**Swap**	**Absolute value of net change**
FOREST	**16.92**	1.08	0.36	1.62	0	19.98	3.06
GRASSLAND	0.9	**48.69**	0.72	9.81	0	60.12	11.43
SHRUB	1.44	2.52	**0.72**	3.96	0	8.64	7.92
BARELAND	0.09	0.9	0	**9.63**	0	10.62	0.99
CROPLAND	0	0	0	0	**0**	0	0
Total	19.35	53.19	1.8	25.02	0	**99.36**	**23.4**

Habitat change of LULC

Landscape has interaction with and influence on natural and cultural values (CE, 2000). Due to this, monitoring the changes of landscape dynamics has a key role in this research. In this context, the natural and cultural landscape resources of Mary Valley were examined and habitat potential based on landscape sensitivity was determined to observe changes of LULC and endemic plant populations.

The quantification of landscape dynamics of a landscape pattern using landscape metrics is a basic way to determine habitat function and landscape changes (Forman and Godron, 1986; Turner, 2001; Fan et al., 2018). Therefore, the habitat function of the research area was evaluated using landscape structure metrics for LULCC types from 1987 to 2016 in four categories. The landscape metrics grouped in the area/edge metrics class from 1987 to 2016 indicated that the values of TE, ED and PN of grassland and

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cropland are higher; conversely, PD and MPS values are lower than those of other LULC types. Therefore, grasslands and croplands had smaller and more fragmented patches, which led to a decreased habitat potential.

According to the shape metrics group, the low MSI and AWMSI values of shrublands indicated more regular patch shapes that conduce to higher habitat values than other LULC types. On the contrary, bare land and croplands have small and irregular geometric patch shapes, which means lower habitat potential.

The core metrics group is important for protecting landscape values. According to it, forest areas had the highest values of TC and TCA, because forest areas lie in the middle of the watershed valley with low PN, MPS values, and low fragmentation, thus they had a higher habitat potential. Core areas of shrubland and bare land types were quite small, resulting in low habitat values.

According to landscape metrics results of isolation/contiguity metrics, croplands had the highest CONTIG_MN and ENN_MN values, which led to a decreased habitat potential. On the contrary, shrub areas had the lowest continuity and isolation values. Grasslands, forest and bare land, respectively, have similar habitat potential due to the topographic shape of deep valley.

The low ED of forest and grassland indicated that they had higher habitat values with more interior differences in the richness of species. PN of bare land was the highest with a low PD. Therefore, this LULC type became more fragmented than other types. Grasslands and croplands with high CONTIG_MN, ENN_MN and FRAG_MN values demonstrate more heterogeneous and fragmented habitats. Furthermore, croplands with low PN and AWMSI had a potential to shrink. Generally, croplands had the lowest habitat potential in this research, due to the fact that croplands had been decreasing since 1987, and the patches of croplands were quite fragmented and dispersed. The high values of TC, and CAI_MN in forest areas had high habitat potential, conversely, shrublands had the opposite effect. In addition, the low MPAR and FRAC_AM values of shrubs indicated a high habitat potential. In addition, shrublands with high PN, PD and low MSI means improved habitats. However, their habitat potential was lower due to their high ED, CONTIG_MN, ENN_MN and low MPS values, which mean a high level of fragmentation (*Table 4*).

According to the linear normalization of all metrics from 1987 to 2016, the habitat function of the LULC types from highest to lowest were forests, shrublands, croplands, grasslands and bare land, respectively (*Figure 6*). The habitat function of forests decreased suddenly in 1998 compared to other periods. This loss of forest habitat led to an increase in shrubland, cropland and bare land areas. The habitat potential of shrublands was the highest among the LULCC types in 1998, and the habitat value of bare land was the lowest. In 2016, forests experienced the highest habitat function at 1.00 due to uniform, compact and un-fragmented patches, followed by grasslands at 0.540. Bare land experienced the lowest habitat function at 0.00. This landscape structure analysis integrated with the normalization method is a first in terms of ranking the habitat changes of LULC on the same scale to provide a more objective approach.

The values of habitat function assess each LULCC pattern according to ecological integrity (Esbah et al., 2009; Demir and Demirel, 2018; Hussain et al., 2018). External influence should be considered in habitat function because unsustainable use and lack of administrative structure can negatively affect it (Bruton et al., 2016; Demir et al., 2016).

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Table 4. *Landscape metrics of LULC (land use-land cover) from 1987 to 2016*

	Area/Edge Metrics									Shape Metrics						Core Metrics			Isolation/Contiguity		
	LULC	PLAND	TE	ED	PN	PD	MPS	AWMPS	Norm.	MSI	AWMSI	FRAC MN	FRAC AM	MPAR	Norm.	TCA	CAI MN	Norm.	CONTIG MN	ENN MN	Norm.
1987	Shrubland	9.06	344100	44.11	322	0.45	0.68	126.63	0.06	1.19	3.44	1.04	1.16	494.86	1.00	127.53	0.04	0.00	0.27	106.51	0.86
	Grassland	28.57	436110	55.9	221	0.08	4.12	736.2	0.00	1.26	7.2	1.04	1.23	206.61	0.58	539.19	0.3	0.27	0.13	91.98	1.00
	Forest	49.87	308730	39.58	68	0.02	42.76	3711.79	1.00	1.31	11.15	1.04	1.27	82.35	0.00	1900.53	0.65	1.00	0.2	95.17	0.94
	Bare land	11.29	233700	29.96	174	0.14	3.18	128.86	0.20	1.36	4.33	1.05	1.19	285.72	0.04	76.05	0.27	0.05	0.26	119.6	0.86
	Cropland	1.2	19740	2.53	17	0.53	5.52	12.47	0.85	1.38	1.51	1.07	1.08	226.49	0.28	9.9	2.59	0.80	0.69	767.56	0.00
	LULC	PLAND	TE	ED	PN	PD	AREA MN	AREA AM	Norm.	SHAPE MN	SHAPE AM	FRAC MN	FRAC AM	PARA AM	Norm.	TCA	CAI MN	Norm.	CONTIG MN	ENN MN	Norm.
1998	Shrubland	50	460140	30.61	1540	1.18	27.86	3586.5	1.00	1.21	7.72	1.04	1.23	64.47	1.00	2604.42	0.82	1.00	0.27	106.51	0.86
	Grassland	24.62	528030	49.39	1100	0.43	4.03	274.88	0.00	1.29	4.52	1.05	1.2	212.88	0.46	552.87	0.47	0.23	0.13	91.98	1.00
	Forest	14.12	304890	49.65	234	0.05	2	127.4	0.43	1.33	4.32	1.05	1.2	359.48	0.00	122.22	0.07	0.00	0.2	95.17	0.94
	Bare land	10.25	187800	25.6	672	0.55	3.11	171.83	0.82	1.31	4.68	1.05	1.2	268.3	0.24	105.84	0.24	0.03	0.26	119.6	0.86
	Cropland	1.01	14880	1.92	14	0.16	6.07	12.96	0.85	1.34	1.48	1.06	1.07	207.53	0.71	9.99	4.22	0.84	0.69	767.56	0.00
	LULC	PLAND	TE	ED	PN	PD	AREA MN	AREA AM	Norm.	SHAPE MN	SHAPE AM	FRAC MN	FRAC AM	PARA AM	Norm.	TCA	CAI MN	Norm.	CONTIG MN	ENN MN	Norm.
2009	Shrubland	13.81	460140	58.98	375	0.34	0.89	185.05	0.23	1.19	4.18	1.03	1.19	435.65	1.00	187.29	0.04	0.00	0.26	105.75	0.87
	Grassland	29.85	528030	67.69	247	0.08	4.29	806.42	0.20	1.28	10.18	1.04	1.27	237.25	0.00	363.24	0.16	0.12	0.13	90.12	1.00
	Forest	46.45	304890	39.08	68	0.02	43.66	3379.73	1.00	1.33	11.29	1.04	1.28	87.19	0.01	1667.79	0.93	1.00	0.19	99.86	0.94
	Bare land	8.92	187800	24.07	159	0.16	2.91	134.22	0.00	1.34	4.43	1.05	1.19	291.22	0.21	56.43	0.3	0.00	0.29	139.59	0.80
	Cropland	0.96	14880	1.91	12	0.4	5.75	13.5	0.21	1.38	1.48	1.07	1.07	217.41	0.54	9.09	3.2	0.76	0.69	550.56	0.00
	LULC	PLAND	TE	ED	PN	PD	AREA MN	AREA AM	Norm.	SHAPE MN	SHAPE AM	FRAC MN	FRAC AM	PARA AM	Norm.	TCA	CAI MN	Norm.	CONTIG MN	ENN MN	Norm.
2016	Shrubland	9.23	128670	16.49	67	0.09	2.56	538.08	0.00	1.15	5.45	1.03	1.21	185.91	1.00	304.38	0.17	0.05	0.12	112.02	1.00
	Grassland	27.61	421050	53.97	185	0.07	4.09	229.93	0.13	1.22	4.87	1.04	1.21	205.1	0.70	522.18	0.55	0.28	0.28	92.91	0.87
	Forest	43.93	295140	37.83	62	0.02	55.27	3235.1	1.00	1.41	11.45	1.05	1.28	89	0.18	1472.04	0.97	1.00	0.18	98.52	0.96
	Bare land	18.29	304170	38.99	180	0.09	6.61	341.9	0.09	1.39	7.65	1.05	1.25	229.93	0.00	155.07	0.36	0.00	0.29	116.82	0.84
	Cropland	0.93	13650	1.75	12	0.7	6.08	12.62	0.24	1.37	1.42	1.06	1.06	205.76	0.61	9.63	3.9	0.80	0.69	693.71	0.00

PLAND: Percentage of Landscape, MPS: Mean Patch Size, AWMPS: Area Weighted Mean, PN: Patch Number, PD: Patch Density, TE: Total Edge, ED: Edge Density, MSI: Mean Size Index, AWMSI: Area Weighted Mean size Index, MPAR: Area Weighted Mean Perimeter-area Ratio, FRAC-AM: Area Weighted Mean Fractal, CONTIG_MN: Mean Contiguity, ENN_MN: Mean Euclidean Nearest Neighbors Distance, TCA: Total Core Area, CAI_MN: Mean Core Area Index

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DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

For example, more than 40% of the uncontrolled wood cutting of local people and road networks in 1998 represented a key factor in the decline of forest habitats in Mary Valley. The habitat function of landscape patterns is calculated using different types of landscape metrics and indicates direct or inverse effects (Malaviya et al., 2010; Fichera et al., 2012). All metrics were evaluated separately, depending on their direct or inverse relationships with the habitat function changes of each LULC types through the linear normalization (Norm.) called Min-Max method (*Figure 6*). As a result, objective values for the habitat function of all LULC types were calculated. This normalization statistical technique was an effective and easy way to enhance our understanding and to measure the effects of metrics on habitat function. A high number of metrics creates difficulties for the evaluation of habitat function (Esbah et al., 2009; Fichera et al., 2012; Bruton et al., 2016). A research was suggested to limit and categorize them using a statistical method such as factor or cluster analysis with the assistance of an expert (Mairota et al., 2013). Thus the 16 metrics selected were categorized in 4 groups based on their characteristics and were evaluated objectively using linear normalization method.

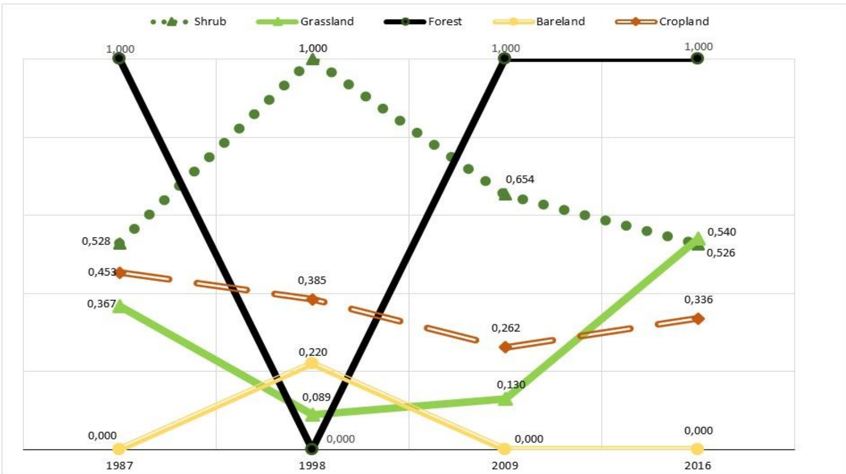

***Figure 6.** Total habitat changes (ha) of LULC (land use-land cover) by linear normalization*

LULC change of endemic plant areas

Changes in the endemic plant habitats were compared with the habitat function of each LULC type. The changes in these species' distribution areas were inversely related to the habitat function of bare land and forest areas, but directly related to habitat function of shrublands (*Figure 7*). In the grasslands, the endemic plants habitats were inversely related to habitat function. The results of these analyses, conducted along the distribution areas of endemic plants, has allowed detecting a significant relationship with the habitat function of LULC.

The decline in shrublands diminished the habitat function of endemic plant distribution areas. Generally, shrublands have a valuable habitat status for endemic plants (Salapaş, 2002; Estes et al., 2012; Bruton et al., 2016). Thus, the transformation of shrublands should be stopped to protect the species. Bare land areas have increased over 29 years and adversely affected the habitat function of sensitive, unique and rare endemic plant habitats. The rise in the total area of bare land should also be reduced to prevent negative effects. Unlike the bare land trend, forest area inversely affected them.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

This method is a first in terms of identifying changes in endemic plant habitats to prove the effect of LULC type changes on sensitive habitats.

Endemic plants are located in different LULC types (Uzun, 2002; Estes et al., 2012; Scullion et al., 2014). Due to this reason, future conservation decisions can be prepared based on the separate environmental requirements of endemic plants to sustain each habitat function. Uncontrolled tree cutting and infrastructure expansion connect the decline of the habitat function of forest areas to the increase in bare land and grassland habitat functions based on area/edge, shape, core and isolation/contiguity metrics. Since 1998, the habitat function of forest areas for endemic plants has increased. This result showed that forest habitat renewed itself with ecological processes. Conservation and management plans should respect ecological processes to ensure the ecological integrity of the research area.

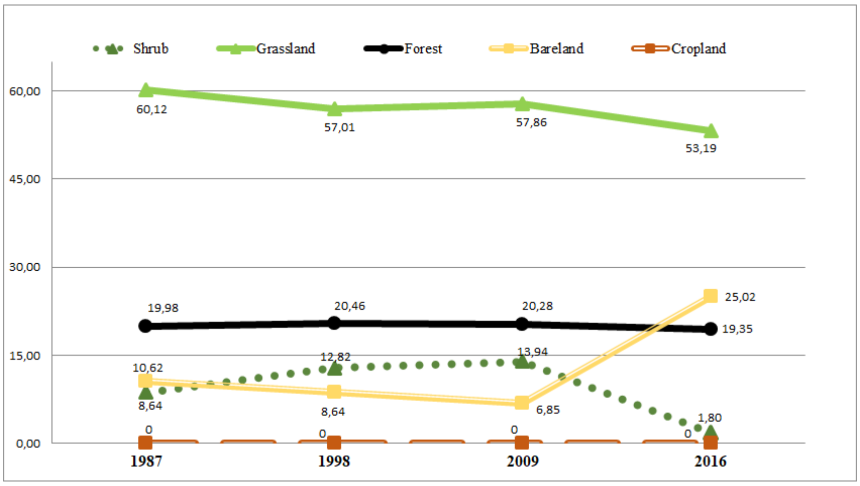

Figure 7. *LULC (land use-land cover) change of endemic plant areas (ha) from 1987 to 2016*

Other related changes

The spatial and temporal monitoring model supplied quantitative results of past to present day changes and can estimate the direction and extent of future transformation (Malaviya et al., 2010; Fichera et al., 2012; Mairota et al., 2013) However, this research has some limitations. It is still too difficult to determine the unpredictable effect of other changes such as decreasing local population and insufficient visitor management. Local people play an important role in the protection of their own landscape resources (Chaminuka et al., 2012; Estes et al., 2012; Fichera et al., 2012; Scullion et al., 2014; Demir et al., 2016). Nevertheless, according to data of TURKSTAT (2016), the population decreased from 4,142 in 1987 to 1,605 in 2016 in Mary Valley's protected area. Demographic projections for Mary Valley (Demir et al., 2015; Demir, 2017) forecast that this declining trend will continue until 2040 (1,373 in 2020 and 743 in 2040). This decline was due to restrictions within the boundaries of the national park banning forestry and agricultural activities. The forestry area had constantly decreased since 1987 due to mostly uncontrolled deforestation and new road infrastructure. Another limitation was related to climate, which restricts tourism activities to July and August.

The research area is part of a valuable national park with tourism and recreation potential (Demir et al., 2015; Pirselimoglu and Demirel, 2015a, 2015b). Consequently,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

a future conservation plan and ecotourism activities with local cooperation in participatory management can be offered as a way to partially solve the problems of the natural environment and the populace (Estes et al., 2012; Francisco and Hochshil, 2012). Such a plan and ecotourism can create socio-economic prosperity for local people to return to the valley and protect its natural and cultural landscape. Local awareness on the protection of ecological values can be raised. All protection rules and ecotourism activities can be controlled by an interdisciplinary scientific council responsible to the related ministries, experts and other stakeholders. This can make it possible to provide special local protection and effectively prevent smuggling of the area's fauna, flora and historical resources.

In the study area, there is no capacity control or monitor visitors of the national park or tourism and recreation activities in general. These problems can put pressure on its ecological and cultural values and diminish its environmental characteristics. Thus, while conservation oriented plans are being made, tourism management and land resource management strategies based on landscape planning can be carefully considered not only for the national park, but for the entire catchment area in a future research. This method can be an efficient and effective way for future researches to monitor LULCC and habitat function worldwide and to determine conservation and management strategies.

In relevant literature, there are many studies in which LULC change detection and habitat change is studied. However, there researches on protected areas are quite limited, especially where changes of endemic plants habitat and the evaluation of landscape metrics by grouping are involved. Similar to Mary Valley’s research, the Mairota et al. (2013) developed a quantitative method based on landscape structure metrics to monitor protected areas. It only analyzed the landscape structure of protected areas, but LULC changes from past to present day could be an important key element to monitor habitat function change of landscape dynamics to support the findings. On the contrary, in the research of Mary Valley, the effect of LULC changes on endemic plants was monitored with change detection and landscape metrics (individually and in four groups) which has an important role in improving sustainable planning and management strategies for the protected area. Malaviye et al. (2010) monitored 34 years of LULC changes to determine changes of habitat diversity of a mining area. Fichera et al. (2012) analyzed and monitored 50 years of LULC and habitat changes. The oldest image dated back to only the year 1987 of Marry Valley, because the satellite images of this study area were limited due to cloudy days and snow cover on highlands. Therefore, for the further research, the images of the study area should date back to before the conservation status was established, to provide an exhaustive analysis of want went on before and after that date. Although these researches analyzed LULC and habitat change in spatial and temporal scales using change detection and a few landscape structures metrics, they did not consider the relationship of LULC and habitat change with the endemic plants of the area, and neither did they group landscape metrics to qualify the landscape structure of LULC and endemic plant habitats. Furthermore, the main difference between Mary Valley’ research and these sample studies is that the spatial and temporal LULC and habitat changes of protected area under four landscape metric groups, paired with the relationship of endemic plants were all analyzed. Therefore, these methods were developed to contribute guidance to future researches and practices worldwide.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

Conclusions

The objectives of this study were to determine temporal habitat function based on landscape structure analysis using landscape metrics and to identify the relationships between the change of LULC, habitat function and endemic plant distribution areas by temporal and spatial monitoring and analysis. First of all, changes of LULC and endemic plant areas were monitored, followed by habitat function of LULC types and endemic plant distribution areas, based on related studies conducted nationally and internationally. Through landscape habitat and landscape structure analysis, the temporal changes of LULC, endemic plants and habitats were determined for the research area. Mary Valley protected area has a catchment and archeological site. However, this research area has been under pressure due to habitat changes of LULC types and endemic plants distribution areas. Therefore, connectivity of landscape patches of each LULC types can be improved for sustainability, and ecological and cultural integrity of the study area, through establishing healthier conservation and management strategies by improving existing ones.

This research examined habitat function of LULC and endemic plants distribution areas based on landscape sensitivity, using landscape dynamics. The spatial and temporal landscape pattern was investigated on four different dates (over 29 years), which enabled us to monitor the changes of landscape dynamics. According to the findings on LULC change with change detection, forest areas lost the most area, whereas bare land areas significantly increased, gaining most of it from lost forests, grasslands and croplands. Furthermore, the most endemic plants are located in grasslands, although the highest loss and net change amounts were also indicated in grasslands. In addition, shrublands were under threat with a sharp constant decline trend. In the context of habitat function of LULC types and endemic plant distribution areas evaluated using landscape structure metrics with linear normalization scale, the endemic plant distribution areas had adverse effects on habitat function due to the edge effect, which diminished environmental resources. The forest had the highest, while bare land had the lowest habitat potential with high fragmentation. This study evaluated temporal LULC and habitat changes. In this research, the landscape structure analysis integrated with normalization method and the determination of the effects of LULC changes on sensitive endemic plants habitats were a first, and thus these new approaches may set an example for future studies related LULC and LULC changes on sensitive areas. These methods and findings can help control sensitive landscape dynamics for further sustainable development of protected areas. Thus, this research can set an example for Turkey and other developing countries in supporting the sustainability of ecological and cultural integrity of protected areas.

Acknowledgements. I am grateful to Professors Jiquan Chen and Assoc. Professors Ranjeet Kumar for their valuable and constructive comments on this paper.

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APPENDIX

Appendix 1. *The endemic plants of Mary Valley based on IUCN category (Salapaş, 2002; Uzun 2002; Demir, 2017)*

IUCN	Taxonomy	Family
VU	Alchemilla oriturcica	Rosaceae
VU	Symphytum longipetiolatum	Boraginaceae
VU	Jasione supina subsp. pontica	Campanulaceae
VU	Crocus aerius	Iridaceae
LR(nt)	Sempervivum minus var. glabrum	Crassulaceae
LR(nt)	Cirsium trachylepis	Asteraceae(Compositae)
LR(nt)	Alchemilla sintenisii	Rosaceae
LR(nt)	Potentilla cappadocica	Rosaceae
LR(nt)	Astragalus lineatus var.jildisianus	Fabaceae
LR(nt)	Sempervivum minus var. minus	Crassulaceae
LR(nt)	Alchemilla sintenisii	Rosaceae
LR(lc)	Ranunculus dissectus subsp. huetii	Ranunculaceae
LR(lc)	Cerastium gnaphalodes	Caryophyllaceae
LR(lc)	Draba rigida var. rigida	Brassicaceae (Criciferae)
LR(lc)	Trifolium pannonicum subsp. elongatum	Fabaceae
LR(lc)	Heracleum platytaenium	Apiaceae (Umbelliferae)
LR(lc)	Galium fissurense	Rubiaceae
LR(lc)	Tripleurospermum monticolum	Asteraceae(Compositae)
LR(lc)	Cirsium sommieri	Asteraceae(Compositae)
LR(lc)	Geranium asphodeolides subsp. sintensii	Geraniaceae
LR(lc)	Geranium ibericum subsp. jubatum	Geraniaceae
LR(lc)	Muscari aucheri	Liliaceae
LR(lc)	Muscari coeleste	Liliaceae
LR(lc)	Dactylorhiza osmanica var. osmanica	Orchidaceae
LR(lc)	Cyclamen parviflorum	Primulaceae
LR(lc)	Phlomis russeliana	Lamiaceae(Labiatae)
LR(lc)	Lamium ponticum	Lamiaceae(Labiatae)
LR(lc)	Campanula betulifolia	Companulaceae
LR(lc)	Lonicera caucasica subsp. orientalis	Caprifoliaceae
LR(cd)	Cerastium lazicum	Caryophyllaceae
LR(cd)	Festuca amethystina subsp. orientalis var. turcica	Poaceae (Gramineae)

VU: Vulnerable, LR(nt): Near Threatenes, LR(lc): Least Concern, LR(cd): Critically Endangered

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_35913613

ASSESSING THE CLIMATE FORECAST SYSTEM REANALYSIS WEATHER DATA DRIVEN HYDROLOGICAL MODEL FOR THE YANGTZE RIVER BASIN IN CHINA

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(Received 10th Dec 2018; accepted 20th Feb 2019)

Abstract. It is a challenge to adequately represent meteorological data for river basin modelling in data-scarce regions. Hydrological models may benefit from alternative climate data sources such as the Climate Forecast System Reanalysis (CFSR) weather data. A proposed method was applied to evaluate the applicability of CFSR data in the Soil and Water Assessment Tool (SWAT) eco-hydrological model for the 1.8×10^6 km^2 Yangtze River Basin (YRB) in China. The CFSR data were first validated by ground-based meteorological station (GMS) data to confirm sufficient accuracy to predict the hydrology for the YRB. It was determined that the CFSR simulation was able to generate acceptable accuracy with calibrated parameters of GMS-based model. The R^2 and ENS of the monthly results from both GMS- and CFSR-SWAT simulations were > 0.70 in both calibration and validation periods. The monthly streamflow hydrograph from GMS and CFSR simulations showed two different patterns: distinct differences in baseflow and less discrepancy for the peak flows, such as the patterns at Cuntan and Yichang gauge stations, whereas the results are characterized as close matches the peaks and the recession periods at Hankou and Datong gauge stations. The CFSR simulation generated better results in the middle and lower reaches than in the upper reach of the basin. To a certain extent, the CFSR provides an alternative to quickly build hydrological model in data scarce area for large basins.
Keywords: *SWAT model, data scarce area, model calibration, meteorological forcing data, large-scale watershed*

Introduction

Traditionally, weather data collected at ground-based meteorological stations (GMS) are utilized to drive eco-hydrological models; therefore, it is important to collect adequate and representative weather data. It is difficult and time-consuming to obtain adequate meteorological data for hydrological models to accurately represent climate patterns for some watersheds, especially those located in data-scare regions, due to inadequate overall coverage of the watershed area provided by ground-based meteorological station (GMS) data. In developing countries or ungauged areas (Lu et al., 2018), weather data are also unavailable because of financial limitations and/or inadequate technology (Vu et al., 2012). In addition, there are shortcomings related to GMS data in regards to supporting rapid development of watershed models. For a small-scale watershed, GMS data are sometimes located outside of the investigated watershed and consequently, the collected records from GMS may not accurately represent the actual weather that occurred at the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36153632

watershed (Fuka et al., 2014). The scarcity of GMS, especially in developing countries, increases the uncertainty in precipitation inputs for each model sub-basin (Worqlul et al., 2014). In addition, because of the data management policies of governments for state security in some countries, it is usually difficult to collect sufficient data for hydrological models.

The availability of other sources of data (i.e., satellite precipitation and reanalysis data) where there is no conventional GMS has recently attracted the interest of hydrologists. The hydrological model can benefit from satellite observation data and global reanalyzed weather data such as TRMM (Tropical Rainfall Measuring Mission), ECMWF (European Centre for Medium-Range Weather Forecasting), ERA-40 (40-year reanalysis), CFSR (Climate Forecast System Reanalysis) produced by National Center of Environmental Prediction (NCEP), APHRODITE (Asian Precipitation-Highly-Resolved Observational Data Integration towards Evaluation of Water Resources), and PERSIANN-CDR (Precipitation Estimation from Remotely Sensed Information using Artificial Neural Network-Climate Data Record) to overcome data shortages (Behrangi et al., 2011; Ward et al., 2011; Worqlul et al., 2015; Tan et al., 2017).

Concerns have been increasing on the subject of climate change as a driver of hydrological changes within the Yangtze River Basin (YRB) in China (Zhang et al., 2008; Zhu et al., 2011) based on satellite observation data and global reanalysis weather data. Previous studies were carried out to evaluate the remotely sensed weather products and their reanalysis datasets by application in different basins. Yang et al. (2014) found that gridded precipitation products poorly reproduced hydrologic processes except that the APHRODITE and trend surface data can give stable and desirable results in a relatively flat test basin, in an evaluation of a gridded precipitation-driven Soil and Water Assessment Tool (SWAT) eco-hydrological model application for the upper reaches of Three Gorges Reservoir. Sun et al. (2013) investigated the spatiotemporal rainfall patterns of the YRB using TRMM/PR (TRMM/ Precipitation Radar) data in a decadal scale, which reveals that there are possible linkages between major precipitation patterns and hydrological extremes. Li et al. (2015) conducted water balance and streamflow studies for multiple time scales using three global satellite rainfall products [TMPA (TRMM Multi-satellite Precipitation Analysis) 3B42 V7, TMPA 3B42 Real Time (RT), and CMORPH (Climate Prediction Center morphing technique)] during 2003 to 2012 in the YRB. They found that the 3B42 V7 data generally resulted in improved annual water budgeting and monthly streamflow performance. The 3B42 RT tends to overestimate streamflow in the upper Yangtze River, and, in contrast, CMORPH shows serious underestimation in downstream sub-basins. Worqlul et al. (2014, 2015) compared to rainfall estimations by TRMM 3B42, MPEG (Multi-Sensor Precipitation Estimate–Geostationary) and CFSR with ground-observed data for the Lake Tana basin and Upper Blue Nile in Ethiopia. The MPEG and CFSR satellites provided the most accurate rainfall estimates, and both the gauged data and the CFSR precipitation estimates were able to reproduce the hydrology well for both models and both watersheds. Tan et al. (2017) analyzed the accuracy of three long-term gridded climate products (APHRODITE, PERSIANN-CDR and NCEP-CFSR) for two tropical river basins in Malaysia. The APHRODITE data resulted in strong results for streamflow simulations, while NCEP-CFRS data showed unsatisfactory performance.

The satellite-based precipitation products and their reanalysis weather data provide an alternative data source to study hydrology and climate change. The application of remotely-sensed data and corresponding reanalysis precipitation data within eco-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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hydrological models is gradually increasing in many regions; however, use of such remains limited in many areas of the globe including the YRB. Therefore, gridded precipitation products must be evaluated to improve the performance of the watershed model in this data-scarce area. In addition, the SWAT eco-hydrological model (Arnold et al., 1998; Arnold et al., 2012; Gassman et al., 2007, 2014) has recently been applied to parts or all of the YRB to predict the water balance, sediment transport, and nutrients. Ouyang et al. (2008) used SWAT to evaluate the impact on streamflow and nonpoint source pollutants from the conversion of cropland to forest in Bazhong City, located in the central YRB region. Zhang et al. (2011) investigated the water balance of Three Gorges Reservoir in the upper reach of the Yangtze River with the aid of SWAT model. Sun et al. (2016) mapped the water vulnerability of the YRB using SWAT for 1994 to 2013; the statistical results showed that the simulated output accurately replicated the observed data during both the calibration and validation periods. Chen et al. (2017) and Su et al. (2017) assessed the impacts of climate change on river discharge in the upper reaches of the YRB based on hydrological models under different climate projections. Several other studies reported predicted water budget, water quality, and/or the impacts of climate change or land use management using SWAT for various YRB sub-regions (Yang et al., 2014; Huang et al., 2009, 2014; Zhou et al., 2013). The applicability of global reanalysis climate data for hydrological model predictions has not so far been adequately investigated in such a large watershed: the entire Yangtze River basin.

Therefore, the aim of this study was to assess the performance of CFSR weather data versus GMS data for predicting YRB hydrology using SWAT. To evaluate the applicability of CFSR weather data in the watershed model, the same hydrological parameters of the model calibrated using GMS weather data were used in both models, and then both the results were validated against observed data. The simulated results of streamflow from the SWAT model driven by CFSR weather data with and without calibration were compared. Finally, the monthly averaged streamflow simulated by the models driven by CFSR and GMS weather data were investigated to evaluate the performance of CFSR as input weather data.

Materials and Methods

Study area

The Yangtze River, also called the Changjiang, which means ‘Long River’ in Chinese, is the longest river in China and the third longest in the word, with a length of approximately 6,380 km and a drainage area of 1.8×10^6 km^2. Originating from the Tibetan Plateau, the Yangtze River flows across the Qinghai-Tibet Plateau in west China, Sichuan basin in the middle China, and east China Plain with an average elevation of 3000 m, 1000 m, and 100 m, respectively (Gong et al., 2006). Generally, the Yangtze River is divided into three reaches: upper, which drains the area controlled by the Yichang gauge station, middle, which captures drainage between the Yichang and Hukou gauge stations, and lower, which covers the area downstream of the Hukou gauge station to the estuary (*Figure 1*).

The YRB is located in the Asian monsoon region, which manifests as two independent components: the Indian summer monsoon and the East Asian summer monsoon which affect the upper and mid-lower YRB sub-regions, respectively (Ding et al., 2005). Because of the large area and extensive range of elevation, precipitation varies considerably between the different climatic zones. The annual precipitation in most areas

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of the YRB is 800-1600 mm, whereas it is less than 400 mm in the river source areas in the arid zone. The area with precipitation larger than 1600 mm is mainly located in the Sichuan, Jiangxi, Hunan and Hubei provinces. Large floods are frequent in the summer due to the heavy monsoon rainfall influence. Precipitation declines across the basin from east to west, ranging on average annually from 1528 mm in the east to 859 mm in the west. The upper Yangtze region is characterized by the highest elevation and the least precipitation (Chen et al., 2014). The weather data of GMS over the YRB for 65 weather stations (*Figure 1*) were collected for model setup. The mean annual temperature in the middle and the lower Yangtze basin is 19 and 15°C from the west to east respectively (Zhang et al., 2006). The annual average streamflow of the Yangtze River is 9.5×10^{11} m^3, and the average runoff depth is 526 mm, which is the largest streamflow in China, comprising 35% of the streamflow of all of China's rivers.

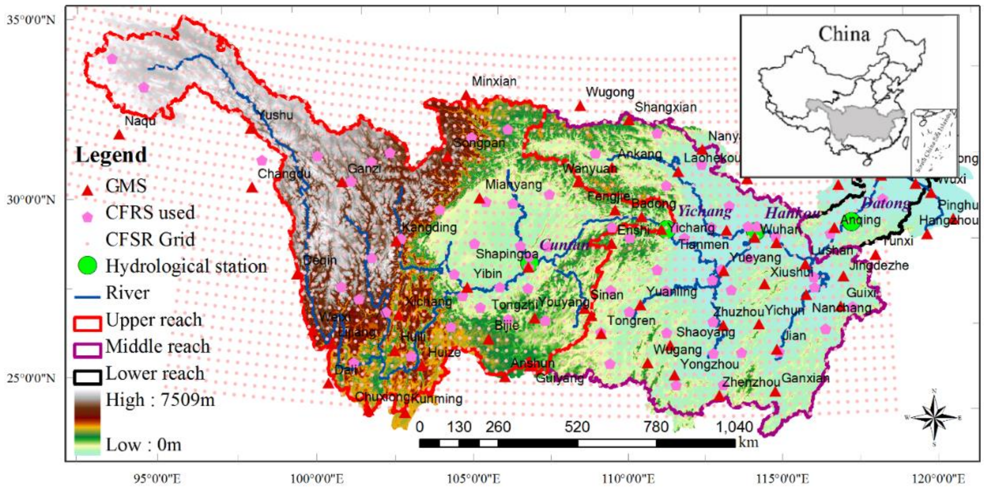

Figure 1. *The Digital Elevation Model (DEM) of the YRB, its river networks, hydrological stations, Ground-based Meteorological Station (GMS) and Climate Forecast System Reanalysis (CFSR) grids. The location of the basin with respect to China is shown in the upper right panel*

Datasets and model setup for the YRB

Spatial data

The spatial databases used to establish the SWAT model included the Digital Elevation Model (DEM), land use and soil type. The DEM describes the spatial distribution of regional topography and serves a fundamental role in the definition of the basin boundary, division of sub-basin, generation of river network, extraction of various hydrological parameters and division of HRUs (Tao et al., 2015; Lu et al., 2017). The DEM (*Figure 1*) with 90-m resolution used in this study was generated from the Shuttle Radar Topography Mission (SRTM) DEM (http://srtm.csi.cgiar.org/SELECTION/inputCoord.asp) processed with ArcGIS 9.3 software. Land use affects the runoff process on the land surface and has an important influence on the results of the simulation. The land use data used here were produced by the Department of Geography, University of Maryland (Hansen et al., 2000). This land use / cover products were generated using the AVHRR (Advanced Very High Resolution Radiometer) images which were analyzed to distinguish fourteen land use and cover classes with a spatial scale of 1 km pixel resolution. These images were analyzed to distinguish fourteen land use and cover classes with a spatial

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DOI: http://dx.doi.org/10.15666/aeer/1702_36153632

scale of 1 km pixel resolution. The land use was then reclassified to nine classes for the SWAT model input (*Figure 2a*). The main land use type is forest, accounting for 36% of the area, whereas pasture (23.5%) and crop land (23%) make up the next largest proportions of the YRB.

The original China soil data that are parts of the HWSD (Harmonized World Soil Database) dataset were produced by the Institute of Soil Science, Chinese Academy of Sciences based on the Second National Soil Survey of China (Shi et al., 2006). Soil properties were extracted by 1:1,000,000 HWSD version 1.1 (*Figure 2b*). The main soil type is Haplic Luvisols with higher organic matter, Cumulic Anthrosols with human improvement of water and fertility conservation, and Gelic Leptosols with thin layer that are sensitive to the temperature with the percentage of 16.1%, 13.8%, and 11.3% respectively in the YRB.

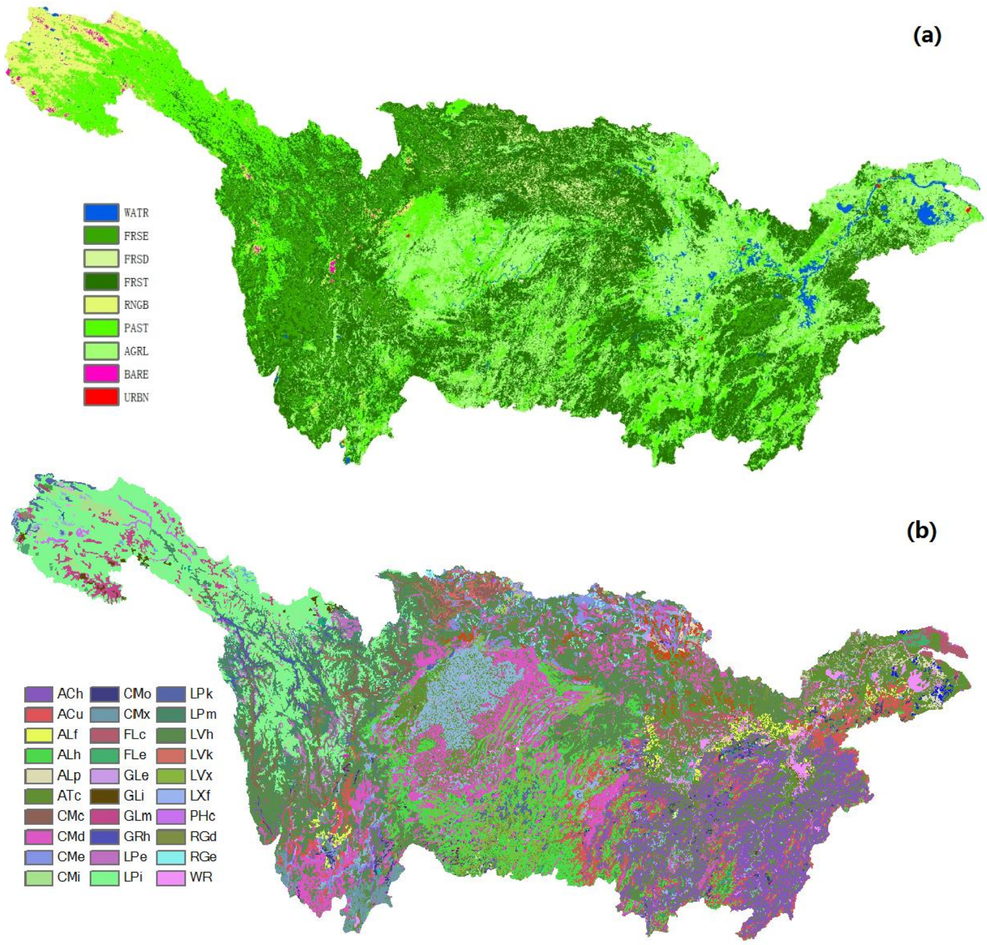

Figure 2. *Land use (a) and soil type data (b) for the YRB*

Hydro-meteorological data

Four main hydrological gauge stations (Cuntan, Yichang, Hankou, and Datong), which controls the runoff from different tributaries in the upper, middle and lower reaches of the YRB, were selected for measured streamflow data (*Figure 1*). Monthly streamflow were acquired for these four hydrological gauge stations for the time period 1981-2002 from the Bureau of Hydrology, Changjiang (Yangtze River) Water Resources Commission for China

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(http://www.cjh.com.cn/). The GMS weather data covered over the YRB were downloaded for 1981 to 2005 from the China Meteorological Data Sharing Service System (http:// http://data.cma.cn/). Weather data for each station included observed daily precipitation, average temperature, minimum and maximum temperature, relative humidity, wind speed and direction and solar radiation data that were all the input data required for the hydrological model.

The CFSR weather data are produced using cutting-edge data assimilation techniques, (which reflects both conventional meteorological station observations and satellite irradiances) as well as highly advanced atmospheric, oceanic, and surface-modelling components. These data are available globally at an hourly time step since 1979. The CFSR dataset consists of hourly weather forecasts generated by the NCEP Global Forecast System of the U.S. National Weather Service. The horizontal resolution of the CFSR is ~38 km (Saha et al., 2010). CFSR data (precipitation, wind, relative humidity, and solar) over the YRB for the period of 1981 to 2005 were downloaded in SWAT file format (Dile and Srinivasan, 2014) from the website (https://globalweather.tamu.edu/). More than 2,000 CFSR gridded points were initially imported (*Figure 1*), and data for 69 points were selected automatically for the SWAT simulations based on the sub-basin delineations.

Model setup

The SWAT model, a river basin or watershed scale eco-hydrological model, was developed by United States Department of Agriculture (USDA) and Texas A & M University (Gassman et al., 2007; Williams et al., 2008). SWAT is able to predict water, sediment, nutrient, pesticide, and fecal bacteria yields using spatial distributed data on topography, soils, land cover, land management, and weather. The hydrological parameters are calculated by SWAT model based on the minimum research units, hydrologic response units (HRUs) which consist of homogeneous slope, land cover, and soil characteristics. Since SWAT has already been widely applied to watersheds around the world for stream flow simulation, it will also present sophisticated performance on the simulation in the YRB. One of the SWAT versions ArcSWAT 2009.93.3 integrated in ESRI ArcGIS 9.3 was used in this study which is one the major releases of SWAT version 2009.

In this study, the YRB was divided into 69 sub-basins and 613 HRUs according to land use, soil type and slope characteristics in the watershed. The performance of the SWAT model for the YRB was evaluated by the following three statistical indexes: The coefficient of determination (R^2), Nash-Sutcliffe Efficiency Coefficient (E_{NS}) and Percent bias (PBias) (Moriasi et al., 2007, 2015). In the period of sensitivity analysis, the Automated Sensitivity Analysis tool integrated in SWAT was applied to the YRB, which is based on the LH-OAT (Latin Hypercube Sampling-One at A Time) analysis method (van Griensven et al., 2006). SWAT usually must be calibrated before being applied to analyze the hydrology of a given watershed, considering the complex hydrological processes and limitations of the model (Gassman et al., 2007). Therefore, following a sensitivity analysis, model calibration was carried out and was executed using external SWAT-CUP software in which the SUFI-2 (Abbaspour et al., 2007) procedure was applied to calibrate SWAT in this study.

The SWAT parameter sensitivity analysis was initially conducted as a function of the GMS weather data, to compare the predicted streamflow for each hydrological gauge station to select the most sensitive parameters for hydrology prediction at each station. In order to maintain consistency, both the GMS- and CFSR-driven models were calibrated separately within SWAT-CUP at the four hydrological gauge stations (Cuntan, Yichang, Hankou and Datong). The parameters identified as being adjusted were based on the most

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DOI: http://dx.doi.org/10.15666/aeer/1702_36153632

sensitive aspects of predicted hydrographs in comparison with observed monthly streamflow from 1981 to 1992, and the simulated results were validated with observed data from 1993 to 2002. The calibrated parameters in the GMS simulation were then applied in the CFSR-driven SWAT model in order to compare to the result of GMS simulation at the same condition. Finally, the CFSR-driven SWAT model was completely calibrated and validated to investigate the differences of the hydrological parameters and modelling results between the GMS- and CFSR-based models.

Results

CFSR Precipitation and temperature validation by GMS weather data

The CFSR data at grid points that involved in SWAT model computation was firstly validated by the GMS data including precipitation and maximum and minimum temperature. CFSR grid points near the GMSs Huize (2110 m.a.s.l. (meters above sea level)), Mianyang (522 m.a.s.l.), Badong (334 m.a.s.l.), Ji'an (78 m.a.s.l.) and Yuanling (143 m.a.s.l.) (Their locations can be found in *Figure 1*), which represent different elevations in this area, were selected to compare the precipitation and temperature between the two data sources.

The scatter plots of the monthly precipitation and the linear regression lines at Huize, Mianyang, Badong, Ji'an and Yuanling for the GMS versus CFSR weather data from 1981 to 2005 are shown in *Figure 3*.

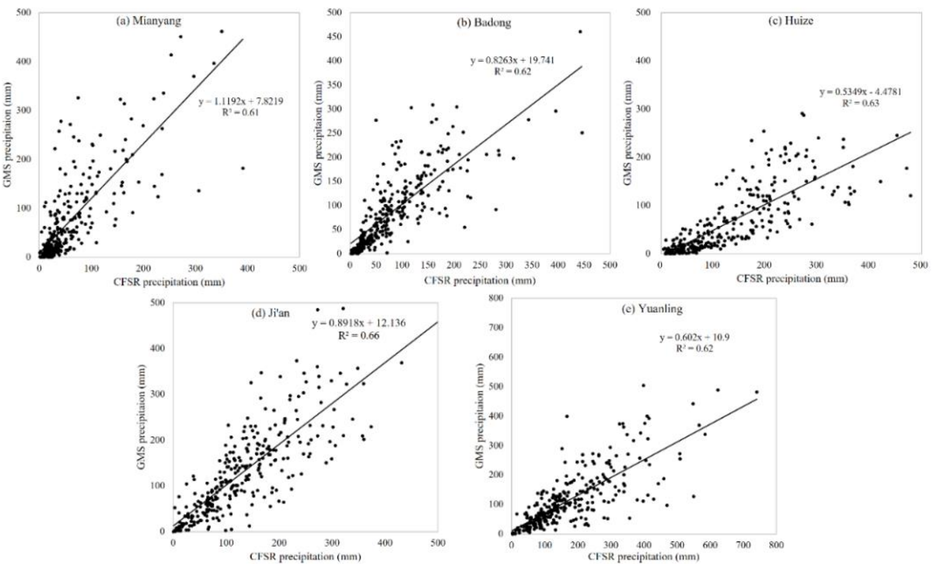

Figure 3. *Comparison of monthly precipitation between the CFSR and GMS climate data sources during 1981 to 2005 at: (a) Badong, (b) Mianyang, (c) Huize, (d) Ji'an, and (e) Yuanling meteorological stations*

The CFSR precipitation underestimated the GMS observed precipitation, based on the regression comparison for the Mianyang station. The precipitation was underestimated when it was < 113 mm and overestimated when > 113 mm. The most extreme underestimations of CFSR precipitation, as compared to the GMS data, occurred at the Huize station. The Huize station is located at much higher altitude than the Mianyang and

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Badong stations, which may be the reason for the greater discrepancy. According to the coefficient of the regression equation the accuracy of the estimated precipitation at the Mianyang station, which is characterized by high elevation, was lower than the computed accuracy for the Ji'an station. However, the R^2 between the GMS and CFSR monthly average precipitation was larger than 0.60 at all five meteorological stations from 1981 to 2005, which showed that the CFSR weather data provide sufficient accuracy to build the hydrological model.

To validate the CFSR temperature, the monthly average minimum and maximum temperature were calculated to compare with the corresponding GMS temperature. *Figure 4* shows the comparison between the monthly average minimum and maximum temperature at Mianyang, Badong, and Huize meteorological stations and corresponding CFSR grid points during 1981 to 2005. The CFSR monthly average minimum temperature was slightly underestimated at all five meteorological stations, especially at the Badong and Huize stations, although the R^2 at both meteorological stations was > 0.96. Both the monthly average maximum and minimum temperature was underestimated by the CFSR weather data at the Badong meteorological station, and the monthly average minimum temperature was underestimated at the Mianyang, Huize and Yuanling meteorological stations. However, the estimated temperature from the CFSR weather data was highly correlated with that from the GMS weather data, with an R^2 larger than 0.9 at all five meteorological stations.

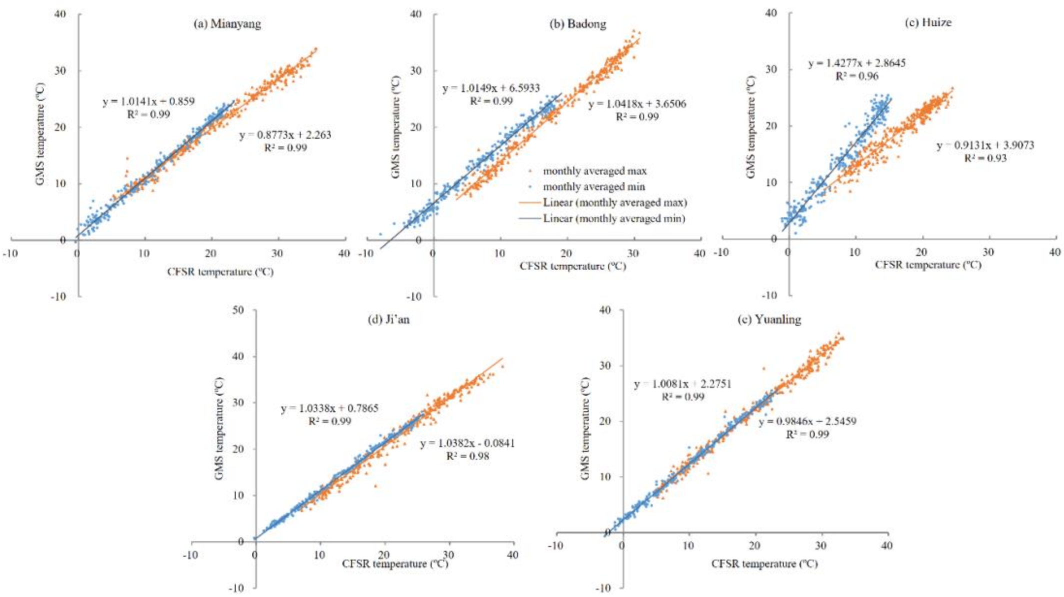

***Figure 4.** Comparison of the monthly average maximum and minimum temperature from the CFSR and GMS weather data at (a) Badong, (b) Mianyang, (c) Huize, (d) Ji'an and (e) Yuanling meteorological stations in the years 1981-2005*

Based on the validation of the CFSR weather data with the GMS weather data at these five representative meteorological stations from the year 1981 to 2005 in the YRB, the CFSR weather data have reasonable accuracy to represent the weather conditions occurring in the watershed. Therefore, to a certain degree, the CFSR weather datasets representing the weather occurring over the watershed are able to drive hydrological model.

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Parameter sensitivity analysis and calibration in the GMS- and CFSR-driven models

To decrease model uncertainty, streamflow was calibrated at each of the four gauge stations. For each simulation, with models driven by GMS and CFSR weather data, parameter sensitivity analysis and parameter calibration were conducted at each gauge station separately. After sensitivity analysis was performed, the most sensitive parameters were selected as calibration parameters at each gauge station. In this study, the following parameters were calibrated in both models using SWAT-CUP: CN2, CH_K2, ESCO, CANMX, RCHRG_DP, ALPHA_BF, GW_DELAY, HRU_SLP, SLSUBBSN, SFTMP, and CH_N2 (their descriptions can be found in *Table 1*). According to the parameter calibration by SWAT-CUP, the values of the most sensitive parameters were determined in the different models. The most sensitive parameters calibrated in each simulation at each stations and their values are listed in *Table 1*.

Table 1. *Hydrological parameters calibrated during 1981 to 1992 in the SWAT models driven by GMS and CFSR weather data for simulations at different hydrological gauge stations*

Parameters	Parameter description	GMS-driven model				CFSR-driven model			
		Cuntan	Yichang	Hankou	Datong	Cuntan	Yichang	Hankou	Datong
CN2	runoff curve number for moisture condition II	94.00	89.00	97.87	35.00	87.00	87.50	82.55	44.38
CH_K2	effective hydraulic conductivity in the main channel alluvium, unit: mm/h	10.12	4.02	—	—	15.91	6.57	—	12.49
ESCO	soil evaporation compensation factor	0.95	0.85	0.09	—	0.80	0.35	—	0.725
CANMX	maximum canopy storage, unit: mm	—	—	—	—	1.08	6.47	—	42.50
RCHRG_DP	deep aquifer percolation fraction	0.26	0.54	0.29	—	0.25	0.15	0.91	0.33
ALPHA_BF	baseflow alpha factor, unit: day	0.03	0.04	0.63	—	—	—	—	—
GW_DELAY	groundwater delay, unit: days	—	—	313.75	—	—	—	466.25	—
HRU_SLP*	average slope steepness, unit: m/m	—	—	—	0.48	—	—	0.53	—
SLSUBBSN*	average slope length, unit: m	—	—	—	-0.09	—	—	0.35	—
SFTMP	snowfall temperature, unit: °C	—	—	—	1.68	—	—	—	—
CH_N2	Manning's "n" value for the main channel	—	—	—	0.01	—	—	—	—

* multiply original value by (1+ the value shown in table)

Calibration and validation of the SWAT model driven by GMS weather data

The SWAT model for the YRB was firstly driven by GMS weather data. After parameter sensitivity analysis, the observed streamflow and simulated results at Cuntan, Yichang, Hankou and Datong hydrological gauge stations were presented during the calibration (1981-1992) and validation period (1993-2002) in *Figure 5*. For the calibration period, there was general agreement between the simulated and observed monthly streamflow from 1981 to 2002. From the evaluation indexes presented in *Table 2*, the R^2 are greater than 0.80, while the E_{NS} are greater than 0.70 during both calibration and validation periods. The results can be rated as "good" according to suggested performance ratings for a monthly time step (Moriasi et al., 2007, 2015). There were several months in which the peak simulated streamflow under predicted the observed peak streamflow at the Datong and Cuntan stations, respectively. In contrast, at Hankou and Cuntan stations, the peak simulated streamflow replicated the observed peak streamflow. There were fewer discrepancies of the peak streamflow between the simulated and observed data at Hankou and Datong stations than that at Cuntan and Yichang stations.

As the purpose of the study was to compare the performance of CFSR simulation in relation to conventional GMS simulation, all parameters calibrated in the GMS-driven

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model were hold constant and used in the CFSR-driven model. The results from the CFSR-driven SWAT model with Calibrated parameters of GMS model (hereafter called CSWCG) were obtained. The observed streamflow was used to validate both modelling results: the results from the GMS and CSWCG simulations and the CSWCG results. The SWAT simulation with the GMS data generated good results for baseflow conditions, whereas the CFSR results replicated observed peak streamflow compared with the observed data, particularly at Hankou and Datong gauge stations. There were larger discrepancies between the observed and simulated results at Cuntan and Yichang gauge stations than at Hankou and Datong gauge stations. However, the evaluation indexes (*Table 2*) at Yichang gauge station were superior to that at Hankou and Datong stations in both the calibration and validation periods. From *Figure 5*, the simulated streamflow from CSWCG closely match peak streamflow for the lower reaches of the Yangtze River, which was demonstrated by the comparison of the simulated results and the observed streamflow at the four gauge stations in the upper, middle, and lower reaches of the Yangtze River.

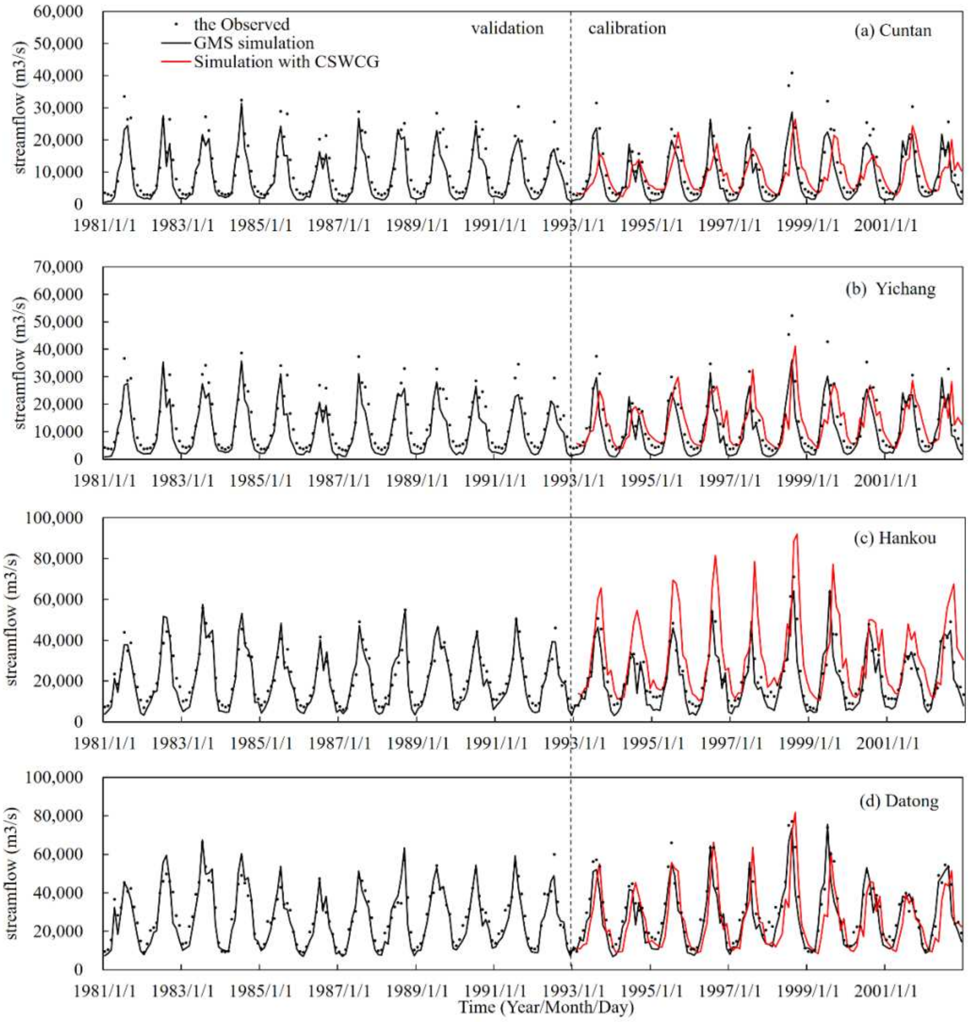

Figure 5. *Calibration (1981-1992) and validation (1993-2002) of the ground-based meteorological station (GMS) simulation and comparison to the results from the CFSR-driven SWAT model with calibrated parameter of GMS model (CSWCG) using the monthly observed streamflow at (a) Cuntan, (b) Yichang, (c) Hankou and (d) Datong gauge stations*

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Table 2. *Performance evaluation for the SWAT model driven by the ground-based meteorological station (GMS) weather data in the calibration and validation period and comparison to the results from the CFSR-driven SWAT model with calibrated parameters of GMS model (CSWCG)*

	Calibration			Validation			Validation of CSWCG		
	R^2	E_{NS}	PBias/%	R^2	E_{NS}	PBias/%	R^2	E_{NS}	PBias/%
Cuntan	0.91	0.81	23.30	0.83	0.73	24.44	0.78	0.71	11.22
Yichang	0.92	0.78	25.00	0.85	0.71	27.03	0.85	0.83	1.43
Hankou	0.92	0.76	6.00	0.88	0.82	12.5	0.89	0.82	-12.01
Datong	0.89	0.74	5.00	0.88	0.82	12.6	0.88	0.81	-5.82

To explicitly present the performance of the GMS simulation in the YRB, the evaluation indexes for the SWAT model driven by GMS weather data in the calibration and validation periods and comparison to the results from the CFSR simulation with calibrated parameters of GMS model (CSWCG) are listed in *Table 2*. The R^2 and E_{NS} were greater than 0.80 in both the calibration and validation periods except Cuntan station. The model underestimated streamflow at Cuntan and Yichang stations while overestimated at Hankou and Datong. The comparison between the observed data and the results from the CFSR simulation showed rather high accuracy, with R^2 and E_{NS} larger than 0.80, except that at Cuntan gauge station. As for the values of PBias, it is slightly underestimated at Yichang gauge station and overestimated at Hankou and Datong gauge stations. The E_{NS} and R^2 of the validation of the simulation with GMS data are all larger than 0.80, and PBias is less than 10%; therefore, the results in this validation period can be rated as "very good" according to the research on performance ratings for a monthly time step (Moriasi et al., 2007, 2015). However, the results of validation in CSWCG at Cuntan and Hankou gauge stations are rated as "good" because their absolute values of PBias are larger than 10%. The E_{NS} value is less than 0.75 only at Cuntan station, and it is "good" at Yichang and Datong gauge stations.

Calibration and validation of the SWAT model driven by CFSR weather data

The sufficiency of the modelling performance is highly dependent on the calibrated parameters. Therefore, it is necessary to investigate the performance of the SWAT model setup with CFSR global weather data in the YRB. The CFSR weather data were used to establish a SWAT model for the YRB, which was calibrated and validated in CFSR-driven SWAT model by the discharge data at Cuntan, Yichang, Hankou, and Datong hydrological gauge stations (*Figure 6*). From the tendency of monthly streamflow comparison from 1981 to 2002, the simulated results from the CFSR-driven SWAT model fit closely to the observed data, especially at Hankou and Datong gauge stations, with E_{NS} and R^2 greater than 0.75. The simulated baseflow closely matched observed, and there was little discrepancy between the peak streamflow, especially in July and August of 1998, during which heavy floods occurred along the Yangtze River. The daily discharge of 87,000 m^3/s, with the largest water level in history, was recorded at Datong gauge station on July 27, 1998, and exceeded the average monthly discharge of 56,800 m^3/s in the wet season. From the validation of the CFSR precipitation data at Huize, Mianyang and Badong stations, the CFSR precipitation was underestimated in the period of heavier rainfall especially at the peak rainfall at Huize and Mianyang station compared with the GMS observation. The underestimated precipitation in the upper reaches with higher elevation led to

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difficulty to reach the observed peak streamflow. The simulated peak streamflow nearly reached the observation at Hankou and Datong gauge stations in the middle and lower reaches of the Yangtze River. From the illustration of the *Figure 6*, the CFSR-driven SWAT model driven matches the observations in the lower streamflow period for the whole area and generates better results in the middle and lower reaches of the Yangtze River than in the upper reaches, because the CFSR precipitation has better accuracy for representing weather occurring in the middle and lower reaches than in the upper reaches of the Yangtze River basin.

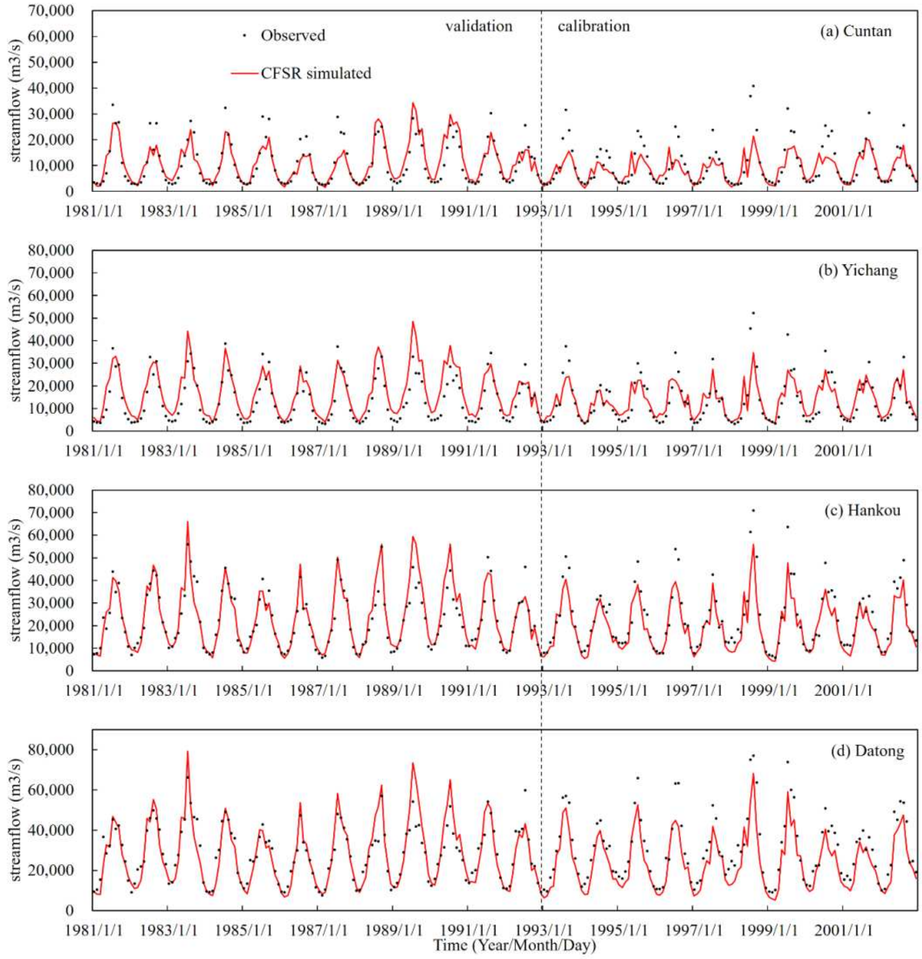

Figure 6. *The Climate Forecast System Reanalysis (CFSR) driven SWAT model calibration (1981-1992) and validation (1993-2002) using monthly observed streamflow at (a) Cuntan, (b) Yichang, (c) Hankou, and (d) Datong gauge stations from 1981 to 2002*

To demonstrate the model performance, *Table 3* shows the evaluation indexes of the SWAT model driven by CFSR weather data in calibration and validation at Cuntan, Yichang, Hankou, and Datong hydrological gauge stations. The R^2 and E_{NS} are larger than 0.70 in both calibration and validation periods, which are superior to the simulated result based on NCEP (National Centers for Environmental Prediction of United States) data in upper reach of Yangtze River basin by the study (Yang et al., 2014). In addition, the model tended to overestimate the streamflow, except at Datong gauge station in calibration period, and it underestimated the streamflow in validation except slightly overestimated

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at Yichang station. According to performance ratings (Moriasi et al., 2007, 2015), the calibration results at all of the hydrological stations can be rated as "good" because E_{NS} and R^2 are larger than 0.75.

Comparing *Table 2* and *Table 3* for the model performances of the GMS- and CFSR-driven SWAT model calibrated and validated separately alone, the performance of the model driven by GMS weather data is better than that of the CFSR data for calibration, as well as for validation. The comparison shows the GMS-driven SWAT model is more robust than the CFSR model. However, the CFSR-driven model can also generate rather satisfactory results.

Table 3. *Performance evaluation for the SWAT model driven by the Climate Forecast System Reanalysis (CFSR) weather data in calibration and validation period at different hydrological gauge stations*

	Calibration			Validation		
	R^2	E_{NS}	PBias/%	R^2	E_{NS}	PBias/%
Cuntan	0.75	0.75	-1.91	0.71	0.70	14.9
Yichang	0.83	0.71	-23.6	0.76	0.72	-0.14
Hankou	0.88	0.84	-4.8	0.89	0.80	15.34
Datong	0.88	0.79	0.00	0.91	0.74	21.05

Although the R^2 of the precipitation validation was approximately 0.6 between from CFSR and GMS weather data, the CFSR-driven SWAT model produced a streamflow with R^2 and E_{NS} greater than 0.70 in both calibration and validation periods. The established SWAT model provides parameter adjustment to improve the accuracy of hydrology prediction through parameter sensitivity analysis and model calibration. The streamflow simulated with GMS weather data is better than that simulated with CFSR weather data, and the results simulated with CFSR weather data are reasonable.

From the perspective of the whole large-scale watershed, the Datong gauge station records the discharge for the whole basin. The simulation with the CFSR weather data produced better results for the watershed scale than the simulation with the GMS data based on the indexes of E_{NS} and R^2 during validation period at Hankou and Datong station. From the perspective of the sub-watershed-scale, the discharge recorded at Cuntan, Yichang and Hankou gauge stations represented the hydrological regimes of different sub-watersheds. Comparing the results in *Table 2* and *Table 3*, the GMS weather data simulation generated better results than the simulation with CFSR weather data at Cuntan and Yichang stations during both calibration and validation periods. Therefore, the GMS weather data are superior to the CFSR weather data for predicting hydrology at Cuntan and Yichang gauge stations at the sub-watershed-scale. In addition, a group of parameters calibrated in the GMS-driven model in future years with the scarce data for the Cuntan and Yichang gauge stations were proposed because the E_{NS}, R^2 and PBias in these simulations are all superior to the simulation with parameters calibrated with the CFSR model.

Comparison of the monthly average streamflow hydrograph (1981-2002) among the observed data, simulated results by CFSR- and GMS-driven model

Hydrological regime of the monthly average streamflow from 1981 to 2002 were simulated to investigate the performance of the GMS- and CFSR-driven SWAT model which were calibrated and validated separately, respectively (*Figure 7*). For Cuntan gauge

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station, the simulation with GMS underestimated the streamflow during March to July, and it was a better match to the observations than the CFSR weather simulation at the recession curve from August to December. The results from the CFSR weather-driven model more closely matched the observations during January to June, whereas the GMS data simulation more closely matched the peaks than the CFSR simulation in July and better replicated the recession curve during September to December at Yichang gauge station. The GMS weather model generated better results and replicated the observations better than the CFSR model throughout the year at Hankou and Datong gauge stations.

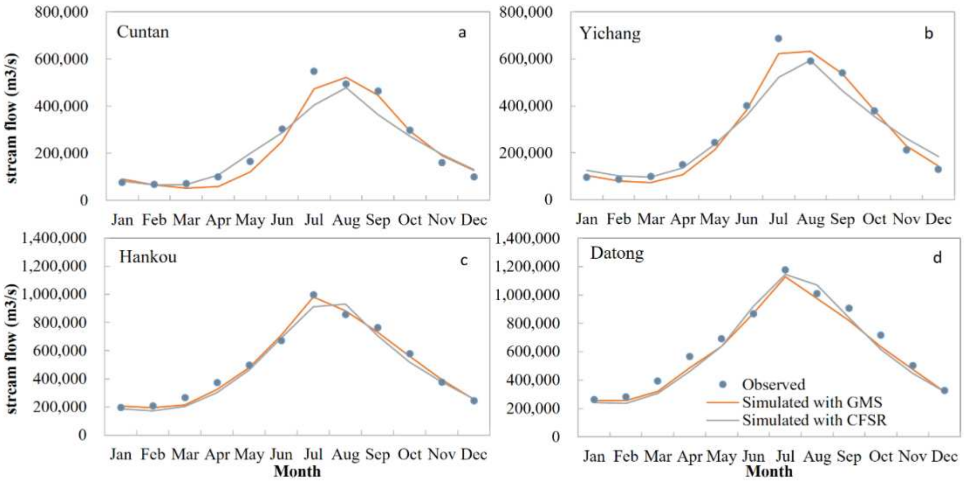

Figure 7. *Average monthly streamflow hydrograph (1981-2002) for observed and simulated results from the SWAT model driven by the Climate Forecast System Reanalysis (CFSR) and the ground-based meteorological station (GMS) weather data at (a) Cuntan, (b) Yichang, (c) Datong and (d) Hankou gauge stations*

From these four hydrological gauge stations, there were two patterns in the comparison curves. Firstly, there was a distinct difference in the lower streamflow between the results from the GMS and CFSR simulations, and there was less discrepancy for the peak streamflow with the GMS simulation. Secondly, the results from the GMS weather simulation more closely matched the peaks and the recession period than the CFSR weather model. From the perspective of gauge station distribution in the basin, the results from the GMS and CFSR simulations in the upper reaches of the Yangtze River are characterized as distinct difference in the lower streamflow and less discrepancy for the peak streamflow, such as the patterns at Cuntan and Yichang gauge stations, whereas the results are characterized as close match the peaks and the recession period in the middle and lower reaches of the Yangtze River, such as the patterns at Hankou and Datong gauge stations.

Discussion

From the investigation of the CFSR-driven SWAT model with Calibrated parameters of GMS model (CSWCG), although, the R^2 and E_{NS} were greater than 0.80 in both the calibration and validation periods except Cuntan station, The CSWCG resulted in acceptable results. It proves that it is feasible to apply the CFSR global data to the establishment of a SWAT model in a large-scale watershed. In addition, the CFSR and

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GMS data driven SWAT model can share the same hydrological parameters to a certain extent for hydrology prediction in the YRB. That is to say, the CFSR dataset can be used to quickly established hydrology model for real time flood prediction when conventional weather data are absent in urgently, as well as the former calibrated hydrological parameters is also applicative.

Performance of the CFSR data using as hydrological model driven data is reasonable accepted, which is in agreement with the findings of Dile et al. (2014), Fuka et al. (2014), and Yang et al. (2014). However, the results found here conflict with the findings of Tan et al. (2017), who report that the original CFSR weather data is not suitable to apply for streamflow simulations in Malaysia. Differences in climate and geographical conditions are the most likely explanation for such differences between different studies. In any case, data accuracy and representativeness is more important (Yang et al., 2014). Therefore, It is important to validate the data representativeness of the weather occurred in study area before hydrological prediction.

The input weather data density is an influence, and alters the hydrologic model results. the GMSs and CFSR grids is show in *Figure 1*, in which the spatial distribution patterns of GMSs, CFSR grids and used CFSR were clearly demonstrated. The gridded data usually contain weather data of high density for hydrology simulation in the large-scale basins. Although, the density of CFSR grids is superior to the GMSs available in this study, the performances of the hydrologic simulations in this study strongly suggest the spatial density has no major influence on model simulations of this study area. Because of the automatically selected by SWAT model, the number of used CFSR data grids happened to be the same of GMS data in this study. The advantage of intensive distributions of the CFSR grids will be take effects in many large-scale and data-scarce regions.

Conclusions

In this study, the performance of CFSR global data for quickly building a hydrological model was investigated for the Yangtze River, the largest river basin in China. The study demonstrated that the CFSR weather data could modestly replicate weather conditions occurring in the YRB to a certain degree. In addition, the accuracy of the CFSR meteorological data was stronger at low elevation conditions versus high altitude. With reasonable accuracy of CFSR data validated by GMS observed weather data, it is feasible to predict hydrology with the CFSR weather datasets to drive the hydrological model of the YRB.

The R^2 and E_{NS} were larger than 0.8 in both the calibration and validation periods for the GMS weather data driven SWAT model in the YRB. However, the model showed a tendency to underestimate streamflow at all four gauge stations. Because of the reasonable accuracy of the CFSR-driven SWAT model without calibration, the CFSR- and GMS driven SWAT models can share the same hydrological parameters for hydrology prediction in the YRB, and the prediction results were better in the middle and lower reaches than in the upper reaches of the Yangtze River. Using only the CFSR global weather data, the SWAT model R^2 and E_{NS} are larger than 0.70 in both calibration and validation periods. Although the R^2 of the precipitation validation is only 0.6 between the CFSR and GMS weather data, the established SWAT model provides parameter adjustment to improve the accuracy of hydrology prediction through parameter sensitivity analysis and model calibration. Comparing the monthly average streamflow hydrograph,

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the results from the GMS and CFSR simulations are characterized by two patterns in the upper, middle, and lower reaches.

This paper presented the performance of the CFSR driven hydrological model in the Yangtze River basin during 1981-2002. As we know, the Three Gorges Dam commenced impoundment since 2003, which would influence hydrology regimes of the Yangtze River. It will be an interesting topic for the further research based on CFSR driven hydrological model. Although, the CFSR-driven hydrological model generated results are less accurate than that of GMS-driven model, the CFSR global data are an alternative data source for quickly building a SWAT model for hydrology prediction in such a large-scale river basin. We can conclude that the CFSR weather data provides new opportunities to meet the challenges of driving the hydrological model in data-unavailable river basin. The CFSR weather data can be a viable option for simulating the hydrology where traditional GMS networks do not satisfied the given spatiotemporal resolution, or the GMS weather data are unavailable.

Acknowledgements. This work was funded by the National Key Research and Development Program (2017YFB0504103), the National Natural Science Funding of China (NSFC) (41331174), the Open Foundation of Jiangxi Engineering Research Center of Water Engineering Safety and Resources Efficient Utilization (OF201601), the Fundamental Research Funds for the Central Universities (2042018kf0220) and the LIESMARS Special Research Funding.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36153632

POSSIBILITY OF INCREASING MICRONUTRIENT CONTENTS IN POTATO TUBERS WITH FOLIAR APPLICATION OF TITANIUM

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(Received 14th Dec 2018; accepted 22nd Feb 2019)

Abstract. This paper analyses the effect of titanium foliar application on the Fe, Zn, Mn and Ti content in early crop potato tubers. The titanium source was the plant growth stimulant Tytanit® (8.5 g Ti per litre). Tytanit® was applied once at the leaf development stage (BBCH 14-16) or at the tuber formation stage (BBCH 41-43) and twice at the leaf development and tuber formation stages, at the dose of 0.2 L ha^{-1} or 0.4 L ha^{-1} in each treatment. Potatoes were harvested 75 days after planting (the end of June). Following Tytanit® application, the content of Fe was higher by 6.150 mg kg^{-1} and Mn by 0.409 mg kg^{-1} of dry matter. Titanium foliar application did not affect the content of Zn, whereas the content of Ti was higher by 0.364 mg kg^{-1} of dry matter. The dose and date of Tytanit® application slightly affected the content of Fe. The content of Mn was greatest with a single Tytanit® application at the tuber formation stage at the dose of 0.2 L ha^{-1}, whereas the Ti content was greatest with a double Tytanit® application, at the leaf development and tuber formation stages, at the dose of 0.2 L ha^{-1}.
Keywords: *early potatoes, plant growth stimulant, Tytanit®, nutritional value*

Introduction

Although micronutrients are used in relatively small amounts, they play an important role in plant metabolism and in the human body assisting in the prevention of many diseases and the maintenance of good health. On the other hand, an excessive amount of some micronutrients can be toxic to a human (Welch, 2002; Martinez-Ballesta et al., 2010; Soetan et al., 2010; Al-Fartusie and Mohssan, 2017; Nieder et al., 2018). The Recommended Dietary Allowance (RDA) of Fe for men and postmenopausal women is 8 mg and for premenopausal women 18 mg per day. The RDA of Zn is 11 mg per day for men and 8 mg for women, and the RDA of Mn for men and woman is 2.3 mg and 1.8 mg per day, respectively (Institute of Medicine US, 2001). Micronutrient deficiency in humans occurs in all regions of the world (Martinez-Ballesta et al., 2010; Nieder et al., 2018). Plant based food provide most of the micronutrients required for a healthy human diet. Soil is the main source of micronutrients for plants. The micronutrient availability to plants depends on their chemical form, soil properties, particularly pH and organic matter content, and environmental conditions. Abiotic stresses such as drought or extreme temperatures affect the mineral element content in the field growing crops (Tack, 2014). Micronutrient deficiencies in plants not only limit crop productivity but also affect crop quality as a human food. In sustainable agriculture, the most environmentally-friendly way to increase the amount of essential human micronutrient in plant based food is breeding of genotypes that have a naturally higher micronutrient content and proper agronomic practices (Frossard et al., 2000; Khoshgoftarmanesh et al., 2010; Haynes et al., 2012).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

In modern plant production focusing on high quality of plant based food, biostimulants have been gaining increasing importance (Calvo et al., 2014; Bulgari et al., 2015). Titanium is categorized as a plant growth stimulant. Water-soluble chelate forms of titanium, such as ascorbate, citrate or malate applied via roots or leaves in low concentrations have a beneficial effect on various plant physiological processes and increase the crop yield, and can improve the crop quality. The beneficial effect of titanium is a result, among others, of its interaction with other elements, primarily with Fe (Du et al., 2010; Bacilieri et al., 2017; Lyu et al., 2017). Titanium foliar application resulted in an increase in Fe, Cu and Zn contents in bean (Ram et al., 1983), Fe in red pepper and in barley grain (Carvajal and Alcaraz, 1995; Tlustoš et al., 2005) and Zn in wheat grain (Tlustoš et al., 2005). To date, few studies have been focused on the effect of titanium on micronutrient contents in root and tuber plants. Titanium foliar application resulted in an increase in Zn content and a decrease in Fe and Mn contents in potato tuber (Tlustoš et al., 2005; Mystkowska, 2018).

Potato (*Solanum tuberosum* L.) is a one of the most important crops ensuring food security in the world and a valuable source of nutritional compounds in the human diet. Potatoes do not contain a large amount of micronutrients, with the exception of Fe, however, due to the amount consumed per capitata, they can make up a significant source of micronutrients in the human diet (Burgos et al., 2007; Haynes et al., 2012; Tack, 2014). Consumption of 100 g potatoes can provide 12-15% of the Recommended Dietary Allowence (RDA) of iron, 6-8% of manganese and 2-3% of zinc (Leszczyński, 2012; Wierzbicka, 2012). Micronutrient contents in potato tubers are affected by a cultivar, environmental conditions and agronomical practices (Rivero et al., 2003; Martinez-Ballesta et al., 2010; Wierzbicka, 2012; Lombardo et al., 2014; Tack, 2014; Wierzbowska et al., 2018). In the our study it was hypothesised that the stimulation of potato plant growth by titanium foliar application would contribute to an increase in essential human micronutrient content in tubers. It was assumed that the potato response to titanium depend on the dose and date of titanium application and that the results of titanium application depends on the potato cultivar and environment in which the potato grow. The aim of the study was to determine the effect of the dose and date of titanium foliar application on essential human micronutrient contents in early crop potato tubers.

Materials and Methods

Plant material and experimental design

The study material included tubers of two very early potato cultivars ‘Lord’ and ‘Miłek’ (Polish cultivars which are registered on the Common Catalogue of Varieties of Agricultural Plant Species CCV) obtained from a field experiment carried out in central eastern Poland (52°03'N; 22°33'E) during three growing seasons (2009, 2010 and 2012) with a varied the weather conditions (*Table 1*). Over the three-year period of the study, the mean air temperatures in the beginning period of potato growth were above the long-term average. In 2009 total precipitation in potato growing season was above and in 2010 and 2012 similar to long-term average, however, unevently distributed during potato growth period (Wadas and Kalinowski, 2018). The field experiment was carried out on Luvisol with an acidic-to-slightly-acid reaction, with a low content of available Zn, a medium content of Mn as well as a high content of Fe (*Table 2*).

The titanium (Ti) source was the mineral plant growth stimulant Tytanit® (8.5 g Ti per litre, 0.8% m/m, in the form of Ti-ascorbate) produced by INTERMAG Ltd., Poland. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

effect of dose (0.2 L ha^{-1} and 0.4 L ha^{-1}) and date of Tytanit® application (a single foliar application at the leaf development stage – BBCH 14-16 or at the tuber formation stage – BBCH 41-43, and a double foliar application at the leaf development and tuber formation stages – BBCH 14-16 and BBCH 41-43) on the essential human micronutrient and titanium contents in early crop potato tubers was investigated. The field experiment was established in a split-block-split-plot design with a control object without Tytanit® in three replications. Potato cultivation was carried out according to common agronomical practice. In successive years, 6-week pre-sprouted seed potatoes were planted on 15th, 13th and 12th April. Potatoes were harvested 75 days after planting (the end of June). For laboratory studies 50 different-sized tubers of each treatment were taken.

Table 1. *Selected soil chemical properties in the experimental site*

Years	C_{org} (g kg^{-1})	pH_{KCl}	Available forms (mg kg^{-1} of soil)					
			P	K	Mg	Zn	Mn	Fe
2009	8.7	6.6	128	104	38	7.2	95.3	730
2010	14.2	6.1	88	120	45	9.3	79.2	550
2012	7.9	4.7	122	208	22	4.1	65.2	645

Table 2. *Mean air temperature and precipitation total in the potato growing season*

Years	Temperature (°C)			Rainfalls (mm)		
	April	May	June	April	May	June
2009	10.3	12.9	15.7	8.1	68.9	145.2
2010	8.9	14.0	17.4	10.7	93.2	62.6
2012	8.9	14.6	16.3	29.9	53.4	76.2
Many-year mean (1981-2005)	8.1	11.9	16.7	45.5	49.7	63.0

Chemical analysis

The contents of Fe, Zn, Mn and Ti were determining by atomic emission spectroscopy with the inductively-coupled plasma method (ICP-AES) following previous mineralization of potato tuber samples. One gram of the oven-dried potato sample was oxidized dry at 450-550°C in a muffle furnace, and the ash was dissolved in a 10% HCl. The total contents of Fe, Zn, Mn and Ti were determined on an atomic emission spectrometer with inductively-coupled plasma Optima 3200 RL produced by Perkin-Elmer. All assays were performed in duplicate and the mean results were expressed in milligrams per kilogram of potato tuber dry matter (DM).

Statistical analysis

The results of the study were analysed statistically by means of analysis of variance (ANOVA) for the split-block-split-plot design. The analysis of the results was conducted using orthogonal contrast to compare the control object without Tytanit® with the remaining objects. The significance of differences was verified using Tukey's test at $p \leq 0.05$.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

Results

Tytanit® application caused an increase in the content of Fe, Mn and Ti in potato tuber but did not affect the content of Zn (*Table 3*). Over the three-year period of the study, the Fe content in tubers was higher, on average, by 6.150 mg kg^{-1} of DM, Mn by 0.409 mg kg^{-1} of DM and Ti by 0.364 mg kg^{-1} of DM compared to the cultivation without the growth stimulant. Tytanium foliar application had a greatest effect on the Mn content in tubers in 2012 with the lowest content of available Mn in soil (data not presented). In that year, with the Tytanit® application, the Mn content in tubers was higher by 0.939 mg kg^{-1} of DM, on average, compared to the control object without the growth stimulant.

Table 3. *Effect of Tytanit® on micronutrient contents in potato tubers (mg kg^{-1} of DM); mean for years and cultivars*

Treatment	Fe	Zn	Mn	Ti
Without Tytanit®	73.33b	24.72a	6.774b	1.803b
With Tytanit®	79.48a	24.64a	7.183a	2.167a

Means within columns followed by the same letters do not differ significantly at p≤0.05

The Tytanit® dose did not affect the content of Fe in tubers of either tested potato cultivars (*Table 4*). The study demonstrated a significant interaction effect of the potato cultivar and the dose of Tytanit® on the Mn content in tubers. The dose of Tytanit® application had a significant effect on the Mn content only in the 'Lord' cultivar tubers (*Table 4*).

Table 4. *Effect of Tytanit® dose on Fe, Mn and Ti contents in potato tubers in relation to cultivar (mg kg^{-1} of DM); mean for years and date of Tytanit® application*

Tytanit® dose	Fe		Mn		Ti	
	Lord	Miłek	Lord	Miłek	Lord	Miłek
0.2 L ha^{-1}	81.74a	77.97a	7.293a	7.291a	2.293a	2.239a
0.4 L ha^{-1}	80.38a	77.82a	6.808b	7.339a	2.096b	2.041a

Means within columns followed by the same letters do not differ significantly at p≤0.05

Following the application of 0.2 L ha^{-1} of Tytanit®, the Mn content in tubers of 'Lord' was higher, on average, by 0.485 mg kg^{-1} of DM compared to the dose of 0.4 L ha^{-1}. The Tytanit® dose had the greatest effect on the Mn content in tubers of 'Lord' in 2012 with the lowest content of available manganese in soil (data not presented). In that year, following the application of 0.2 L ha^{-1} of Tytanit®, the manganese content in tubers of this cultivar was higher, on average, by 1.338 mg kg^{-1} of DM compared to the dose of 0.4 L ha^{-1}. The dose of Tytanit® application had a significant effect on the Ti content in tubers of both tested potato cultivars (*Table 4*). After the application of 0.2 L ha^{-1} of Tytanit®, the Ti content in tubers was higher, on average, by 0.198 mg kg^{-1} of DM compared to the dose of 0.4 L ha^{-1}. The difference was greatest in the warm and moderately wet growing season of 2012 (*Table 5*). In that year, after the application of 0.2 L ha^{-1} of Tytanit®, the Ti content in tubers was higher, on average, by 0.455 mg kg^{-1} of DM.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

Table 5. *Effect of Tytanit® dose on Mn and Ti content in potato tubers in relation to year (mg kg^{-1} of DM); mean for cultivars and date of Tytanit® application*

Tytanit® dose	Mn			Ti		
	2009	2010	2012	2009	2010	2012
0.2 L ha^{-1}	7.562a	6.901a	7.413a	1.557a	3.004a	2.237a
0.4 L ha^{-1}	7.194a	7.139a	6.887a	1.413a	3.011a	1.782b

Means within columns followed by the same letters do not differ significantly at p≤0.05

The date of Tytanit® application had a slight effect on the content of Fe and Mn in tubers of both tested potato cultivars (*Table 6*). The date of Tytanit® application had a significant effect on the Mn content only in 2012 with the lowest content of available Mn in soil (*Table 7*).

Table 6. *Effect of date of Tytanit® application on Fe, Mn and Ti contents in potato tubers in relation to cultivar (mg kg^{-1} of DM); mean for years and Tytanit® dose*

Date of Tytanit® application	Fe		Mn		Ti	
	Lord	Miłek	Lord	Miłek	Lord	Miłek
Leaf development stage	81.72a	76.70a	6.972a	7.040a	2.010b	1.829c
Tuber formation stage	80.08a	78.40a	6.864a	7.360a	2.088b	2.165b
Leaf development and tuber formation stages	81.38a	78.60a	7.315a	7.545a	2.485a	2.425a

Means within columns followed by the same letters do not differ significantly at p≤0.05

Table 7. *Effect of date of Tytanit® application on Mn and Ti content in potato tubers in relation to year (mg kg^{-1} of DM); mean for cultivars and Tytanit® dose*

Date of Tytanit® application	Mn			Ti		
	2009	2010	2012	2009	2010	2012
Leaf development stage	7.418ab	6.963a	6.673b	1.303b	2.702b	1.755b
Tuber formation stage	6.932b	6.808a	7.596a	1.503ab	2.679b	2.195a
Leaf development and tuber formation stages	7.784a	7.289a	7.218ab	1.649a	3.642a	2.080a

Means within columns followed by the same letters do not differ significantly at p≤0.05

In that year, with the single Tytanit® application in the leaf development stage the Mn content in tubers was lower, on average, by 0.959 mg kg^{-1} of DM as compared with the single application in the tuber formation stage. With double Tytanit® application, in the leaf development stage and with a repeated treatment in the tuber formation stage, the Mn content in tuber was similar to the single treatment in the tuber formation stage. The Ti content in tuber was greatest with double Tytanit® application (*Table 6*). With double Tytanit® application, the Ti content in tuber was higher, on average, by 0.535 mg kg^{-1} of DM compared to the single treatment in the leaf development stage and by 0.325 mg kg^{-1} of DM compared to the treatment in the tuber formation stage. The differences were greatest in 2010 with heavy rainfall in May and the periodically water shortage in June, 0.940 mg kg^{-1} and 0.963 mg kg^{-1} of DM, respectively (*Table 7*). The performed study demonstrated a significant interaction effect of the dose and date of Tytanit® application

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

on the Mn and Ti contents in tubers (*Table 8*). The content of Mn was greatest with a single Tytanit® application in the tuber formation stage at the dose of 0.2 L ha^{-1}, whereas the content of Ti was greatest with the double Tytanit® application, in the leaf development stage and with repeated treatment in the tuber formation stage at a dose of 0.2 L ha^{-1} in each treatment.

Table 8. *Effect of dose and date of Tytanit® application on Mn and Ti content in potato tubers (mg kg^{-1} of DM); mean for years and cultivars*

Date of Tytanit® application	Mn		Ti	
	Tytanit® dose		Tytanit® dose	
	0.2 L ha^{-1}	0.4 L ha^{-1}	0.2 L ha^{-1}	0.4 L ha^{-1}
Leaf development stage	6.777b	7.235ab	1.901c	1.938b
Tuber formation stage	7.567a	6.657b	2.200b	2.060ab
Leaf development and tuber formation stages	7.531a	7.329a	2.700a	2.210a

Means within columns followed by the same letters do not differ significantly at $p \leq 0.05$

The content of examined micronutrients in the potato tuber depended to a great extent on the cultivar and environment conditions during the potato growing season (*Table 9*). Regardless of the treatment, 'Lord' tubers contained more Fe, on average, by 3.20 mg kg^{-1} of DM and Zn by 2.50 mg kg^{-1} of DM and less Mn by 0.30 mg kg^{-1} of DM than tubers of 'Miłek'. The content of Ti in tubers of both cultivars was similar. The most Zn and Mn were accumulated by potato tubers in the cool and very moist growing season of 2009, whereas the most Fe and Ti were accumulated in tubers in 2010, with havy rainfall in May and the periodically water shortage in June.

Table 9. *Micronutrient contents in potato tubers in relation to cultivar and year (mg kg^{-1} of DM); mean for objects with and without Tytanit®*

Cultivar and year	Fe	Zn	Mn	Ti
Lord	80.20a	25.90a	6.974b	2.152a
Miłek	77.20b	23.40b	7.274a	2.078a
2009	83.12b	30.06a	7.354a	1.420c
2010	94.90a	22.04b	7.003b	2.958a
2012	57.78c	21.87c	7.016b	1.967b

Means within columns followed by the same letters do not differ significantly at $p \leq 0.05$

Results presented in this study demonstrate that a typical meal with 200 g of boiled potatoes (at least three medium-sized tubers) from the cultivation without Tytanit® provided up to 32% of the Recommended Dietary Allowance (RDA) of Fe for men and postmenopausal women and up to 14% of the RDA for premenopausal women, and up to 10% of the RDA of Mn for men and 13% for woman. Potatoes derived from the cultivation with Tytanit® provided up to 35% of the RDA of Fe for men and postmenopausal women and up to 16% of the RDA for premenopausal women, and up to 11% of the RDA of Mn for men and 14% for woman. Potatoes from the cultivation with or without Tytanit® provided up to 8% of RDA of Zn for men and 11% for woman.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

Discussion

The beneficial effect of titanium on plant metabolism include, among others, an increase in the uptake of other elements (Du et al., 2010; Lyu et al., 2017; Bacilieri et al., 2017). A foliar application of Ti during potato growth increases the activity of peroxidase, however, during tuberization the activity of superoxide dismutase and peroxidase. Tytanit® did not affect the content of dry matter and nitrogen, phosphorus, potassium, calcium, magnesium or sulphur in potato tubers. Following Tytanit® application, there was only an increase in sodium content (Wadas and Kalinowski, 2018). The application of Ti favored Fe uptake (Bacilieri et al., 2017), which was confirmed in the present study. A single or a double Tytanit® (Ti-ascorbate) application during potato grown on Luvisol with an acidic-to-slightly-acid reaction at the dose of 0.2 L ha^{-1} or 0.4 L ha^{-1} in each treatment caused an increase in the contents of Fe, Mn and Ti in early crop tubers of very early 'Lord' and 'Miłek' cultivars. Titanium foliar application did not affect the content of Zn. A study carried out in the Czech Republic showed that titanium foliar applied three times in the form of Ti-citrate during potato grown on Chernozem with an alcaline reaction (at the beginning of June with a plant height of 30 cm and repeated treatment in two-week intervals) in the dose of 10 g Ti ha^{-1} in each treatment resulted in a slight decrease in Fe and an increase in the Zn in mature tubers of 'Cordoba' cultivar (Tlustoš et al., 2005). A study carried out in Poland showed that three times Tytanit® application during potato grown on the soil with a slightly-acid or alcaline reaction (beginning of plant flowering, full flowering and after plant flowering) in the dose of 0.2 L ha^{-1} in each treatment resulted in a decrease of Mn and Fe content in mature tubers of three medium-early ('Honorata', 'Jelly', 'Tajfun') potato cultivars (Mystkowska, 2018), which was not confirmed in the present study. Titanium application up to a dose of 25.5 g Ti ha^{-1} is not toxic for the potato crop (Bacilieri et al., 2017). A negative correlation was revealed between soil pH and iron and manganese concentration in potato tubers (Rogóż, 2009; Tack, 2014), which was confirmed in the present study. Fe and Ti have sinergistic and antagonistic relationships (Lyu et al., 2017). In the presented studies titanium was applied in the chelate forms as Ti-citrate or Ti-ascorbate. Leaf ability to uptake chelated nutrients is related to various properties of a chelate, such as the molecular weight of the complex, dissociation constant and stability of the complex at various pH (Wójcik, 2004). The effect of titanium depends on the plant age and the tissue concentration of other minerals (Lyu et al., 2014; Bacilieri et al., 2017). Potato plants uptake mineral elements primarily from the soil solution through their roots. Direct uptake of minerals into the developing tubers across the periderm is also possible, however in a mature tuber it will be limited due to the suberized nature of the periderm (Subramanian et al., 2011). The dose and date of Tytanit® application slightly affected the content of Fe in tubers of both tested potato cultivars and the content of Mn in the 'Miłek' tuber. The Mn content in the 'Lord' tuber was greater with Tytanit® application at the dose of 0.2 L ha^{-1}. The results showed a differential response of the examined very early potato cultivars to titanium foliar application. The response of crop plants to a foliar supply of nutrients differed between crop species as well as cultivars within the same species (Kannan, 2010), which was confirmed in the present study. The age of the leaf is important for nutrients foliar absorption. The young leaves absorbed more nutrients than the older ones (Wójcik, 2004). This study demonstrated a significant effect of the interaction of the dose and date of Tytanit® application on the Mn and Ti contents in potato tubers. Regardless of the potato cultivar, the content of Mn was greatest with the single Tytanit® application in the tuber formation stage at the dose of 0.2 L ha^{-1}, whereas the Ti content was greatest with a double

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Tytanit® application during potato grown at the leaf development stage and at the tuber formation stage at a dose of 0.2 L ha^{-1} in each treatment. Leaf-absorbed Ti is distributed approximately equally between the leaves and roots (Kelemen et al., 1993; Lyu et al., 2017). The study carried out by other authors, showed a trace amounts of Ti in potato tuber after foliar application of titanium-containing fertilizers (Ti-ascorbate). The content of Ti in sugar beet roots was considerably higher. The content of Ti in the green parts of a plant (leaves, stems) was greater than in the roots or tuber (Świerczewska and Sztuder, 2004). A positive correlation was found between the Fe and Mn content in potato tubers (Wierzbicka, 2012), which was confirmed in the present study. Tytanit® caused an increase in the content of both Fe and Mn in early crop potato tubers. An opposite correlation between the Fe and Mn are in human body; Fe deficiency can increase in Mn availability (Jędrzejczak, 2004). Following Tytanit® application, the Fe/Mn ratio in the early crop potato tubers (11.1/1) was similar to that for potatoes with the cultivation without the growth stimulant (10.8/1), and was on the same level or broader than those reported for mature tubers of early and medium-early potato cultivars (Lombardo et al., 2014; Wierzbowska et al., 2015; Mystkowska, 2018).

The content of Fe, Zn or Mn in potato tubers depended to a great extent on the cultivar and environmental conditions, which was confirmed by other authors (Rivero et al., 2003; Burgos et al., 2007; Haynes et al., 2012; Lombardo et al., 2014; Rivadeneira et al., 2016; Sharma et al., 2017; Wierzbowska et al., 2018). In the present study, 'Lord' tubers contained more Fe and Zn and less Mn than 'Miłek' tubers grown under the same conditions, although the content of Ti in tubers of both tested cultivars did not differ significantly. Genotype variation in nutrient uptake from soil is associated with the size of the root system and root morphology (Trehan and Singh, 2013). Nutrient uptake by plants depends on their levels in the soil and phytoavailability. The most significant environmental factors affecting the nutrient uptake by plant are soil moisture and pH. The availability of micronutrients present in a soil solution as cations (e.g. Fe^{2+}, Zn^{2+} and Mn^{2+}) increases with increasing soil acidity. The potential of roots to uptake mineral elements generally declines in water-stressed plants (Martinez-Ballesta et al., 2010; Trehan and Singh, 2013; Rivadeneira et al., 2016), which was confirmed in the present study. Under drought conditions, the greater presence of oxygen in the soil induces a decrease in the Fe^{2+}/Fe^{3+} ratio, leading to a decrease in Fe available for plants (Silva et al., 2011). Under a limited plant supply with Fe, the application of an appropriate concentration of Ti may induce the expression of genes related to iron acquisition, enhancing iron uptake (Lyu et al., 2017), which was confirmed in the present study. The most Ti was contained in potato tubers in the year with the highest air temperature and a heavy rainfall in the leaf development and tuber formation stages. Generally, it is believed that high light intensity and high air temperatures during rapid leaf expansion favours the absorption of mineral nutrients by the leaves. High air humidity also stimulates the absorption of leaf-applied nutrients (Wójcik, 2004), which was confirmed in the present study.

Titanium foliar application may be one approach to increasing the amount of essential human micronutrients in potato tubers. It has been estimated that human body take in about 0.8 mg Ti per day (Kabata-Pendias and Pendias, 1999). The amount of Ti provided with the examined potatoes does not exceed 10% of the estimated daily intake.

Conclusions

This study showed that it is be possible to increase Fe and Mn content in early crop potato tubers by foliar application of an appropriate dose of titanium, particularly under abiotic stress conditions. Following Tytanit® application, the content of Fe was higher, on average, by 6.150 mg kg^{-1} of DM and Mn by 0.409 mg kg^{-1} of DM compared to the cultivation without the growth stimulant. Titanium foliar application did not affect the content of Zn, whereas the content of Ti in tuber was higher, on average, by 0.364 mg kg^{-1} of DM. The dose and date of Tytanit® application slightly affected the content of Fe in potato tuber. The content of Mn was greatest with a single Tytanit® application at the tuber formation stage at the dose of 0.2 L ha^{-1}, whereas the Ti content in tuber was greatest with a double Tytanit® application during potato growth, at the leaf development stage and with a repeated treatment in the tuber formation stage at the dose of 0.2 L ha^{-1} in each treatment. This study showed a differential response of very early potato cultivars to titanium foliar application. So, determination of the possibility of increasing micronutrient contents in potato tubers with foliar application of titanium needs the more researches. Determining of optimal dose and date of titanium application for various potato cultivars is very important in the optimisation of potato production.

Acknowledgements. This study was supported by the Polish Ministry of Science and Higher Education under the statutory activities of the Siedlce University of Natural Sciences and Humanities [grant number 218/05/S].

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DOI: http://dx.doi.org/10.15666/aeer/1702_36333643

MOLECULAR APPROACH TO UNCOVER THE FUNCTION OF BACTERIA IN PETROCHEMICAL REFINING WASTEWATER: A MINI REVIEW

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(Received 16th Dec 2018; accepted 12th Feb 2019)

Abstract. Water is one of the most important resources in the universe. This is due to the fact that all life processes require the use of water. The rate at which petrochemical refineries release wastewater is alarming, and needs to be stoped. Many attempts to improve the efficiency of treatment methods of petrochemical refining wastewater have been made, among which bioremediation is considered an emerging advancement. Bioremediation incentivized the identification and characterization of most bacteria responsible for the breakdown of petrochemical refinery wastewater pollutants. The use of genomic approach (DNA and RNA sequence) in the identification of these bacteria is widely accepted. However, proteins and metabolites as functional entities in a cell are gaining less attention but could still be used to give an accurate insight into how Bacteria respond to pollutants in refinery wastewater. This review aims to find out the present trend and predicting the future prospect of the concept of a molecular genetics approach to uncover the function of bacteria present in refinery wastewater. The review presents literature that identified and characterized bacteria responsible for the degradation of pollutants in petrochemical refining wastewater using culture methods and molecular methods, such as the genomic, metabolomic and proteomic approach. The research hereby recommends the need for further research on a molecular approach to uncover the function of bacteria in petrochemical refinery wastewater using metaproteomic and metabolomic approach.
Keywords: *biostimulation, bioremediation, metabolites, metaproteomic, metagenomic, adsorption*

Introduction

Water is the most important asset in the universe. This is based on the fact that all life procedures require the utilization of water (Long et al., 2019; Longyang, 2019). The ever expanding world population and adoption of industrial lifestyle have inevitably lead to an increase in water shortage and water-borne diseases (Jimena Zambrano et al., 2018). Petrochemical refineries produce enormous amounts of wastewater consisting of both organic and inorganic pollutants, due to its refining process which involves cracking, resulting in the formation of simpler organic substances including benzene, toluene, biphenyl, cresol, cumene, ethylbenzene, hexane and methyl-butyl, etc. (Serio et al., 2018). The wastewater created contains oxygen depleting organisms and can lead to the production of toxic compounds e.g. heavy metals, phenol, oil and grease, bicarbonate etc. which, if not properly treated, could be discharged into the environment (Jimena Zambrano et al., 2018). These pollutants are created in an effort to increase the standard of living for man, but ironically the unplanned intrusion of these pollutants can reverse the same standard and affect the environment in a negatively way (Zhao et al.,

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

2018). These pollutants have the capacity to pollute underground water, or where it is released without proper treatment it affects Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of surface water.

Refinery wastewater contains microbial world which has the ability to withstand the pollutants of petrochemical wastewater (Gunawardena et al., 2018; Karbassi et al., 2018; Liu et al., 2018). Bioremediation is rising in importance owing to the way that the technique is has been efficiently used for remediation of the polluted site over other biological and conventional methods (Pettit et al., 2018; Ribeiro et al., 2018; Singh et al., 2018; Zhang et al., 2018). There is an increasing trend in the quantification of a molecular cellular component such as Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), Protein and metabolites of an organism (Vendramel et al., 2015; Asl et al., 2018; Dalman et al., 2018). The use of DNA and RNA sequence has been dominantly used. However, proteins and metabolites are functional entities in cell and therefore could be used to give an accurate insight into how Bacteria respond to pollutants in refinery wastewater (Rafat et al., 2018; Al-Namnam et al., 2019; Megarajan et al., 2019). This review is aimed at knowing the present trend and predicting the future prospect of the concept of a molecular genetics approach to uncover the function of bacteria present in refinery wastewater.

Petrochemical refinery pollutants

The water consumption rate of petrochemical refining industries is worrisome owing to the fact that large volumes of water are used in the refining of petroleum thus leading to the creation of wastewater (Licht and Isebrands, 2005). Factors such as crude oil composition and extraction mechanisms play a great role in the determination of the properties of wastewater resulting from crude oil refining attributing to the reason why was petrochemical refining wastewater varies from one refinery to the other (Colborne et al., 2019). Research worldwide has shown that refinery wastewater contains diverse pollutants that are either organic or inorganic (Donner et al., 2019). Organic pollutants can be subdivided into aliphatic or aromatic pollutants (Ghazouani et al., 2019). These pollutants influence the production of the aquatic environment by facilitating the process of oxygen depletion which leads to increased chemical oxygen demand (COD) and biological oxygen demand (BOD) (Mkhinini et al., 2019; Sancho et al., 2019; Sousa et al., 2019; Williams et al., 2019). Many studies have associated carcinogenic and mutagenic effect caused to microorganisms, macro-organism, and even human beings to be due to exposure to organic pollutants particularly BTEX benzene (toluene, ethylbenzene, and xylene) (Colborne et al., 2019), PAH (polycyclic aromatic hydrocarbon) (Chen et al., 2019), MTBE (methyl tert-butyl ether) (Krizman-Matasic et al., 2019; Nie et al., 2019; Zhang et al., 2019). Some organic pollutants that are likely found in petrochemical refining wastewater and the possible environmental effects associated with them are summarized in *Table 1*.

Inorganic pollutants in petrochemical refinery wastewater range from heavy metals to phosphorus. Heavy metals are one of the major problems associated with petrochemical refining wastewater owing to their non-biodegradable ability (Chen et al., 2019; Derakhshan et al., 2019). Though most times these metals are in minute concentration measured in microgram, they tend to accumulate till they get to toxic level. Research has demonstrated that heavy metals are harmful in both elemental and soluble form. The most reported toxic metals include Cd, Pb, Hg, Ag, Cr and As

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

(Derakhshan et al., 2019). Heavy metals such as Cr, Cd, and Pb are highly toxic to plants because they are needed by plants in either minute quantity or not needed, whereas heavy metals such as Cu and Zn are used by plants but can be highly toxic to plants also at higher concentration (Ioannou-Ttofa et al., 2019). Iron which is a vital mineral needed by the body becomes toxic at a higher concentration of above 1.6 mg/l leading to hemorrhagic and sloughing of the stomach particularly the mucosa area. Some heavy metals needed for plants growth are carcinogenic to human e.g. Ni (Kolecka et al., 2019). Another pollutant present in petrochemical refining wastewater is a nitrogen compound which is sometimes in combine state with hydrogen forming ammonia (Long et al., 2019). Phosphorus is also another pollutant present in petrochemical refining wastewater (Tahtouh et al., 2019). The worrisome situation associated with the presences of phosphorus and nitrogen compound in petrochemical refinery wastewater is the fact that both contribute immensely to eutrophication, a process that encourages the growth of algae leading to a scenario refers to as algae blooms. Algae blooms tend to deplete oxygen and some of the algae are directly poisonous to fishes and other aquatic organisms. The situation thereby leads to a drastic reduction in aquatic productivity (Costa et al., 2019; Tahtouh et al., 2019; Tang et al., 2019). It is thereby of paramount importance to remove Nitrogen and phosphorus from petrochemical refining wastewater (Costa et al., 2019).

Table 1. *Organic pollutants and environmental impacts*

S/N	Pollutant	Environmental impact	References
1	Benzene	It hinders the growth of plants and other terrestrial animals and could lead to their death through extended exposure. The presence in the aquatic environment leads to behavioral changes, health instability of the organisms and low reproductive rate	Cuo et al. (2018), Staehelin et al. (2018)
2	Toluene	Causes mutagenic and carcinogenic effects to micro organisms and other macro organisms	Lagoviyer et al. (2018)
3	Biphenyl	In humans, exposure to biphenyl leads to eye and skin irritation, toxic effect on liver, kidney and nervous system. In the aquatic environment, exposure to biphenyl leads to behavioral changes and low reproductive rate	Lauren Hayashi (2014), Li Z (2016)
4	Cresol	Exposure can lead to severe gastrointestinal damage which could eventually lead to the death of an organism	Shi et al. (2019)
5	Cumene	No observable effect has been recorded in a mammal and some larger vertebrates but exposure to cumene inhibits photosynthetic activities in some algae and also shows positive effects on some invertebrates	Junqueira et al. (2018)
6	Ethylbenzene	Long time exposure causes the damage of the kidney can lead to an irreversible damage to the inner ear	El-Hashemy and Ali (2018)
7	Hexane	Exposure causes body weight lost, neurological, reproductive and developmental defects in exposed organisms	El-Hashemy and Ali (2018)

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8	Methyl-butyl ether	Causes leukemia and lymphoma (blood cancer), cancer of the kidney and nerve dysfunction. In an aquatic environment, it rapidly leads to the depletion of dissolved oxygen	El-Hashemy and Ali (2018)
9	Xylene	Causes the dysfunctions of the liver, kidney and nervous system. A lot of neurological effects have also been reported	El-Hashemy and Ali (018)
10	Phenol	Causes muscles tremor resulting into walking difficulty and gastrointestinal damage and even death	Wang et al. (2018)
11	Styrene	Exposure could lead to impaired learning and reproductive defects particularly damage in sperm. Possible carcinogenic effects have been reported to result as a result of exposure to styrene	Arabjafari et al. (2017)
12	Nepthalene	Causes mutagenic and carcinogenic effects to micro organisms and other macro organisms and also associated with damage of red blood cells	Wang et al. (2018)
13	1,2,3 Trimethylbenzene	No normal background level adverse effect to human have been reported but on a high exposure lead to Vomiting, tension, wheezing and may be injurious to the blood	Kostrzewski et al. (1997)

Remediation of petrochemical refining wastewater by bacteria

The use of Bacteria for bioremediation can be dated to 1972 when soil pollution resulting from the bursting of Ambler pipeline was bioremediation (Raymond, 1976). Bioremediation technology in the remediation of petroleum wastewater holds a promising start owing to the fact that its earliest research has shown high pollutants removal efficiency compared to other biological and conventional methods (Bako et al., 2008; Peng et al., 2017; Panasia and Philipp, 2018).

Bioremediation of organic pollutants in refinery wastewater can occur naturally by the degradation of the pollutants to a minimum concentration to exert toxicity except for persistence organic compound such as polycyclic aromatic hydrocarbon (PAHs) and benzene, toluene, ethylbenzene and xylene (BTEX) (Horemans et al., 2017; Buryska et al., 2018; Nikolaivits et al., 2018). Although, the rate at which organic pollutants degrade naturally is very slow hence the reason why bacteria are used to engineer the process (Wang et al., 2017).

Petrochemical refining wastewater treatment using bioremediation techniques is less energy, pressure, and temperature consuming if compare with another convectional method (Silva et al., 2012; Ojha et al., 2013; Banerjee and Ghoshal, 2016). Bioremediation potential of bacteria to degrade refinery wastewater depend on its metabolic activities (Eberlein et al., 2013; Boll et al., 2014; Nikel et al., 2016) which involves the ability of the bacteria to introduce molecular oxygen into the wastewater to catabolize the hydrocarbon present in the wastewater utilizing it as a source of energy and carbon (Moghadam et al., 2016; Banerjee and Ghoshal, 2017; Iqbal et al., 2018). Thapa et al. (2012) stated that the bacteria with the potentiality to degrade petroleum pollutants includes *Pseudomonas sp, Beijerinckia sp, Chlorobacteria sp, Antinetobacter sp, Nocardia sp, Lavobacteria sp, Streptomyces sp, Cyanobacteria sp, Baccilus sp,*

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Moraxella sp, Corynecbacteria sp, Aeromonas sp, Mycobactena sp etc. Although, in the treatment of petrochemical refining wastewater these bacteria must not be indigenous bacteria inhabiting the wastewater because nonindigenous bacteria isolated from other sources can be used to treat this wastewater (Chaudhary and Borah, 2011; Singh et al., 2013; Rodriguez-Mateus et al., 2016). Remediation of petrochemical refining wastewater using bacteria is associated with little or no adverse effect to the ecosystem on like in the case of phytoremediation where the disposal of the plants use for remediation becomes another problem to the ecosystem (Boroujeni et al., 2014; Bahobail et al.,, 2016; Duniya, 2016). Many researchers have used indigenous and non-indigenous bacteria in the treatment of refinery petrochemical wastewater as summarized in *Table 2*.

Table 2. *Literature on bacterial consortia effectively used for bioremediation*

SN	Bacterial consortia	Pollutant removed	Reference
1	*Aeromonas punctata* *Aeromonas caviae* *Stenotrophomonas maltophilia* *Ochrobactrum intermedium* *Rhodococcus sp*	Crude oil Chemical Oxygen Demand (COD) Biological Oxygen Demand (BOD) Total petroleum hydrocarbion (TPH)	Gargouri et al. (2011)
2	*Bacillus cereus*	COD and BOD	Banerjee and Ghoshal (2016)
3	*Alcaligenes odorans* *Bacillus subtilis* *Corybacterium propinquum* *Pseudomonas aeroginosa*	Crude oil and phenol	Singh et al. (2013)
4	*Alcanivorax borkumensis* *Alcanivorax dieselolei* *Anderseniella baltica* *Devosia albogilva* *Gordonia alkalivorans* *Gordonia amicalis* *Gordonia paraffinivorans* *Haloferax volcanii* *Marinobacter gudaonensis* *Methylopila capsulata* *Mycobacterium aromaticivorans* *Mycobacterium aubagnense* *Mycobacterium chelonae* *Mycobacterium doricum* *Mycobacterium llatzerense* *Mycobacterium monacense* *Mycobacterium rutilum* *Petrimonas sulfuriphila* *Proteiniphilum acetatigenes* *Saccharospirillum impatiens* *Williamsia marianensis* *Williamsia muralis*	Total petroleum hydrocarbion Electrical Conductivity (EC) Total Solid Volatile Solid Total Nitrogen Total Kheldhal Nitrogen	Gargouri et al. (2014)
5	*Rhodococcus pyridinivorous* *Advenella faeciporci* *Pseudomonas aeroginosa*	Phenol	Moghadam et al. (2016)

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6	*Pseudomonas sp* *Acinetobacter sp* *Kelibsiella sp* *Citrobacter sp* *Shigella sp*	Phenol	Ojha et al. (2013)
7	*Diaphorobacter sp* *Pseudomonas sp* *Thauera sp* *Comamonas sp*	Biphenyl Naphthalene Benzoate	Silva et al. (2012)
8	*Diaphorobacter sp* *Paracoccus sp*	Polycyclic aromatic hydrocarbons (PAHs)	Sanches et al. (2017)
9	*Bacillus subtilis* *Micrococcus luteus* *Pseudomonas aeroginosa*	Oil and grease, COD and BOD	Musa (2015)
10	*Pseudomonas aeroginosa* *Penicillium janthinellum*	Crude oil	Bako et al. (2008)
11	*Achromobacter sp* *Pseudomonas sp* *Alcaligenes sp*	Phenol Benzene	Iqbal et al. (2018)
12	*Bacillus licheniformis* *Bacillus shaericus* *Bacillus brevis*	Total petroleum hydrocarbon	El-Borai et al. (2016)
13	*Rastonia picketti* *Alcaligenes piechaudi* *Bacillus subtilis*	Total petroleum hydrocarbon	
14	*Burkholderia cepacia* *Cornebacterium sp*	COD BOD Phenol Total petroleum hydrocarbon Oil and grease	Ajao et al. (2013)
12	*Serratia marcescens*	Trichloroethylene Tetrachloroethylene Polyvinylchloride	Li et al. (2008)
13	*Pseudomonas aeruginosa* *Bacillus thermosaudia*	PAHs (Benzo-pyrene, benzo (k) fluoranthene, naphthalene, phenanthrene, fluorine, and anthracene)	Pugazhendi et al. (2017)
14	*Pseudomonia putida*	Hexadecane Anthracene Naphthalene	Yalcin et al. (2011)
12	*Alcanivorax,* *Marinobacter,* *Alphaproteobacteria* *Pseudomonas*	Oil	Kostka et al. (2011)
13	*Pseudomonas stutzeri,* *Rhodococcus erythropolis* *Alcanivorax borkumensis,*	Crude oil	Santisi et al. (2015)

It will be noticed from the *Table 2* that despite the fact that benzene, toluene, biphenyl, cresol, cumene, ethylbenzene, hexane, and methyl-butyl ether are very poisonous pollutants present in petrochemical refining wastewater, there is still scanty literature with respect to the bioremediation of these pollutants in refining wastewater

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using bacteria. Instead, a significant number of researches on bioremediation of petrochemical wastewater centered the research on total petroleum hydrocarbon which encompasses all. Quite a number of researches have shown the potentiality of some bacteria to bioremediate total petroleum hydrocarbon in petrochemical refining wastewater but it is of paramount importance to be case specific with respect to the potentiality of bacteria that can bioremediate benzene, toluene, biphenyl, cresol, cumene, ethylbenzene, hexane and methyl-butyl ether in the free state because these pollutants varied in state and testing site and so also do their ecological effects. *Table 2* also shows that scanty literature exists for the bioremediation of heavy metals from petrochemical refining wastewater. The poor literature in the bioremediation of heavy metals from petrochemical refining wastewater could be attributed to the non-biodegradable behavior of heavy metals. Although the literature has shown the potentiality of bacteria to remove heavy metal from a polluted site by biosorption and enzymatic degradation, the process of biosorption is illustrated by *Figure 2*. The poor literature in the bioremediation of phosphorus and nitrogenous compound in petrochemical, refinery wastewater is also worrisome because degradation catalyst by bacteria is dependable on environmental conditions.

Biodegradation of pollutants in petrochemical refining wastewater

Petrochemical and refining wastewater contains pollutants that are either organic or inorganic as shown in *Table 2*. The degradation of organic pollutants depends on the aerobic and anaerobic pathway as shown in *Figure 1* while in the case of inorganic pollutants it occurs by adsorption, absorption or the action of enzyme release by the bacteria as shown in *Figure 1* (Fuchs et al., 2011).

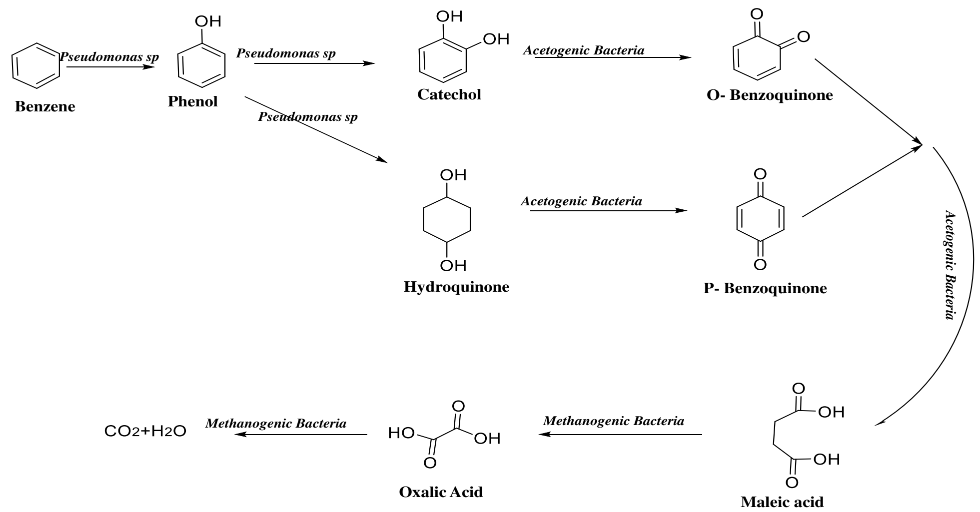

***Figure 1.** Proposed process of biodegradation of benzene by bacteria*

Bacteria possess high potentiality in the biodegradation of hazardous petrochemical wastewater as shown in *Figure 1*. To further enhance the Biodegradation by bacteria particularly in a contaminated site, there is the need to study the condition in which bacteria best perform it functions and the enzymes involved in this process. The role of enzymes in the degradation of petrochemical pollutants particularly organic compounds

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was reported by Scoma et al. (2017) to be due to Rieske non-heme iron oxygenases, flavoprotein, Monooxgenases or soluble di iron monooxygenases.

Heavy metal present in petrochemical wastewater is nonbiodegradable due to their persistence nature as such this persistence pollutants are removed from petrochemical wastewater by adsorption as shown in *Figure 2*. Researchers such as Gutnick and Bach (2000), Feng et al. (2012) and Oves et al. (2013) have used the dead and live culture of bacteria for heavy metal removal by adsorption. The efficiency of heavy metal adsorption by bacteria depends on the speciation, behavior, transportation mechanism and the fate of heavy metal in binding with the functional group of the bacteria as shown in *Figure 2*. To understand the mechanism involved in adsorption of heavy metals by bacteria, studies on cell surface characterization, functional group identification, acidic and basic active site identification etc have been performed by researchers such as Vecchio et al. (1998), Banerjee et al. (2018).

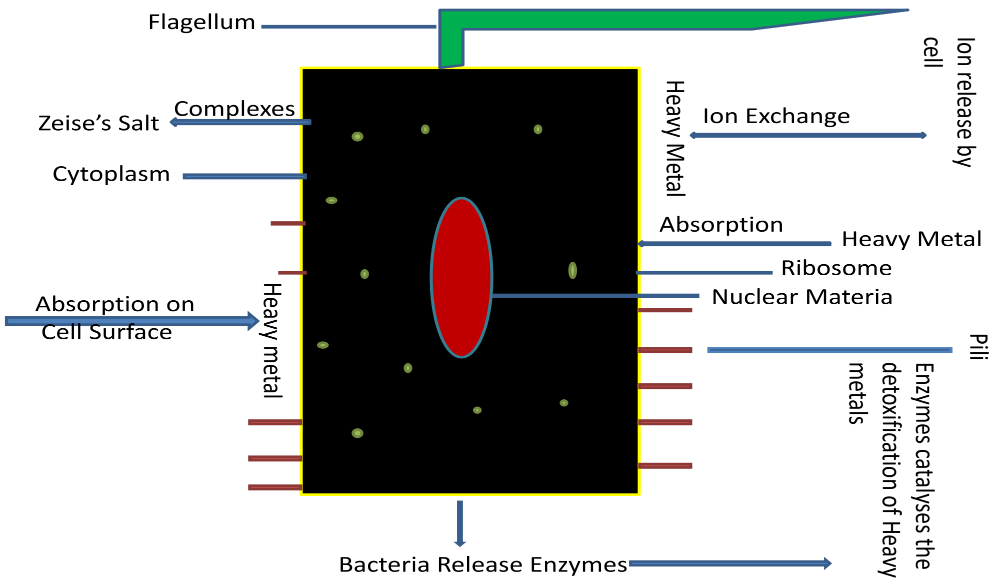

***Figure 2.** Process of heavy metal removal by bacteria (Jin et al., 2018)*

Petrochemical refining wastewater remediation strategies and principles

The strategies used in the bioremediation of petrochemical refining wastewater include biostimulation, bioaugmentation, bioattenuation and the use of genetically modified bacteria.

Biostimulation

This is a strategy that involves the use of indigenous bacteria for the treatment of petrochemical refining wastewater. The indigenous bacteria used for biostimulation are supported by nutrients which maybe inform of cations or anions to enhance the biodegrading capability of the bacteria (Jemli et al., 2017). The addition of nutrient supports the metabolic process of the bacteria thereby facilitating the degradation of pollutants present in refinery wastewater. Sarkar et al. (2016) reported high total petroleum hydrocarbon degradation of about 80% after the retention time of 90 days after bio stimulating indigenous bacteria by enhancing the nitrate constituent of the wastewater (Lin et al., 2012).

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

Bioaugmentation

This is a strategy employ in the treatment of petrochemical wastewater in which foreign bacteria is introduced into petrochemical refining wastewater to enhance the biodegradation ability of the indigenous bacteria (Yan et al., 2014). To have a successful bioaugmentation of petrochemical refining wastewater, the adaptability of the foreign bacteria must be considered owing to competition that may exist between indigenous and foreign bacteria, another problem that the foreign bacteria may face include predation by protozoa. Dueholm et al. (2014) recorded high removal efficiency of methyl ethyl ketone remediation using bioaugmentation assisted bioremediation using Rhodococcus pyridinivorans. Yan et al. (2013) also recorded high cresol removal efficiency using Pseudomonas sp supported by bioaugmentation.

Bioattenuation

Bioattenuation is a type of strategy which employs natural biodegradation mechanism of bacteria to treat petrochemical wastewater (Huling et al., 2002). The presence of other refinery wastewater pollutants such as phosphate, sulfate, nitrate etc stimulates the process of bioattenuation leading to the degradation of BTEX and PAHs as shown in *Figure 1*. Bioattenuation is the process that occurs when wastewater is stored in stabilization pond. The bioattenuation process is achieved either aerobically or anaerobically by acetogenic bacteria and methanogenic bacteria (Nijenhuis et al., 2004).

The use of genetically modified bacteria

Genetic modification of bacteria genome was among the earliest contribution in the field of genetic engineering owing to their simple structures. The use of the genetically modified bacteria in the degradation of hydrocarbons and chlorinated hydrocarbon is a cost-effective an emerging trend in bioremediation. The degradation pathway of organic compounds as shown in *Figure 1* have been reported by researchers such as Scoma et al. (2017). Also Duarte et al. (2014) reported that enzymes such as dioxygenase, flavoprotein monooxygenase, catechol 2-3 dioxygenases, protocatechuate, gentisate dioxygenase etc are responsible for the degradation organic compound as shown in *Figure 1*. The used of genetically modified bacteria in degradation of petrochemical wastewater is beyond just the use of the natural biodegrading potential of bacteria. genes are introduced into bacteria by recombinant DNA technology to enhances the bioremediation of both organic and inorganic Pollutant.

Molecular characterization of bacteria present in petrochemical refinery wastewater

Molecular characterization of bacteria present in petrochemical refinery wastewater involves the investigation of the chemical functional groups or constituent in bacteria cell that allow the bacteria to be able to survive in petrochemical refinery wastewater and even bring about the bioremediation by degradation of both organic and inorganic pollutants presents in the wastewater (Chaudhary and Borah, 2011; Singh et al., 2013). The technique used in the molecular characterization of bacteria present in petrochemical refinery wastewater includes the following approaches:

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

- Genomic approach
- Proteomic approach
- Metabolomic approach (Bahobail et al., 2016; Duniya, 2016; Rodriguez-Mateus et al., 2016)

The methods stated above are changing the understanding of bacteria functions in petrochemical refinery wastewater from a simple study of the sequences of gene, protein or metabolites to a more complex and detailed approach showing the role of bacterial in petrochemical wastewater environment (Hobert, 2010). The molecular approach to petrochemical wastewater bacteria characterization is achieved at the microbial community rather than a single bacteria species due to the fact that biodegradation activities of bacteria depend on consortia (Santisi et al., 2015).

Genomic approach in the uncovering of bacteria function in petrochemical refining wastewater

Bacteria genome simply refers to as the total sets of DNA found in bacteria. Genomic investigation of bacteria genome is centered towards the study of the bacteria genomic structure, bacteria genomic evolution, bacteria genomic mapping, and editing of a bacterial genome (Gilbert and Scott, 2017).

This technique has been helpful in the determination of how bacteria cell is able to successfully leave in petrochemical refinery wastewater and the function they play in this wastewater. This has also helped in the determination of the most efficient bacteria to be used in the remediation of petrochemical wastewater (Larsen et al., 2012).

The aspect of genomic, that is the concern with the study of environmental samples such as petrochemical refining wastewater, is referred to as metagenomic or ecogenomic. This technique employs the use of 16S rRNA gene (16S rDNA) sequences analysis method for the identification of bacteria strain. These methods have shown a better advantage over the culture method of the function of bacteria in petrochemical refinery wastewater. The major sequential method employed includes extraction of DNA, PCR amplification of the 16S rRNA gene, nucleotide sequence determination (sequencing) and sequences comparison with available data (Fuks et al., 2018). The sequencing and analysis of bacteria genome by the use of DNA sequencing, RNA sequencing, and bioinformatics techniques have been utilized in the assembling and analysis of the function and structure of the entire organism genome of bacteria presents in petrochemical refinery wastewater (Toshchakov et al., 2017).

Metabolomic approach in the uncovering of bacteria function in petrochemical refining wastewater

The metabolomic approach of studying the function of a living organism in an environment is highly complex because it tends to give more detail or complex cellular pathways and biological mechanisms in organisms (Vinaixa et al., 2016; Beale et al., 2018a; Beale et al., 2018b). This approach employs the use of small bacteria metabolites including metabolic intermediates hormones, signaling molecules and secondary metabolites to uncover the function play by bacteria (Bonifay et al., 2017).

This approach is used as a basic tool used in functional genomics studies to ascertain the function of the gene (Tang, 2011). This approach holds a promising future towards the ascertaining the function play by bacteria in petrochemical wastewater owing to the

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

fact that they are involved in normal growth and development of bacteria as such could provide important detail to why the organism was able to survive and develop fully in the presence of petrochemical wastewater pollutants (Foght, 2008). Metabolites are also involved in activities carried out by antibodies and pigments which is also an important tool for the determination of the reaction pathway in the degradation of organic and inorganic pollutants in petrochemical refining wastewater and biosorption of heavy metals by the bacterial cell (Tang, 2011).

Table 2 summarized some recent contribution done by researchers in the uncovering the function of bacteria in the degradation of petrochemical wastewater making references to metabolomics.

Proteomics approach in the uncovering of bacteria function in petrochemical refining wastewater

This approach involves the use of proteins to uncover the function of bacteria in petrochemical wastewater. The use of this technique has made possible to know the function of bacterial consortia. The proteomic study that a deal with the study of protein for the particular environmental function is refers to as metaproteomic. The use of metaproteomics approach in the characterization of bacterial consortia has given a better insight to which bacteria consortia is best to be used in the wastewater treatment.

Recent findings on molecular identification and characterization of refinery wastewater

Genomic DNA extraction from bacteria isolate was performed by Chaudhary and Borah (2011) who shows that *Bacillus* and *Pseudomonas* species have the maximum capacity to degrade oil but gave upper hand advantage to *Bacillus species*. Singh (2013) shows the presence of *Alcaligens odorans, Bacillus subtilis, Corynebacterium propinquum* and *Pseudomonas aeruginosa* in petrochemical refinery wastewater. The researcher positively collated high pollutant reduction efficiency to lipolytic activities of the bacteria. Rodriguez-Mateus et al. (2016) using wizard genomic DNA purification Kit (Promega, USA) identified bacterial isolates using amplification and sequencing. Rodriguez-Mateus et al. (2016) detected the present of 10 species of bacterial isolate stating that the bacteria isolates belong to the genus *Candida* and *Bacillus*. Rodriguez-Mateus et al. (2016) also show that the bacterial isolates show visible lipolytic activity evidenced by the reduction of in the pollutants of the palm oil refining wastewater. Iqbal et al. (2018) performed molecular characterization by 16S rRNA gene sequencing and find out that phenol was degraded completely by *Achromobacter species, Pseudomonas specie*, and *Alcaligenes species*. The researcher also reported the significant degradation of benzene by *Bacillus species, Enterobacter sp,* and *Acinetobacter sp*. This study is supported by the previous work performed by Bahobail et al. (2016) who shows that *Pantoea agglomerans, Acinetobacter iwoffi* and *Bacillus thuringiensis* where fully characterized to be lipolytic to hydrocarbons. Duniya (2016) shows the presences of the gene for hydrocarbon enzymes catechol 2,3 dioxygenase in *Enterobacter hormaechei*, *Escherichia coli*, and *Shigella flexneni*. Boroujeni et al. (2014) reported that the sequences of the gene bank and the highest homology were identified for bacteria belonging to the *Nitratireductor aquamarinus* and *Pseudomonas stutzeri* were identified. Igwaran et al. (2018) shows the molecular characterization and antimicrobial resistance pattern by 31 genes of *Escherichia coli*. Chikkanna et al. (2018) using

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polyphasic taxonomy approach identify bacterial strain including Halomonas *Mauramaura, Halomonas nitoreduceus, Halomonas ventose, Halomonas trenophila* and *Halomonas methanobrevibacter*. Azadi et al. (2017) screen and characterized Mycobacteria from the diverse range of Iranian aquatic and terrestrial ecosystem with a harsh and unfavorable environmental condition that can be used in biodegradation of refinery product. The presences of *Mycobacterium fortuitum, Mycobacterium flavescens, Mycobacterium paragordonae, Mycobacterium monacense, Mycobacterium fredriksbergense, Mycobacterium aurum, Mycobacterium conceptionense, Mycobacterium porcinum, Mycobacterium aurum, Mycobacterium celeriflavum, Mycobacterium novocastrense, Mycobacterium obuense* and 12 isolates belonging to an unknown potentially novel bacterium species. Obi et al. (2016) performed molecular characterization using rRNA gene to indicate the presence of bacteria belonging the genera *Stenotrophmonas, Bordetella, Pseudomonas, Brucella, Bacillus, Achromobacter, Ochrobactrum, Advenella, Mycobacterium, Mesorhizobium, Klebsiella, Pusillimonas* and *Raultella sp* with potential to degrade polycyclic aromatic hydrocarbons (PAHs) from oil sludges. Abo-State et al. (2018) was able to isolate Naphthalene tolerance bacteria belonging to *Bordetta avium* including *MAM-P14, MAM-P22, MAM-P25, MAM-P26, MAM-P9*. Nascimento et al. (2018) was able to characterize microbial diversity in sewage sludges from Sao Paulo, Brazil. The presence of high bacterial diversity was noticed with proteobacteria as dominant phylum then Bacteroidetes and Firmicutes, Clostridium were the dominant genus followed by Treponema, Propionibacteria, Syntrophus, and Desulfobulbus. Ma et al. (2015) isolated Strain DN002 from petroleum-contaminated soil which was identified as *Achromobacter xylosoxidans.* The research shows that during fluoranthene biodegradation, catechol 2, 3 dioxygenases (C23O) activity was augmented 1.5 times more than catechol 1,2 dioxygenase (C12O), which indicated that C23O played a major role in fluoranthene degradation by DN002. The dominant general was *Propionibacterium, Comamonas, Brevundimonas, Methylobactenum, Stenotrophomona*s, and *Cloacibacterium.* Jamal and Pugazhendi (2018) identify the halophilic bacteria trains present in the consortium to include *Ochrobacterium halosaudis, Stenotrophomonas maltophilia, Achromobacter xylosoxidans* and *Mesorhizobium halosaudis*. Katyal and Kaur (2018) were able to isolate chromate tolerance bacteria from a treatment plant. The isolates belonging to the *Micrococcus luteus* include HM 16, *HM, 2, HM 3, HM 15*. Ibarbalz et al. (2016) used function encoded in metagenome to establish a link between microbial community structure and function in activated sludge. The bacteria detected include Actinobacter, Planctomycetes, Verrucomicrobia, and Chlorobi. Rosso et al. (2016) also use a proteomic approach to investigate the changes of bacteria community overtime, Cyanobacteria, Bacteriodetes, and Proteobacteria were the bacteria that express extracellular hydrolases. Navarro-Locsin and Lim-Jurado (2018) use geno-wide transcriptome and proteome data to confirm most highly expressed organohalide respiration (OHR) rate. He concluded that hydrogenase (HUPL) transcript is the most robust activity biomarker across multiple Dehalcoccoides mccartyi (DMC) strain and in mixed communities including DMC co-culture such as bioaugmentation culture (KB1^{TM}). Cernava (2017) used multi-omics approach to characterize functional guilds in an unconventional model system. The community structure of the microbiota was found to be highly similar irrespective of the omics approach. The abundant bacterial orders include Sphingomonadales, Rhodospirillales, Myxococcales, Chthoniobacterales, and Sphingobacteriales. The function played by these Bacterias identified is the

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provision of vitamins and cofactors to the degradation of phenolic compounds like phenylpropanoid, xylenols, and cresols. Matilda and Shanti (2017) isolated trivalent chromium resistant bacterium and identify it by 16S rRNA gene sequencing and designated as *Alcaligenes faecalis* VITSIM2. The organism also showed resistance to copper, cadmium, and certain antibiotics and also shows changes in cell wall content.

Nzila (2018) Isolated and characterized a bacterial strain capable of metabolizing the four fused aromatic ring polycyclic aromatic hydrocarbons (PAH). The analysis of 16S rRNA gene revealed that the bacterium is called Achromobacter xylosoxidans. The proteins involved in the degradation of PAH are 4-hydroxyphenylpyruvate dioxygenase and homogentisate 1,2-dioxygenase. Otzen et al. (2018) isolated a caprolactam-degrading strain of Pseudomonas jessenii from soil and identified proteins and genes putatively involved in caprolactam metabolism using quantitative mass spectrometry-based proteomics. He demonstrated that caprolactamase consist of two subunits and demonstrated high sequence identity to the 5-oxoprolinases. Escherichia coli cells expressing this caprolactamase did not convert 5-oxoproline but were able to hydrolyze caprolactam to form 6-aminocaproic acid in an ATP-dependent manner. The research concluded that the presence of a caprolactam catabolism gene cluster comprising a set of genes involved in the conversion of caprolactam to adipate.

Conclusion

Molecular characterizations of bacteria responsible for the bioremediation of petrochemical refining wastewater have a promising future for the control of petrochemical engineered pollution. Bacteria consortia consisting of bacteria which have shown bioremediation capacity for phenol, benzene and other constitutes of petrochemical refining wastewater should be properly utilized as an advantage to properly degrade these pollutants completely from the wastewater before discharge. Molecular characterization will help in the proper identification of the bacteria and enzyme released by the bacteria responsible for the pollutant degradation. The technique will also lead to the identification of the mechanism involved in the ecological adaptation of the bacteria to polluted environment and the role of antibiotic, antimicrobial compound (metabolite and proteins) and bacteria gene in the degradation of petrochemical refining wastewater.

Acknowledgements. The first author appreciate Petroleum Development Fund (PTDF) Nigeria and College of New Energy and Environment, Jilin University for Providing the fund and enabling environment to carry out the research.

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DOI: http://dx.doi.org/10.15666/aeer/1702_36453665

GREEN ROOF PLANT RESPONSES TO GREYWATER IRRIGATION

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(Received 20th Dec 2018; accepted 26th Feb 2019)

Abstract. Water is an essential natural resource and is even known to be the cause of many wars of history. While people depend on it to survive, they also use it for everyday activities and several other purposes. As for plants, water is an essential factor, one they need for growth. Although irrigation is really important for certain agricultural plants, many criticise its water consumption especially on scientific platforms and media. This naturally pushes the world to find alternative ways to decrease the water consumption where possible. Greywater (GW) use is one of these alternative ways. This study aims to focus on two different but ecologically useful recent phenomena; GW use and green roofs. GW use makes it possible to decrease water consumption by replacing irrigation water, while green roofs (GRs) are known to increase biodiversity in urban areas and to decrease heat island effect and as a result reduce the costs of air conditioning. Since the main purposes of this study are to evaluate the use of GW on GRs to examine if there might be an ecologically and economically more beneficial way to keep the roof green, we used a prepared greywater simulation (PGWS) to measure green roof plants' responses to it. Two different greywater models, non-diluted prepared greywater simulation (N-D.PGSW) and 50% diluted prepared greywater simulation (%50-D.PGWS), were used for irrigation; while tap water (TW) irrigation and no irrigation (N-I) were the other two conditions to make the study more reliable.
Keywords: *Sedum irrigation, urban landscape, water management, environmental management, sustainable water use*

Introduction

Water is a life-sustaining natural resource and has been regarded as inexhaustible until quite recently, but the accelerating population growth and development of industrial activities have led to its depletion, especially in areas facing drought (Illan, 2011). After the recognition of water as an exhaustible resource, innovative technological and agronomic practices such as storage and regulation methods for water, installation of irrigation automation systems, and reuse of wastewater were adopted. Despite these innovations and cultural practices, cities currently suffer from water deficit. Water deficit is defined as the exceedance of the currently-available renewable sources by the sum of the water use requirement of different fields (urban, agricultural, industrial, and environmental uses) (Zavala and Estrada, 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36673680

GW is defined as the urban wastewater consisting of the waters used for domestic purposes except for the toilet water (water from bathroom sink, shower, lavatory, dishwasher, washing machine, and kitchen) (Mohamed et al., 2013; Li et al., 2009; Ottoson and Stenströn, 2003; Eriksson, 2002; Jefferson et al., 1999). GW constitutes 50-80% of the total wastewater use of a household (Antonopoulou, 2013; Friedler and Hadari, 2006; Eriksson et al., 2003). Previous studies reported that depending on the population structure, tradition and habits, structure of the plumbing system, and water abundance, the use of GW vary between 90-120 l/p/d (liter/person/day) in developed countries, whereas in low-income and water-scarce countries the rate drops to 20-30 l/p/d (Morel and Diener, 2006).

In addition to avoiding river pollution, the use of GW as a valuable fertilizer and for irrigation purposes in landscape and agricultural areas in Rome, Greece, China, England, and Germany dates back to ancient times. The use of wastewaters in irrigation has emerged as an effective mechanism to regulate water resources in water-scarce areas since the 16th century (Zavala, 2016; Negahban-Azar et al., 2012).

One of the main factors adding to the importance of GW is undoubtfully the expanding place of its generators in everyday use. In addition, since GW do not contain fecal waters, they can be easily treated and potentially reused (Yalçınalp et al., 2018). Moreover, GW is increasingly used due to the lower polluting effects of pathogens and nitrogen (Li et al., 2009; Li et al, 2003). The use of GW in gardens, car washing, laundries, and toilet flushes has been a long-employed practice in various regions of the world. The use of treated GW also greatly contribute to the protection of high-quality water resources. Depending on the popularization of these practices, this contribution brings along a great cumulative potential as well (Li et al., 2003).

It is clear that the reuse of waste water poses a health risk both for the public and system employees due to the high exposure rate to pathogenic microorganisms and toxic materials. The health concerns regarding the increases in these activities generally involve the extent of human exposure to treated wastewater, the type and quality of the treated wastewater, and the safety of the treatment processes (Zavala and Estrada, 2016; Benami et al., 2013). Nonetheless, in the case of landscape plants, since they are not edible and thus, the water used in their irrigation can have a lower microbiological quality, the use of wastewaters is advantageous in the irrigation of landscape plants.

The great amounts of the GW generated by the recent large-scale urban development in Trabzon have led to its emergence as a new recyclable water resource. GW will be an important resource to partially mitigate the water deficiency and prevent the pollution of environment by these waters.

Despite its huge popularity in recent years due to its great potential and the environmentally destructive course of the world, pessimistic and refraining voices have been raised against the use of GW. The most prominent concern is the doubts about its possible use in the irrigation of edible plants. Furthermore, the possibility and rapidity of bacterial growth during the storage of GW, which contains bacterial growth-facilitating organic wastes, have long been among the underlying causes of the reluctance towards its use. However, scientific studies evaluating it within the limits of universal standards have indicated that GW was a safe and sustainable resource.

Analysis of the detailed information gleaned in this study helps (1) to evaluate green roof plants' response to GW irrigation, (2) to make a contribution to green roof phenomena by decreasing the estimated irrigation costs in the long term as GW has a huge potential to take tap water place for irrigation and maybe the most importantly (3)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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to create a more sustainable green roof approach to prevent water waste while green roof is already considered as an ecological way to make urban areas healthier and more livable.

Material and Method

Study area

The roof of the Landscape Architecture Department of the Karadeniz Technical University (40°59'27.02"N, 39°46'30.80"E) was selected as the study area (*Figure 1*). After obtaining the necessary permissions, the materials were transferred to the roof using a crane installed on the rooftop and the irrigation equipment were prepared for use. The city covers an area of approximately 4.685 km2 and has a population of about 758.237 inhabitants, making it the second largest principal city in the region (Anonymous, 2013a). The city is within the A8 of the grid system created by Davis 1965 and Davis 1985 and the annual mean rainfall is about 760 mm, while the mean temperature is about 14.6 °C. The monthly mean temperature ranges from 7.3 °C in January and from 13 to 23.1 °C in August.

Figure 1. *Study area*

The Installation of the Experiment

A review of the relevant literature revealed that Sedum species were used in various studies on GRs. *Sedum album* and *Sedum sediforme*, as two species from one of the genera that first come to mind when GRs are considered, were used as the plant materials in this study as well. *Sedum album* and *Sedum sediforme* plants that were brought to the study area, derived from the same origin, and as identical as possible were planted in 60cmx40cm pots and on 10cm blockage for drainage and 20cm soil. To obtain data for the comparisons, experiments were installed in a total of 80 pots comprising 10 pots for each *Sedum* species and each irrigation type (N-I, TW, 50%-D.PGWS, N-D.PGWS). The irrigation rate was planned to be 1 L/w (Liter/Week) for each pot. The plants were planted

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in the middle of the pots in an area of 24 cm2 (about 10% of the total area) to achieve the adequate examination of the plant propagation. According to the monthly precipitation reports of the Trabzon General Directorate of Meteorology for 2016-2017, during the experiment, the plants were exposed to a monthly precipitation ranging from 6.3mm to 160.2mm and corresponding to an annual precipitation of 88.37mm.

The plant and soil samples were prepared for analyses at the end of the experiment in December 2017 by collecting and blending the samples from each pot that contain plants from the 8 groups examined using different irrigation systems. Soil samples were randomly taken in a depth of approximately 0–15 cm from an open-air field in a nursery. Then the samples were dried at 110 °C and transferred to polyethylene bags until the installation. To make sure that each pot will have the same amount of soil, a bascule was used just before putting the soil into the pots. Trying to pick similar in size, plant samples in 2 different *Sedum* species were also taken from the same nursery and kept on the roof top for the last 3 days to the installation.

Because one of the main aims of the study is to make a practical, sustainable and significant contribution to the green roof existence apart from the evaluating of the plants' responses to greywater, periodic watering was chosen as the irrigation method just like how it is done in the traditional gardening in the area to simulate the conditions on the roof top as close as possible to the daily life.

The Preparation of the Greywater Simulation

The daily water use in Turkey is 216 L/p/d (liter/person/day), whereas the daily water use in Trabzon, Turkey, was determined to be 333 L/p/d (Anonymous, 2013b). No reliable data concerning the average consumption of the chemicals involved in the formation of GW in Turkey were found. Thus, developing an average model for GW has emerged as one of the most important steps of the study. For the simulation of average GW, a "Household greywater scenario" (HGWS) that can provide an insight into the average consumption levels was constructed by combining statistical data such as the average number of individuals in a household and empirical data such as hand washing, teeth brushing, shaving, etc. By grouping the activities such as bathing, cleaning, and personal care, etc. according to the scenario, we were able to derive estimates about the weekly frequency of the activities and by whom the activities were performed, the amount of shampoo, detergent, and cleaning product use, and the contents of the greywater generated by water use. According to the simulation, the amounts of the materials in 1 L GW generated within the scope of the study are given below (*Table 1*).

The cleaning products were randomly procured from a grocery at the city center and during the study, same products were used in the same amounts. The storage of GW is one of the most important issues in industrial GW use, the main reason being the emergence of a bacterial growth-facilitating habitat during the storage due to the organic wastes in its content. To evade this problem, GW was not stored in the study and instead, a new HGWS was created using measuring cups before each irrigation. Attention was given to the complete dissolution of each material added to the HGWS and continuous stirring was applied; then, the GW was rested for an hour, followed by it use for irrigation. Although not using storage for the GW used in irrigation may seem like a disadvantage in terms of the accurate representation of the standard case, this concern is minimized by the currently present measures taken against the bacterial growth in the GW generated by the use of industrial products.

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***Table 1.** The amounts of the materials in non-diluted and half-diluted greywaters*

	N-D.PGWS	50%-D.PGWS
Shampoo	0,077 ml/l	0,038 ml/l
Shower gel	0,038 ml/l	0,019 ml/l
Liquid soap	0,225 ml/l	0,113 ml/l
Toothpaste	0,027 ml/l	0,014 ml/l
Dish soap	0,135 ml/l	0,068 ml/l
Surface cleaner	0,027 ml/l	0,014 ml/l
Glass cleaner	0,233 ml/l	0,117 ml/l
Shaving foam	0,167 ml/l	0,083 ml/l
Bleach	0,013 ml/l	0,007 ml/l
Detergent	0,750 g/l	0,375 g/l

In the study, a group of plants was not irrigated and left by themselves after installation, while the other groups were irrigated for two years from March 2015 to March 2017 in every Saturday when no rainfall had occurred. For irrigation, 1 L water was used for each case containing the plants and drip irrigation was preferred. The HGWS was directly applied to one plant group, while the other group received 50%-D.PGWS and the final group directly received TW.

In addition to the irrigation with the GW, all samples were exposed to natural rainwater. Although this may raise a doubt about the accurate uncovering of the relationship between GW and the two Sedum species examined in the GR, since the main concern of the study is the performance of GW in GRs and GRs are already exposed to rainwater, this issue is believed to enhance the ability of the study to reflect the realities of practice. In addition, as all cases containing the plants were exposed to rainfall, the effect of precipitation on GW-plant relationship, if there is any, most certainly takes place under the same conditions as each plant.

The Analyses of Soil, Water and Plants

To determine the plant propagation rate at the end of the two-year experiment in the pots, separate photographs of each pot from each group were taken 1m above the intersection of the diagonals and prepared for examination in an office setting. Each photograph was divided into 5cmx5cm grids in the computer environment and the *Sedum* cover for each pot was established by determining the frames containing the *Sedum* species. The growth of wild weeds both through the soil in which the experiment was installed and through pollination was also observed in the experimental areas during the vegetation period. Weeding was not carried out to observe the competition between the *Sedum* species and wild weeds.

Then, the plant samples were pressed and dried between blotting papers and prepared for identification. The dried and identified plants were then ground in a plant grinding mill and the wet digestion method was employed using HNO_3 to prepare the samples for analysis. The soil samples were laid and dried on blotting papers for one week and the

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blotting papers were replaced three times during this period. Upon drying, the soil was prepared for analysis after sieving with a 2mm sieve.

The heavy metal ratios in the plant, soil, and water samples prepared for analyses were determined and entered in the database using the atomic absorption device of the Research and Application Center of the Central Laboratory of Bingol University. As the same water samples were used for N-D.PGWS and 50%-D.PGWS, water analysis was not separately performed for the each group.

All parameters (soil particle size; pH; EC; organic matter; $CaCO_3$, Fe, Zn, Cu, Mn, Cr, Ni, and Pb contents) were determined by common methods used in practice. The particle size analysis was done using standard hydrometer method described by Gee and Bauder (Gee and Bauder, 1986). The pH and EC of soil samples was measured in the saturation extract by a pH and EC meter (McLean, 1982; Rhoades, 1996). In addition, pH and EC of water samples was measured directly by a pH and EC meter (Tüzüner, 1990). Organic matter was determined by using Walkley– Black method (Nelson and Sommers, 1982). The $CaCO_3$ content was measured with a Scheibler calcimeter after addition of dilute acid to the samples (Nelson and Sommers, 1982). The contents of Fe, Zn, Cu, Mn, Ni and Cr extractable by DTPA in the soil were determined as described by Lindsay and Norwel 1987. In addition, the Pb content was determined by the ammonium acetate method. The total Fe, Cu, Zn Mn, Ni, Cr and Pb contents of plant samples were measured as described by Kaçar and inal (Kaçar nad İnal, 2010).

Results

There is no doubt that evaluating ecological conditions that affect plants on a GR and in a pot on a conventional roof is not exactly the same thing as the conventional one can could warm up faster and more intensively, which probably makes the conditions a bit more challenging. However, because all the plants in the research area was in the same conditions and because performances of the plants in harder conditions are important while rooftops are knowns as tough habitats, this micro climatic situation was ignored. The propagation rates of the *Sedum* species and the chemical content of the plants are the two most important parameters in the evaluation of the effect of the use of GW as the irrigation water on herbal life in GRs, which was the starting point of this study as well. Thus, the analyses for each pot was carried out by considering these two parameters and the results are given below (*Table 2*).

According to the field measurements, the highest coverage (67.622%) was observed in the trial with N-DPGWS irrigation, while the lowest coverage (34.376%) was observed in the N-I trial. As for the case of *Sedum* species-specific coverage, the *Sedum album* species irrigated with N-D.PGWS had the highest cover rate (60.922%), while the lowest cover rate (12.830%) was observed in the *Sedum sediforme* species irrigated with TW (*Figure 2*).

Wild weed coverage ratio was calculated by comparing it with the whole plant coverage within the 5cm-5cm grids on each pot. The comparison of the total cover rates with the differences between the cover rates of the *Sedum* species revealed that the trial with the TW irrigated *Sedum sediforme* species had the highest wild weed content (47.786%), while the trial with the N-D.PGWS irrigated *Sedum album* species had the lowest wild weed content (6.7%) (*Figure 3*).

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Table 2. *The soil, plant, and water analysis results with respect to the irrigation types*

	N-I		TW		50%-D.PGWS		N-D.PGWS	
	Sedum album	***Sedum sediforme***	***Sedum album***	***Sedum sediforme***	***Sedum album***	***Sedum sediforme***	***Sedum album***	***Sedum sediforme***
Total Coverage (%)	45,608	34,376	65,086	60,616	61,900	50,662	67,622	52,112
Sedum Coverage (%)	36,940	15,886	19,124	12,830	38,546	33,506	60,922	24,350
Fe* (ppm)	16,639	16,569	16,986	17,640	10,353	16,401	16,807	19,257
Cu* (ppm)	0,476	0,511	0,651	0,686	0,357	0,504	0,483	0,952
Mn* (ppm)	10,948	12,999	1,323	1,358	6,636	10,871	10,647	1,568
Zn* (ppm)	1,253	1,477	12,341	12,509	1,204	1,344	1,512	9,023
Ni* (ppm)	0,266	0,490	0,385	0,238	0,378	0,497	0,420	0,455
Cr* (ppm)	8,890	1,267	9,982	10,052	13,391	7,462	12,810	11,396
Pb* (ppm)	2,400	2,330	2,400	2,330	2,320	2,360	2,550	2,620
O. Matter (%)	1,550	1,570	1,580	1,620	1,650	1,630	1,490	1,470
Ph*	6,630	7,000	6,780	6,940	7,000	7,130	7,220	6,970
EC* (µS/cm)	152,900	164,500	152,200	165,300	189,500	196,800	159,500	148,000
$CaCO_3$ S (%)	0,840	0,880	0,840	0,850	0,920	0,330	1,050	0,760
Fe** (ppm)	833,000	1693,500	1512,500	1053,500	172,550	1687,500	150,100	1028,500
Cu** (ppm)	43,450	78,450	19,550	45,200	19,850	65,800	16,150	43,400
Mn** (ppm)	4,250	11,300	5,800	6,950	3,850	9,950	3,900	8,900
Zn** (ppm)	18,900	55,200	21,200	41,300	16,650	38,750	9,300	59,700
Ni** (ppm)	6,500	1,800	6,600	9,950	5,350	18,550	7,250	1,900
Cr** (ppm)	134,000	125,550	94,200	134,900	14,350	20,500	56,750	115,300
Pb** (ppm)	0,880	0,890	1,010	0,900	0,960	1,020	0,110	1,140
Fe*** (ppm)	-		0,23		-		1,05	
Cu*** (ppm)	-		0,09		7,000		7,65	
Mn*** (ppm)	-		0,04		1,700		1,9	
Zn*** (ppm)	-		-		-		-	
Ni*** (ppm)	-		-		-		-	
Cr*** (ppm)	-		0,25		125,000		121,3	
Pb*** (ppm)	-		0,02		0,470		0,59	
Ph***	-		6,952		7,130		7,27	
EC*** (µS/cm)	-		178,22		1021,000		1098	

*: Soil analysis; **: Plant analysis; ***: Water analysis

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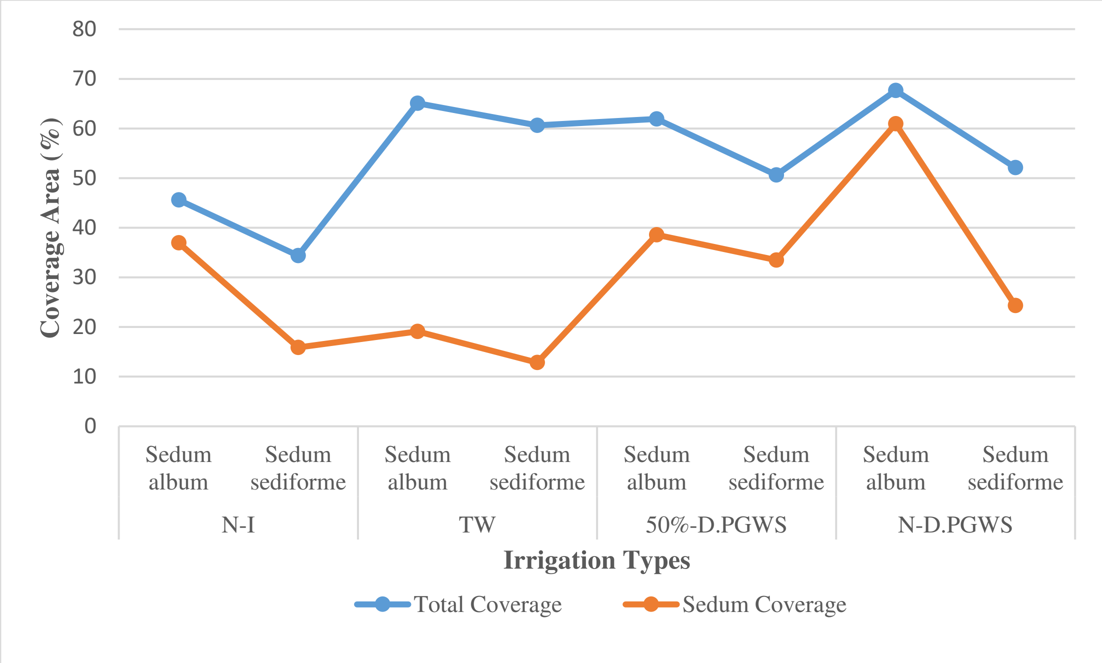

Figure 2. *The cover rates with respect to the irrigation types*

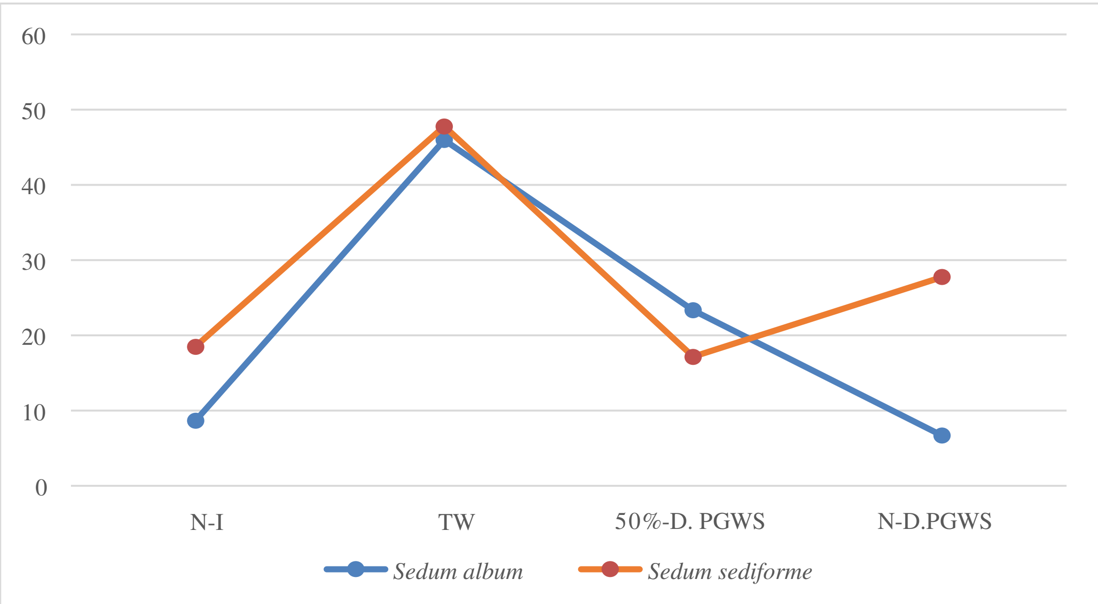

Figure 3. *The wild weed content of the trial pots with respect to the irrigation types*

Conclusion and Recommendations

Making a classification according to the irrigation types, non-irrigated plants were coded as ‘N-I’, the plants irrigated with tap water were codes as ‘TW’, the plants irrigated with 50 % diluted water were coded as ‘50%-D.PGWS’, and the plants irrigated with non-diluted water were coded as ‘N-D.PGWS’ while, in terms of *Sedum* species, *Sedum album* was coded as ‘*S. Al.*’and *Sedum sediforme* as *‘S. Sed.’* and recorded into the database.

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DOI: http://dx.doi.org/10.15666/aeer/1702_36673680

In *Table 3*, in a comparison between the irrigation type and the cover density of *Sedum* species, it is seen that the highest average value was obtained in N-D.PGWS *(S. al.)* group (x=60,400). This rate was followed by 50%-D. PGWS *(S. al.)* (x=38,000), N-I *(S. al.)* (x=36,600), N-I *(S. sed.)* (x=34,000), 50%-D. PGWS *(S. sed.)* (x=33,000), N-D.PGWS *(S. sed.)* (x=23,800) and TW *(S. al.)* (x=18,600). The irrigation type that *Sedum* species exhibited the lowest propagation was determined to be TW *(S. sed.)* (x=12,400).

Table 3. *Results of the One-way Analysis of Variance performed to determine the differences between the coverage area of Sedum species according to the irrigation variable*

	Irrigation Type	N	X	SE	F	Sig.
Coverage Area of *Sedum* Types	N-I *(S. sed.)*	10	34,000	5,568	6,767	0,000
	N-I *(S. al.)*	10	36,600	2,337		
	TW *(S. sed.)*	10	12,400	3,385		
	TW *(S. al.)*	10	18,600	5,354		
	50%-D. PGWS *(S. sed.)*	10	33,000	5,797		
	50%-D. PGWS *(S. al.)*	10	38,000	9,338		
	N-D.PGWS *(S. sed.)*	10	23,800	1,497		
	N-D.PGWS *(S. al.)*	10	60,400	7,325		

To determine if different irrigation types have a significant effect on the propagation types of *Sedum* species, the statistical analysis One-Way Anova test was utilized. As a result of the statistical analysis, a statistically significant difference was determined F=6.767, ($p<0.05$). To determine the source of the difference, the Tukey test of the Post Hoc tests was utilized. The direction of the difference was determined to be N-D.PGWS *(S. sed.)*-TW *(S. sed.).*

To examine whether different irrigation types significantly affect the overall propagation rates, One-Way Anova test was used *(Table 4).* As a result of the statistical analysis, no statistically significant differences were found. In the study, for the *Sedum* species used to determine the performance of GW in GRs, it was determined that irrigation with GW had a positive effect on the coverage area.

Table 4. *One-way ANOVA results to determine the differences between total coverage area according to irrigation variables*

	Irrigation Type	N	X	SE	F	Sig.
Total Coverage Area	N-I *(S. sed.)*	10	34,376	5,590	2,147	0,67
	N-I *(S. al.)*	10	45,590	4,784		
	TW *(S. sed.)*	10	60,616	7,362		
	TW *(S. al.)*	10	65,186	9,575		
	50%-D. PGWS *(S. sed.)*	10	50,662	4,732		
	50%-D. PGWS *(S. al.)*	10	61,900	10,902		
	N-D.PGWS *(S. sed.)*	10	52,112	8,630		
	N-D.PGWS *(S. al.)*	10	67,622	7,361		

When the same analysis was performed for the wild weeds in the pots and all the areas covered green, it was seen that the irrigation type had no effect on the propagation area. GRs are known as surfaces where living conditions are not so easy due to many conditions including solar energy and intense winds that they are exposed to. Therefore, certain

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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species with high resistance to these challenging conditions are very popular in both scientific researches and practice studies in this field. *Sedum album* and *Sedum sediforme* are two of the most known species of these species and are selected as the materials in this research. Numerous wild weeds that came to the area after these species were brought to the area, since they can easily adapt to and tolerate these difficult conditions, were found abundantly even in the N-I trial and, therefore, inhibited the effect of the green area associated with irrigation. At the point, the best answers to the question "So, why are *Sedum* species primarily preferred for the GRs?" are that they are always green compared to these wild species, they cover the area in a more compact way and they form a more aesthetic and successful layer in terms of thermal insulation. In addition, the contribution of these natural *Sedum* species to the biological diversity and the effect of feeding the wild life is inherently higher.

Examining the correlation tests, it was determined that the propagation of *Sedum* species is inversely proportional to Fe (Fe **) and Pb (Pb **) elements in the plant *(Table 5).*

Table 5. *Sedum cover density correlation analysis*

		Fe**	Pb**
***Sedum* Coverage**	Pearson Correlation	-,716*	-,745*
	Sig. (2-tailed)	,046	,034
	N	8	8

Iron has an important feature since it can easily form clay with organic complexes in soil and plants. Therefore, iron can become soluble in the upper layers of the overly washed and low-drainage soils, and it adheres to the lower soil layer (Karaman, 2012). In the nutrient environments such as the roof gardens where the depth of the soil is low, Fe can be transported to the plant via the plant roots even if it adheres to the lower soil.

Excessive Fe intake forms a toxic effect in plants. Tanning is the most important example of this toxic effect in plant leaves. This is observed when Fe content in leaves is 700mg/kg (Anonymous, 2005; Yamauchi, 1989). Tanning in iron toxicity results from oxidized polyphenols (Peng and Yamauchi, 1993). K application can be carried out to eliminate iron toxicity. In this case, K both reduces the Fe^{2+} uptake and increases oxidative potential of roots (Güneş et al., 2000).

Lead is not absolutely required for plants, but it is found in the soil at a dose of 15-40 ppm. It is potentially dangerous for human health when it exceeds 300 ppm (Özkan, 2017; Kaçar et al., 2013; Dürüst et al., 2004). Lead affects the plant water regime due to its negative effects on cell turgor and cell wall stability, and its reducing effect on stoma movements and leaf area. Also, it reduces the uptake of cations and anions since it is held by the roots and it reduces root development, affecting nutrient uptake (Tao et al., 2007; Sharma, 2005).

Examining the propagation of the species in total, it was determined that the cover density was directly proportional to Cr (Cr *) in the soil and pH value (pH ***) of the irrigation water and inversely proportional to Cu (Cu **) in the plant *(Table 6).*

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Table 6. *Total cover density correlation analysis*

		Cr*	Cu**	Ph***
Total Coverage	Pearson Correlation	,850**	-,765*	,807*
	Sig. (2-tailed)	,008	,027	,015
	N	8	8	8

Chrome is naturally found in soils in amounts ranging between 5-100 mg/kg (Asri and Sönmez, 2006; Özbek et al., 1995). The first physiological process affected by the toxic chrome levels in plants is seed germination (Knezevic et al., 2009; Jain et al. 2000). Although chrome reportedly inhibits the development of roots through obstructing cellular division in roots and root growth and thus reduces plant growth and development through decreased water and nutrient intake from soils (Khan et al., 2000), the statistical analysis of the results obtained with the experiment installed in this study showed that the total cover density increased with increased chrome levels in soils.

The toxicity level of copper differ among plants (Knezevic, 2009; Robson and Reuter 1981; Hodenberg and Finck, 1975). The first symptoms of copper toxicity in copper-sensitive plants include decreased root growth, damaged plasms and membranes, and release of potassium (K) ions (De Vos et al., 1991; Baker and Walker, 1989). The decreases in the indole-3-acetic acid (IAA) oxidase activity in plants due to exposure to high Cu concentrations lead to certain changes such as reduced root growth and lateral root formation (Karaman, 2012).

The pH of the irrigation waters directly or indirectly affects various physical, chemical, and biological phenomena that occur in soils. There is a close relationship between soil reactions and soil biota; for example, fungi are more active at pH 4-5, while bacteria are more active at pH 6-8. Moreover, pH levels also play an important role in the availability of the nutrients found in soils to vegetables. The most suitable pH range for nitrogen, phosphorus, and potassium intake by vegetables is between 6.5 and 7.5. At pH levels below 6.0, phosphorus bonds with Al and at pH levels above 7.5, phosphorus bonds with Ca, which obstructs its intake by plants. Al and Mn have toxic effects on plants at pH levels below 5.0, while at pH levels below 7.5, microelements such as Fe, Cu, Zn, Mn convert to a non-soluble form and their availability to plants significantly decreases (Url 1; 2018). As a result of the statistical analyses, it was seen that the Cu taken from the soil was inversely proportional to the plant growth. It is expected that the pH of the irrigation water will increase the plant propagation since the pH of the water makes the element of Cu insoluble and decrease its intake by the plants, preventing the toxic effect of Cu.

Evaluating the study as a whole, it was seen that there was a significant difference between irrigation and non-irrigation of *Sedum*, one of the most popular GR plants, in terms of the roof surfaces they cover. In addition, since clean water is one of the most valuable items in the world, considering GW as an alternative irrigation method, it was seen that this usable waste water can decrease, even eliminate, the use of important and expensive TW. It was also seen that the use of GW provides more positive effects on the plant growth compared to that of the TW. In this sense, by using GW in GRs, it would be possible to achieve both economical and ecologically more favorable results, by both reducing water consumption and providing positive effects on plant growth. Moreover, it is also clear that utilizing this waste water will reduce the irrigation costs of the GR systems and consider the GR as a more sustainable and economic alternative in the long run, thus becoming more widespread and having an impact on urban sustainability. Yalçınalp at al. (2018) has stated that the use of the industrial system required for GW use would prove to meet the GR cost in the long run and that greywater should be used as irrigation water. Despite its ecological and economic benefits, the incentives and scientific studies on using GW in the irrigation for the widespread use of GR systems, which are still not widespread as it should be, will greatly contribute to making the world a more sustainable place.

Acknowledgements. This research was designed as a part of a master thesis of which the title is "Performance of Greywater Irrigation on Two *Sedum* Species", which was supervised by Emrah Yalçınalp.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36673680

EFFECT OF ORGANIC AND INORGANIC SOURCES OF NUTRIENTS ON THE BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF TOMATO

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(Received 21st Dec 2018; accepted 20th Feb 2019)

Abstract. The study was conducted to compare the levels of bioactive compounds and antioxidant activities of (*Solanum lycopersicum* var. cerasiforme) fruits cultivated on different soil amendments (cow dung, chicken droppings and ([40 g/kg N: 60 g/kg N: 40 g/kg N] Nitrogen-Phosphorus-Potassium) in a shaded house. Tomato seedlings were transplanted into pre-treated soils and watered daily in the afternoon for a period of five months. The general growth and yield performances were in the order of NPK > Chicken droppings > Cow dung. The fruits were harvested at maturity (red-ripened stage) and subjected to bioactive compounds analysis using High Performance Liquid Chromatography (HPLC). The results of the study showed strong relationship between the level of macronutrients like nitrogen, phosphorus and potassium in the soil and the level phenolics, flavonoids, beta-carotene and lycopene contents in tomato. The level of beta-carotene was significantly higher ($p < 0.05$) in the fruits cultivated in the NPK amended soil which was totally different to the result of other bioactive compounds like phenolics, flavonoids, and lycopene which recorded their highest concentrations in the fruits harvested from control and cow dung amended soil. Antioxidant activity was determined by adding 2,2-diphenyl-1-picrylhydrazyl to an aliquot of methanolic extract. The ability to scavenge radical was measured by reading the decrease in purple coloration of the solution using Ultraviolet-Visible (UV) Spectrophotometer. The tomato fruit from cow dung showed highest percentage of radical scavenging activities.
Keywords: *abiotic and oxidative stress, acetate shikimate pathway, cow dung, physiological changes, reactive oxygen species*

Introduction

The use of chemical fertilizer has been a popular and widespread practice in the effort to address global food security challenge resulting from low fertile agricultural soil. The dependence on these chemical fertilizers, particularly nitrogen fertilizer has become necessary to replenish soil nutrients and invariably improve the quantity and quality of agricultural produce. High crop yield and high biomass of plant from N-fertilizer application are responsible for increasing dependent on N-fertilizer (Camara et al., 2003; Guo et al., 2010). However, intensive usage of N-fertilizer has been reported to cause accumulation of nitrogen residues, nutrient toxicity, metal pollution, greenhouse gas emission, groundwater contamination and soil acidification (Han and Zhao, 2009; Sierra et al., 2015). Furthermore, research has shown that increase in applied nitrogen leads to increase in the soil electrical conductivity, available nitrogen, distortion of C/N ratio, distorting soil microbial community and reduction in nutritional quality of agricultural produce (Stuart et al., 2014; Sierra et al., 2015; Norman and Dazzo, 2016; Wei et al., 2018). It has been established that crops only take up 30-50%

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

of chemical fertilizers and the remaining percentage are lost to the environment (Norse, 2005; Mózner et al., 2012).

Conversely, the use of organic manure, has been reported to improve biological, chemical and physical properties of the soil and invariably increase plant growth and yield because of its high organic matter content due to high microbial activity (Stephen et al., 2014; Mitran et al., 2017). Organic manure, as opposed to inorganic fertilizer has also been reported to increase the level of secondary metabolites like phenolic, flavonoid, and antioxidant activity in plant (Zeinab et al., 2013; Fließbach et al., 2007). For example, Wiebel et al. (2000) reported a 19% higher phenolic content for organically produced apples compared to the inorganically produced ones. Higher level of phenolic content was also recorded for organically grown strawberries compared to inorganically cultivated ones (Hakkinen and Tomonen, 2000). All these reported cases have led to growing preference by consumers for organic agricultural produce (Hughner et al., 2007; Mie, et al., 2016). All over the world, attention has now shifted towards the use of organic manures because of the perceived health benefits and higher nutritional contents believed to be derived from products from these types of farming (Worthington, 2001; Magkos et al., 2001; Tarozzi et al., 2006). This submission is supported by the studies which reported that the positive attitude of the consumers towards organic food is based on their perception of higher nutritional contents and better tasting of organic food compared to the conventional or inorganic foods (Hunter et al., 2011; Smith-Springer et al., 2012). Organic manures are said to enhance antioxidant activity and bioactive compounds like phenol, flavonoid, beta-carotene and lycopene contents of fruits and vegetables (Dumas et al., 2003; Mohd et al., 2013). The beneficial effects of these plant bioactive compounds range from giving fruits their quality tastes and distinct colors in the case of phenolic and lycopene compounds, to their nutraceutical properties such as prevention of degenerative and carcinogenic diseases (Johnson et al., 2014; Canene-Adams et al., 2005). These reported reasons and benefits of organic farming have prompted scientists and farmers to embark on the use of organic manures in the cultivation of agricultural crops, fruits and vegetables.

However, there are a lot of reports which contradicts the suggestion of superior nutritional quality of organic produce over conventional ones. This study was therefore setup to investigate the effects of these different nutrient sources on the nutritional quality of *Solanum lycopersicum* var. Cerasiforme commonly known as tomato.

Tomato is a fruit bearing herbaceous plant grown annually for its many nutritional contents. It is an important fruit with a wide range of reported health benefits and cultivated in all continents except Antarctica (Ilahy et al., 2011; Pinela et al., 2012). It is one of the cheapest and most readily available sources of proteins, minerals, vitamins and essential amino acids (Stephen et al., 2014), and is said to be rich in antioxidants and bioactive compounds such as phenolics, flavonoids, beta-carotene and lycopene contents which are reported to serve as endogenous defense mechanisms produced in response to pathogens (Pinela et al., 2012; Bhowong et al., 2009; Simova-Stoilova et al., 2008). The concentration level of these bioactive compounds has been linked with the system of production. The effort to reduce the negative impacts of mineral fertilizers on the environment and the increasing demands of consumers for tomato fruit due to its nutritional and health benefits have caused scientists and growers to device means of meeting the demand for quality produce. This study was therefore conducted to compare the effect of organic manures and chemical fertilizer on the growth rate and the levels of bioactive compounds in tomato.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

Methodology

A farming experiment was set up at the production unit of Sefako Makgatho Health Sciences University located in the northern part of Pretoria (Tshwane metropolitan) South Africa, on a coordinate (25°37’8” and 28°1’22” E). The study area experiences a climate of long hot, rainy summer and a short cool and dry winter with an average annual temperature of 18 °C (65.7 °F).

Experimental design and planting

The farming experiment was carried out in the summer period of September 2015 – March 2016. The study was conducted on red beefsteak tomato with a globe shape fruit, smooth and glabrous skin and weighs between 0.12 – 0.18 kg. The design comprised three different soil amendments (cow dung, chicken droppings and Nitrogen-Phosphorus-Potassium (NPK) fertilizer with a control experiment of soil with without any amendment. The experimental design consisted of comparable amounts of 0.15 kg of inorganic amendment (NPK fertilizer) manufactured by Omina Fertilizers Johannesburg, South Africa was purchased from a registered marketer, Plantland Kwikery Akasia Pretoria North, South Africa. The dry organic amendments (cow dung and chicken droppings) were collected from a registered livestock farm De-Wildt Brits Road North west South Africa. The 0.15 kg weight of the $N_{40}P_{60}K_{40}$, i.e. N_{40} is 40% Nitrogen, P_{60} is 60% phosphorus and K_{40} is 40% potassium used as the basis for the comparison of the organic amendments falls within the recommended doses of NPK fertilizer for the cultivation of tomato (Hebbar et al., 2004). The experiment was a randomized block design which contained 32 pots, each with a 22 cm diameter (Alain et al., 2013) divided into four groups which were each filled with 5 kg of sandy-loam soil mixed with 0.15 kg of the amendment. The first group were each filled with an equal quantity of soil mixed with dry 0.15 kg of cow dung; the second group contained equal quantity of soil thoroughly mixed with dry 0.15 kg of chicken droppings; the third group were filled with the same quantity of soil thoroughly mixed with 0.15 kg of pellet $N_{40}P_{60}K_{40}$ fertilizer and the last group contained pot plants each filled with equal quantity of soil without any treatment which served as the control. The soils were continuously watered and turned over at two weeks interval with garden forks and left for over a month after the addition of the treatments for proper mineralization of the amendments to take place (Ayeni, 2014). The Pot plant saucers/ trays were placed under the perforated bottom pots to prevent nutrient loss by runoff during irrigation. The seedlings of tomato at two weeks old purchased from a registered nursery, Plantland Kwikery Nursery Akasia Pretoria North were transplanted into each pot plant and watered daily using a measuring cylinder with each plant receiving the same volume of water enough to moist the soil and the plants and preventing runoff.

Soil nutrients

The soil is a sandy loamy with 71% sand, 15% silt and 14% clay. The control and cow dung amended soils were low in total N, available P and exchangeable K compared to NPK and chicken droppings treated soils (*Table 1*).

Data collection

Parameters such as plant height, stem girth and leaf area index were recorded for the determination of growth performance of tomato plants from different amendments and the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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control following the procedure of (Stephen et al., 2014). The Plant height was measured using a meter rule. Stem girth was recorded by using a vernier caliper and the leaf area index was measured by determining the average of the distances between the two longest and the two shortest branches using a meter rule (Witness et al., 2016).

Table 1. *Composition of soil nutrients*

Treatments	Total N (%)	Available P (µg/g)	Exchangeable K (µg/g)
Cow dung	0.8	552	535
Chicken droppings	1.2	647	443
NPK	1.8	670	456
Control	0.6	518	376

Preparation of tomato fruit for phytochemical and antioxidant activity analyses

Tomato fruits from soil treated with different amendments and that of the control were harvested after ripening. Visually selected injury free, ripened fruits were picked and immediately transferred to the laboratory where they were cut into small pieces and homogenized. The homogenates were then frozen in a -80 °C refrigerator and freeze-dried for the phytochemical and antioxidant activity analyses.

Extraction and determination of free radical scavenging activity

The methanolic extraction was carried out by sonicating the mixture of 100 ml of 50% methanol into an Erlenmeyer flask containing 6 g of freeze-dried powdery sample for 60 min. The resulting solution was thereafter filtered through a Whatman No. 1 filter paper and the filtrate (extract) placed in fume cupboard for 24 h for a complete evaporation of methanol. The determination of free radical scavenging activity was done by following the procedure described by (Biehler et al., 2010). An aliquot (10 mg) of methanolic extract was dissolved in 1 ml of 50% methanol from which 30 µl of the solution was pipetted into an Eppendorf tube. This was made up to 750 µl volume to which 750 µl of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was added. The solution was incubated for 40 min in the dark at room temperature. The decrease in the purple coloration of the reaction mixtures was read at 517 nm using an Ultraviolet-Visible Spectrophotometer (SPECORD 210 PLUS). Ascorbic acid was used as the standard and methanol used for extraction served as a negative control. The assay was performed in triplicate and the radical scavenging activity (RSA) was calculated using *Equation 1:*

$$RSA(\%) = A_0 - \frac{A_1}{A_0} \cdot 100 \quad \text{(Eq.1)}$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Beta-carotene and lycopene contents

Extraction and quantification of β-carotene were done using the method described by (Moyo et al., 2017). Samples were extracted with ice-cold hexane: acetone (1:1, v/v). The mixture was vortexed before centrifuging at 2000 rpm for 2 min and the organic phase decanted into another test tube containing 5 ml sodium chloride solution. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

residue was similarly re-extracted and was combined in the saturated sodium chloride solution each time until the extract was colorless. The separated organic phase was injected into a High-Performance Liquid Chromatography (HPLC) equipped with a Photodiode Array Detector (PDA) and a mobile phase consisted of (7:2:1) acetonitrile-dichloromethane-methanol with an injection volume of 20 µl and detection at 450 nm. Peak identification and quantification were achieved with comparison to authentic β-carotene standard which was used for the plotting of the calibration curve (Moyo, et al., 2017).

Total phenolic

The method described by Singleton et al. (1999) was used for the extraction and the determination of the total phenolic content. An aliquot (10 ml) of 50% methanol was added into an Erlenmeyer flask containing 0.2 g of the freeze-dried tomato sample and the mixture sonicated for 30 min. The resulting solution was subsequently centrifuged in a HERMLE Z513 at 2000 rpm for 2 min. About 50 µl of the supernatant was pipetted into a new test tube and 450 µl of distilled water, 450 µl of 1N folin ciocalteu reagent, 125 µl of sodium carbonate solution were added. The solution was vortexed and incubated for 40 min at room temperature. Different aliquots of gallic acid (0.1 mg/ml) was used as the standard for plotting the calibration curve and total phenolic content was expressed in mg gallic acid equivalents (GAE) per g dry weight.

Flavonoid content

The flavonoid content was quantified using aluminum chloride colorimetric method as described by (Zhisten et al., 1999). An aliquot (0.2 g) of the freeze-dried tomato sample was weighed into an Erlenmeyer flask into which 10 ml of 50% methanol was added and the mixture sonicated for 30 min. The solution was then vortexed and centrifuged at 2000 rpm for 2 min and the supernatant pipetted into a new test tube. About 250 µl of the supernatant was pipetted into a new test tube and 1 ml of distilled water, 75 µl of 5% $NaNO_2$ were added. After 5 min, 75 µl of 10% $AlCl_3$, 500 µl of 1 M NaOH and 600 µl of distilled water were added and the solution vortexed. Different aliquots of gallic acid (1 mg/ml) catechin was used as standard for the calibration curve and flavonoid content was expressed in mg catechin equivalents (CE) per g DW.

Statistical analysis

All statistical analyses were performed using SPSS 24.0 by carrying out analysis of variance to determine the statistical significance and post hoc test using Tukey for the separation of the means of growth performance, bioactive compounds and scavenging activity from the different treatments.

Results

Effect of different soil amendments on the growth and yield performances

The results of the growth and yield performances were presented in *Tables 1* and *2* (Aina et al., 2018). Although, plants from control soil and cow dung amended soil recorded higher plant height and canopy size at the early stage after transplanting. This observation according to literature, may be due to the physiological adjustment of plant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

to the new environment (Ayeni et al., 2014). Overall, the tomato cultivated in inorganic fertilizer (NPK) recorded the best growth rate which was determined by recording the height, canopy size and stem girth of the plant. Similarly, the yield results showed that NPK produced the highest number of tomato fruits, followed by chicken droppings and with a yield pattern of NPK > chicken droppings > cow dung > control. This yield result is also consistent with other study which suggested that it may be a result of deficiency and or slow mineralization of nutrient of organic manure during the plant growing and developmental stages (de Ponti et al., 2012).

Effect of different soil amendments on the level of bioactive compound concentrations

The varied level of bioactive compounds of the tomato fruits as influenced by different soil amendments are presented in *Table 2*. The results showed that the level of phenolics, flavonoids, and lycopene were lower in the fruits harvested from inorganic NPK fertilizer compared to their counterparts from the control and cow dung amended soil. The difference in the level of phenolics flavonoids and lycopene in the fruits cow dung manures, NPK and control soil were statistically significant, the difference in the lycopene across different treatments was statistically significant. However, in reverse pattern, beta-carotene (*Fig. 1*) was significantly higher in fruit harvested from the soil treated with NPK fertilizer compared to the ones from the rest of the amendments and control. Finally, although the tomato fruit from cow dung amended soil recorded the highest concentration of potential radical scavenging activity, there was however no significant difference in the radical scavenging activity of the fruits across the group (*Fig. 2*).

Discussion

It has been suggested that nitrogen stimulates more roots production which in turn enhance plant growth and yield performance (Sultana et al., 2012; Rai et al., 2013). The study done by Mani et al. (2002) reported higher plant height and yield in plant cultivated with NPK fertilizer and attributed this observation to higher N and P contents. Chemical fertilizer like NPK has been reported to increase plant biomass and crop yield due to its richness in N and P contents which are reported to promote growth in plant (Ekbic et al., 2010; Zeng et al., 2011; Stephen et al., 2014; Mishra and Singh, 2006). The growth result of the current study is consistent with all these previous observations. In this study, application of NPK fertilizer enhances plant height, stem girth and leaf area index considered to be key factors in determining growth vigor. The higher growth response of tomato may be due to the higher nitrogen (N) and phosphorus (P) contents in the NPK compared to other treatments (*Table 1*).

This observation corroborates previous findings that NPK and poultry manure recorded better yield compared to control and cow dung (Znidarcic et al., 2007; Hague, 2012). The better growth rate and yield performance of NPK and chicken droppings compared to the cow dung and control could be due to the high content of essential nutrients like N and P in poultry manure which have been reported to enhance photosynthetic activity capable of promoting root development, vegetative growth and crop yield (John et al., 2004; Lima et al., 2012; Isah et al., 2014; Nafiu et al., 2011; Meysam et al., 2017).

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Table 2. *The mean concentration of bioactive active compounds in response to different soil treatments with different letter(s) indicating significant difference at 95% confidence interval*

Treatments	Phenolics (mg GAE/g DW)	Flavonoids (mg CE/g DW)	Lycopene (mg/100 g DW)
Cow dung	3.90[a]	0.40[b]	129.76[b]
Chicken droppings	3.20[b]	0.27[c]	114.50[d]
NPK	3.81[a]	0.34[b]	123.21[c]
Control	3.91[a]	0.56[a]	132.66[a]

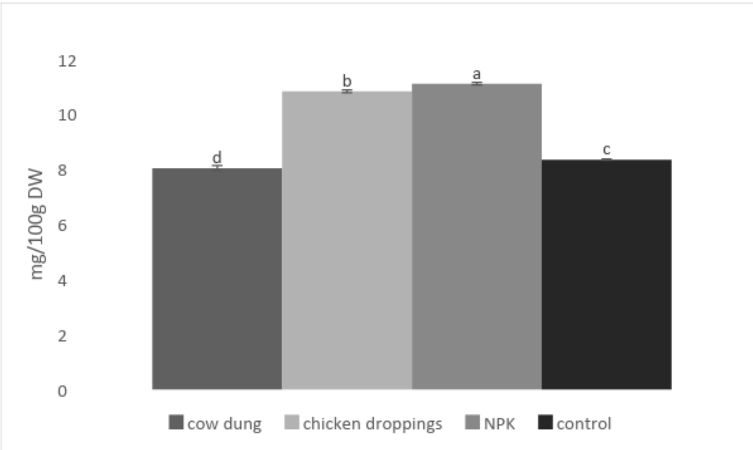

Figure 1. *The level of beta-carotene in tomato fruits with different letters indicating significant differences (p < 0.05)*

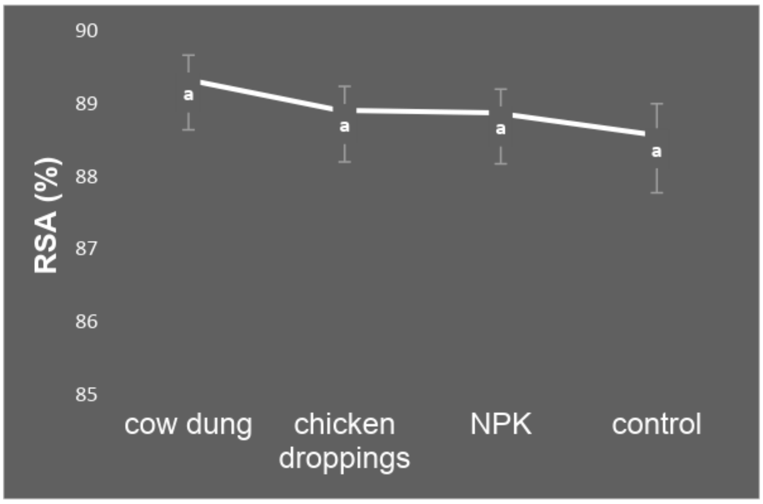

Figure 2. *Free radical scavenging activity of tomato fruits same letter indicating no significant difference (p > 0.05)*

On the other hand, organic manures are reportedly less concentrated nutrient sources compared to mineral or chemical fertilizers and are noted to be very slow in mineralization rates, leading to lower nutrient bioavailability during growth and development nutrients demanding stages (Zhao, et al., 2009; Lester and Saftner, 2011;

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DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

Seufert et al., 2012). This condition causes organically cultivated tomato to experience; 1. oxidative stress from superoxide dismutase which is a key enzyme in plant defense and development (Chang et al., 2008), and 2. abiotic stress which causes accumulation of Reactive Oxygen Species (ROS) that inhibits enzymatic activity, disturbs cellular homeostasis and ruptures membrane with deleterious effects on plant growth (Luis, 2015; Baxter et al., 2014). Consequently, organically grown vegetables react to this condition by activating their own defense mechanisms which leads to the synthesizing of more bioactive and antioxidant compounds (Vallverdu-Queralt et al., 2011). They activate signaling pathway for the detoxification of ROS by synthesizing antioxidant that scavenges on ROS (Sharma et al., 2012).

It is therefore conceivable that the result of this study which showed the tomato fruits cultivated in control and organic cow dung amended soils producing higher phenolic, flavonoid and lycopene contents in comparison to their counterparts from chicken droppings and especially inorganic NPK fertilizer amended soil corroborates these reports. The cow dung amended soil as well as the control were low in essential nutrients like N and P in comparison to the soil treated with chemical (NPK) fertilizer (*Table 1*). Several literatures have linked the accumulation of phenolics, flavonoids and lycopene to the level of available N in the soil. For instance, Oliveira et al. (2013) and Mitchell et al. (2007) in their separate studies which investigated the effects of organic and inorganic fertilizers on plant secondary metabolites, reported that organically produced tomatoes accumulated higher concentrations of bioactive compounds like total phenolics and flavonoids compared to inorganically grown ones. These compounds have been reported to accumulate more in plants cultivated on organic manures due to low level of N (Mittler, 2002; Abd El-Moniem et al., 2012; Mohd et al., 2013). Numerous studies have explained that plants tend to produce more or higher level of bioactive compounds and antioxidant as a preventive or protective measure against oxidative and abiotic stress which may result from low level or slow release of macronutrient like N (Caris-Veyrat et al., 2004; Toor et al., 2006; Wang et al., 2008; Vallverdu-Queralt et al., 2012; Oliveira et al., 2013). This conclusion is backed up by several other studies on the nutritional quality of fruits and vegetables, which reported that organically produce have approximately three times less nitrate in comparison to conventional crops due to lower availability of nitrogen in organic farming systems (Worthington, 2001; Williams, 2002; Tuomisto et al., 2012).

The result of the current study is consistent with the above submissions as evident in the concentrations of phenolics, flavonoids and lycopene in the tomato fruits from control and cow dung amended soils, both growing media with lower level of available N compared to NPK fertilizer and chicken droppings. Whereas lower concentrations of total phenolic, flavonoid and lycopene were detected in the fruits harvested from soils amended with chicken droppings and NPK fertilizer, both growing media with higher level of available N. It was therefore assumed that there is an inverse relationship between the concentration of bioactive compounds like phenolics, flavonoids, lycopene and the level of available N in the soil.

Other studies concluded that organic manures have stimulatory effect on the accumulation of bioactive compounds by inducing acetate shikimate pathway in biosynthesis, resulting in higher production of secondary metabolites like flavonoids and phenolics (Sousa et al., 2008; Yoldas et al., 2008). It must be noted that although the results of this study showed a significantly higher lycopene content in both the control and the organic cow dung amended soils, some studies have argued that, types

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of soil fertilization have no effect on lycopene content of fruits. It was explained that lycopene content is accumulated more in the deep ripen stage (Toor et al., 2006; Ilahy et al., 2011). The current study therefore identified this area for further studies in future research.

This study however showed a result of opposite pattern for beta-carotene which was significantly higher in the fruit cultivated in the soil treated with NPK fertilizer and closely followed by that from chicken droppings. This could be due to the higher content of available P in the NPK fertilizer and chicken droppings manure which has been reported to increase beta-carotene contents in fruits and vegetables (George and Zhou, 2002; Abd El-Baky, 2010). Furthermore, the action of phosphorus on enzymes like phosphofructokinase, pyruvate kinase and precursors of pyruvate has been linked with the biosynthesis of carotenoid (Bramely, 2002; Black et al., 2008). The study by Meysam et al. (2017) also reported that increased level of P application led to increase level of carotenoid production.

Although, there was no statistical significance in the potential ability to inhibit and retard oxidation processes, also known as radical scavenging activity of the fruits, it was however slightly higher in the fruits from the soil treated with cow dung. There is no conclusive report on what is responsible for higher production or stimulation antioxidant activity in plant. While some reports have suggested that organic production increases plant antioxidant activity (Janzantti et al., 2012; Vinha et al., 2014), other studies with similar observation to the current study have reported no significant difference in the antioxidant activity of plants from organic and conventional system of production (Fischer et al., 2007; Reche et al., 2019).

Conclusion

In general, this study demonstrated the responsiveness of bioactive compounds in tomato to the level of soil macronutrients which are relatively low or slowly released in organic amendments. It was observed that nitrogen content in the soil has a significant effect on the level of certain bioactive compound synthesis. Although, inorganic NPK fertilizer produced highest growth, yield and beta-carotene, control and organic cow dung amended soils had a higher stimulatory effect on the bioactive compounds like phenolic, flavonoid, lycopene and antioxidant activity. This study therefore concludes that in the effort to reduce environmental degradation through minimum dependence on chemical fertilizer, more usage of free and readily available organic manures as a source of soil fertility management should be recommended. The study also identifies that the link between method of farming system and other secondary metabolites as well as radical scavenging activity requires extensive and detailed study in future research.

Acknowledgements. The authors would like to thank the staff and management at the Sefako Health Sciences University as well as Agriculture Research Council (Vegetables and Ornamental Plants Unit). The funding from National Research Foundation (NRF) is also greatly appreciated.

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DOI: http://dx.doi.org/10.15666/aeer/1702_36813694

DECOMPOSITION CHARACTERISTICS AND NUTRIENT RELEASE RULES OF MAIZE STRAW UNDER DIFFERENT RETURNING AMOUNTS

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(Received 21st Dec 2018; accepted 20th Feb 2019)

Abstract. The decomposition and nutrient release of maize (*Zea mays* L.) straw under the four returning amounts of 10.0 g (CR1), 30.0 g (CR3), 50.0 g (CR5), and 70.0 g (CR7) were studied by nylon mesh bag method, exploring the decomposition characteristics and nutrient releases rules of maize straw, and providing a theoretical basis of rational use of straw resources and nutrient resources management. The results showed that decomposition and the nutrients release of straw are fast in the early stage (0-20 days) and slow in the late stage (20-100 days). In the first 20 days, the C/N ratio of the straw decreased the fastest, and the decomposition and nutrient release was the fastest. At the end of the experiment, the C/N ratio is relatively stable, the characteristics of decomposition rate and P and K releases rules was CR3 > CR5 = CR7 > CR1, the C and N release rate rules were CR3 = CR5 > CR7 > CR1; the release order of the different nutrients was K > P > N > C; the decomposition and nutrient release of CR3 was the fastest. In summary, a reasonable amount of returning promotes straw decomposition and nutrient release, which are conducive to the rational use of straw resources, and put forward measures of nutrient resource management.
Keywords: *straw returning, maize planting, decomposition stage, nutrients, C/N ratio, rational use, nutrient resources management*

Introduction

In recent years, the extensive application of chemical fertilizers has led to agricultural non-point source pollution, soil ecosystem damage and other issues. The crop straw is an important biological in crop production and contains rich plant nutrients and organic carbon. Straw returning is of great significance for improving soil fertility, reducing fertilizer application, reducing agricultural non-point source pollution and improving soil ecosystem (Jin and Zhou, 2018). Many studies have shown the straw returning to the field reduces the use of chemical fertilizers by releasing nutrients from crop straw decomposing, which is an important technology for green agriculture, ecological agriculture and environmental agriculture (Chen et al., 2018; Streets, 2006; Shibu et al., 2006). Straw returning is of great significance for improving soil nutrient, increasing resource use, reducing environmental pollution and improving ecological environment (Jin and Xing, 2018; Witt et al., 2000; Lal, 2010; Hu et al., 2012), so, it is an important way to the rational use of straw resources and sustainable development of agriculture.

To speed up the development and promotion of straw returning technology, some research on straw decomposition is increasing. Studies have shown the crop straw requires the combined action of microorganisms and enzymes to decompose (Becker et al., 2014; Ji et al., 2014). When the straw enters the soil, the decomposition rate of straw increases gradually with time, and the speed of straw decomposition decreases

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DOI: http://dx.doi.org/10.15666/aeer/1702_36953707

gradually, which is faster in the early stage and slower in the latter stage (Dai et al., 2010; Huang et al., 2016; Ji et al., 2013). The study found the different crops have different speeds of decomposition, for example, maize straw is faster than wheat (Zheng et al., 2004). Some studies have shown the amount of straw returning is negatively correlated with the rate of decomposition, and the application of decomposition agents can significantly speed up the decomposition and mineralization rate of straw (Jusoh et al., 2013; Chen et al., 2011). However, the application of chemical fertilizers in the soil can also promote to decompose of straw (Zheng et al., 2004). Soil fertility affects the decomposition rate of maize straw and high fertility promotes decomposition (Wang et al., 2009). The decomposition rate of crop straw mainly depends on the C/N ratio, and the decomposition is obviously accelerated after the C/N ratio is lowered (Zhang et al., 1994). About nutrient release rate of straw, studies have shown that characteristic is P > K > N > C (Dai et al., 2010; Ji et al., 2013), but some studies have found that is K > P > C > N (Zhong et al., 2017). The depth of straw application also influences on the release of nutrients, the release rate of N, P and K in the deep application of straw is faster than the surface application (Douglas et al., 1980). It can be seen that are many studies on straw decomposition, but a few studies on the amount of straw returning. Thus, it is of great significance to study the straw decomposition at different returning amounts.

Most studies on straw decomposing use the nylon mesh bag method (Olson and Krawczyk, 1963; Dai et al., 2010; Nakajima et al., 2016). Our study was carried out in the arid area using the nylon mesh bag method, because it has little effect on soil water transport, it is close to the actual situation in the field, and is more suitable for studying the decomposition of crop straw in arid and semi-arid areas (Lin et al., 1992). Yining County is the major maize producing area in Xinjiang, China. It has a large planting area, excessive use of chemical fertilizers in production, rich maize straw resources but low use rate; therefore, straw returning has enormous potential. Exploring the decomposition characteristics and nutrient (C, N, P, and K) releases rules of maize straw under different returning amounts of maize planting, and providing a theoretical basis of rational use of straw resources and nutrient resource management.

Materials and methods

Experimental site description

This experimental site was located in Yining County Modern Agricultural Technology Demonstration Park in Xinjiang, China (43°55'N, 81°33'E; altitude is 843 m). The county is temperate desert climate, which is mild and humid in the winter and spring, and warm and dry in the summer and autumn. The annual average temperature is 10.6 °C, and the annual maximum temperature is 35.8 °C. Annual precipitation is from 250 mm to 550 mm, the frost-free period is 154 days to 184 days, the annual average sunshine hours are 2792.7 h, and the effective accumulated temperature ≥10 °C is 3621.2 °C (10 years of data). Air temperature, precipitation and soil temperature during the experimental period in 2018 (*Fig. 1*).

The curve represents air temperature (°C) and soil temperature (°C), and the bar represents 20 days of accumulative precipitation (mm). Air temperature (°C) and daily average soil temperature (°C) are recorded once a week and recorded 20 days of accumulative precipitation (mm) every 20 days. The meteorological data are from local meteorological departments.

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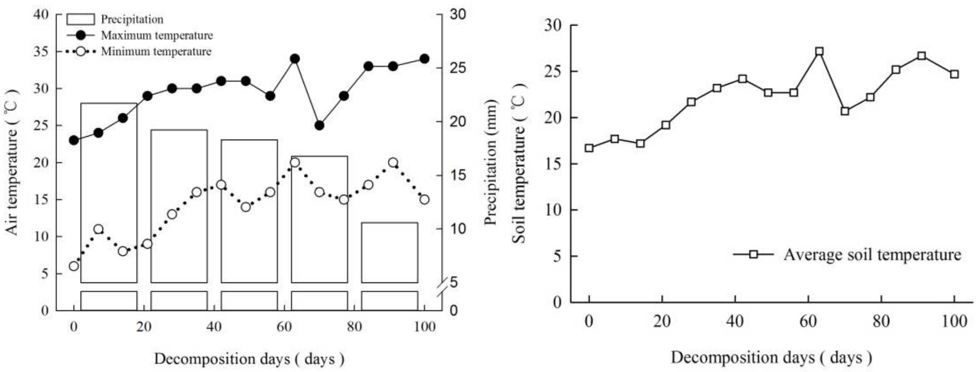

Figure 1. *Temperature and precipitation during the experimental period*

Experimental materials

The soil type of the experiment area is the *irrigated sierozem*, and the basic physical and chemical properties of the ploughed soil (0-20 cm): pH of 7.71 (1:2.5 soil/water ratio), soil organic matter (SOM) of 12.73 g kg^{-1}, total nitrogen (TN) of 0.96 g kg^{-1}, alkali-hydrolyzable nitrogen (AN) of 85.95 mg kg^{-1}, available phosphorus (AP) of 23.94 mg kg^{-1}, and available potassium (AK) of 229.24 mg kg^{-1}, soil C/N ratio of 13.26.

The experimental straw was maize (*Zea mays* L.) straw (comprised the stems and leaves) preserved after harvest in 2017, and its initial nutrient contents were as follows: C/N ratio of 28.13, organic C (C) of 413.50 g kg^{-1}, total N (N) of 19.47 g kg^{-1}, total P (P) of 3.09 g kg^{-1}, total K (K) of 17.53 g kg^{-1}. Fertilizers used included urea (46.0% N), diammonium phosphate (18.0% N, 48.0% P_2O_5), potassium sulfate (51.0% K_2O), and organic fertilizer (1.77% N, 1.95% P_2O_5, 0.53% K_2O).

Experimental design

The plot experiment was conducted from May 8, 2018, to August 16, 2018. There were three plots with a plot area of 33.2 m^2 (4 m × 8.3 m). Maize (*Zea mays* cv. Xinyu 50) was planted with the plot, the row spacing was 50 cm (8 rows), the plant spacing was 21.5 cm, and the planting density was 9.3 × 10^4 plants ha^{-1}. 469.5 kg urea ha^{-1}, 333.0 kg diammonium phosphate ha^{-1}, 103.5 kg potassium sulfate ha^{-1} and 1350.0 kg organic fertilizer ha^{-1} were applied to the soil (total fertilization amounts: 300.0 kg N ha^{-1}, 180.0 kg P_2O ha^{-1}, 60.0 kg K_2O ha^{-1}). Each plot had four different straw returning amount treatments: 10.0 g (CR1), 30.0 g (CR3), 50.0 g (CR5) and 70.0 g (CR7), with 5 repetitions. Maize straw (comprised the stems and leaves) is dried and pulverized into 3 cm to 5 cm, mixed and weighed (10.0 g, 30.0 g, 50.0 g, and 70.0 g, respectively), and placed them in a nylon mesh bag (length × width is 30 cm × 20 cm, pore diameter 0.125 mm) and tightened the bag mouth, and the net bag is about 2 cm high when leveled. Each returning amount is 15 bags, a total of 60 bags (3 plots × 4 treatment × 5 repetitions).

The specific operation steps of the buried bag: at the center of each plot, between the 2-6 rows (5 rows × 4 treatment), the nylon mesh bags of each treatment is completely randomly buried below the plough layer for 10 cm (*Fig. 2A*). When landfilling, the soil 10 cm depth between rows of maize is taken out, the soil deep in the 2 cm is dug out (no landfill), then the nylon mesh bag is laid horizontally (the distance between the two

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rows of maize is 15 cm), and then the soil is filled back and compacted to the level with the ground (*Fig. 2B*).

The experimental period was 100 days, and 12 nylon mesh bags were taken every 20 days (4 bags were randomly taken out from each plot). To avoid interference from topdressing on determines the nutrient content of maize straw residue; no topdressing was carried out in the area where straw was buried.

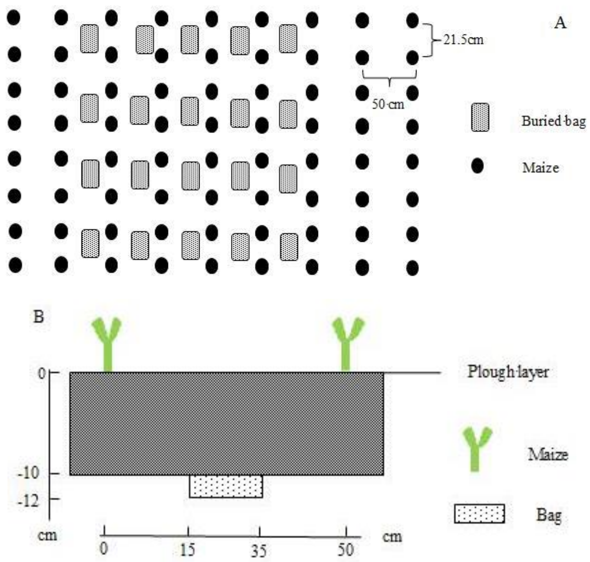

Figure 2. *Schematic diagram of the buried bag of maize straw in the plot. A and B are schematic diagrams of buried mesh bags in the plot and vertical cross-sections of the buried mesh bags, respectively*

Sampling and chemical analysis

Sampling began on May 28, 2018, and ended on August 16, 2018, the sampling is 5 times (20, 40, 60, 80, and 100 days of decomposition days). After each sampling, remove the floating soil and root debris on the surface of the nylon mesh bag, rinse the nylon mesh bag and dry it in an oven (65 °C) (KJ-DY02, Kejie Company, China) to constant weight. The straw was removed, weighed, pulverized and sieved (pore size of 1 mm) to determine the contents of C, P, N and K.

Determination of organic C (C) by $K_2Cr_2O_7$ dichromate-external heating method (133 mmol L^{-1} $K_2Cr_2O_7$ at 170-180 °C), The straw samples (0.25 g) were digested by the H_2SO_4-H_2O_2 method, the total N in the digestate was measured with an automatic Kjeldahl nitrogen determiner (K9840, Hanon Company, China), the total P (P) in the digestate determined by vanadium molybdate yellow-visible spectrophotometric (722N, JKI Company, China), the total K (K) in the digestate was measured with automatic digital flame photometer (FP640N, OEM Company, China). All methods have been described by Bao (2000).

Calculation and statistical analysis

Calculate the decomposition rate, decomposition speed, nutrient release rate, and C/N ratio methods (Dai et al., 2010; Yue et al., 2016) (*Eqs. 1-4*):

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$$\text{Decomposition rate (\%)} = \frac{M_0 - M_t}{M_0} \times 100\% \qquad \text{(Eq.1)}$$

$$\text{Decomposition speed (g day}^{-1}) = \frac{M_0 - M_t}{t} \qquad \text{(Eq.2)}$$

$$\text{Nutrient release rate (\%)} = \frac{M_0 \cdot C_0 - M_t \cdot C_t}{M_0 \cdot C_0} \times 100\% \qquad \text{(Eq.3)}$$

$$\text{C/N ratio} = \frac{C_t\ (\text{C})}{C_t\ (\text{N})} \qquad \text{(Eq.4)}$$

where M_0 (g) is the initial dry weight of the straw; t (day) is the decomposition day; M_t (g) is the dry weight of straw at t; C_0 (g kg^{-1}) is the initial nutrient (C N, P, and K) content of the straw; C_t (g kg^{-1}) is the nutrient (C N, P, and K) content of the straw at t.

All analyses were carried out on the replicates. Statistical analysis was completed using the SPSS 17.0 software. Statistically significant differences were identified using analysis of variance (one-way ANOVA) and Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

Results

Decomposition characteristics of maize straw

With the increase of decomposition days, the decomposition rate of maize straw gradually increases, and the decomposition speed gradually decreases, it is fast in the early stage (0-20 days) and slows in the late stage (20-100 days) (*Table 1*). In the first 20 days, the decomposition speed was the highest and the decomposition rate was higher. The decomposition rates increased to the increase in returning amounts, ranging from 17.07% to 27.55%, accounting for 36.97% to 58.20% of the total decomposition rate, and the characteristics of decomposition rate were CR7 = CR5 = CR3 > CR1. At the 40th days, there was no significant ($P > 0.05$) difference in the decomposition rate of each treatment, range from 28.43% to 36.68%. The decomposition speed gradually decreased, CR7 and CR5 decreased faster than CR1 and CR3. After 100 days of decomposition, CR3 was the fastest, the decomposition rate reached 53.94%, CR1 was the slowest, and the decomposition rate was 46.17%, and CR3 decomposition rate was 7.77%, 3.79%, and 6.60% higher than CR1, CR5, and CR7, respectively, and the characteristics of the decomposition rate was CR3 > CR5 = CR7 > CR1. The entire decomposition process, when the same decomposition days, the decomposition speed increases significantly ($P < 0.05$) with the increase in returning amounts, and the characteristics of decomposition speed were CR7 > CR5 > CR3 > CR1.

Nutrient releases rules of maize straw

With the increase of decomposition days, the nutrient releases rate of the straw increased, and the C, N, P and K release rate was fast in the early stage (0-20 days) and slow in the later stage (20-100 days) (*Fig. 3*), which was similar to the characteristics of decomposition (*Table 1*). In the first 20 days, the nutrient releases rate increased from the increase in the returning amount. The C, N, P and K released 16.95% to 29.16%, 17.35% to 29.87%, 23.55% to 33.93%, and 46.04% to 53.13%, respectively, accounting for 36.18% to 59.26%, 34.76% to 57.11%, 42.45% to 59.97%, and 51.43% to 58.85%

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of the total release rate, respectively, the rules of C, N and P releases rate were CR7 = CR5 = CR3 > CR1, the K release rate was not significant ($P > 0.05$). As the decomposition progresses, the releases speed decreased slowly, at 40th days, the nutrient releases speed of CR7 and CR5 decreased faster than CR3, and was gradually lower than CR3, and at 60th days, and the nutrient release rate of CR3 was higher than CR1, CR5, and CR7. After 100 days of decomposition, the C, N, P, and K release rates reached 46.85% to 56.51%, 49.92% to 59.69%, 55.48% to 63.23%, and 86.64% to 100.00%, respectively. Among them, the nutrient release rate of CR3 was the highest, and the release rates of C, N, P, and K of CR3 were 3.61% to 9.66%, 3.73% to 9.77%, 3.76% to 7.75%, and 6.58% to 13.36% higher than CR1, CR5, and CR7, respectively. The rules of C and N release rate were CR3 = CR5 > CR7 > CR1 (*Fig. 3A-B*), and the rules of P and K release rate were CR3 > CR5 = CR7 > CR1 (*Fig. 3C-D*).

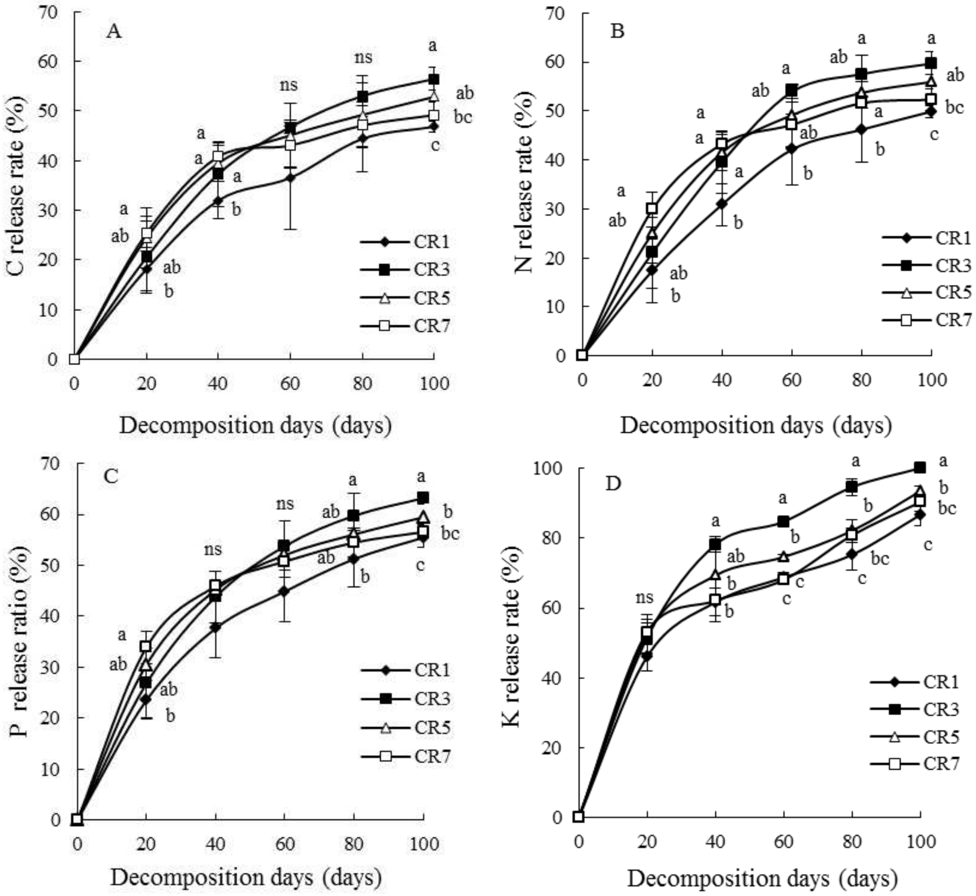

Figure 3. *Nutrient releases rate of maize straw under different straw returning amounts. A, B, C, and D is the nutrient release rate of C, N, P, and K, respectively. The data represent the mean values of three replicate ± SD (n = 3), and different lowercase letters next to the mean value show a significant difference at P < 0.05, ns = not significant. Error bars show the SD (n = 3)*

In the whole decomposition, according to the nutrient release rules of different treatments (*Fig. 3*) and comparing the nutrient release rates of different treatments, we can be found the release order of different nutrients was K > P > N > C (*Table 2*).

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Table 1. *Decomposition characteristics of maize straw under different straw returning amounts*

Treatment	20th days	40th days	60th days	80th days	100th days
Decomposition rate (%)					
CR1	17.07±3.89 b	28.43±6.79 a	37.03±7.55 b	44.13±5.39 b	46.17±2.04 c
CR3	20.40±6.83 ab	33.79±5.08 a	46.47±2.78 a	51.04±2.77 a	53.94±0.25 a
CR5	24.05±3.59 ab	35.37±5.87 a	43.37±3.82 ab	47.21±2.32 ab	50.15±2.13 b
CR7	27.55±3.15 a	36.68±2.70 a	40.21±2.15 ab	44.05±0.82 b	47.34±1.50 bc
Decomposition speed (g day^{-1})					
CR1	0.08±0.02 d	0.07±0.02 d	0.06±0.02 d	0.05±0.01 d	0.05±0.01 d
CR3	0.31±010 c	0.25±0.04 c	0.23±0.02 c	0.19±0.01 c	0.16±0.00 c
CR5	0.60±0.09 b	0.44±0.08 b	0.36±0.03 b	0.29±0.02 b	0.25±0.01 b
CR7	0.96±0.11 a	0.64±0.05 a	0.47±0.03 a	0.39±0.01 a	0.33±0.01 a

Values are means ± SD (n = 3). Mean values followed by different letters in the same column show signification differences at $P < 0.05$

Table 2. *The order of nutrient release at different decomposition days*

Decomposition day	The ranking order of nutrient release			
	1st	2nd	3rd	4th
20th days	K (46.04%-53.13%)	P (23.55%-33.93%)	N (17.35%-29.87%)	C (16.95%-29.16%)
40th days	K (61.65%-78.11%)	P (37.65%-45.93%)	N (30.91%-43.27%)	C (28.76%-40.86%)
60th days	K (68.74%-84.70%)	P (44.88%-53.80%)	N (42.14%-53.92%)	C (38.26%-49.77%)
80th days	K (75.25%-94.55%)	P (51.26%-59.75%)	N (46.22%-57.56%)	C (44.42%-53.06%)
100th days	K (86.64%-100.00%)	P (55.48%-63.23%)	N (49.92%-59.69%)	C (46.85%-56.54%)

The values in parentheses show the range of nutrient (C, N, P, and K) content in different treatments

Dynamic change of C/N ratio of maize straw

In the first 20 days, the C/N ratio decreased rapidly with the increase of decomposition days and the decomposition and nutrient releases of straw were the fastest, the C/N ratio ranged from 21.15 to 22.72, and it is 5.41 to 6.98 lower than the initial value (*Table 1; Fig. 3*). After 40 days, the C/N ratio of CR5 and CR7 increased first and then gradually stabilized, ranging from 22.71 to 22.81, CR1 and CR3 increased first and then decreased, and gradually stabilized after 80 days. At the end of the decomposition, the C/N ratio ranging from 22.64 to 23.02, among them, the C/N ratio of CR3 is higher than CR1, CR5 and CR7 by 0.38, 0.21 and 0.31, respectively (*Fig. 4*). Similar to the decomposition and nutrient release of CR3 was higher than other treatments at the end of decomposition (*Table 1; Fig. 3*).

Discussion

Decomposition characteristics of maize straw

With the increase of decomposition days, the decomposition rate increased, and the decomposition speed decreased, and the decomposition characteristics of straw were fast in the early stage (0-20 days) and slow in the late stage (20-100 days), which was

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consistent with some researches (Dai et al., 2010; Ji et al., 2013; Zheng et al., 2004; Che et al., 2013; Chen et al., 2011). The reasons may be: on the one hand, maize straw contains a large amount of easily decomposed organic substances in the early stage, such as polysaccharides, amino acids, organic acids. It provides plentiful energy and nutrients for the life activities of soil microorganisms, and improves the activity and quantity of soil microorganisms, thus promoting the decomposition of maize straw. As the straw is decomposed, the remaining major organic substances are difficult to decompose, such as lignin and cellulose, which are difficult to be used by soil microorganisms, resulting in a decrease in microbial activity and a slow decomposition of straw in the later stage (Summerell and Burgess, 1989; Li et al., 2009). On the other hand, the experiment began in May, because of the climate characteristics of the experimental site *(Fig. 1)*, the air temperature and soil temperature gradually increased, and the average precipitation was high, which improved microbial activity, promoted the decomposition of straw. With the increase of decomposition days, the temperature and soil temperature was too high, the precipitation decreases, which weaken microbial activity and metabolic activity, inhibiting the decomposition of straw (Summerell and Burgess, 1989; Nakajima et al., 2016; Azam et al., 1991). Besides, it applied organic fertilizer and chemical fertilizer in maize planting in the plot, which improved soil nutrients. In the early stage of decomposition, maize grew slowly. With the increase of decomposition days, maize grows rapidly and reduces the content of available nutrients, thus inhibiting microbial activity and slowing decomposition. We can see that the decomposition of maize straw is affected by the climate such as temperature, precipitation, and field management, which is consistent with research by Wang et al. (2016). Some studies have shown that pre-decomposition speed is fast, it too slows the medium-term decomposition speed down, and the late decomposition is stagnant (Kuang et al., 2012; Li et al., 2001).

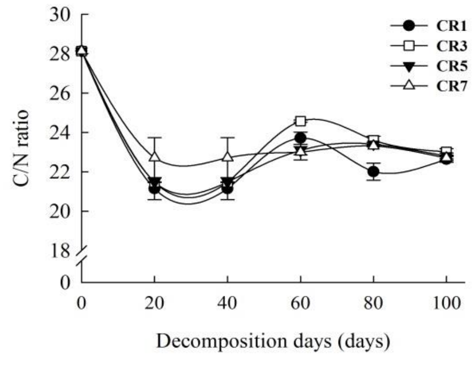

Figure 4. *Variation characteristics of the C/N ratio of maize straw under different straw returning amounts. The data represent the mean values of three replicate ± SD (n = 3). Error bars show the SD (n = 3)*

Nutrient releases rules of maize straw

This study found the release rules of nutrients (C, N, P, and K) of different treatments were fast in the early stage (0-20 days) and slow in the late stage (20-100 days), which is similar to the characteristics of decomposition. In the first 20 days, the decomposition rate, decomposition speed and nutrient release rate of different treatments increased with the increase of the amounts of returning, which is consistent

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with Yue et al. (2016) research on the decomposition experiment of maize straw and wheat (*Triticum aestivum* L.) straw. At the end of the experiment, the nutrient release rate of the treatments with higher decomposition rate was also higher, which is the same result as some studies have found a positive correlation between decomposition rate and nutrients release rate (Dai et al., 2010; Ji et al., 2013; Zheng et al., 2004). We found that when the returning amounts were greater than 30.0 g, the decomposition rate was negatively correlated with the returning amounts, some studies have also shown this result (Zheng et al., 2004). We found the returning amounts were 30.0 g, and its decomposition and nutrient release was the fastest, which is similar to Yue et al. (2016) result of the decomposition and nutrient release of maize straw was the fastest when the amount of maize straw returned was 26.3 g. Therefore, the proper amount of returning is conducive to the decomposition and nutrient release of straw.

This study showed the release of nutrients under different returning amount was K > P > N > C, which is consistent with some studies (Dai et al., 2010; Xu et al., 2010; Gong et al., 2018). In addition, Ji et al. (2013) studies showed that nutrient release of rapeseed (*Brassica napus* L.) and wheat (*Triticum aestivum* L.) straw was K > P > N ≈ C. Some studies have also found the release rate of maize straw by different decomposers was K > P > C > N (Zhong et al., 2017; Yang et al., 2016). Therefore, nutrient release rules of different crop straw are different. The release rate of nutrients depends mainly on the morphology of the elements in the straw. Among them, the K is mainly in the ionic state in cells or plant tissues and is easily soluble in water, so the release rate is the fastest, final cumulative release rate is also the highest; The content of P is less than K, which is mainly in the insoluble organic state, so the rate of K release is slower than P (Wang et al., 2013; Dai et al., 2017); The N mainly comprises insoluble organic matter, which is decomposed faster in the early stage, and is more difficult to decompose in the later stage, resulting in a decrease in decomposition rate (Murayama et al., 2010); The C mainly exists in various organic compounds, the decomposition rate is different (Tang et al., 2016). The C and N are the main ingredients of straw, the degree of cementation is high, and it is difficult to decompose, resulting in the slower release (Ji et al., 2013; Devêvre et al., 2000). Therefore, there are differences in the nutrient release. This study shows the rule of nutrient release was K > P > N > C.

Dynamic change of C/N ratio of maize straw

The speed of decomposition of straw mainly depends on C/N ratio, and the decrease of C/N ratio has a certain promoting effect on decomposition (Zhang, 1994), when C/N ratio is large, it affects the rapid growth and metabolism of microorganisms, so the process of straw decomposition is inhibited (Ding et al., 2008). The initial C/N ratio of maize straw is 28.13, and the best C/N ratio of microbial activity ranges from 25 to 30 (Devêvre et al., 2000). In addition, fertilization and proper temperature and precipitation also promote microbial activity, which promotes the decomposition of straw and rapid release of C and N; therefore, the C/N ratio decreases rapidly (Summerell and Burgess, 1989; Zhou et al., 2015; Nakajima et al., 2016). As decomposition progresses, the C/N ratio rise first and then are relatively stable, which because of the same as the reason for straw decomposition and nutrients release. Although the C/N ratio is close to the proper range of microbial growth, the C and N in the straw are mainly organic substances that are difficult to be used by microorganisms, and the N content needed for microbial metabolism in the soil is not enough, which reduces the microbial activity, leading to

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slower decomposition and nutrient release (Summerell and Burgess, 1989; Li et al., 2009; Rao et al., 1976). At the end of the decomposition, the decomposition and nutrients release was faster when the C/N ratio was higher. The dynamic change of C/N ratio in the process of decomposition cannot only reflect the characteristics of straw decomposition and the rules of nutrient releases, but also the speed of decomposition and nutrient release of different treatments.

Rational use of straw resources and nutrient resource management

The proper amount of returning is conducive to the decomposition and the nutrients release of straw and promotes the rational use of straw resources. According to this study found the characteristics of decomposition and the rules of nutrients release of straw are fast in the early stage (0-20 days) and slow in the late stage (20-100 days), which can properly reduce the use of base fertilizer, and carry out topdressing during the period when maize needs fertilizer, and effectively use fertilizers. According to the rule of nutrient release (K > P > N > C), the release of P and K are faster, P and K fertilizer can reduce the K fertilizer or delay the application period of P and K fertilizers; Because of the slow release of N and the decrease of soil N content in the later stage, N fertilizers should be applied in time for maize growth and to promote the decomposition of straw.

Conclusions

Our study concludes that, at the end of the decomposition, the decomposition and nutrient release of CR3 (30.0 g) are the fastest under different returning amounts, therefore, the proper amount of returning is conducive to the decomposition and the nutrients release of maize straw; In the whole decomposition, the characteristics of decomposition and the rules of nutrients (C, N, P, and K) release of straw under different returning amount are fast in the early stage (0-20 days) and slow in the late stage (20-100 days), and the release order of the different nutrients was K > P > N > C; Proposed that proper amount of returning in maize planting can reduce the amount of base fertilizer used, and the nutrients resource management measures to reduce or delay the application of P and K fertilizers and timely applied N fertilizers.

Prospect

According to the characteristics of decomposition and the nutrient release rules of straw, guiding scientific straw returning is conducive to the rational use of straw resources and to reduce fertilizer uses. In agricultural production, the characteristics of crops needed for fertilizer should be fully considered, and a reasonable amount of straw returning and nutrient resources management measures should be planned. Therefore, we will conduct a comprehensive study on the characteristics of crop fertilizer need and reasonable straw returning, to further guide the rational use of resources in agricultural production.

Acknowledgements. The National Key Research and Development Program of China (2017YFD0201808), the National Natural Science Foundation of China (31660598, 31360501) supported the research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_36953707

LAND USE CHANGES AND ITS DRIVING FACTORS: A CASE STUDY IN NANPING CITY, CHINA

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(Received 23rd Dec 2018; accepted 14th Feb 2019)

Abstract. Changes in land use affect the living environment of humans, considering even the survival and development of human beings. Study on the driving mechanism of land use plays a vital role in the accurate prediction of the changing trends of land use, and sustainable development of land. In this study, the Landsat images in Nanping City from 1995, 2010 and 2015 were obtained and processed using ENVI 5.2 and Arcgis 10.1. Object-oriented classification was used as the land use classification in this study. Transition matrix was used to study the characteristics, directions and structures of land use changes. The driving factors that led to the land use changes were studied using qualitative analysis and quantitative analysis (principal component analysis). The results showed that the forestland increased greatly and the cultivated land decreased significantly over the 20 years. Population, economy, urban development, policy, spending power, and technical factors were the drivers of the land use changes. The impact of comprehensive factors on the land use changes gradually increased with time.
Keywords: *remote sensing, GIS, transition matrix, principal component analysis, Fujian*

Introduction

Land is an indispensable resource for humans because of food supplies. Land use changes are the process that human beings constantly change land surface for surviving and developing (Aklile and Beyene, 2014; Bestelmeyer et al., 2016; Maimaitijiang et al., 2015). Human behavior changes the coverings of land surface and affects the natural ecosystems on the earth's surface, such as the cycles of energy, water, biochemistry, and atmospheric circulation (Allan et al., 1997; Liu et al., 2010). Therefore, Land Use and Land Cover Change (LUCC) proposed by the International Geosphere-Bisphere Program (IGBP) and the International Human Dimensions Program (IHDP) in the 1990s has become the frontier of global environmental change research (Turner, 1997). Land use changes are one of the three important issues of LUCC research (Alexander et al., 2015). It has a profound impact on the global sustainable development (García-Ruiz et al., 1996; Lovell, 2010; Young et al., 2005). Therefore, the studies of land use changes and its impact on the surrounding environment have become an extremely important research field in sustainable development (Renetzeder et al., 2008).

Studies of the driving mechanism of land use changes are also the core of land use changes research. The shortage of land resources and the deterioration of ecological environment caused by irrational land use are increasingly serious with the continuous development of industrialization and urbanization, and the increase of population, and the continuous expansion of human activities (Meyfroidt et al., 2013; Chaudhary et al., 2015; Song et al., 2015). Examining the driving factors have a highlighted practical

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DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

significance in accurately predicting the trends of land use changes, the rational planning of regional land, and the protection of ecological environment (Liu et al., 2016; Zhang et al., 2015). Driving factors of land use changes can be divided into natural and socio-economic factors (Maimaitijiang et al., 2015). The impact of the natural factors, such as topographic gradient, elevation, climate, etc., on land use was not obvious during a short period of time. However, the impact of socio-economic factors covering population, economic status, policies, on land use was significant during a period of the 20 years (Girma et al., 2014). Therefore, we focused on the socio-economic driver of the land use in this study.

The Economic Zone on the West Side of the Taiwan Straits is a regional economic complex. Its purpose is to cooperate with Taiwan in economy and is to drive the growth of economy in China. Nanping, the study area, is located in the central region of the Economic Zone. Consequently, studies of the driving factors in Nanping provided an important basis for the sustainable development, ecological protection, and the formulation of relevant policies and regulations in the Economic Zone.

Materials and methods

Study area

Nanping, the study area, is located in the southeast coast of China and the west side of the Taiwan Straits (*Fig. 1*). The climate belongs to the subtropical humid monsoon with an average annual temperature of 17-19 °C, an average winter temperature of 6-9 °C, an average summer temperature of 28-29 °C, and an average annual rainfall of 1684-1780 mm.

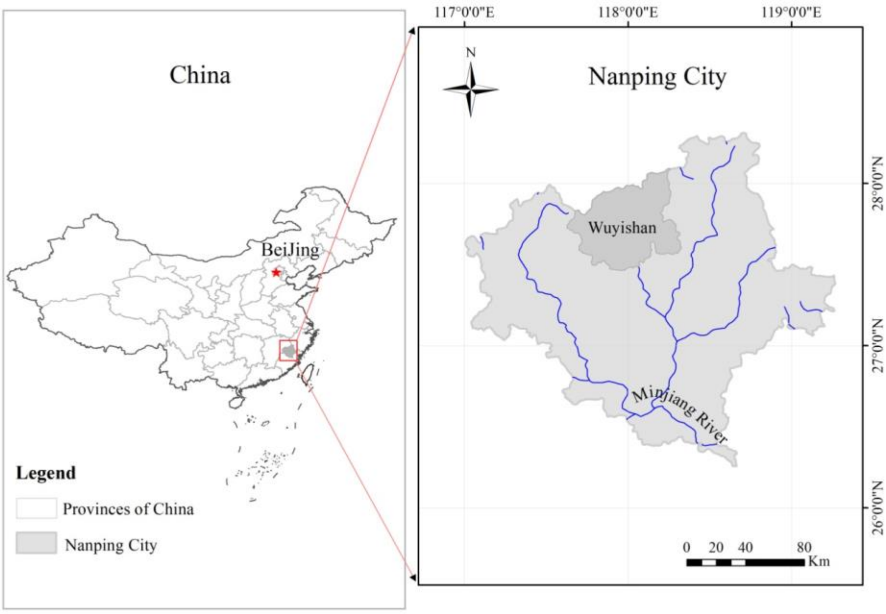

Figure 1. *Map of study area*

It has a history of more than 4000 years, a population of 3.19 million, and an area of 26,300 km^2. The area of the bamboo forest accounts for one-tenth of that of China. In

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2015, the gross domestic product (GDP) achieved 20.933 billion USD, amd the forest coverage was 74.75%. It ranks first in the reserves of niobium and tantalum in Asia. It not only is the birthplace of Minjiang River that is the mother river of Fujian Province but also is one of the best ecological regions in the world at the same latitude. Wuyishan under the jurisdiction of Nanping is one of the four World Natural and Cultural Heritage in China.

Acquisition and processing of the remote sensing data

The remote sensing images from Landsat-5 TM (1995 and 2010) and Landsat-7 ETM (2015) were derived from Chinese geospatial data cloud (*http://www.gscloud.cn/*). The images from Landsat-7 were processed using strip treatment. Cloud coverage of these images is less than 10%. The track numbers were respectively 120/40, 119/40, 119/41, 119/42, 120/42, 121/41, 120/41. Other study materials include the statistical yearbook from 1995 to 2015(NSB, 1995; NSB, 2005; NSB, 2015), the topographic maps of 1: 50000 and the administrative zoning maps obtained by Geographical information monitoring cloud platform (http://www.dsac.cn/).

These remote sensing images had undergone the correction of ground control point geometry and DEM terrain before downloaded. Therefore, the processing of remote sensing data in this study was to do color synthesis, mosaic, clip and object-oriented classification using ENVI 5.2. Types of the land use were divided into six categories: forestland, grassland, cultivated land, construction land, water and unused land. Band 5, 4, and 3 (RGB) was used for object-oriented classification. 250 samples for each type of land use were selected to achieve better classification results. The field investigation and interactive verification using Google Earth were carried out. After field verification, the classification accuracy of the remote sensing images from the three periods was 88.30%, 90.70%, and 92.10%, respectively, which can meet the requirements of this study.

Transition matrix of land use

Transfer matrix is used commonly to study the characteristics, directions and structures of land use changes. It not only can express the structural characteristics of land use, but also indicate the source and composition of each type of land use during study period. In addition, transition matrix can also generate a transition probability matrix that can be sorted by percentage. The major and secondary land use types that causing the changes of land use can be found by the sequence and the reason of the changes can be analyzed.

The mathematical equation of transfer matrix is as follows:

$$P_{ij} = \begin{bmatrix} P_{11} & P_{12} & \cdots & P_{1n} \\ P_{21} & P_{22} & \cdots & P_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ P_{n1} & P_{n2} & & P_{nn} \end{bmatrix} \quad \text{(Eq.1)}$$

where P represents the area; and i and j expresses the land use type at the early and late stages of study period respectively; and n indicates the number of land use types.

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DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

The transfer probability matrix in this study was calculated using post-classification images of the land use and Change Detection Statistics tool of Spatial Analysis Module from ENVI classic 5.1 according to *Equation 1*.

Principal component analysis

Principal component analysis is to recombine variables that have a certain correlation with each other into those with non-correlation. It extracts some new variables from original variables, and represents the information of the original variables as many as possible with minimum amount of the variables.

The mathematical model of principal component analysis is as follows:

$$\begin{cases} F_1 = a_{11}ZX_1 + a_{21}ZX_2 + \cdots + a_{P1}ZX_P \\ F_2 = a_{12}ZX_1 + a_{22}ZX_2 + \cdots + a_{P2}ZX_P \\ \vdots \\ F_i = a_{1i}ZX_1 + a_{2i}ZX_2 + \cdots + a_{Pi}ZX_P \end{cases} \quad \text{(Eq.2)}$$

where $a_{1i} + a_{2i} + \cdots (i = 1, \cdots, m)$ indicates the eigenvector corresponding to X covariance matrix; and $ZX_1 + ZX_2 + \cdots$ donates the value after normalized original variable.

Principal component analysis was used to examine the impact of socio-economic driving factors on the land use changes according to *Equation 2*. However, the selection rules of socio-economic factors needed to be determined at first. The rules and the operation steps are as follows:

1. Review the statistical yearbook of Nanping to ensure that the factors are easy to be quantified and have a significant relationship with the land use changes.
2. Review the two statistical yearbooks of 1995 and 2015 to make sure the data of the chosen factors are complete.
3. The units of the chosen factors are different. Hence, these data need to be processed in a non-dimensional manner for subsequent calculation using SPSS.
4. Confirm the data are suitable for principal component analysis. If appropriate, correlation coefficient among the data is calculated, and Bartlett and KMO are tested. Finally, principal components are extracted using SPSS.
5. Comprehensive score of the driving factors of land use changes is obtained by multiplying the obtained eigenvectors and normalized data.

Results

Areal changes of the land use

The area of various land use types was achieved using ArcGIS 10.1 during the 20 years (*Table 1*). As shown in *Table 1*, the land use types were dominated by forestland, cultivated land and grassland, accounting for over 89% of the total area in Nanping City. The forestland increased rapidly with a total increase of 2075.90 km^2. The construction land had a significant rise with an increase of 329.99 km^2. In contrast, the cultivated land declined with a reduction of 1198.70 km^2. The water, unused land, and grassland also decreased with a reduction of 32.65 km^2, 545.10 km^2, and 528.03 km^2 respectively.

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Table 1. *Areal changes of different land use types during the 20 years (km^2)*

Land use types	1995	2010	2015	Changing value (km^2)
Construction land	771.64	1350.44	1101.63	329.99
Forestland	19304.79	19258.94	21380.69	2075.90
Water area	657.49	645.76	624.84	-32.65
Cultivated land	2305.14	2452.60	1106.44	-1198.70
Unused land	955.46	770.43	410.36	-545.10
Grassland	2172.78	1796.45	1644.75	-528.03

Transfer matrix of the land use

As shown in *Table 2* that the value of outflow of the forestland was the lowest with a value of 16.64%. In contrast, unused land had a highest value (99.5%) in outflow. The order of the outflow of these land use types was unused land (99.5%) > cultivated land (91.86%) > water (84.14%) > construction land (77.64%) > grassland (76.91%) > forestland (16.64%). The main transition directions of all the land use types were forestland, which caused the continuous increase in forestland over the 20 years. In addition, grassland was also one of the transition directions of the unused land. The construction land and grassland were the main outflow types of cultivated land.

Table 2. *Transfer matrix of land use in the study area from 1995 to 2015 (%)*

2015 \ 1995	Construction land	Forestland	Water	Cultivated land	Unused land	Grassland
Construction land	22.36	6.05	10.83	20.60	11.04	7.08
Forestland	57.68	83.36	64.96	47.55	53.74	65.35
Water	11.60	2.55	15.86	3.10	1.85	1.52
Cultivated land	3.36	1.15	1.95	8.14	4.43	2.68
Unused land	0.20	0.10	0.16	0.53	0.50	0.28
Grassland	4.80	6.79	6.24	20.08	28.44	23.09

Driving factors of the land use

Comprehensive driving factors of the society and economy

From *Table 3*, 12 factors were selected in this study. Owing to the greater number of these factors, they were reduced dimensionally in order that most information was expressed with the least number of the factors. The correlation coefficient matrixes were obtained using these dimensionless factors (*Table 4*). As shown in *Table 4*, the correlation coefficients were all higher except GDP and natural population growth rate, which indicated the greater repetitiveness of information expressed by these factors. Accordingly, principal component analysis, and Bartlett and KMO tests were implemented. The value (Sig.) of Bartlett's concomitant probability was 0.000, less than the significance level (0.05), and the KMO value was 0.865, which showed these factors were linearly correlated and principal component analysis were adopted compatibly.

As shown in *Table 5*, one principal component was achieved according to the principle of specifying eigenvalue >1 and initial eigenvalue >80%. The initial eigenvalue of the first principal component was 83.75%, which indicated that it

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reflected 83.75% of the overall factors. Hence, it could explain the reason of the land use changes.

Table 3. *List of socio-economic factors*

Code	Name	Unit	Types of factor
X1	Total population	Person	Demographic factors
X2	GDP	Billion USD	Economic factors
X3	Primary industry	Billion USD	
X4	Secondary industry	Billion USD	
X5	Tertiary industry	Billion USD	
X6	Local fiscal revenue	Billion USD	
X7	Local financial expenditure	Billion USD	
X8	The total retail sales of social consumer goods	Billion USD	Spending power
X9	Resident deposits end of the year	Billion USD	
X10	Natural population growth rate	‰	Social awareness factors
X11	The total social investment in fixed assets	Billion USD	Technical factors
X12	Per capita net income of farmers	USD	Spending power

Table 4. *Correlation coefficient matrix among these driving factors*

Factors	ZX_1	ZX_2	ZX_3	ZX_4	ZX_5	ZX_6	ZX_7	ZX_8	ZX_9	ZX_{10}	ZX_{11}	ZX_{12}
ZX_1	1.000											
ZX_2	-.184	1.000										
ZX_3	-.168	.998	1.000									
ZX_4	-.177	.999	.998	1.000								
ZX_5	-.203	.997	.992	.994	1.000							
ZX_6	-.154	.992	.991	.994	.984	1.000						
ZX_7	-.155	.984	.981	.986	.977	.995	1.000					
ZX_8	-.192	.999	.995	.997	.997	.993	.988	1.000				
ZX_9	-.202	.996	.991	.994	.997	.988	.984	.999	1.000			
ZX_{10}	-.033	.280	.257	.296	.270	.331	.324	.292	.287	1.000		
ZX_{11}	-.156	.978	.973	.980	.974	.991	.996	.984	.982	.365	1.000	
ZX_{12}	-.185	.998	.994	.996	.998	.991	.984	.999	.998	.288	.982	1.000

Table 5. *Eigenvalue and cumulative rate of driving factors*

Principal component	Initial eigenvalue			Rotation square sum loading		
	Total	Variance (%)	Accumulative total (%)	Total	Variance (%)	Accumulative total (%)
1	10.05	83.75	83.75	10.05	83.75	83.75

As shown in *Table 6*, the loads values of the factors exceed 0.9 except for the total population and the natural population growth rate. From *Table 6*, the values exceed 0.9 that represented economic factors, spending power and technical factors. Consequently,

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DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

the three types of the factors were the dominating ones for the land use changes in Nanping during the study period.

Table 6. *List of factors loading matrix*

Index of factors	Factors	Component 1
ZX_1	Total population	-.197
ZX_2	GDP	.997
ZX_3	Primary industry	.993
ZX_4	Secondary industry	.997
ZX_5	Tertiary industry	.994
ZX_6	Local fiscal revenue	.996
ZX_7	Local financial expenditure	.991
ZX_8	The total retail sales of social consumer goods	.999
ZX_9	Resident deposits end of the year	.997
ZX_{10}	Natural population growth rate	.329
ZX_{11}	The total social investment in fixed assets	.989
ZX_{12}	Per capita net income of farmers	.997

A principal component expression was obtained using the principal component load matrix and the result of the eigenvectors multiplying with the normalized data.

$$\begin{aligned}F = &-0.0621ZX_1 + 0.3145ZX_2 + 0.3132ZX_3 + 0.3145ZX_4 \\ &+ 0.3136ZX_5 + 0.3142ZX_6 + 0.3126ZX_7 + 0.3151ZX_8 + \\ &0.3145ZX_9 + 0.1038ZX_{10} + 0.3120ZX_{11} + 0.3145ZX_{12}\end{aligned} \quad \text{(Eq.3)}$$

The comprehensive score of the factors of the land use changes was calculated using *Equation 3.* As can be seen from *Figure 2,* the impact of the factors on the land use changes showed a sharp increase over time. Specifically, the comprehensive score of the factors had increased slowly before 2008, which indicated that economic factors, spending power and technical factors had little effect on the land use changes. However, the score of driving factors had demonstrated an exponential growth after 2008. This was mainly due to the rapid increase in GDP, the continuous development of high-tech industries, the integration of geographical advantages and the adjustment of industrial layout in Nanping after 2008.

Discussion

Population

Population is one of the most active drivers of land use changes (Shoshany and Goldshleger, 2002). The increase of population directly changes spatial distribution of land use. According to the statistical yearbook of Nanping City from 1995 and 2015, it was found that the population in Nanping City was continuously increasing from 2,939,904 in 1995 to 3,198,600 in 2015 with an increase of 258,700 over the 20 years. This phenomenon was bound to cause changes in the land use (Bilsborrow et al., 1992; Miao et al., 2016; Rufino et al., 2017). The large-scale facilities, such as water,

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electricity, early warning facilities, and the daily facilities, such as landfill, recreational facilities, water supply system, resident health information system and school informatization, and other convenient service facilities were constructed during this time in order to deal with the increase of population (Liu et al., 2006; Sun et al., 2013). The construction of these facilities profoundly affected the spatial distribution of the land use in Nanping.

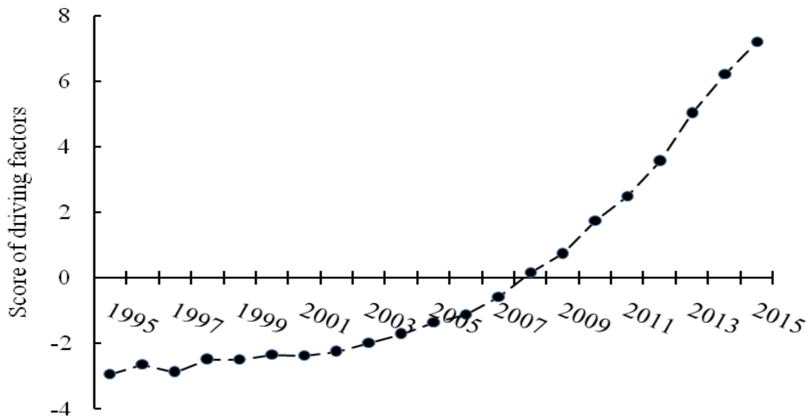

Figure 2. *Comprehensive score of socio-economic driving factor of the land use change*

The growth in the population led to a marked increase in the construction land and a significant decrease in the cultivated land over the 20 years. The construction land and cultivated land increased and decreased by 329.99 km^2 and 1198.7 km^2 respectively. It was found that the construction land was one of the main transfer directions of the cultivated land, which indicated that population growth inevitably increased the construction of large-scale convenient services facilities, resulting in more the cultivated land being transferred to the construction land. However, cultivated land is the main source of food for human beings. The rapid population growth led to the conversion of more cultivated land into the construction land, which inevitably led to insufficient food supply. Therefore, it is crucial to solve the contradiction between population and cultivated land. In summarize, population was one of the main factors causing the changes in the land use.

Economy

GDP refers to the status of regional economy. The growth of GDP represents the improvement of people's living level, which is the prevalent aspiration and struggle target of people. The activities of human beings for living affect changes in land use types. Hence, economic development inevitably leads to changes in land use (Weber et al., 2001). GDP in Nanping was continuously increasing from 2.28 billion USD in 1995 to 20.94 billion USD in 2015 (*Fig. 3*). The citizens began to seek spiritual enjoyment after satisfying their food and clothing needs. For raising the living quality and meeting spiritual needs of the citizens, the municipal government accelerated the construction of infrastructure buildings, which led to the significant changes of the land use in Nanping (Liu et al., 2015).

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According to the Classification of Chinese Economic Industries, agriculture, forestry, animal husbandry, and fishery were classified as the primary industry, and the supply and construction industries such as mining, manufacturing, electricity, and gas were classified as secondary industry, and service industry as the tertiary industry.

As shown in *Figure 3,* the rankings of the three industries in Nanping City significantly changed during the 20 years. The proportion of the primary industry decreased from 34.44% in 1995 to 21.59% in 2015, the secondary industry increased from 35.82% in 1995 to 43.16% in 2015 and the tertiary industry only increased by 5.52% over the 20 years, which showed that the agriculture, forestry, animal husbandry and fishery gradually turned to service industry and manufacturing industry.

In this study, the decrease in the proportion of the primary industry (agriculture, forestry, animal husbandry and fishery) indicated the decline in the cultivated land and grassland. With the increase in the proportion of the secondary and tertiary industry, more land resources were occupied by the facilities, which means that the increase in the construction land and the decrease in the unused land. In summarize, the changes of the economy undoubtedly affect the spatial distribution of the land use (Fan et al., 2017). Economy is one of the driving factors of the land use changes in Nanping.

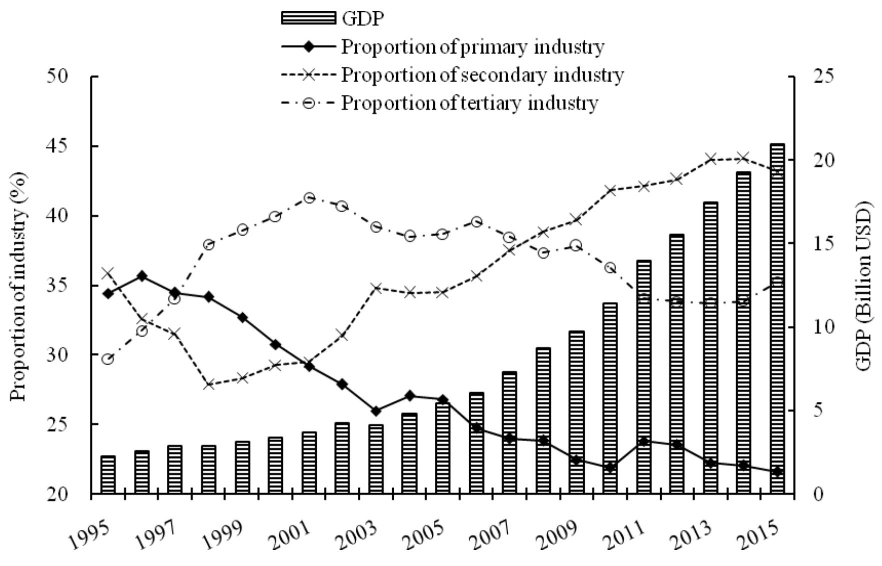

Figure 3. *The proportion of GDP and three major industries in the study area*

Urbanization and transportation

Urbanization is a symbol of the improvement of people’s living level (Sigel, 1978). It is a kind of development trends to shift from simple agricultural life to multiple city life for human beings. However, a series of environments problems, such as biodiversity loss, heat island effect, environmental pollution, and water and soil loss, have arisen due to the rapid urbanization and irrational use of land resources (Wang et al., 2012). The urban construction in Nanping continued to expand outward over the 20 years. The urbanization rate in Nanping increased from 29.0% in 1995 to 54.0% in 2015 with an increase of 27.0% (NSB, 1995; NSB, 2015), which led to the changes of the land use.

Accordingly, the staggered road networks resulted in the serious destruction of the landscape (Mo et al., 2017). In 2015, the total mileage of the roads was 15554.24 km

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

and mileage of freeway was 929.67 km, and the pavement with asphalt and cement concrete were 877.48 km and 10846.19, respectively (NSB, 2015). The intensity of the fragmentation in the landscape was obviously enhanced, which led to the decrease in the types of the landscape such as cultivated land, water and grassland (Jaeger et al., 2007). In short, the development of the urbanization and transportation led to the dramatic changes of the land use.

Policy

The main pillars of the development plan of the Economic Zone issued by the state council were as follows.

1. Strengthen exchanges and cooperation between mainland China and Taiwan by virtue of the construction of Pingtan Experimental Zone which is a nearest island in mainland China to Taiwan.
2. Accelerate infrastructure construction and improve sustainability in economic development in the Economic Zone.
3. Accelerate optimization in industrial layout and improve innovation capability in order to build a modern industrial system of the Economic Zone.
4. Realize the coordinated development of the urban and rural areas.
5. Focus on improving people’s livelihood and build a harmonious atmosphere in the Economic Zone.
6. Improve ecological environment of the Economic Zone and strive to build a sustainable development area with beautiful living environment.

According to these pillars, it is necessary to construct the economic zone into a beautiful, ecological, and friendly region, and focus on the coordinated development of the economy and the ecological environment. It is therefore indispensable to increase the forestland so as to maintain the stability of the ecosystem and improve the ecological environment (Wang et al., 2017). In this study, the forestland had decreased by 45.85 km^2 from 1995 to 2010 before the “Plan” was promulgated. However, from 2010 to 2015, the forestland had increased by 2121.75 km^2 after the “Plan” was issued, which further illustrated the decisive role of policies in the land use changes.

The Development Plan for Wuyishan was promulgated in 2010, which is of great significance to further improve the economy in Nanping. Accordingly, new land policies were introduced by the government. The forestry reform was executed and inappropriate cultivated land was retreated. As a result, the forestland continued to increase after 2010. Especially, the cultivated land had increased before 2010. In contrast, it had decreased sharply after 2010. Consequently, the promulgation and implementation of the policies had a profound impact on the spatial distribution of the land use (Dempsey et al., 2017).

Conclusion

The main conclusions are as follows:

The percentage of forestland in overall study region increased significantly from 73.77% in 1995 to 81.39% in 2015 with an increase of 2075.90 km^2 over the 20 years. The cultivated land decreased sharply from 8.81% in 1995 to 4.21% in 2015 with a decline of 1198.70 km^2.

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DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

The unused land, cultivated land, and water had a higher outflow over the 20 years. The forestland was the main outflow types for the land use types. Hence, the forest coverage rate remarkably increased.

The study found that the growth of population and economy, and urban expansion resulted in the sharp increase of construction land, and the decline of cultivated land, grassland and unused land. National and local policies led to a notable increase in the forestland and the decline in the cultivated land over the study period. In a word, the comprehensive impact of population, economy, urban development and policies was the main reason for the land use changes in Nanping during the study period.

Economic factors, spending power and technical factors were the driving factors of the land use changes. The impact of the three drivers on land use changes demonstrated an exponential rise after the year of 2008.

In summary, population, economy, urban development, policy, spending power, and technical factors were the driving factors of the land use changes in Nanping over the 20 years.

Acknowledgements. The authors are so grateful to Specialized Research Fund for the Doctoral Program of Shaoyang University (Fund No. 17ZX03), and National Science and Technology Major Project of the Ministry of Science and Technology of China (Fund No. 2017YFD0601304) for financial support.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37093721

PHYTOREMEDIATION AS A TOOL FOR THE REMEDIATION OF WASTEWATER RESULTING FROM DYEING ACTIVITIES

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(Received 24th Dec 2018; accepted 14th Feb 2019)

Abstract. The production of dye is usually associated with several intermediate products and waste which are usually toxic and bio-hazardous and often disturb the environmental equilibrium. The present study is aimed at selecting macrophytes with the highest efficiency in the remediation of wastewater generated from dyeing activities. The test macrophytes were introduce into three different troughs for each plant containing 100% wastewater, 50% wastewater and control (100% borehole water). After 21 days the macrophytes were removed for further analysis. The reduction efficiency, Heavy metal bioconcentration and bio translocation factor of the macrophytes was determined. The result obtained shows that the test macrophytes improved the pH but were not able to improve the color of the wastewater. *Lemna minor* have the highest reduction efficiency for BOD, COD, Oil, and Grease while *Pistia stratiotes* have the highest reduction efficiency for TDS and TSS, *Eicchornia crassipes* have the highest reduction efficiency for Cl^- and *Salvinia molesta* have the highest reduction efficiency for phenol and ammonical Nitrogen. All macrophytes recorded high reduction efficiency for heavy metal removal but *Pistia stratiotes* and *Eicchornia crassipes* had the highest bioconcentration and translocation. These macrophytes should be introduced to the local dye users as a costless and environmentally friendly way of treating wastewater resulting from dyeing activity.
Keywords: *Azolla pinnata, Lemna minor L., Eicchornia crassipes, Pistia stratiotes, Salvinia molesta*

Introduction

The world ever increasing population and adoption of industrial based lifestyle have led to an increasing in anthropogenic activities which has lots of effects on the biotic community. The availability of water, both in quality and quantity, is one of the prime factors in deciding the growth of towns and cities as well as industries (Amare et al., 2018a; Costa et al., 2018; Fernandes et al., 2018). It is thereby in the best interest of the world if water is properly treated for the purpose of reuse. In Northern Nigeria, different communities and ethnic groups use numerous plants as dyeing agents. This has greatly influenced cultural acceptability and easy method of survival. The synthesis of organic dye was introduced in 1871 and was practiced in Egypt, Persia, China and India thousands of years ago using natural materials derived from insects, plants, shellfish etc. as dyestuff. Dyes are generally fast as they retain their color in the fiber throughout the textile making process and under exposure to normal wear including sunlight, water, and detergent washing. Dye is a soluble compound that can either be absorbed or retained by the fiber. The production of dye is usually associated with several intermediate products and waste which are usually toxic and bio-hazardous and often disturb the environmental equilibrium. Local dye users of Faskari road (*Fig. 1*) who use dye in the dyeing of cloths and other garments create a large quantity of wastewater which contains different pollutants. The wastewater generated during dying activities is

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DOI: http://dx.doi.org/10.15666/aeer/1702_37233735

characterized by high BOD, COD, Acidity, Deep color of Different Shades, levels of chlorides etc (Haddad et al., 2018; Lyu et al., 2018; Pandian et al., 2018). These pollutants are produced in an effort to improve the looks of the inhabitants of Kaduna south local government but ironically the lack of treatment facilities reverse the same standard by impacting negatively on the environment (Amare et al., 2018b; Carvalho et al., 2018).

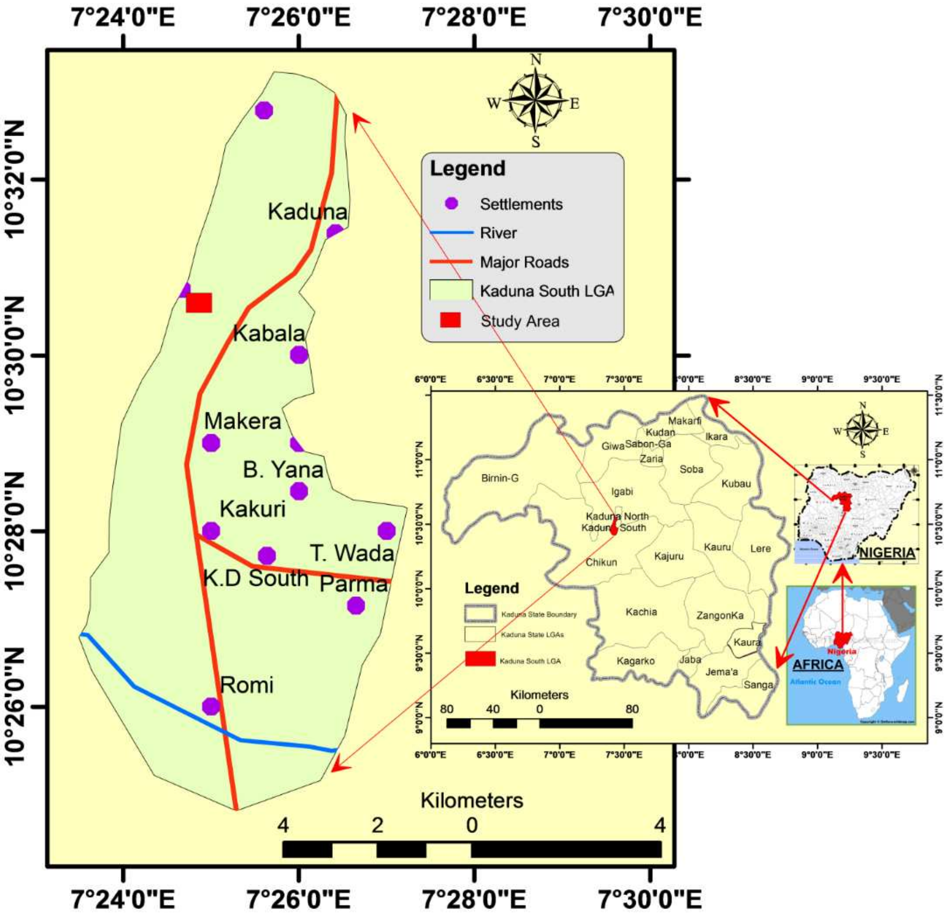

Figure 1. Map showing Study Area

The wastewater is released into drainage system without treatment, eventually, this toxic wastewater makes it way into River Kaduna which is the nearby water body (Bununu et al., 2015; Ogwueleka, 2014, 2015). Some of the wastewater can seep into aquifers and pollute underground water since they cannot be confined within specific boundaries (Braun et al., 2018; Demarco et al., 2018; Fernandez San Juan et al., 2018). The untreated wastewater tends to have an ecological impact on the nearby water bodies which is river Kaduna leading to increased nutrient load leading to eutrophication, which is open water can progressively lead to oxygen deficiency, algae blooms, and death of aquatic life. Several technologies are available to remediate this wastewater before discharge. However, many of these technologies are costly, so not easily accessible (Jiang et al., 2018; Osti et al., 2018; Zhao et al., 2018). Phytoremediation can provide a long lasting, cost effective, long lasting and aesthetic solution to the remediation of this wastewater, since macrophytes such as *Lemna minor* L., *Azolla*

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DOI: http://dx.doi.org/10.15666/aeer/1702_37233735

pinnata, Pistia stratiotes, Eicchornia crassipes and Salvinia molesta which are easily accessible have been proof to have phytoremediation potentials by researchers such as (Saraswat and Rai, 2018; Ting et al., 2018; Vanhoudt et al., 2018) etc. The present study is aimed at selecting macrophytes with the highest efficiency in the remediation of wastewater generated from dyeing activities.

Materials and Methods

Plant Sampling and Identification

Young macrophytes of *Lemna minor* L., *Azolla pinnata, Pistia stratiotes, Eicchornia crassipes, and Salvinia molesta* were collected from different pond located at Kaduna, Kaduna State, Nigeria and identity using relevant guide. The young plants were selected for these studies due to their ability to double their size in 3 weeks. The plants were authenticated at the herbarium of the department of botany (*Table 1*), Ahmadu Bello University Zaria, Kaduna State, Nigeria.

Table 1. *Test Macrophytes Used for Phytoremediation*

SN	Plants	Common Name	Accession Number
1	*Lemna minor* L.	Duckweed	L.6935
2	*Eichhornia crassipes*	WaterHyacinth	3268
3	*Pistia stratiotes*	WaterLettuce	1977
4	*Azolla pinnata*	WaterVelvet	0678
5	*Salvinia molesta*	Kariba Weed	01689

Water Sampling

Wastewater was collected from dyeing point of Faskari road located in TudunWada of Kaduna South Local Government, Kaduna State, Nigeria from March 2017-March, 2018 (*Fig. 1*). The collection, storage, transportation and analysis of the sample for the parameters such as BOD_5, COD, pH, electrical conductivity, Chlorides, Ammonical Nitrogen, phenol, phosphate, total suspended solids, total dissolved solids, oil and grease before treatment was done according to (AFNOR, 2001).

Phytoremediation Setup

To access the phytoremediation efficiency of these macrophytes in the improvement of dyeing wastewater, an offsite culture experiment was performed in the general biology laboratory of Kaduna State University. The test plants were thoroughly washed using borehole water and distilled water then kept on a filter paper to remove excess water before transferring into fifteen different troughs having a capacity of five liters. The experiments were grouped into 5 groups with each group consisting of three troughs as shown below:

Group 1: *Lemna minor* L. was inoculated in wastewater of different concentration below:

- 100% of wastewater.
- 50% of wastewater.
- 0% wastewater (control).

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Group 2: *Eicchornia crassipes* was inoculated in wastewater of different concentration below:

- 100% of wastewater.
- 50% of wastewater.
- 0% wastewater (control).

Group 3: *Pistia stratiotes* was inoculated in wastewater of different concentration below:

- 100% of wastewater.
- 50% of wastewater.
- 0% wastewater (control).

Group 4: *Azolla pinnata* was inoculated in wastewater of different concentration below:

- 100% of wastewater.
- 50% of wastewater.
- 0% wastewater (control).

Group 5: *Salvinia molesta* was inoculated in wastewater of different concentration below:

- 100% of wastewater.
- 50% of wastewater.
- 0% wastewater (control) (Qin et al., 2016, Qu et al., 2017)

After 21 days the plants were removed from the treated water, the treated water was thus filtered before the re-determination of BOD_5, COD, pH, electrical conductivity, Chlorides, Ammonical Nitrogen, phenol, phosphate, total suspended solids, total dissolved solids, oil and grease by using standard method as described by (AFNOR, 2001), the color change before and after treatment of the wastewater was also observed. The removal efficiency of each of the macrophyte was thus calculated according to the method employed by Ugya (2015) as represented below:

$$x = \frac{B-A}{B} X \ \frac{100}{1} \quad \text{(Eq.1)}$$

where:
B = Final Concentration.
A = Initial concentration.
X = ReductionEfficiency.

The experiment was repeated for nine months to enable the determination of the mean removal efficiency of each of the macrophyte (Akhtar et al., 2016; Bokhari et al., 2016; Zhao et al., 2016).

Heavy Metal Bioconcentration and BiotranslocationFactor

After remediation macrophytes were removed and separated into leaves, stems, and roots. These parts were washed using tap water, ionized water then oven dried at 70^0C before grounding. The grounded plant materials were then subjected to acid digestion using HNO_3 and $HClO_4$. After digestion, the sample was then analyzed using Atomic Absorption Spectrophotometer for the concentration of heavy metals (Ni^{2+}, Cr^{3+}, Cd^{2+}, and Pb^{2+}) present in theroot and shoot (leave and stem) of the macrophyte. These were then used in the determination of Bioconcentration and biotranslocation factor using *Equations (2)* and *(3)* below:

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DOI: http://dx.doi.org/10.15666/aeer/1702_37233735

$$\text{Bioconcentration Factor} = \frac{a}{b} \quad \text{(Eq.2)}$$

$$\text{Biotranslocation Factor} = \frac{c}{d} \quad \text{(Eq.3)}$$

where:
a = metal concentration in root.
b = metal concentration in wastewater.
c = metal concentration in shoot.
d = metal concentration in root.

The concentration of heavy metals present in the wastewater was also determined before and after the treatment process for the determination of heavy metal reduction efficiency using *Equation (1)*.

Data Analysis

With the objective of determining the macrophyte with the highest reduction efficiency, biotranslocation factor and bioaccumulation factor, Data were analysed using IBM SPSS statistics version 23 for two-way analysis of variance (ANOVA).

Result and Discussion

Wastewater Colour and pH Reduction Efficiency

The result obtained shows that all the test plants were not able to improve the color of the wastewater from dark brown although *Lemna minor* L and *Pistia stratiotes* were able to change the color of the wastewater to pale yellow after treatment. The pH of the wastewater was improved by all the test plants as the pH was not reduced but increase. On the mean scale, the macrophytes were able to increase the pH of the water from 4.2 to 7.3 (i.e from acidic to neutral pH). The pH of the dyeing wastewater was increased from high acidity to neutral by all the aquatic macrophytes, this increase in pH could be attributed to the utilization of CO_2 by the test plants for photosynthesis (Irawati et al., 2017; Singh and Rai, 2016; Victor et al., 2016). Some recent studies of the success of increase in pH resulting from the use of macrophytes include workdone by researchers such as Galal et al. (2017), Klink (2017), Maleva et al. (2016), Zhao et al. (2017) with references to different wastewater. The color removal efficiency of *Pistia stratiotes* could be attributed to the property of proper particle sedimentation by *Pistia stratiotes* or the ability of the root of *Pistia stratiotes* to retain both coarse and fine particle (Kaminski et al., 2014; Neagu et al., 2014).

The efficiency of Macrophytes in BOD, COD and EC Reduction

The BOD and COD reduction by the macrophytes is in the trend of Lemna minor, Pistia stratiotes, *Eicchornia crassipes, Salvinia molesta,* and *Azolla pinata* according to decreasing efficiency. *Lemna minor* was able to reduce BOD and COD from 645 mg/l to 75 mg/l and 1604 mg/l to 352 mg/l, respectively (*Fig. 2a*). The pattern for EC reduction is different that of COD and BOD because *Pistia stratiotes* show a maximum EC reduction compare to other macrophytes (*Fig. 2a*). Similar pattern of COD, BOD and EC reduction by the macrophyte recorded in 100% wastewater was followed by the macrophytes in 50% wastewater and control (0% wastewater) (*Figs. 2b, 2c*) signifying

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that *Lemna minor* has the highest reduction efficiency for COD and BOD while *Pistia stratiotes* is a has the hieghest reduction efficiency for EC. The high BOD and COD removal by macrophytes could be linked to the increase in pH. Ng and Chan (2017) and Qin et al. (2016) reported an increase in pH by macrophyte and concluded that the macrophytes can be used to increase low pH. This pH increase canalso be linked to COD and BOD reduction since studies by Qu et al. (2017) and Riaz et al. (2017) has shown that the presence of plants in water contributes to the growth of microbes which in turn causes the degradation of organic compounds. The reduction of EC is attributed to the growth of the macrophytes. Higher EC, COD and BOD removal has been reported by Augustynowicz et al. (2014), Di Luca et al. (2014), Török et al. (2015) using different macrophytes to remediate various wastewaters. The reduction of EC is attributed to the growth of the macrophytes (Haddad et al., 2018).

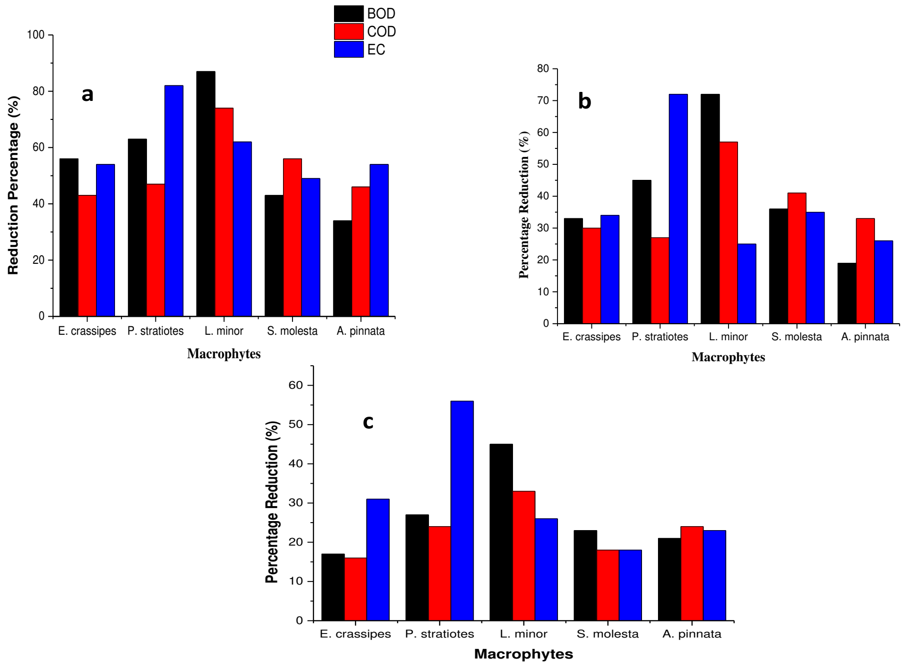

Figure 2. *Efficiency of Macrophytes in BOD, COD and EC Reduction from (a) 100% Wastewater (b) 50% Wastewater (c) Control*

The efficiency of Macrophytes in Oil and grease, Total Dissolved Solids and Total Suspended Solid Reduction

The pattern of TDS and TSS removal was heighest in the control than 100% wastewater and 50% wastewater, it was also noticed that the performances of all the plants were without any significant differences (*Fig. 3c*). *Pistia stratiotes* and *Eicchornia crassipes* have no significant differences in term of their reduction efficiency of TDS and TSS but both have a high significant difference to the removal efficiency of *Lemna minor, Salvinia molesta* and *Azolla pinnata* for TDS and TSS removal from both 75% wastewater and 100% wastewater (*Figs. 3a, 3b*). The removalefficiency of *Lemna minor* was found to be higher for oil and grease removal from 100% wastewater and 50% wastewater. Little significant differences exist between

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the removal efficiency of *Lemna minor, Pistia stratiotes* and *Eichhornia crassipes* in oil and grease removal if compare to oil and grease removal efficiency of *Salvinia molesta* and *Azolla pinnata*. The ability of the macrophytes to reduce TDS and TSS have been reported to be due to the particle sedimentation ability of the macrophytes which is due to there taining of the particle by the roots of macrophytes hence the reason why *Pistia stratiotes* and *Eicchornia crassipes* have higher TDS and TSS reduction efficiency than other macrophytes (da Silva et al., 2018; Osti et al., 2018). The reduction of oil and grease by the macrophytes is attributed to the enhancement of microbial growth caused by the presences of themacrophyteswhich in turncausesthedegradation of oil and greases (Amare et al., 2018b; Liu et al., 2010).

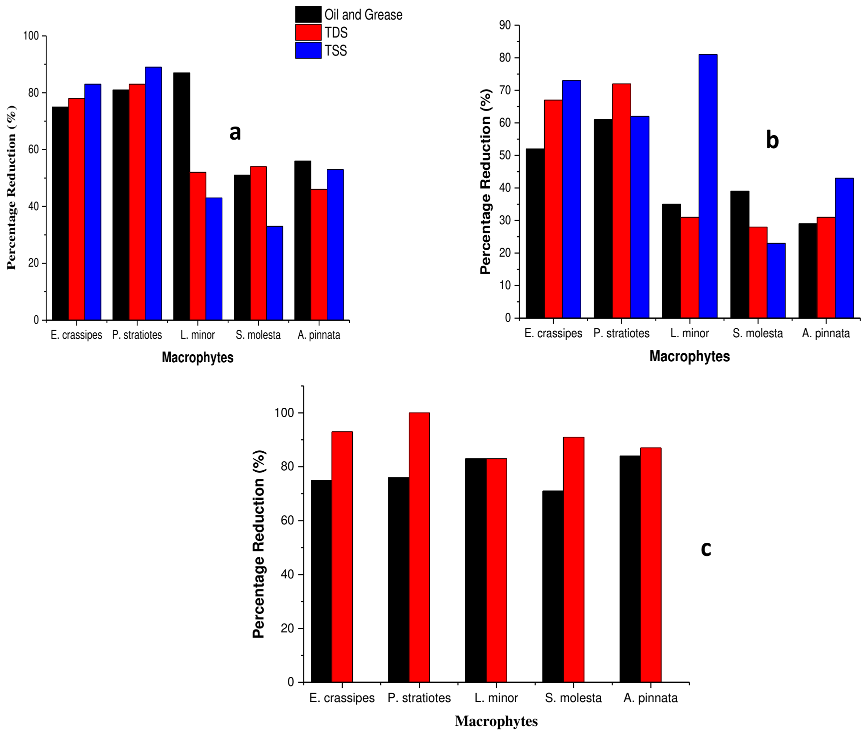

Figure 3. *Efficiency of Macrophytes in Oil and grease, Total Dissolved Solids and Total Suspended Solid Reduction (a) 100% Wastewater (b) 50% Wastewater (C) Control*

The efficiency of Macrophytes in Chloride, Phenol, and Ammonical Nitrogen Reduction

The result obtained shows a similar pattern for the removal of chloride, phenol and ammonical nitrogen present in 100% wastewater and 50% wastewater (*Figs. 4a, 4b*) by the macrophytes, with *Eicchornia crassipes* having the highest efficiency for chloride removal (780 mg/l to 107 mg/l) and *Salvinia molesta* having the highest efficiency for phenol and ammonical nitrogen removal (13 mg/l to 3 mg/l and 87 mg/l to 14 mg/l, respectively). The high phenol removal efficiency could be attributed to the fact that the presences of the macrophytes favor the growth of some microorganisms which help in the degradation of phenol (Mustapha et al., 2018; Suyamud et al., 2018). The high

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DOI: http://dx.doi.org/10.15666/aeer/1702_37233735

ammonical nitrogen and chloride % reduction by the macrophytes is attributed to the fact that but nitrogen and chloride are needed for plant growth although chloride are needed in minute quantity, so there as on for the high chloride removal efficiency could be due to the fact that the concentration of chloride in the wastewater was minute (Han et al., 2018).

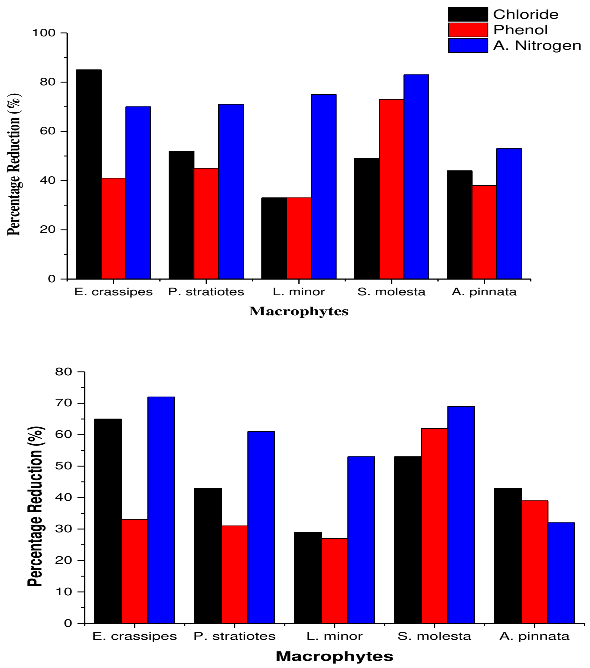

Figure 4. *Efficiency of Macrophytes in Chloride, Phenol and Ammonical Nitrogen Reduction (a) 100% Wastewater (b) 50% Wastewater*

Heavy Metal Removal Efficiency, Bioconcentration and BiotranslocationFactor

The result obtained shows high heavy metal removal efficiency by the entire test macrophytes (*Fig. 5*). This result obtained could be due to the fact that the heavy metal present in the wastewater was in low concentration with the highest been 10 ug/l. Several researchers such as Malar et al. (2014) have show that most macrophytes have the ability to effectively utilized heavy metal to a concentration of within 10 mg/l-15 mg/l (Sharma et al., 2015; Teles Gomes et al., 2014; Török et al., 2015). The BTF and BTC factor obtained in *Tables 2 and 3* respectively show that *Eicchornia crassipes* have the highest bioconcentration and biotranslocation factorfor Cd^{2+} and Ni^{3+} signifying that the plant can effectively absorb Cd^{2+} and Ni^{2+} from the wastewater and successfully transport the heavy metal totheshoot (stem and leaves) for utilization. *Pistia stratiotes* effectively absorbed and translocated Cr^{3+} and Pb^{2+} due to the high biotranslocation and bioconcentration factor recorded in *Tables 2 and 3*, respectively. This also shows that the plants were able to properly utilize the absorbed heavy metals. *Lemna minor, Azolla pinnata,* and *Salvinia molesta* were found to have accumulated the heavy metals, they still are not better accumulators if compared to *Eicchornia crassipes* and *Pistia stratiotes* (Amare et al., 2018b).

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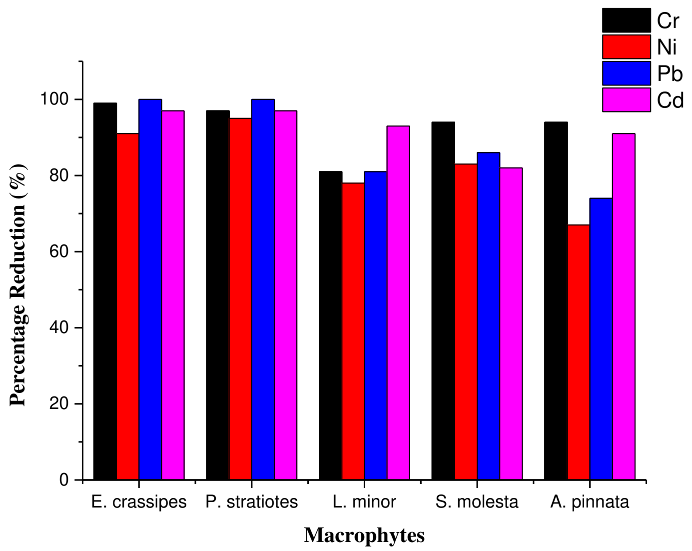

***Figure 5.** Efficiency of Macrophytes in heavy metal Reduction*

***Table 2.** Biotranslocation Factor of Macrophytes*

SN	Plant Sample	Cd^{2+}	Ni^{2+}	Pb^{2+}	Cr^{3+}
1	*Eicchornia crassipes*	2.3	1.7	1.4	1.6
2	*Pistia stratiotes*	1.8	1.5	1.7	1.8
3	*Lemna minor*	0.4	0.8	1.4	0.4
4	*Salvinia molesta*	1.2	1.1	0.9	1.5
5	*Azolla pinnata*	0.7	1.6	1.6	0.9

***Table 3.** Mean Bioconcentration Factor of Macrophytes*

SN	Plant Sample	Cd^{2+}	Ni^{2+}	Pb^{2+}	Cr^{3+}
1	*Eicchornia crassipes*	1.2	1.4	0.9	0.7
2	*Pistia stratiotes*	1.1	1.3	1.6	1.9
3	*Lemna minor*	0.7	0.8	0.5	0.6
4	*Salvinia molesta*	0.4	0.3	0.4	0.6
5	*Azolla pinnata*	0.3	0.5	0.3	0.4

Conclusion

Although, all the test plants show the ability of removal of pollutants from wastewater resulting from dying activities when compare to the control and 50% dilution, *Eicchornia crassipes*, *Lemna minor* L., and *Pistia stratiotes* proof to be more efficient in the the remediation of wastewater resulting from dying and can be used in dyeing wastewater sedimentation and treatment before discharge into drainage system to prevent effects on aquatic flora and fauna.

Acknowledgements. The first author is thankful Nigeria Petroleum Development Fund (Nigeria) for providing the fund and enabling the environment to carry out the research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37233735

THE CORRELATION BETWEEN URBAN OPEN SPACE OCCUPATION DIFFERENCES AMONG GENERATIONS X, Y, AND Z AND OCCUPANT WELL-BEING

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(Received 25th Dec 2018; accepted 20th Feb 2019)

Abstract. As spatial occupants, humans expect the space to be designed to meet their needs and satisfy their goals. Thus, designers should identify the changing spatial uses and preferences of several different groups. The present study aimed to determine intergenerational differences among generations x, y, z in their preferences in open space occupation and activities and their well-being levels based on occupation types. The study was conducted in Trabzon province in Turkey. It aimed to determine the occupation types for different generational groups in urban open spaces. For this purpose, a survey was conducted with 70 individuals from each generation (x, y, z), a total of 210 individuals in urban open spaces and their well-being levels were determined based on their occupational objectives and usage. The analyzes and tests conducted on the collected data demonstrated that the generations x, y, and z had different objectives and conducted different activities in open urban spaces. It was determined that generation x used the urban open spaces for sitting, resting, chatting, reading newspapers, etc. activities, generation y used the urban open spaces for meeting their peers, socialization, shopping, eating-drinking, etc. activities, while generation z occupied the urban open spaces for activities such as entertainment, sports, spending time with friends, meeting, etc. It was found that when they spent time in spaces that offer adequate activities, the level of well-being improved. It was concluded that different generations occupied outdoor spaces for different purposes, performed different activities in these spaces and their well-being improved when they spend time in adequate spaces.
Keywords: *age groups, activities, preferences, outdoor spaces*

Introduction

The group of individuals that were born in a particular period of time is called a generation. Generation is defined as a group of people who were born during the same period, sharing similar age, experiencing similar problems, fate and responsibilities. Sociological definitions of generation describe the concept as group of individuals who were born in certain period, affected by similar social, political and economic factors in the socialization process, assumed similar responsibilities due to similar conditions, and thus share common values, beliefs, expectations and behavior (Kon, 2017; Alpak et al., 2018; Glass, 2007; Inglehart, 1997). Nowadays, the term generation is generally used to emphasize the social or cultural structure of a period. Differences between generations are evident in every century, every period, and every époque. Intergenerational conflicts led to important social problems, the attempt of older generations to understand the new ones is very important for the society to live together and occupy a common space.

Auguste Comte initiated the scientific research on generation studies between 1830 and 1840. Comte suggested that generational deviations are forces that evolve in the historical process and social progress is possible only with the knowledge that one generation would transfer to the next (Comte, 1974). As the generations age, the behavioral characteristics of individuals differ. This is called the ageing effect and explains the different behavior of different generations within the same timeframe. Each generation has its own history and knowledge that distinguishes it from other generations. This difference stems from the socio-cultural and political legacy of previous generations (Toruntay, 2011; Dereli and Toruntay, 2015).

Strauss and Howe (1991), who conducted generation studies based on the work by John Stuart Mill, August Comte and Karl Manheim, used the following criteria to describe the concept of generations:

- There should be a common historical period shared by the members of a generation. These individuals experience similar historical events and social trends when living the same periods of their lives.
- Generation members tend to share behavior and common beliefs, including attitudes such as taking risks, culture, values, professional and domestic life that were shaped by their experiences in childhood or adulthood.
- They are aware of the experiences and skills they share with their peers and share this belonging with the members of the same generation. Several studies demonstrated that individuals in different generations define the traits of their generation as original and unique, although these traits are not observed as such by others. Known and frequently used generations in recent history are classified as follows:
  - Traditionalists1925-1945 (Lehto vd, 2006).
  - Baby boomers 1946-1964 (Pekala, 2001), 1944-1960 (Arsenault, 2004) and 1943-1960 (Families & Work Institute, 2002), 1945-1965(Roberts and Manolis, 2000; O'Bannon, 2001; Smola and Sutton, 2002; Wallace, 2006).
  - Generation X 1966-1979 (Alwin, 2002; Roberts and Manolis, 2000; Smola and Sutton, 2002; Wallace, 2006).
  - Generation Y (Millennials) 1980-2001 (Lower, 2008), 1980-1995 (Cogin, 2012).
  - Generation Z (Millennial/Internet Generation) 2000-2020 (Haeberle et al., 2009; Kuran, 2010).

The classification of the generations in the 21st century included traditionalists, baby boomers, generations X, Y and Z. Individuals' perceptions, expectations, priorities, perspectives on life, and ultimately their behavior vary based on time. The fact that individuals born on similar timeframe have similar characteristics and each generation have distinct characteristics led to the studies on the concept of generation and the characteristics of generations (Davis et al., 2006; Adıgüzel et al., 2014). Currently, there are at least 3 generations that must spend time together and occupy the urban spaces as peers. The conflicts between these generations are undoubtedly an important social issue. As spatial occupants, humans expect the space to be designed to meet their needs and satisfy their goals. Thus, designers should identify the changing spatial uses and preferences of several different groups. Children, young individuals and adults, i.e., generations x, y, and z are groups of occupants with different ages whose needs should be identified.

Ultimately, different generations have different demands, requirements and preferences. It is important to determine the differences between the generations to construct suitable designs for the occupants and create open spaces that the occupants could use, heal the occupants and increase their prosperity (Zhang et al., 2018). The present study aimed to determine the different needs of the generations x, y and z and the differences in outdoor

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DOI: http://dx.doi.org/10.15666/aeer/1702_37373751

space use among these generations. Determination of the different uses and activities by different generations in urban open spaces was also aimed in the present study. Thus, design criteria for the open space occupancy by different generations were determined to design therapeutic and prosperous spaces for the occupants in order for the spaces to attract individuals of all current generations.

Generation X and its attributes

The generation x was defined as the generation born between 1965 and 1978 (Okay, 2001) in certain resources and between 1961 and 1980 (Arsenault, 2004) and between 1965 and 1979 (Families and Work Institute: 2002) in others. This generation was raised in a world where the economy was in decline and a completely different value system was constructed (Acar, 2010). The decisive events for this generation were the Challenger Disaster in the US, divorces, AIDS, Sesame Street, MTV, Game Boy, and the first personal computers (Pekala, 2001). X generation possess a lower level of communication skills and technology knowledge when compared to younger generations (Elmore, 2011; Jianrui, 2011). Their technology knowledge is generally limited to general skills such as e-mailing and online job search. They are usually children of working parents and are defined as "latchkey kids" in various studies since they grew up with house keys hanging on their necks (FWI, 2002). They learned to grow up on their own and going home from school alone (Toruntay, 2011).

In this period, oil crisis, economic shocks, the generation of '68, university revolts, political conflicts and television were the most important events in Turkey. Women began to participate in the labor activities in this period in Turkey. Parents started to have fewer children for a better life. They focused more on finance and individualism gained importance (Mengi, 2011).

Generation x is often described as indifferent and aimless. Researchers argued that this may be due to a phenomenon derived from a collective cynicism among Generation X, a generation who witnessed more violent and negative events with the introduction of television into homes (Wolburg, 2001; Smola and Sutton, 2002).). They tend to take more risks and question authorities such as common rituals and traditions when compared to previous generations, as well as being family-oriented, self-confident, open-minded and fun-loving (Zhang and Bonk, 2010; Etlican, 2012). Generation X is defined as a generation that is compliant with the rules, with a strong sense of belonging, respect for authority, loyal and hardworking. this age range, they experienced a feeling of alienation as a result of being the first generation to witness the divorce of their parents (Montana and Petit, 2008). According to Elmore (2011), members of Generation X do not endeavor to understand other generations. They usually work to live. Furthermore, this generation witnessed a number of inventions and discoveries. The members of the generation X, who opened their eyes to the world with washing machines with a roller press, transistor radios, cassette players and turntables, experienced several transformations. Specifically, they tried to adapt to the transformation to computer systems and the changing business processes due to technological advances. Today, they are 22% of the population in Turkey (URL 2).

Generation Y and its attributes

The generation y was defined as the generation born between 1981 and 2000 in certain resources (Arsenault, 2004), between 1980 and the present (Families and Work Institute, 2002), between 1979 and 2001 (Pekala, 2001), and between 1982 and 2004 (Strauss and

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Howe, 1997) in others. This generation has different names such as Millennials, Generation Next, Digital Generation, Echo Boomers and Nexters (Morgan and Ribbens, 2006).

The principal events of the period that affected the generation y in Turkey included increasing terrorist acts, the Gulf War, Iraq War, the Internet, globalization and mobile phones (Aktan, 2011). Similar to generation x, generation y had working parents. However, the age of parenthood increased to 27. This generation, which had quite different parents when compared to previous generations, had more active parents who had a more active role in raising their children under better conditions, their education and safety. Members of Generation Y are the children of parents who regularly check the children's grades, question bad grades, meet their teachers, and visit the school frequently. The generation y, who had to defend themselves against their parents constantly on issues they failed, learned how to deal with the system at an early age (Wendover, 2001; Zempke et al., 2013).).

Generation Y, the busiest generation ever, were raised with micro programs by their overprotective parents and grew up within close relationships, however they are still the most stressed generation ever (Raines, 2002). In the literature, the parents who monitor this generation closely and are constantly around are called "Helicopter Parents" (Howe and Strauss, 2007). Globalization also had a great impact on this generation. This generation is the first generation to be born to Internet with the highest daily interaction with different ethnicities and cultures, and its members possess more cultural richness when compared to previous generations. They grew up under the media and commercial influences since their birth, and thus possess higher brand awareness when compared to their parents. When the economic conditions of the members of this generation are compared, it is observed that the difference between the members with high and low purchase power is the greatest among the studied generations (Toruntay, 2011). Sheahan (2005) defined the generation y as the generation that could easily express their thoughts and emotions, who love social relationships and with high emotional intelligence.

This generation in Turkey that includes young adults who are free-spirited, adaptable, fickle, well-educated, technology-oriented and like to defy the authority live in a period where the impact of globalization are felt intensely and economic and intercultural interaction is at a maximum due to the facilities available (Türk, 2013). It was claimed that this generation is approximately three times larger than the Generation X (Schroder and Warren, 2005). 35% of the population living in Turkey currently are the members of this generation (TUDK). In Turkey, there are more Generation Y members when compared to the total population in several European countries (Toruntay, 2011).

Generation Z and its attributes

This generation is believed to include individuals who are born between 2000 and 2020 and will join the labor force within the next 5 years (Kuran, 2010).

Generation Z children live in a society where novel technological means of communication and transportation are in abundance. They could communicate with each other via verbal or even visual communication using digital tools even when they live far away. The members of this generation play with tablet computers instead of toys, are fast-consuming, and have the ability to multitask. Unlike previous generations, although they are considered as 'network' youth, they are estimated to be physically alone and tend to live alone since they could communicate in long distance. Since their multitasking skills are extremely developed, they are considered as the generation with the highest motor skill synchronization such as manual, sight and auditory skills (Toruntay, 2011). According to

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Senbir (2004), this generation would be open to technology, and will not consider technology as a tool, but as a natural part of life.

However, it is considered that these advantages could turn into disadvantages due to attention and concentration difficulties. They like creative activities. Their long-term memory could be activated through games, storytelling and imagination rather than memorization. They are result oriented. Since the members of this generation are unsatisfied, unstable and innate consumers, it is considered that the concept of authority is not important in their lives. The networks they live in since their birth would play a major role in their identity.

This generation that includes the children born after 2000 in Turkey is also called the 'Crystal Generation'. Experts consider this generation as 'deeply emotional' and it includes 17% of the Turkish population (Adıgüzel et al., 2014). It is estimated that the share of generation z in the Turkish population reached 18.000.000 in 2015. Since they dislike hierarchy and are open to communications, it is considered that they would alter the organizational structures of the spaces they live in and work. Children who are the members of this generation expect everything to be personalized (Toruntay, 2011). The fact that their high education level, their independence and individuality would increase their creativity, their immediate honesty could lead to a motivated environment, the decrease in intergenerational differences, their social character and openness to communications, leading to a good understanding of each other, destruction of geographical borders due to the Internet, their higher self-expression levels could be considered among the positive attributes of this generation. Similarly, the disloyal, nondriven and unambitious, and fickle characteristics of the generation z and their desire to personalize everything could be considered among the negative attributes of this generation (Toruntay, 2011). It is also expected that they would have several diplomas and become experts, and would be innovative, malcontent and indecisive.

As a result, previous study findings demonstrated that there are different personality development, requirements and perceptions of these three generations. Thus, are the outdoor activities that these 3 generation occupants with different attributes conduct or desire to conduct different? The present study aimed to answer this question. Whether the well-being levels of the occupants increase when they spend time in spaces adequate for different activity requirements was another question that the present study aimed to respond.

Materials and methods

The study area included the outdoor spaces in the Eastern Black Sea Region and Trabzon province. Trabzon was established in early 2000's BC at the beginning of the historic Silk Road, which passes through Erzurum and reaches the Iranian border, connecting Europe and Asia. Trabzon, which is an important historical city, possesses several cultural assets due to its location. This led to the privileged position of Trabzon province among Eastern Black Sea Region provinces (*Fig. 1*).

Application

A survey was conducted with 210 Trabzon residents from the generations x, y, and z (70 individuals from each generation) in urban open spaces. The survey form included two sections. In the first section, different generation occupants were provided a list of activities

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and they were asked to mark the most frequent activity they conduct in open spaces. The second section was designed to determine the correlation between space occupancy and well-being of the occupants. In this section, the following statements were tested with 5-point Likert attitude scale (5 = very frequently, 1 = never):

- I feel happy when I can conduct different activities that suit my needs in spaces.
- I feel happy when the spaces provide facilities for the activities I desire to conduct.
- I feel better in spaces that allow me to conduct active activities.
- I feel better in spaces that allow me to conduct passive activities.
- The activities I conduct in urban spaces make me happy.

Snowball sampling was used to select the survey participants. This method starts with the selection of a subject based on the research criteria and the other subjects are accessed using the initially selected subject. The subject is asked to recommend someone or some people with the same characteristics (Lawrence, 2016). Thus, individuals in the adequate generation were accessed and 210 individuals (70 in each generation) were interviewed. Initially, demographic data about the 210 participants were obtained, then the activity preference rates for the generations were determined and One-way ANOVA was conducted to determine the differences between the distribution of activity levels of the generations in urban open spaces. In the second stage, the questions asked to determine the correlation between the activities in open spaces and occupant well-being was analyzed separately for each generation and percentage and mean values are presented. Then, t-test statistics were applied to determine the distribution of well-being level for each generation (x, y, z).

Figure 1. *Study area*

Findings and discussion

Demographic findings

The survey form included the age of the participants to determine their generation (*Table 1*).

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Table 1. *Participant demographics*

	Age range	%	frequency	
Generation X	45-50	11,0	23	70
	44-40	11,4	24	
	39-36	11,0	23	
Generation Y	35-31	10,5	22	70
	30-26	11,0	23	
	25-19	11,9	25	
Generation Z	18-16	12,4	26	70
	16-14	10,5	22	
	14-12	10,5	22	

Activity findings

Gehl (1987, 2010) categorized the activities conducted in open spaces in three categories: compulsory activities, optional-selective activities and social activities:

- The compulsory activities include more or less obligatory and non-selective activities such as going to work, transportation, and waiting for someone. Since the activities in this group are mandatory, they occur under all conditions throughout the year. The participant has no right to choose.
- Optional activities are voluntary activities conducted when the time and space is suitable. The activities in this group take place only in favorable external conditions, when suitable whether and space are available. There is no obligation to conduct these activities and an individual conducts these activities compleyelt voluntarily such as taking a walk.
- Social activities take place based on the other two types of activities and are also called "eventual activities." Social activities are scrutinized in two categories: passive and active activities. Passive activities include short-lived and single superficial activities such as watching and listening to other people that one is not familiar with, watching the environment, reading newspapers or books, playing games such as backgammon, etc. (Gehl, 1987) These passive relations are important. Because the weak relationships established via the passive relations could be used as a first step in establishing a strong structural interaction (Peters et al., 2010). Active activities include activities where individuals interact with each other such as chatting, playing games, eating and drinking, sports, socializing, shopping and meeting.

In studies conducted to determine human behavior, attitudes and behavior, the optional and social activities that people conduct on their own without any requirement are investigated rather than the mandatory activities conducted without any choice. Various studies reported these activities conducted by occupants of various age groups in public open spaces (Bäckman and Rundqvist, 2005; Whtye, 1980; Mehta, 2007, 2009; Yuen and Chor, 1998; Kärrholm, 2008; Lofland, 1998). These activities included passive or active activities such as watching and listening to other people, talking, sitting with friends, chatting, reading newspapers, playing games, listening to street performers, etc. In the present study, behavior and attitudes of different generations, in other words age groups, were investigated. Thus, the present study scrutinized social and optional activities conducted in open spaces. The activities determined based on this

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criteria included 15 activities (going out with friends, spending time with peers, sitting-resting, eating-drinking, spending time with family members, to breath the fresh air, shopping, walking, meeting with peers, viewing, reading books and newspapers, playing backgammon, playing games, socializing, having fun, playing sports, chatting) is provided in the question posed to determine the activities that the occupants from different generations conducted in urban open spaces and they were asked to mark the activity they conducted most. The highest value was observed for sitting-resting activity for generation x (22.9%). The highest value was observed for being with peers activity for generation y (28.6%). The highest value was observed for sports activities for generation z (24.3%) (*Fig. 2*).

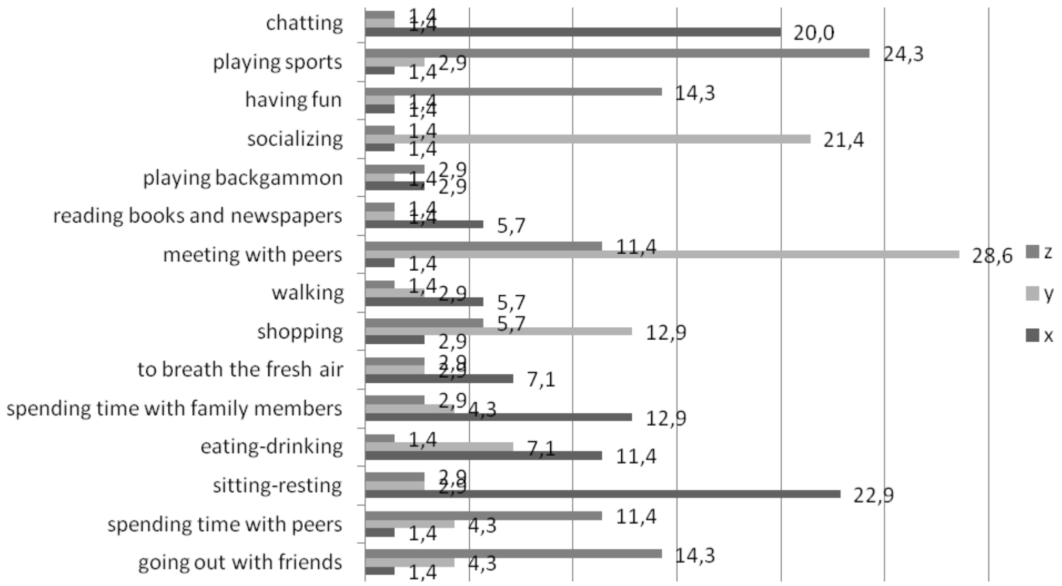

Figure 2. *Activity distribution for generations x, y, and z*

Since the age range of generation x includes adults who are more serene when compared to the new generations, the preference for passive activities such as sitting-resting was higher among these individuals. According to Elmore (2011), members of the Generation X do not endeavor to understand generations other their own. The fact that they are inclined to conduct activities alone increases the possibility that they would be challenged in crowded activities. This could explain why they preferred passive activities in the study. It was observed that the generation y members preferred to be among peers the most, since they are between the ages of youth and adulthood and due to the social attributes of the generation. It could be suggested that the fact that their lives are generally fast, versatile and extremely intense (Zhang and Bonk, 2010) could be due to their environment of growth where these rapid changes occurred. Thus, they live in fast, active and crowded environments to meet their expectations. This explains the fact that they preferred social activities conducted with their peers in the study. Finally, generation z preferred active activities the most since this group includes the youngest individuals between childhood and youth and the sportive activities were the most preferred activities. Because this generation has high self-esteem, and the fact that their parents cherished them and considered them different increase their self-esteem.

They are independent and free, there is nothing impossible for them (Haeberle et al., 2009). Thus, it was expected that they would prefer active activities in the study.

The One-Way ANOVA test conducted with SPSS (v. 17.0) software demonstrated that the distribution of the activity levels among different generations in urban open spaces was significant (*Table 2*).

Table 2. *Distribution of urban open space activities based on generations*

	Sum of squares	df	Mean square	F	Sig.
Between groups	138.867	2	69.433	3.383	.000
Within groups	4248.657	207	20.525		
Total	4387.524	209			

Occupant well-being findings

In order to determine the correlation between the open space activities and occupant well-being, the answers provided to 5 questions by each generation were analyzed separately.

The generation x preferred to conduct passive activities with an average of 3.94, they were satisfied with the activities they conducted in urban outdoor spaces with an average of 3.80, they were satisfied with conducting various suitable activities with an average of 3.61, they stated that they were satisfied when the spaces allowed them to conduct the activities they desired with an average of 3.39, however their preference on conducting active activities was low with an average of 2.93. Since individualism and competitiveness are more important in this generation that prioritizes materialism (Reynolds et al., 2009), it was determined that they conducted more passive individual activities and their open space satisfaction was also high. The percentage graph for the answers of generation x members is presented in *Figure 3*.

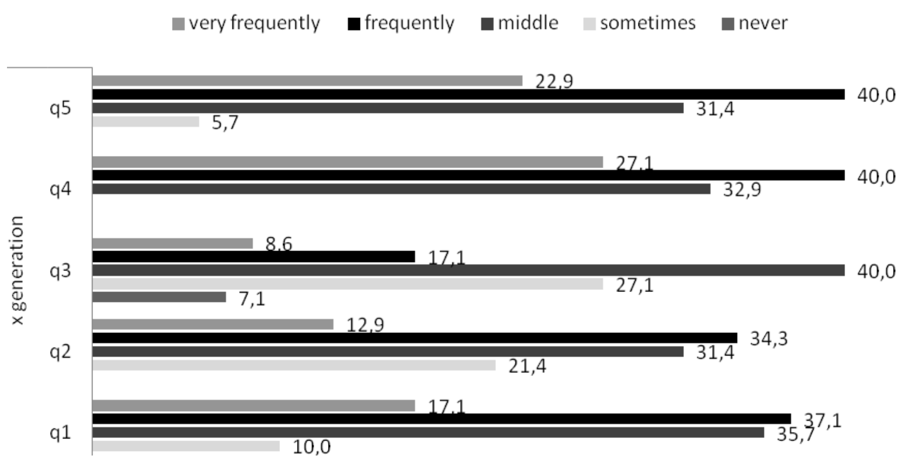

Figure 3. *Percentages of the generation x responses*

Then, it was determined that the above-presented distribution of the generation x responses was significant based on the t-test results conducted with SPSS (v. 17.0) (*Table 3*).

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Table 3. *Distribution of the generation x responses*

	t	df	Sig. (2-tailed)	Mean difference	95% confidence interval of the difference	
					Lower	Upper
s1	33.998	69	.000	3.614	3.40	3.83
s2	29.279	69	.000	3.386	3.16	3.62
s3	23.558	69	.000	2.929	2.68	3.18
s4	42.398	69	.000	3.943	3.76	4.13
s5	36.908	69	.000	3.800	3.59	4.01

It was determined that generation y was satisfied with the activities they conducted in urban outdoor spaces with an average of 3.96, preferred to conduct passive activities with an average of 3.56, they were satisfied with conducting various suitable activities with an average of 3.51, they preferred to conduct active activities with an average of 3.31, that is, the answers to all questions were at higher levels. As demonstrated with the literature review, peers and families are very important for Generation Y. They can come together very quickly due to their peer acceptance traits. This is the generation where the difference between the generations is felt the most. This is a technology-friendly, individualist, entrepreneurial, comfortable and globalized generation. Changes in living conditions introduced different preferences for this generation (Zempke et al., 2013). In the present study, it was determined that urban outdoor spaces have positive effects on their preferences and increase their well-being levels. The percentage graph for the answers of generation y members is presented in *Figure 4*.

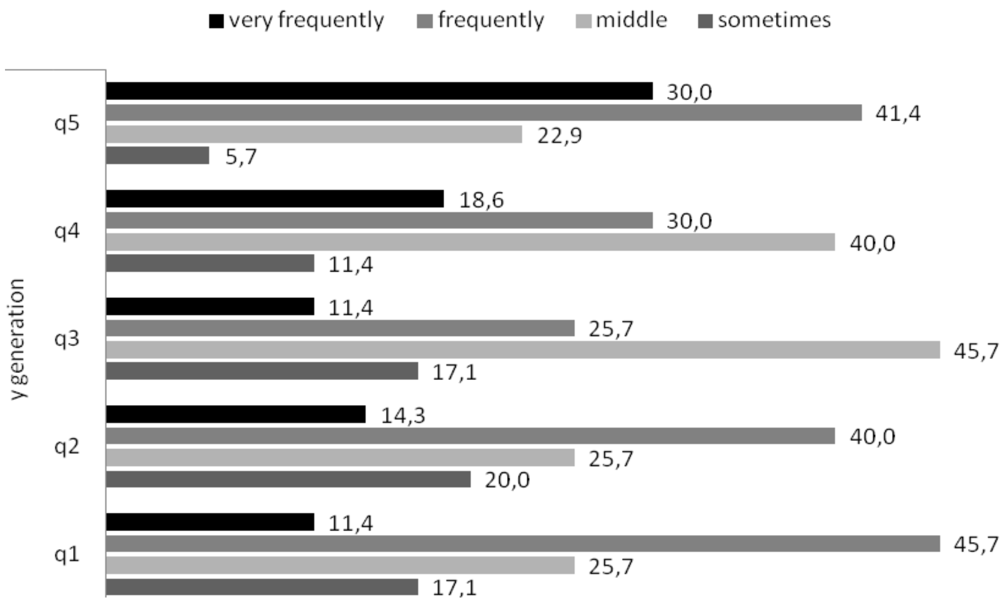

Figure 4. *Percentages of the generation y responses*

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DOI: http://dx.doi.org/10.15666/aeer/1702_37373751

Then, it was determined that the above-presented distribution of the generation y responses was significant based on the t-test results conducted with SPSS (v. 17.0) (*Table 4*).

Table 4. *Distribution of the generation y responses*

	t	df	Sig. (2-tailed)	Mean difference	95% confidence interval of the difference	
					Lower	Upper
s1	32.213	69	.000	3.514	3.30	3.73
s2	29.936	69	.000	3.486	3.25	3.72
s3	31.034	69	.000	3.314	3.10	3.53
s4	32.111	69	.000	3.557	3.34	3.78
s5	37.822	69	.000	3.957	3.75	4.17

It was determined that generation z preferred to conduct active activities with an average of 4.26, they were satisfied with conducting various suitable activities with an average of 4.06, satisfied with the activities they conducted in urban outdoor spaces with an average of 4.03, they stated that they were satisfied when the spaces allowed them to conduct the activities they desired with an average of 4.00, and the responses to all questions were close to very high level, however their preference for passive activities was low with an average of 2.31. This was due to the fact that this generation is still very young and with active-mobile attributes. This generation is a fast-consuming generation. However, since they are familiar with the Internet, they are expected to develop the ability to deal with more than one subject at the same time (Senbir, 2004). The present study findings demonstrated that this generation preferred active activities due to their age group and they were happy when they conducted different activities and open spaces were effective on their well-being levels. The percentage graph for the answers of generation z members is presented in *Figure 5*.

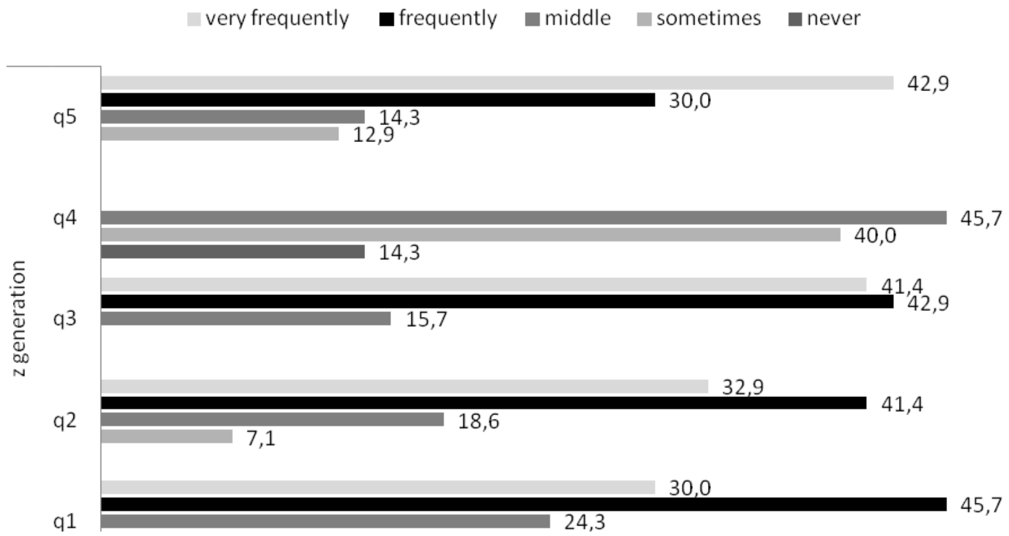

Figure 5. *Percentages of the generation z responses to well-being questions*

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Then, it was determined that the above-presented distribution of the generation z responses was significant based on the t-test results conducted with SPSS (v. 17.0) (*Table 5*).

Table 5. *Distribution of the generation z responses*

	t	df	Sig. (2-tailed)	Mean difference	95% confidence interval of the difference	
					Lower	Upper
s1	45.879	69	.000	4.057	3.88	4.23
s2	37.148	69	.000	4.000	3.79	4.21
s3	49.747	69	.000	4.257	4.09	4.43
s4	27.153	69	.000	2.314	2.14	2.48
s5	32.128	69	.000	4.029	3.78	4.28

Conclusion and recommendations

In conclusion, the present study conducted to determine open space occupancies of three generations with different desires, requirements and preferences revealed the following results:

- The generation x predominantly preferred passive outdoor activities such as sitting-resting,
- The generation y predominantly preferred social activities such as being with their peers,
- The generation z predominantly preferred more active activities such as sports.
- It was determined that all three generation members were happy when they conducted activities that were adequate for their needs and when the spaces allowed activities that were adequate for their desires.
- It was observed that urban open space activities were satisfactory for the members of all three generations and increased their well-being.
- Generation x felt better when they conducted passive activities, generation y felt better when they conducted both passive and active activities, and generation z felt better when they conducted active activities.

The different preferences and occupations determined based on the generations should be used to develop user-friendly designs and to create functional outdoor spaces that are therapeutic and increase the well-being of the occupants. The study investigated the different requirements of generations x, y and z and to determine the differences between these generations in outdoor occupancy. The study also aimed to guide future designs by determining the outdoor occupancy types suitable for different generational groups and the activities preferred by these groups. The study findings are quite important to design open spaces that needs to be renewed, renovated or built and would appeal to all generations, thus would enable creation of adequate spaces to improve the health and well-being of the occupants.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37373751

EFFECTS OF GLYCINE BETAINE CONCENTRATIONS ON THE AGRONOMIC CHARACTERISTICS OF STRAWBERRY GROWN UNDER DEFICIT IRRIGATION CONDITIONS

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(Received 29th Dec 2018; accepted 14th Feb 2019)

Abstract. Glycine betaine (GB) is a quaternary ammonium compound that can be found in a wide range of bacterial, plant and animal species. The exogenous application of GB is a convenient method for the induction of crop tolerance to various abiotic stresses. We examined the effects of different exogenous GB concentrations to deficit irrigation conditions (15% drainage) under soilless condition in terms of morpho-physiological, yield, quality and biochemical features. The different GB concentrations (0, 10 and 20 mM) were used in Fortuna and Albion strawberry cultivars. As a result of the research, GB treatments increased plant growth and yield under deficit irrigation conditions. Fruit weight inclined by 26.29-49.50% and fruit yield by 17.17-39.59% at GB treatments as compared to control. Regarding biochemical content, increasing GB concentrations from 0 mM to 20 mM caused total anthocyanin to decrease, whereas the ascorbic acid increased. Furthermore, the highest yield, fruit firmness, chroma and total anthocyanin were determined in the Fortuna; the highest total soluble solid and ascorbic acid content in the Albion cultivar. Our results showed that the increase in yield and quality was due to exogenous GB applications. Indeed, water stress tolerance varied depending on cultivars.
Keywords: *water stress, ascorbic acid, colour, total anthocyanins, yield*

Introduction

Many strategies aimed at saving water are practiced in horticulture (Tunc et al., 2019). Drip irrigation method increases the efficiency of water use while decreasing the applied amount (Tunc et al., 2019). Mulch usage is a very important cultural practice to decrease evapotranspiration in conventional agriculture. Recent years, soilless culture techniques have expanded to increase the efficiency of water use in many agriculture countries. In addition, soilless culture enables lower water usage, lower fertilizer and higher yield per unit area than conventional method. Therefore this technique is known as a sustainable agricultural method. In fact, in Turkey, while 390 tons of water/decare is used under soil conditions with clay soil in the Mediterrenean region, in soilless systems much less water is used.

Turkey, with a strawberry production of 415150 tons in a production area of 15431 ha, was ranked fifth in 2016 in global production according to the FAOSTAT statistics (FAO, 2018). The strawberry protected production quantity increased by 40.51% (168,191 tons) in total production (TURKSTAT, 2017). Under protected conditions, there is a remarkable increase in general soilless production areas and the total soilless strawberry production area has reached 70 ha (Adak et al., 2018a). In soilless cultivation about 1.5–2 L of growing media/plant is used and even short time water stress causes loss of turgor (Adak, 2009). Thus, abiotic stress experiments, for example water stress, salinity stress, are conducted easier, faster in soilless culture technique as compared to conventional agriculture technique.

Recently, the most important environmental and agricultural problems are global warming and water shortage (Cramer et al., 2007; Fereres and Soriano, 2007; Osakabe

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

et al., 2014; Bakhsh and Hussain, 2015; Zhu, 2016). In particular, salinity and drought are two major constraints that limit agricultural production worldwide. Mainly drought stress causes to decrease yield and quality and affects biochemical features in arid or semi-arid climate, for example Mediterrenean conditions (Souza et al., 2004; Zlatev and Lidon, 2012; Adak et al., 2018b; Tunc et al., 2019). Adak et al. (2018b) stated that some of the fruit's physical features were affected negatively by stress conditions whereas many of the biochemical features such as total anthocyanin content, total phenolic content and antioxidant activity were positively modulated in strawberries. Barreales et al. (2019) stated that deficit irrigation applications drastically affect the phytochemical composition and antioxidant activity of grapevine leaves. Controversaly, Centofanti et al. (2017) indicated that the deficit irrigation strategies did not affect the yield, fruit color, pH, concentration of soluble solids, total phenolic compounds, anthocyanin and non- anthocyanin compounds, and mineral elements in pomegranate fruit.

Plants have evolved various protective mechanisms that allow them to acclimate to unfavourable environments for continued survival and growth (Wani et al., 2013). Osmotic adjustment is an adaptive mechanism involved in drought or salinity tolerance, which permits the maintenance of turgor under water deficit conditions (Wani et al., 2013). Different strategies have been improved for management of these stresses (Wani et al., 2013). Recently, some exogenous agent applications, such as aminoacids, plant nutrients, hormone-like compounds, have been tried in terms of practical usage for producer (Ahmad et al., 2007; Shi et al., 2014; Osman and Salim, 2016). In this regard, osmoprotectants or compatible solutes are small molecules that act as osmolytes and help organisms survive extreme osmotic stress (Lang, 2007).

Glycine betaine, an amino acid derivate from sugar beet (*Beta Vulgaris L. v. altissima*), is purified, enriched and crystallised (Makela et al., 1998; Rajashekar et al., 1999). The known role of GB is maintaining water content in plant cells by lowering solute potential under osmotic stress in osmotic adjustment (Makela, 2004; Dutta et al., 2018). Both the exogenous application of GB and the genetically engineered biosynthesis of GB in such crops is a promising strategy to increase stress tolerance (Wani et al., 2013). As a matter of fact, it ameliorated the harmful effects to various abiotic stress conditions, such as drought (Makela et al., 1998; Rajashekar et al., 1999; Rezaei et al., 2012; Zamani et al., 2014; Dawood, 2016; Rady et al., 2018), high salinity (YingYun et al., 2012; Yildirim et al., 2015), low temperature (Rajashekar et al., 1999; Zhang et al., 2008) and heat stresses (Oukarroum et al., 2012) in many crops. In addition, exogenous application of GB in non-accumulator plants may be a possible alternative approach for tolerance improvement against multiple abiotic stresses (Harinasut et al., 1996; Diaz-Zorita et al., 2001; Yang and Lu, 2005). In fact, tomato plants (*Lycopersicon esculentum* Mill.) do not naturally accumulate GB (Wyn Jones and Storey, 1981). However, Park et al. (2006) stated that exogenous application of glycine betaine increases chilling tolerance in tomato plants.

Glycine betaine can be phytotoxic, when applied either at higher concentration or by increasing the number of applications. The species such as bean, tomato, and grape are more sensitive to high concentrations of GB than grass species/cereals. Therefore, it is important to determine optimal concentration of GB, number of applications, and time of application for each crop species (Muhammad et al., 2006). In this regard, Rajashekar et al. (1999) determined exogenous application of glycine betaine (2 mM) to strawberry plants increased the cold tolerance of leaves within 72 h of application. Likewise, Armin and Reza Miri (2014) examined different application types of glycine betaine

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

(seed-treating, seed-treating+foliage spraying, spraying at flowering and non-application of glycine betaine) under rain-fed and irrigated conditions. 100 mM GB concentration was used in both seed treating and spraying. The result of this study, the seed application+spraying of glycine betaine was superior to other application methods in terms of yield and yield components increasing in cumin plants. Yildirim et al. (2015) observed that exogenous GB treatments at 10 and 25 $mmol \cdot l^{-1}$ elevated the concentrations of gibberellic acid, salicylic acid and indole acetic acid levels and it could ameliorate the harmful effects of salt stress in lettuce. Shams et al. (2016) suggested that exogenous GB treatments could ameliorate the tolerance of lettuce to salt stress by increasing the total antioxidants and total phenolics. Likewise, Youssef et al. (2018) foliar GB at 10 mM to cucumber plants ameliorated the harmful effects of NaCl stress on the vegetative growth and yield through enhancing both leaf relative water content and leaf membrane stability. In additon, Denaxa et al. (2012) stated GB and kaolin clay particles treatments increased CO_2 assimilation rates drought stress as compared to control in olive (*Olea europaea* L.) plants.

In this regard, a little is known about the effects of exogenously applied glycinebetaine concentrations on quantitative and qualititative characteristics in different strawberry cultivars and deficit irrigation conditions with soilless cultivation. As a matter of fact, in strawberries, the degree of water stress tolerance varies according to plant growth stage, stress duration, growing system, growing medium and cultivars (Adak et al., 2018b). Therefore, optimal GB concentrations and number of application and time of application should be determined in strawberry as well. The aim of the study were to assess the effectiveness of GB concentrations in different strawberry cultivars in terms of morpho-physiological, yield, quality and biochemical characteristics. Thus, the most suitable GB concentrations will be determined for drought conditions and it will be recommended to producers working under water stress conditions.

Materials and Methods

Plant Materials, Growing Conditions and Treatments

Experiments were conducted between October 2015-February 2016 in high plastic greenhouse owned by the private research center located in Serik-Antalya (36° 50' 37" N; 30° 50' 31" E; at an altitude of 50 m). Climatic data during the experiment are given in *Table 1*.

Table 1. *Monthly averages of temperature (°C) and relative humidity (%) inside the experimental greenhouse*

	October	November	December	January	February
Temperature (°C)	25.9	22.5	17.9	16.8	18.2
Relative humidity (%)	72.23	68.54	69.65	65.30	69.30

Treatments consisted of two strawberry cultivars (Fortuna and Albion) and three glycine betaine concentrations with control (0, 10 and 20 mM GB) under deficit irrigation condition (15% dranaige fraction). Seedlings were planted in grow bags containing cocopeat (100 cm in length × 15 cm in width × 7 cm in height) on the gutter at the height of 75 cm on 20 October 2015 (14.2 $seedlings \cdot m^{-2}$). A control irrigation regime (30% drainage) was applied to the plant for the first 2 weeks. Later, deficit

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

irrigation (DI) regime (15% dranaige) was followed from 03 November 2015 to 05 February 2016. In the system, a separate irrigation programme was implemented by using separate irrigation valves. Three different concentrations (0, 10, 20 mM) of glycine betaine (GB) (Sigma-Aldrich, 98%). were applied twice (during planting and 30 days after planting) through spraying the foliar part of plants. Ionized pure water was used in control treatment (0 mM GB).

The nutrient concentrations in the nutrient solution named as Starter Solution (from October to December) were in all treatments as follows: 4.50 mM K^+, 4.00 mM Ca^{2+}, 1.25 mM Mg^{2+}, 0.50 mM NH_4^+, 11.50 mM NO_3^-, 1.00 mM $H_2PO_4^-$, 1.50 mM SO_4^{2-}, 20 µM Fe, 10.0 µM Mn, 7.0 µM Zn, 0.75 µM Cu, 25.0 µM B, and 0.50 µM Mo. The EC and pH in the above NS were 1.55 $dS \cdot m^{-1}$ and 5.6, respectively. On reproductive stage, fertigation solution was replenished with different nutrient concentrations than in the initial nutrient solution. In this stage, the ion concentrations in the reproductive solution applied (from December to February) in all treatments were as follows: 5.50 mM K^+, 3.50 mM Ca^{2+}, 1.50 mM Mg^{2+}, 11.00 mM NO_3^-, 1.50 mM $H_2PO_4^-$, 1.50 mM SO_4^{2-}, 20 µM Fe, 10.0 µM Mn, 7.0 µM Zn, 0.75 µM Cu, 25.0 µM B, and 0.50 µM Mo. The EC and pH in the above NS were 1.70 $dS \cdot m^{-1}$ and 5.6, respectively. In the experiment, irrigation schedule was programmed depending on solar radition (3-8 irrigation/day with 3 min/irrigation) and irrigation programmes were done by automatic fertilising system (INTA Crop Technology S.L, Murcia, Spain).

Data Collection and Statistical Analysis

In the experiment, the morpho-physiological features of plants (crown diameter, chlorophyll index), pomological features in fruits (fruit weight, total soluble solid, titratable acid, firmness, colour) and biochemical features (ascorbic acid, total monometric anthocyanin) were examined. While morpho-physiological observations were carried out from November to February, all pomological and biochemical analysis were done from samples taken at the end of January. The yield per plant was determined from the total yield values obtained during the whole season.

Determination of crown diameter and chlorophyll index

In plants, crown diameter and chlorophyll indexes were measured monthly with digital calipers, and with chlorophyll meter (Field Scoot CM1000), respectively. Results were expressed as average monthly taken datas from November to February.

Determination of fruit weight and yield

Fruit weight and fruit yield were determined throughout the growing season (2 December 2015 to 05 February 2016). Fruit weight was presented as grams (g), and yield as yield per plant.

Determination of total soluble solids, titratable acidity and firmness

Total soluble solids content (TSS) were measured by a digital refractometer (REF121; Atago, Guangzhou, China) and expressed as %. The titratable acidity (TA) was determined by juice titration with 0.1 N NaOH up to 8.1 pH and expressed in % of citric acid per 100 mL juice (AOAC 1980). Firmness was determined by the equatorial region of fruit, using a texture analyser (FT-011, with 7 mm probe, Effegi, Italy) and expressed as $kg \cdot cm^{-2}$.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

Colour measurements

Fruit skin colour were measured by a Minolta CR-200 (MINOLTA Camera Co, LTD Ramsey, NJ) chromameter in terms of L* (whiteness = darkness), a* (redness) and b* (yellowness) values. Hue angle (colour shade) was calculated from arc tan (b*/a*) and chroma (colour saturation) was from square root ($a^{*2}+b^{*2}$). High values of hue angle indicate more red–orange and low values more red–bluish colour. Chroma shows transition from grey (low values) to pure colour (high values) (Selcuk and Erkan, 2005).

Determination of ascorbic acid (AA) and total monomeric anthocyanins (TMA)

Ascorbic acid (AA) content of strawberry samples was analyzed according to Bozan et al. (1996) with HPLC method. Results were expressed as mg/100 g fresh weight basis (FW). Total monomeric anthocyanins (TMA) were determined by the pH-differential method. After 30 min incubation at room temperature extracts were diluted with buffers (pH 1 or 4.5), the absorbance was measured at 520 and 700 nm (Agilent 8453 spectrophotometer, Agilent Technologies). The anthocyanins were quantified as mg Pg-3 glk/kg fresh weight (FW) basis (Giusti and Wrolstad, 2001).

Statistical analysis

The treatments were arranged in randomised block design (RBD) using a 2 × 3 factorial arrangement with three replications and 26 plants per treatment. The data were analysed using the Statistical Analysis System software programme, version 9.0 (SAS Institute, Cary, NC, USA) by ANOVA and treatment means were statistically compared using LSD test 5% of error probability.

Results and Discussion

Crown diameter and chlorophyll index

The determined data on crown diameter and chlorophyll index are given in *Table 2*. As can be seen in *Table 2*, the effects of the GB concentrations, cultivar and GB concentrations x cultivar interactions on crown diameter were found to be statistically insignificant. Crown diameter values, depending on the treatments, ranged from 2.30 mm to 2.58 mm.

In the case of chlorophyll index, there were statistical differences among the GB treatments and the highest chlorophyll index was observed at 10 mM GB concentrations (341.83). In the experiment, while cultivars insignificantly influenced the chlorophyll index, interactions were determined to be significant (*Table 2*). As a matter of fact, the highest chlorophyll index was indicated at 10 mM GB concentration x Albion cultivar interaction (346.00), while the lowest was at 20 mM GB x Fortuna cultivar interaction (254.33) (*Table 2).*

Consequently, according to our results, 10 mM GB concentration was the best concentration determined in terms of chlorophlly index. As for crown diameter, although statistical differences are insignificant, the highest values were founded at 10 mM application. In this concern, no literature was found regarding the effect of different exogenous glycine betaine concentrations under deficit irrigation conditions on morpho-physiological features in soilless strawberry cultivation. However, regarding cold stress conditions, Aras and Esitken (2013) stated that GB applications in strawberries affected leaf-relative water content, chlorophyll index, membrane

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

permeability criteria. In addition, the same researchers have also reported that concentration of 10 g/L GB has a protective effect on strawberries under cold stress. Likewise, Rajashejar et al. (1999) determined that exogenous application of glycine betaine (2 mM) to strawberry plants increased the cold tolerance of leaves within 72 h of application.

Table 2. *Effects of different glycine betaine concentrations on crown diameter and chlorophyll index at different strawberry cultivars grown under deficit irrigations*

Glycine Betaine Con. (mM)	Cultivars		Treatments
	Fortuna	**Albion**	
	Crown diameter (mm)		
0	2.43	2.34	**2.39**
10	2.58	2.42	**2.50**
20	2.39	2.30	**2.34**
Cultivars	**2.47**	**2.36**	
$LSD_{\%5\ treatment}$:NS; $LSD_{\%5\ cultivar}$: NS; $LSD_{\%5\ treatmentx\ cultivar}$:NS			
	Chlorophyll index		
0	294.00 BC*	294.33 AB	**294.17 b***
10	337.67 AB	346.00 A	**341.83 a**
20	254.33 C	313.33 AB	**283.83 b**
Cultivars	**295.33**	**317.89**	
$LSD_{\%5\ treatment}$:36.708; $LSD_{\%5\ cultivar}$:NS; $LSD_{\%5\ treatmentx\ cultivar}$:51.914			

*The comparison of the means of the cultivars is done within the same row and means of treatment in the same column and the interaction between all the values are significantly different ($P \leq 0.05$) using LSD comparison test. The mean of treatment and cultivar are shown in lowercase letters while interactions are in uppercase.
** $n = 26$

Other experiments examining different abiotic stress conditions and species indicated similar results. As a matter of fact, Mickelbart et al. (2006) stated application of GB (50, 100, 200 mM) increased the average number of days to maximum leaf area, and specific leaf weight on grapevine. However, leaf water potential and leaf relative water content were unaffected by GB. In addition, while 100 or 200 mM GB concentrations were determined as toxic concentrations, the research studies recommend concentrations below 50 mM for further applications to increase stress tolerance of grapevine. Rezaei et al. (2012) investigated effects of exogenous GB concentrations (0, 5 and 10 mM) in three stages with 10 days interval on some morpho-physiological characteristics of tomato plants at different levels of drought stress (weekly, bi-weekly and tri-weekly watering). Results showed vegetative growth parameters inclined as compared to control, that is shoot height by 70%, root length by 73%, leaf number by 187%, leaf area by 193%, total shoot fresh weight by 168%, total shoot dry weight by 9%, relative water content by 72% and stress tolerance index by 122%. Zamani et al. (20131) examined effects of proline (10 mM) and GB (15 mM) were sprayed on the grapevines at four growth stages (before flowering, flowering, sour cluster and veraison) under drought stress. Results showed that GB significantly affected leaf relative water content, canopy temperature, chlorophyll content, leaf area in all the treated cultivars. Shams et al. (2016) examined four concentrations of GB (0, 5, 10, and 25 mM) and two levels of salinity (0 and 100 mM of NaCl) in lettuce plants. Results showed the 25 mM GB treatment increased the dry matter by 44% as compared to control. Youssef et al. (2018) applied foliar GB at 10 mM to cucumber plants which improved both chlorophyll

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

contents and nutrient content under salt stress condition. Similarly, YingYun et al. (2012) reported that exogenous betaine significantly increased the photosynthetic pigments. Likewise, Rady et al. (2018) investigated the effects of different GB concentrations (0, 25 and 50 mM) under 4.80 dS m^{-1} salt stress on onion. GB treatment significantly increased shoot length, leaves area of plant, shoot fresh and dry weights, bulb yields, leaf chlorophyll contents, leaf relative water content and membrane stability index. Research studies determined that a level of 50 mM GB was more effective under moderate salt stress.

Figure 1. *General view from experimental area (left), 20 mM GB application (middle), 10 mM GB application (right)*

Fruit weight and yield

The effects of GB concentrations, cultivars, GB concentration x cultivar interactions on fruit weight and yield under deficit irrigation condition are given in *Table 3*. As seen in Table 3, increasing GB concentrations from 0 mM to 20 mM induced fruit weight to increase. The highest fruit weight was determined at 20 mM GB application (13.49 g). In the case of fruit weight, there were no statistical differences between cultivars. Regarding interactions, the highest fruit weight was found at 20 mM GB concentrations (13.81 g and 13.17 g), the lowest values were determined in control applications (9.74 g and 8.31 g) in all the tried cultivars.

In the case of fruit yield per plant, all tried GB treatments increased yield as compared to the control under deficit irrigation condition. Among the treatments, the highest fruit yield per plant was founded in 10 mM GB (359.00 g·$plant^{-1}$), followed by 20 mM GB concentrations (301.33 g·$plant^{-1}$). Fruit yield were found statistically different according to cultivars. The highest yield per plant was determined in Fortuna cultivar with 351.00 g·$plant^{-1}$ under deficit irrigation conditions (*Table 3*). Regarding interaction between GB concentrations and cultivars, the highest fruit yield was founded in 10 mM GB X Fortuna cultivar (430.67 g·$plant^{-1}$) (*Table 3*).

Consequently, all tried GB concentrations increased fruit weight and yield as compared to control. In fact, fruit weight inclined by 26.29-49.50% and fruit yield by 17.17-39.59% at GB treatments as compared to control. These results were similar to the values reported by other authors (Makela et al., 1998; Rezaei et al., 2012; Rady et al., 2018). Makela et al. (1998) emphasized that 3.36 kg·ha^{-1} GB during mid flowering

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

period increased fruit yield by 36 and 39%, as compared to control in tomato plants under salt and heat stress conditions. Furthermore, Rezaei et al. (2012) reported that the 10 mM exogenous glycinebetaine was the best treatment in terms of reproductive growth parameters on tomato plant grown under drought conditions. Rady et al. (2018) reported that GB treatments remarkably increased bulb yields of onion under 4.80 $dS{\cdot}m^{-1}$ salt stress. However, our results were in contrast to the findings of Meek et al. (2003), who pointed out that the yield components were not affected in drought stress in cotton plants by foliar GB applications. It can be said that differences on determined GB concentrations may be caused by differences in species, cultivar and application.

Table 3. *Effects of different glycine betaine concentrations on fruit weight and yield at different strawberry cultivars grown under deficit irrigations*

Glycine Betaine Con. (mM)	Cultivars		Treatments
	Fortuna	Albion	
	Fruit weight (g)		
0	9.74 BC*	8.31 C	**9.02 c***
10	12.17 AB	10.61 BC	**11.39 b**
20	13.81 A	13.17 A	**13.49 a**
Cultivars	**11.90**	**10.69**	
$LSD_{\%5\ treatment}$: 2.490; $LSD_{\%5\ cultivar}$: NS; $LSD_{\%5\ treatmentx\ cultivar}$:2.512			
	Yield ($g{\cdot}plant^{-1}$)		
0	287.67 B	226.67 B	**257.17 b**
10	430.67 A	287.33 B	**359.00 a**
20	334.67 AB	268.00 B	**301.33 ab**
Cultivars	**351.00 a**	**260.67 b**	
$LSD_{\%5\ treatment}$: 79.54; $LSD_{\%5\ cultivar}$:64.94; $LSD_{\%5\ treatmentx\ cultivar}$:112.49			

*The comparison of the means of the cultivars is done within the same row and means of treatment in the same column and the interaction between all the values are significantly different ($P \leq 0.05$) using LSD comparison test. The mean of treatment and cultivar are shown in lowercase letters while interactions are in uppercase.
** $n = 26$

Total soluble solid (TSS), titratable acidity (TA) and firmness

The effects of GB concentrations, cultivars, GB concentration x cultivar interactions on total soluble solid (TSS), titratable acidity (TA), and firmness under deficit irrigation condition are given in *Table 4*. As seen in *Table 4*, while the effects of strawberry cultivars on TSS, TA and firmness were found statistically different, GB concentrations was insignificant differences. As a matter of fact, the highest TSS value was determined in the Albion (7.39%) and the highest TA was in the Fortuna (0.78%) and the highest firmness was in the Fortuna cultivar (0.57 $kg{\cdot}cm^{-2}$) under deficit irrigation condition. Regarding the interaction between GB concentrations and cultivars, the highest TSS was founded in the Albion x Control (7.56%) and Albion x 10 mM GB (7.36%). In addition the highest TA value was determined in the Albion x 10 mM GB interaction (0.80%) (*Table 4*).

Consequently, exogenous GB applications did not affect to total soluble solid, titratable acidity, firmness whereas cultivars significantly affected these criteria. Between the cultivars, TSS was higher in the Albion, whereas TA and firmness were in the Fortuna under deficit irrigation condition. However, our results differed from those of YingYun et al. (2012) who reported that exogenous betaine significantly increased

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

the photosynthetic pigments and soluble sugar content. This difference is thought to be caused by species, cultivar and growing conditions.

Table 4. *Effects of different glycine betaine concentrations on total soluble solid (TSS), titratable acidity (TA) and firmness at different strawberry cultivars grown under deficit irrigations*

Glycine Betaine Con. (mM)	Cultivars		Treatments
	Fortuna	**Albion**	
	TSS (%)		
0	6.56 C*	7.56 A	**7.07**
10	6.70 BC	7.36 A	**7.03**
20	6.36 C	7.23 AB	**6.80**
Cultivars	**6.54 b**	**7.39 a**	
$LSD_{\%5\ treatment}$: NS; $LSD_{\%5\ cultivar}$: 0.48; $LSD_{\%5\ treatmentx\ cultivar}$:0.593			
	TA (% citric acid)		
0	0.70 C	0.76 B	**0.73**
10	0.72 C	0.80 A	**0.76**
20	0.70 C	0.77 AB	**0.74**
Cultivars	**0.78 a**	**0.71 b**	
$LSD_{\%5\ treatment}$:NS; $LSD_{\%5\ cultivar}$:0.02; $LSD_{\%5\ treatmentx\ cultivar}$:0.038			
	Firmness (kg·cm^{-2})		
0	0.57 A	0.45 B	**0.51**
10	0.56 A	0.41 B	**0.49**
20	0.56 A	0.43 B	**0.50**
Cultivars	**0.57 a**	**0.43 b**	
$LSD_{\%5\ treatment}$:NS; $LSD_{\%5\ cultivar}$:0.052; $LSD_{\%5\ treatmentx\ cultivar}$:0.052			

*The comparison of the means of the cultivars is done within the same row and means of treatment in the same column and the interaction between all the values are significantly different ($P \leq 0.05$) using LSD comparison test. The mean of treatment and cultivar are shown in lowercase letters while interactions are in uppercase.
** $n = 26$

Colour

The effects of GB concentrations, cultivars, GB concentration x cultivar interactions on L*, chroma, and hue (h°) under deficit irrigation condition are given in *Table 5*. As seen in *Table 5*, statistical analysis showed that there were insignificant differences in terms of lightness (L*) values according to cultivars and treatments. Regarding interactions, the highest lightness (L*) value was founded in Fortuna x 10 mM GB (34.38) (more brighter); the lowest L* value was Albion x 10 mM GB (29.57) (less brighter). In terms of chroma value, while the highest value was determined in 10 mM GB (34.32), the lowest was in control treatment (32.21). In *Table 5*, on the basis of cultivars, the highest color intensity (chroma) value was found in the Fortuna cultivar (34.56) and the highest h° values were at 10 and 20 mM GB concentrations. Indeed, the highest chroma and h° values were determined in the Fortuna and 10-20 mM GB treatments (light-colored).

Consequently, GB applications negatively affected fruit colour in terms of chroma and h° values as compared to control. In this concern, no literature was found regarding the effect of GB treatments on fruit colour in any horticultural plant.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

Table 5. *Effects of different glycine betaine concentrations on fruit colour (L*, Chroma, hº) at different strawberry cultivars grown under deficit irrigations*

Glycine Betaine Con. (mM)	Cultivars		Treatments
	Fortuna	Albion	
	L*		
0	30.32 AB*	32.58 AB	**31.45**
10	34.38 A	29.57 B	**31.97**
20	31.23 AB	31.59 AB	**31.41**
Cultivars	**31.98**	**31.25**	
$LSD_{\%5\ treatment}$:NS; $LSD_{\%5\ cultivar}$: NS; $LSD_{\%5\ treatmentx\ cultivar}$:4.46			
	Chroma		
0	32.28 BC	32.14 BC	**32.21 b**
10	34.89 AB	33.76 AB	**34.32 a**
20	36.52 A	30.37 C	**33.29 ab**
Cultivars	**34.56 a**	**31.99 b**	
$LSD_{\%5\ treatment}$: 2.109; $LSD_{\%5\ cultivar}$:1.72; $LSD_{\%5\ treatmentx\ cultivar}$:2.983			
	hº		
0	29.86 C	35.11 BC	**32.48 b***
10	41.67 A	37.44 AB	**39.55 a**
20	37.44 AB	37.84 AB	**37.64 a**
Cultivars	**36.80**	**36.32**	
$LSD_{\%5\ treatment}$: NS; $LSD_{\%5\ cultivar}$:3.769; $LSD_{\%5\ treatmentx\ cultivar}$:5.330			

*The comparison of the means of the cultivars is done within the same row and means of treatment in the same column and the interaction between all the values are significantly different ($P \leq 0.05$) using LSD comparison test. The mean of treatment and cultivar are shown in lowercase letters while interactions are in uppercase.
** n = 26

Ascorbic acid (AA) and total monomeric anthocyanins (TMA)

The data on ascorbic acid and total monomeric anthocyanin (TMA) content determined were given in *Table 6*. As seen in *Table 6*, increasing GB concentrations from 0 mM to 20 mM caused ascorbic acid contents to increase. As a matter of fact, the highest ascorbic acid (AA) content was founded at 20 mM GB application (35.29 mg·100 g^{-1} FW), the lowest was in control (24.79 mg/100 g FW). Furthermore, on the basis of cultivars, Albion strawberry cultivar (34.17 mg·100 g^{-1} FW) was determined higher than the Fortuna strawberry cultivar (27.91 mg·100 g^{-1} FW) in terms of AA. Interactions between cultivar and GB concentrations showed significant effects on AA. In general, Albion x 10-20 mM GB interactions gave the highest values (39.91 and 37.53 mg·100 g^{-1} FW) (*Table 6*).

As seen in *Table 6*, statistical analysis showed that there were significant differences in terms of TMA contents according to cultivars and GB treatments. As a matter of fact, increasing GB concentrations from 0 mM to 20 mM caused TMA to decrease. While the highest TMA content was founded in control with 511.97 mg Pg-3 glk·kg^{-1} FW; the lowest was at 20 mM GB treatment with 480.70 mg Pg-3 glk·kg^{-1} FW. On the basis of cultivars examined for the TMA content the highest value was determined in the Fortuna cultivar (517.66 mg Pg-3 glk·kg^{-1} FW). Interactions between cultivar and GB treatments showed significant effects on TMA. In general, the highest TMA values were obtained in control x Fortuna cultivar (544.23 mg Pg-3 glk·kg^{-1} FW) (*Table 6*).

Consequently, increasing GB concentrations from 0 mM to 20 mM caused total anthocyanin content to decrease, whereas the ascorbic acid content increased. Similar results were obtained in previous studies (Karjalainen et al., 2002; Shams et al., 2016). In

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DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

this regard, Karjalainen et al. (2002) indicated that foliar-applied glycine betaine (50 mM) and benzothiadiazole (60 g/ha) at the young-seedling stage (3-4 leaf stage) enhanced the levels of several phenolic compounds in strawberry leaves under field condition. Remarkable increases were detected in the levels of ellagitannin, ellagic acid and gallic acid derivates, quercetin and kaempferol konjugates. Briefly, the authors suggest that exogenously applied BTH and glycine betaine may be used to increase the levels of protective and health-promoting compounds in strawberries. Likewise, Shams et al. (2016) tried four concentrations of GB (0, 5, 10, and 25 mM) and two levels of salinity (0 and 100 mM of NaCl) in lettuce plants. Results showed that the 25 mM GB treatment increased dry matter and the content of total phenolics compared to the non-GB-treated plants under salt stress. Briefly, the authors suggested that exogenous GB treatments could ameliorate the tolerance of lettuce to salt stress by increasing the total antioxidants and total phenolics. Also, Khadouri (2015) noted the beneficial effects of GB application on biochemical and mineral content on cowpea plant.

Table 6. *Effects of different glycine betaine concentrations on ascorbic acid (AA) and total monomeric anthocyanin content (TMA) at different strawberry cultivars grown under deficit irrigations*

Glycine Betaine Con. (mM)	Cultivars		Treatments
	Fortuna	Albion	
	AA ($mg{\cdot}100\ g^{-1}$ FW)		
0	24.54 C*	25.05 C	**24.79 b***
10	26.13 C	39.91 A	**33.02 a**
20	33.06 B	37.53 AB	**35.29 a**
Cultivars	**27.91 b**	**34.17 a**	
$LSD_{\%5\ treatment}$: 4.75; $LSD_{\%5\ cultivar}$: 3.87; $LSD_{\%5\ treatmentx\ cultivar}$:6.711			
	TMA ($mg{\cdot}Pg\text{-}3\ glk{\cdot}kg^{-1}$ FW)		
0	544.23 A	479.70 C	**511.97 a**
10	520.15 AB	465.16 C	**492.65 ab**
20	488.62 BC	472.78 C	**480.70 b**
Cultivars	**517.66 a**	**472.54 b**	
$LSD_{\%5\ treatment}$:23.58; $LSD_{\%5\ cultivar}$: 19.25; $LSD_{\%5\ treatmentx\ cultivar}$:33.348			

Ascorbic acid content (AA) was determined by HPLC method of Bozan et al.(1997). Values were expressed as mg/100 g fresh weight basis (FW).
Total anthocyanin content (TAC) was determined by the pH-differential method of Giusti and Wrolstad (2001). Values were expressed in $mg{\cdot}Pg\text{-}3\ glk{\cdot}kg^{-1}$ by spectrophotometer.
*The comparison of the means of the cultivars is done within the same row and means of treatment in the same column and the interaction between all the values are significantly different ($P \leq 0.05$) using LSD comparison test. The mean of treatment and cultivar are shown in lowercase letters while interactions are in uppercase.
**The results based on wet basis for fresh fruit.
*** $n = 26$

Conclusion

Considering the rise in global warming, environmental changes and water shortage will affect sustainable agriculture. Therefore, identification of resistant and tolerant cultivars to stress conditions are crucial for future breeding programs. Furthermore, it is very important to determine suitable agents and application concentrations of these agents under drought stress conditions. In this regard, glycine betaine is environmentally safe, non-toxic and water-soluble and can be practically applied on plants.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

In this study, we examined the various effects of glycine betaine concentrations on plant morpho-physiological features, fruit physical and some biochemical features in two different soilless grown strawberry cultivars under deficit irrigation conditions. Our results showed that exogenous GB treatments induced plant growth and development, fruit yield as compared to untreated GB under stress condition. The best GB concentration was 10 mM in terms of fruit weight and yield. Fruit colour was affected negatively by GB concentrations. It can be said that the most important result was that increasing GB concentrations from 0 mM to 20 mM caused total anthocyanin content to decrease, whereas the ascorbic acid content increased. Furthermore, regarding cultivars, Fortuna was the best cultivar in terms of yield, titratable acidity, firmness and total anthocyanin, whereas Albion was the best cultivar in terms of total soluble solid and ascorbic acid content under deficit irrigation condions in soilless strawberry cultivation.

Acknowledgements. The author would like to acknowledge 2K VEG I.C. Foundation for supporting our study during the 2015–2016 production season and M. CIKER and B. TANRIVERDI for their assistance and Akdeniz University Food Safety and Agricultural Research Center for biochemical analysis.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37533767

DEVELOPMENT OF THE EMERGY-GIS METHOD OF SELECTING AREAS FOR SPONGE-LIKE URBAN RECONSTRUCTION

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(Received 30th Dec 2018; accepted 12th Feb 2019)

Abstract. The theory of sponge city has been attracting extensive attention in addressing urban water-related issues especially in China. We discussed on the emergy-GIS method of selecting areas for sponge-like urban reconstruction in Shenzhen, with four main concerns including data source, neglecting the issue of urban waterlogging, potential inaccuracies in emergy calculations and indices in selecting key areas. The corresponding suggestions and solutions are developed including the rational selection of precipitation and anthropogenic heat, consideration of land use types in assumption of the first 50% of stormwater runoff, incorporation of areas with high risk of waterlogging, methods for emergy calculations of runoff and runoff pollution, scientific choice of indices and integration of local planning and actions. This study could help improve the emergy method integrated with GIS in the application of sponge city studies.
Keywords: *sponge city, emergy, GIS, urban waterlogging, anthropogenic heat, runoff pollution*

Introduction

The theory of Sponge City which is related to low impact development (LID) and green infrastructure (GI) has been attracting extensive attention in addressing water-related issues due to rapid urbanization (Jia et al., 2017; Xia et al., 2017; Jiang et al., 2018; Tang et al., 2018; Wang et al., 2018). Both its practice and theoretical research have become exciting topics across interdisciplinary fields for achieving urban sustainability especially in China (Li et al., 2017, 2018; Xu et al., 2018). Recently an important study was published with the title of an emergy-GIS method of selecting areas for sponge-like urban (Zhao et al., 2018). A comprehensive framework composed of stormwater indices and anthropogenic heat was employed to facilitate selection of key areas for sponge-like urban reconstruction based on the integration of emergy analysis and GIS models with a case study in Shenzhen. Zhao et al. (2018) also claimed that the proposed emergy–GIS framework could bridge the gap between empirical studies and standardize research to ensure that the results of the green infrastructure plan. Emergy evaluation integrated with GIS can certainly help the spatial analysis of sponge city planning (Odum, 1996; Pulselli, 2010; Chang and Huang, 2015; Chen et al., 2016; Wang et al., 2016). However, after extensive analysis of the modeling approach, we found four main issues with the study: (1) selection of time scale of data source; (2) neglecting the issue of urban waterlogging; (3) potential inaccuracies in emergy calculations of runoff and runoff pollution; and (4) choice of indices in selecting key

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37693780

areas. Therefore, we investigated these issues and provided the suggestions and solutions correspondingly. It will help develop the emergy method integrated with GIS in the application of sponge city studies.

Main issues and solutions

Time scale of the data source

Zhao et al. (2018) used the precipitation data from 2010 to assess the stormwater indices (stormwater runoff and runoff pollution). The year 2010 can be hydrologically characterized as an average to dry year. For reference, the annual precipitation data with the deviation from the long-time (1958-2017) average of 1935.8 mm is showed in *Figure 1* (WRBS, 2018). Since precipitation is the dominant variable that determines runoff, and stormwater storage and runoff control are main tasks for sponge city construction, comparative analysis on the data from different typical hydrological years (wet, normal and dry) can help good understanding of the performance of sponge city construction (Jia et al., 2017; Jiang et al., 2018; Wang et al., 2018). Thus, it might be not sufficient to only use one year data from 2010, when is even a normal to dry year. It is recommended that the data from a wet year can be selected to enhance the analysis, such as 2013, 2014 or 2016 in *Figure 1*. Moreover, since Zhao et al. (2018) only used one precipitation value from the 2011 Shenzhen statistical yearbook, the temporal and spatial variability of precipitation was neglected (Tao et al., 2010; Ma et al., 2017; Luo et al., 2018). For reference, the monthly precipitation and the distribution of annual precipitation are showed in *Figure 2* (MBSZM, 2011b). The annual precipitation data at typical hydrometric stations are presented in *Figure 3* (WRBS, 2011), which can help improve the calculation of stormwater runoff on the use of one precipitation value by Zhao et al. (2018).

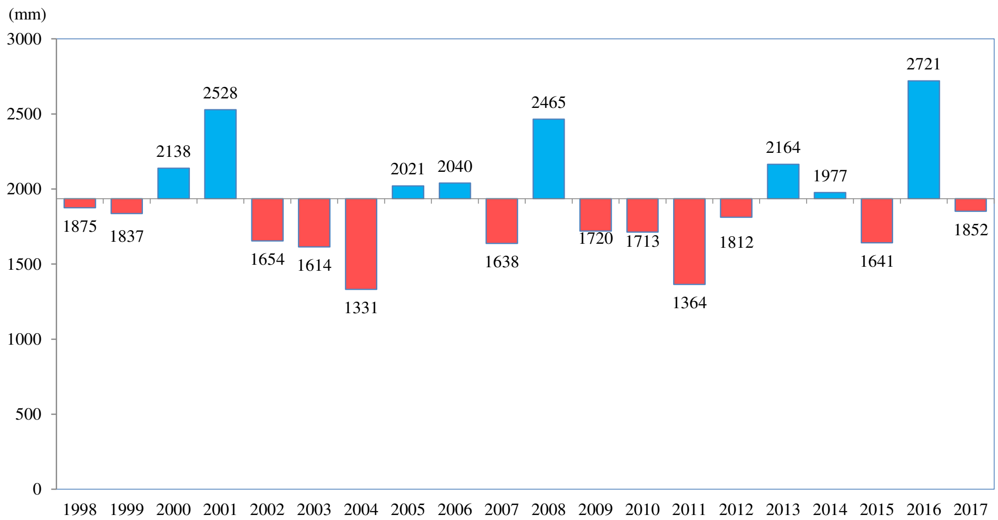

Figure 1. *Annual precipitation data in Shenzhen (1998-2017) (WRBS, 2018)*

Regarding the runoff pollution as the second indicator, Zhao et al. (2018) estimated the pollutant loadings using the first 50% of the stormwater runoff volume, which referred to a study by Qin et al. (2010) in Shenzhen. Yet, Qin et al. (2010) reported that

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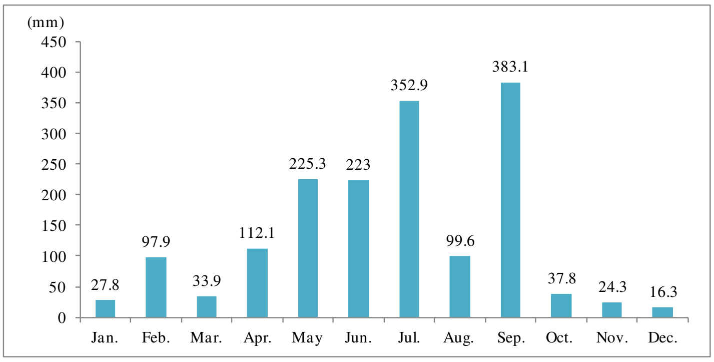
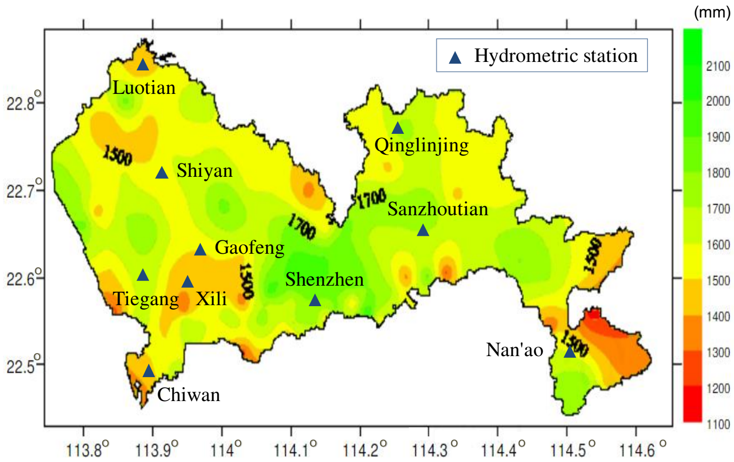
impervious land use contributed significantly to first flush (the first proportion of the runoff volume containing the main proportion of the related pollutant load), whereas pervious land use had low or even zero first flush (Qin et al., 2010). Zhao et al. (2018) applied this assumption of the first 50% of stormwater runoff to all the land use types, which might cause overestimate of the runoff pollution. Thus, it is recommended that only impervious land use types can be considered into the estimation.



a



b

Figure 2. *Temporal and spatial variability of precipitation in Shenzhen (2010) (MBSZM, 2011b). (a) Monthly precipitation in Shenzhen (2010). (b) Distribution of annual precipitation in Shenzhen (2010)*

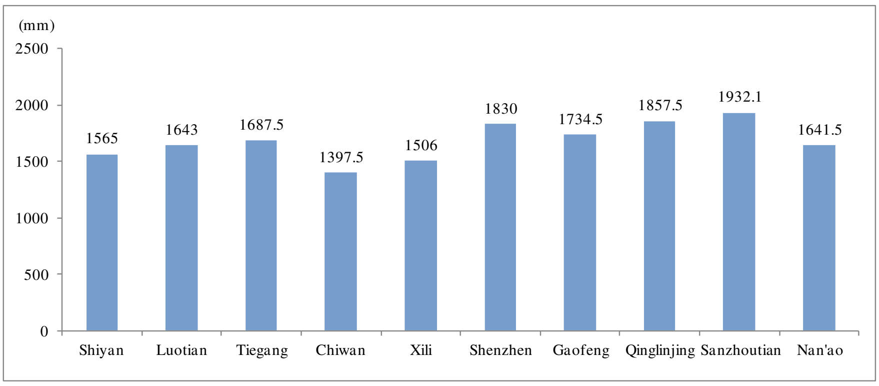


Figure 3. *Annual precipitation data at typical hydrometric stations in Shenzhen (2010) (WRBS, 2011)*

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Concerning the anthropogenic heat as the third indicator, Landsat-5 TM images and meteorological data (temperature) on December 23, 2010 were used by Zhao et al. (2018). The one-day data might be inconsistent with the whole year data for the precipitation and runoff analysis. Moreover, Zhao et al. (2018) did not interpret the representativeness of this day for the anthropogenic heat calculations. Yet, season-specific diurnal profiles of anthropogenic heating for cities had morning and evening peaks in summer and winter (Sailor and Lu, 2004). There are also typical characteristics of the temporal and spatial variation of anthropogenic heat flux in South China (Zhu et al., 2017). Anthropogenic heat has great impacts on the formation of urban heat island (UHI) effect, especially from June to August in Shenzhen (MBSZM, 2011a). Mitigation of the UHI effect has been considered as a major aim for sponge city construction, which was also a key indicator for the performance assessment of China's sponge city construction, issued by the Ministry of Housing and Urban-Rural Development of the People's Republic of China (MHURD) (MHURD, 2015a, 2018). The MHURD also claimed that the average daily data of temperature from June to September should be used to assess the mitigation of the UHI effect (MHURDa, 2015, 2018). However, the day of December 23 was not in this period. The maximum (28 °C), minimum (20.9 °C) and average (24.5 °C) temperatures on December 23 were different from the mean values from June to September (35.3 °C, 22.1 °C and 28.6 °C, respectively). For reference, the monthly mean temperatures in 2010 are shown in *Figure 4* (MBSZM, 2011b). Therefore, the proper date for the anthropogenic heat calculations can be a certain day from June to September, when has the same average temperature with that in this period, rather than December 23.

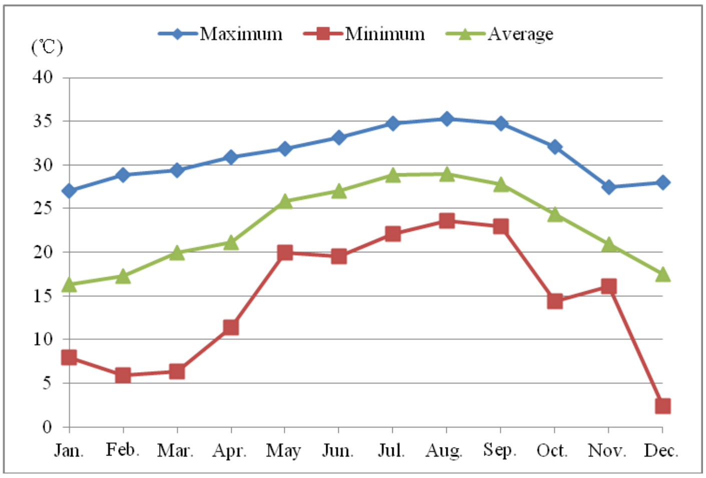

Figure 4. *Monthly mean temperature in Shenzhen (2010) (MBSZM, 2011b)*

Neglect in the situation of urban waterlogging

Mitigation and control of urban waterlogging and flooding is one of the foremost aims for China's Sponge City Construction pilot project programs. For instance, on September 30, 2015 the Chinese government has officially declared to build sponge cities to address urban waterlogging (Wang et al., 2018). It showed that China has been trying to turn its cities into "sponges" to better absorb rain following several fatal bouts of urban inundation in recent years. This target has also been written into two important official documents issued by the MHURD, which are *the Performance Evaluation*

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Indicators of China's Sponge City Demonstration (Pilot version) in 2015 (MHURD, 2015b) and *the Assessment Standard for Sponge City (Discussion version) in 2018* (MHURD, 2018). In the past 60 years, Shenzhen has suffered as much as 111 times from flooding, water lodging and storm surge (Cai, 2017). As for the selection of key areas for sponge-like urban reconstruction, the situation of urban waterlogging in Shenzhen should be firstly incorporated into the evaluation system, including the spatio-temporal distribution, magnitude and frequency of waterlogging events. Investigation of urban waterlogging generally involves several factors, such as the extreme values of rainstorms, rainfall frequency of occurrence, land cover, drainage and runoff, urban drainage system capacity, and location of control structures (Xue et al., 2016; Ma et al., 2017; Sang and Yang, 2017; Lin et al., 2018). Neglecting urban waterlogging and solely using the 2010 precipitation data reduce the applicability of the selected areas for sponge city practices, as these regions could not reveal the extent of urban waterlogging events. In addition, the development of flood and waterlogging risk maps can help in improving the understanding of selecting key areas for sponge-like urban reconstruction (Zhang et al., 2005; Pandey et al., 2010). The Water Resources Bureau of Shenzhen Municipality has identified 446 areas with high risk of waterlogging in Shenzhen in 2015 (*Fig. 5*) (Cai, 2017). It is obvious that these waterlogging concerns have not been incorporated into the study of Zhao et al. (2018).

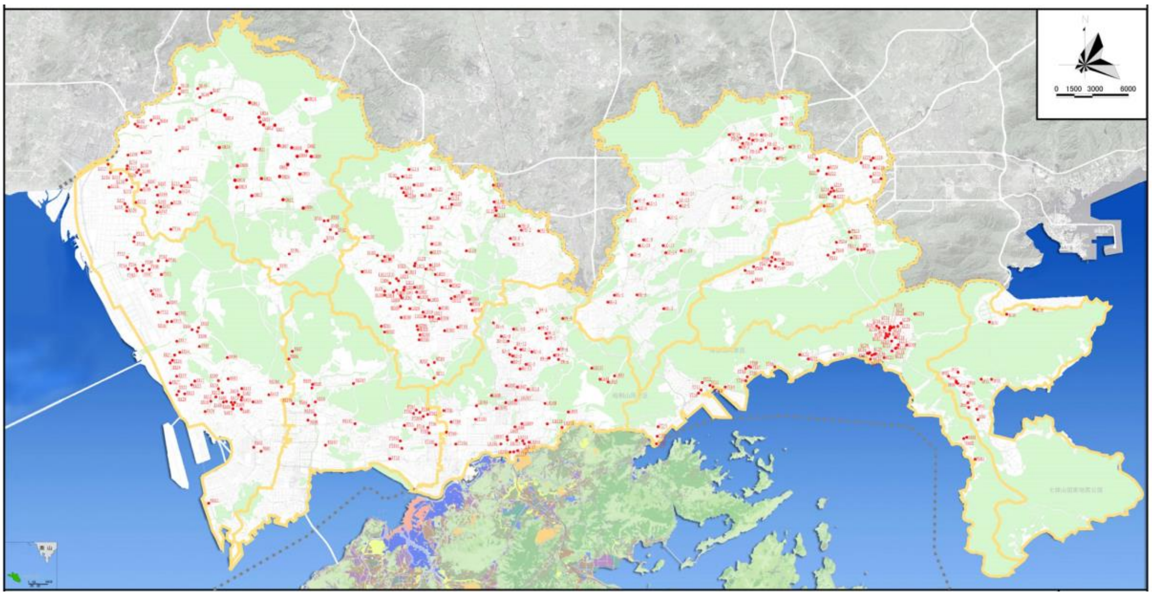

Figure 5. *Locations with high-risk waterlogging in Shenzhen (Cai, 2017)*

Potential implications of the methods used for emergy calculations of runoff and runoff pollution

Since Zhao et al. (2018) calculated the energy of runoff using the volume of runoff water, the water density and the Gibbs free energy, this energy was identified as the chemical potential energy. It appears like there is a misunderstanding in the underlying theory of chemical potential energy and geopotential energy (Kangas, 2002). As per the emergy theory, contributions of inland precipitation can be broken down into physical (runoff) and chemical (evapotranspiration) effects due to its gravitational and diffusive

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chemical potential energy relative to seawater (Odum, 1996; Buenfil, 2001; Chen et al., 2009; Mellino et al., 2014). Runoff represents a kind of elevated potential energy, rather than the chemical potential energy used by Zhao et al. (2018). The available energy associated with runoff can be a product of the mass flow rate, the gravitational constant, the elevation drop or head and the transformity of runoff geopotential (Kangas, 2002; Mellino et al., 2014). Since this geopotential is closely related to the elevation, the Digital Elevation Model (DEM) could be used (Wang et al., 2016). Thus, the equation of runoff energy can be used as *Equation 1* (Mellino et al., 2014):

$$En_{runoff} = A \times \rho_{water} \times R \times g \times h \qquad \text{(Eq.1)}$$

where A is the area in m^2, ρ_{water} is the water density (1E + 6 g/m^3), R is the runoff in meters, g is the gravitational constant (9.81 m/s^2) and h is the vertical distance from the water level.

In addition, Zhao et al. (2018) claimed that the runoff analysis was based on the yearly precipitation data obtained from the 2011 Shenzhen Statistical Yearbook. Since this yearbook (Page 372) only presented a single dataset of the 2010 annual precipitation (1634.0 mm), it showed that Zhao et al. (2018) did not consider spatial and temporal variability in the distribution of precipitation in the runoff analysis. Moreover, according to the data from 1981 to 2010, the Meteorological Bureau of Shenzhen Municipality reported that the average annual evapotranspiration in Shenzhen was 1433 mm, compared to the average annual precipitation of 1935.8 mm. The evapotranspiration should not be ignored in the runoff calculation in the common water budget equation, as annual data was used by Zhao et al. (2018). Evapotranspiration map can be gained implementing Turc equation in GIS, which is an empirical equation with good results estimating evapotranspiration values for areas with annual mean temperatures. Thus, the runoff map can also be generated by the difference between precipitation map and evapotranspiration map (Mellino et al., 2014). Concerning the emergy calculation of runoff pollution, Zhao et al. (2018) obtained the emergy of Chemical Oxygen Demand (COD) by multiplying the energy of COD by the transformity of wastewater as adapted from Bjorklund et al. (2001). Yet, this transformity was based on an emergy analysis of household wastewater in a Swedish town, which was considered to be a by-product of the summation of all activities in terms of water used and food consumed including transport, processing and food storage (Björklund et al., 2001). Therefore, it is possible that the emergy might be orders of magnitude larger than the transformity of COD in natural runoff required by Zhao et al. (2018). Therefore, this potential overestimation of runoff pollution with differences in orders of magnitude can potentially change the integration of the three indices, which can further affect the selection of suitable areas. Due to lack of the proper transformity of COD, a potential solution can be direct calculation of the emergy of runoff pollution in terms of the emergy associated with dilution of COD pollutant to its environmental safety concentration, using *Equations 2* and *3* (Li et al., 2013; Shao et al., 2017).

$$En_{runoff\ pollution} = \rho_{water} \times (M_{COD} / C_{COD}) - M_{runoff\ pollution} \qquad \text{(Eq.2)}$$

$$Em_{runoff\ pollution} = En_{runoff\ pollution} \times G \times UEV_{water} \qquad \text{(Eq.3)}$$

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where M_{COD} is the quantity of COD in grams, C_{COD} is the environmental safety concentration of COD in g/m^3, $M_{runoff\ pollution}$ is the total quantity of the runoff pollution in grams, G is the Gibbs free energy (4.82 J/g), UEV_{water} is the transformity of surface water (4.48 × 10^4 sej/J). According to China's environmental quality standard for surface water (GB 3838-2002), the environmental safety concentration of COD is 15 mg/L (EPB, 2002).

Choice of indices in selecting key areas

In order to select key areas for sponge-like urban reconstruction, Zhao et al. (2018) adopted three indices including stormwater runoff, runoff pollution and anthropogenic heat on the emergy-GIS method. In addition to the incorporation of water logging discussed in the previous section, techno-economic and institutional factors should also be considered in addition to the existing indices. From a practical perspective, the degree of demand, reconstruction costs, demonstration effects and importance of a certain region could have great impacts on whether this region can be selected as a priority area. These concerns have also been written into several official documents about China's sponge city construction plan. For instance, in January 2015, the MHRUD in conjunction with the Ministry of Finances and the Ministry of Water Resources issued a notice on selecting national-level pilot cities for sponge city construction with campaigning rules (Liu et al., 2017). These rules involved the positivity of local government, the rationality of construction demand and targets, the feasibility of construction projects, the innovation of investment and financing mode, and the integrity of supporting measures and infrastructure conditions (MHRUD, 2015). Therefore the three indices proposed by Zhao et al. (2018) might be incomplete for practical application in Shenzhen.

Based on the three indices, Zhao et al. (2018) presented emergy distribution maps and selection results with nine sub-districts. However, the validity of the results presented is questionable due to the aforementioned issues with data source, emergy calculation methods, and indices selection. Moreover, in November 2016 the Planning, Land and Resources Commission of Shenzhen Municipality (PLRCS) released two key planning documents on the sponge city construction in Shenzhen based on abundant data and materials (PLRCS, 2016a, b). The reports included several planning maps involving the spatial patterns, functional divisions and suitable areas (*Figs. 6-8*) (PLRCS, 2016a, b). *Figure 6* presents main urban green spaces, wetland patches, major rivers, ecological corridors and ecological matrix spatial patterns, which can help identify the green cover spatial patterns of the sponge city construction in Shenzhen. *Figure 7* presents the development and protection directions in different functional divisions. The suitable areas for the sponge city construction are shown in *Figure 8,* by identifying uncompleted urbanization areas and unsuitable areas (Karst distribution and slumping area, debris flow distribution area and landslide and slumping area). The Shenzhen Municipal Government selected 24 key areas with a total of 250.12 km^2 for promoting the sponge city pilot construction (PLRCS, 2016b). Therefore, local policies and planning can have a significant influence on future sponge city construction in Shenzhen, however, the implications of these decisions have not been considered in Zhao et al. (2018). These figures and aforementioned information can be incorporated into the discussion section by Zhao et al. (2018), which help assess the validity of emergy distribution maps and improve the areas selection results for sponge-like urban reconstruction.

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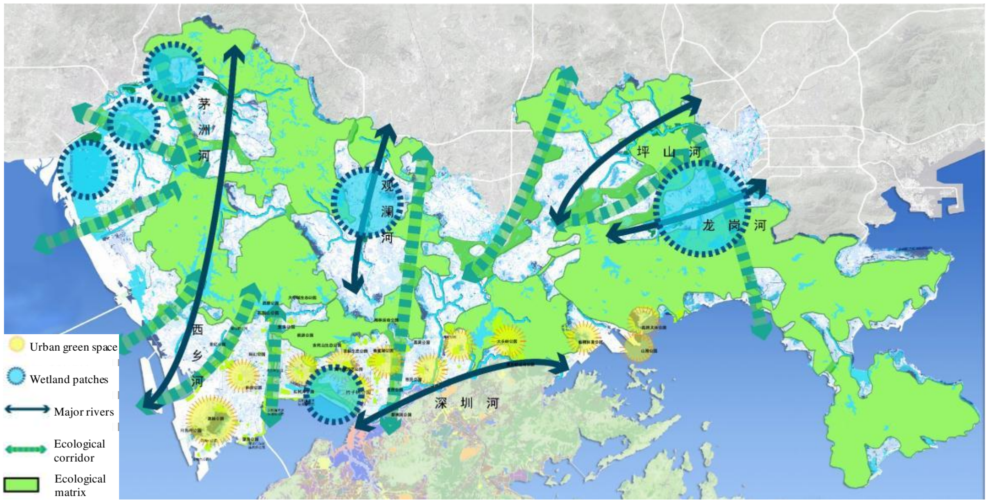

Figure 6. *Spatial patterns of the sponge city construction in Shenzhen (PLRCS, 2016a)*

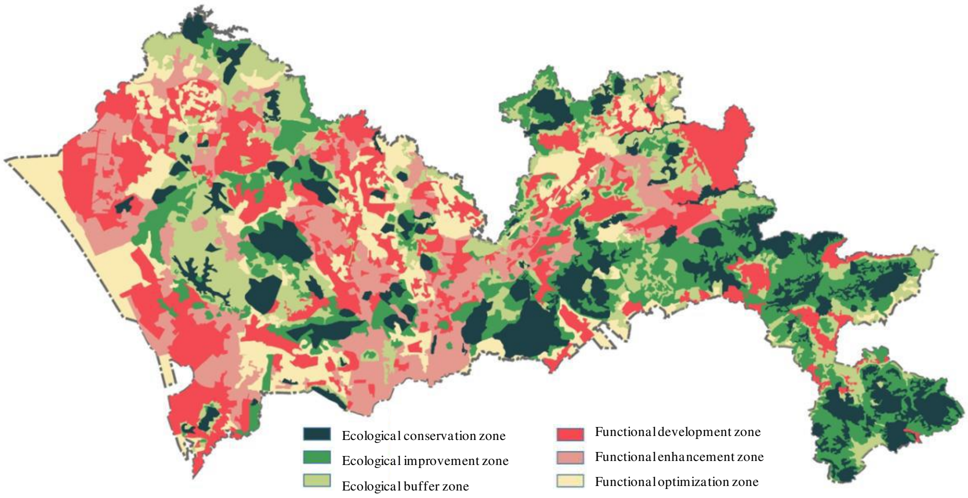

Figure 7. *Functional divisions of the sponge city construction in Shenzhen (PLRCS, 2016a)*

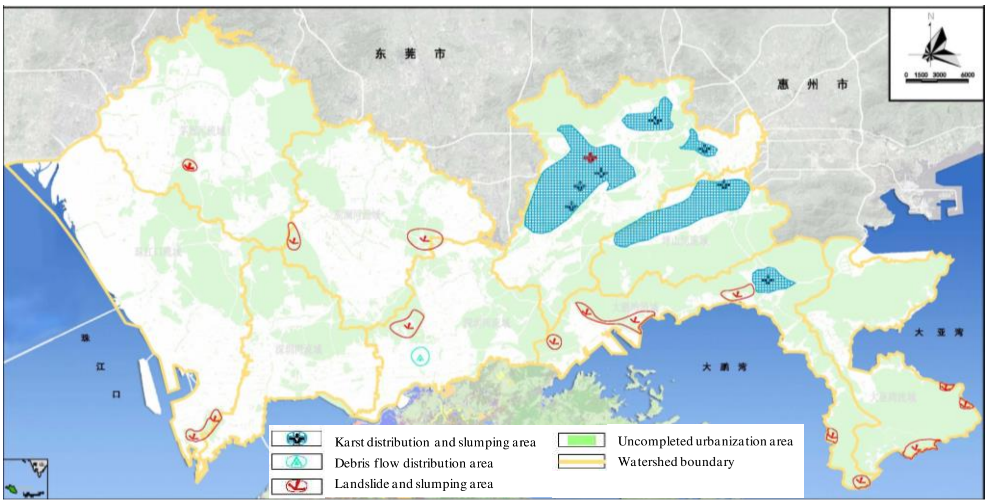

Figure 8. *Suitable areas for the sponge city construction in Shenzhen (PLRCS, 2016a)*

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Conclusions

The theory and practice of sponge city has been attracting extensive attention in addressing urban water-related issues especially in China. This paper focused on a study of the emergy-GIS method of selecting areas for sponge-like urban reconstruction in Shenzhen, with four main concerns including data source, neglecting the issue of urban waterlogging, potential inaccuracies in emergy calculations and indices in selecting key areas. As per the detailed discussion provided in the previous sections, there are four main conclusions that can help improve the analysis further and develop the emergy method integrated with GIS in the application of sponge city studies. These are summarized below:

(1) The analysis of selecting areas for sponge-like urban reconstruction based on an emergy-GIS method needs to be modified due to the improper time scale of data source and inconsistencies in the data itself. The main solutions include the rational selection of precipitation data, consideration of land use types in assumption of the first 50% of stormwater runoff and date selection for the anthropogenic heat calculations.

(2) Since mitigating urban waterlogging is the primary objective for sponge city construction, the spatio-temporal distribution, magnitude, and frequency of waterlogging events in Shenzhen should not be neglected and need to be revaluated while considering these parameters. Incorporation of areas with high risk of waterlogging is needed for the selection of key areas for sponge city construction.

(3) The methods used for emergy calculations of runoff and runoff pollution ignore runoff geopotential, spatio-temporal rainfall distribution, and evapotranspiration, which can potentially weaken the reliability of the emergy distribution maps. The theory and equations which we presened above can improve the methods for emergy calculations of runoff and runoff pollution.

(4) The existing indices for selecting key areas for sponge city construction should also consider techno-economic and institutional factors such as local planning and actions. This can help in providing a more robust spatial-discretization of key areas.

Acknowledgements. This study is supported by a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and Australian Research Council for Discovery Project Grant (DP170104138). We thank the anonymous reviewers and the editors for their constructive comments, which helped us to improve the manuscript.

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DOI: http://dx.doi.org/10.15666/aeer/1702_37693780

ANALYZING THE IMPLEMENTATION OF ENVIRONMENTAL LAWS IN THE SAUDI ARABIAN CONSTRUCTION INDUSTRY

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(Received 30th Dec 2018; accepted 14th Feb 2019)

Abstract. Environmental protection requires adopting and implementing legal, economic, and societal procedures to limit the side effects of the massive real-estate and economic development in today's world. The purpose of this research was to investigate the implementation level of the eight main environmental laws affecting the construction industry in Saudi Arabia, and to identify the difficulties faced by practitioners to implement these laws. Using the descriptive analytical approach, this research described and analyzed the level of implementation of environmental laws using a Likert scale questionnaire distributed to 1000 organizations that work in 29 construction industry fields, in 13 Saudi Arabian regions. These questionnaires were filled out by engineers and professionals, authorized contractors, unauthorized contractors, individuals, and officials and owners. The one-way ANalysis Of VAriance (ANOVA) was applied to the data collected (at <0.05 level of significance), using SPSS Version 23.0 software. The overall environmental legitimacy implementation level in Saudi Arabia was found to be 2.95 out of 5.00 and a standard deviation of 1.13 indicating difficulties in defining the level for environmental legitimacy implementation in the construction industry. Results indicated that more attention was given to regulations related to the handling and disposal of radioactive materials, hazardous waste management, wastewater discharges, hazardous and dangerous substance compliance programs, environmental noise, general environmental requirements, air quality emissions, and external emergency planning with mean values of 3.23, 3.12, 3.10, 3.06, 3.05, 2.95, 2.80, and 2.75, respectively. Further investigation is required to evaluate internal auditing, accounting, and the availability of review teams regarding the environment in the industrial organizations in Saudi Arabia.
Keywords: *environmental laws implementation, statistical analysis, construction industry*

Introduction

Interest in environmental issues is widespread, with climate change and other environmental concerns receiving significant media attention and among the greatest threats facing the world today (White and Hunter, 2009). Today's, economic growth is based on the use of depleted energy resources leads to environmental pollution and stress. One the same time, it works to increase the development of individuals and society. Defining the equilibrium point between economic growth, societal development, and environmental pollution is one of the main points of concern to the world community in general and Saudi Arabia in particular. To coordinate the effort among the countries of the world, several international environmental agreements are

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

signed concerning climate protection, Ozone layer, climate change, marine/water and environmental protection, availability of information and participation, waste management, prevention of environmental damage caused by chemicals, environmental impact assessment, protection of flora and fauna and biological diversity, and landscape besides the bilateral agreements. Moreover, the international effort seeks to include intergovernmental organization involvement as United Nations and World Trade Organization (Millimet and Roy, 2015; Eckersley, 2016; Barrett, 2005; Horn and Mavroidis, 2014; Kellenberg and Levinson, 2014).

Consequently, organizations are heavily implicated in these environmental issues. With greater awareness, the public is now demanding that organizations should take responsibility for voluntarily integrating social and environmental concerns into their operations and into their interactions with stakeholders. Organizations have responded to this call in a variety of ways, complying with regulatory requirements such as ISO 14001 certification and environmental responsibility (To and Lee, 2014). This synthesis of the literature delineates these three important concepts of corporate environmental performance and behavior, explains the causal links between the concepts, and depicts the concepts and causal links diagrammatically in a framework. Jordan et al. (2010) presented a model of environmental accountability and an outline of a process for achieving it and concluded that achieving and maintaining a sustainable environment requires more than accounting. They mention that actions, including legislation, regulation, mitigation, resource management, enforcement, education, and social responses to environmental challenges are the dynamic forces that produce results. Findings are environmental accounting can only tell us what those results are the ecosystem services paradigm, worthiness, and service to humanity. Environmental performance is the most important central concept in the framework. Improvements in environmental performance will eventually lead to the goal, namely sustainability. The general system of environmental protection and its executive regulation aims to protect the environment and prevent pollution, protect public health against the dangers of activities and actions harmful to the environment, conservation, development and rational use of natural resources, and make environmental planning an integral part of comprehensive planning for development in all industrial, agricultural, urban, and other fields (The General Authority of Meteorology and Environmental Protection, 2001).

In this context, an extensive body of literature has emerged recently on environmental legitimacy. The construction industry consumes a large portion of natural resources (Paudel et al., 2014), which has enormous side-effects, such as air and water pollution, deforestation, stratospheric ozone depletion, and increased threat of global warming (Hallett, 2002; Field, 2014). The government and public are setting an increasing number of legislative restrictions on businesses to implement more ecofriendly alternatives. The construction sector in Saudi Arabia includes a variety of activities such as construction, water piping, electrical connections, maintenance activities, and cleaning activities. Under these activities, there are multiple and interrelated sub-activities, which are classified into three basic groups: engineering design and consultancy; project execution, operation, and maintenance; and contracting activities. The fields of activity in this sector include building, roads, water and sanitation works, electrical work, mechanical work, industrial activities, marine works, dams, landscaping, afforestation, slaughterhouses, and building maintenance and operation (Contractor Classification Agency, Ministry of Municipal and Rural Affairs, 2018). The research work done in the field of environmental assessment of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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construction industry has focused on measuring their environmental performance. The researchers noticed that, no research work has been done to assess the implementation of various environmental laws in any industrial sector. The main objective of this article is to present the results from analysis of the environmental legitimacy implementation in the Saudi Arabian construction industry.

Literature review

Legitimacy, as defined by Suchman (1995), is "a generalized perception or assumption that the actions of an entity are desirable, proper, or appropriate within some socially constructed system of norms, values, beliefs, and definitions". Expanding Suchman's (1995) definition of legitimacy into the environmental arena, Bansal and Clelland (2004) define environmental legitimacy as "the generalized perception or assumption that a firm's environmental performance is desirable, proper, or appropriate". Legitimacy is a complex concept, it is generally accepted that organizations have social responsibility towards society. On the other hand, legitimacy is temporally and culturally defined (Deegan, 2002), thus creating an immense challenge for organizations to secure legitimacy. Hutchins et al. (2019) utilized a stakeholder perspective to make the case that firms should focus on multiple constituents in order to be successful specifically with regard to building sustainability programs. They proposed a conceptual model and research propositions that can serve as a foundation for future research to empirically investigate the role of legitimacy in reducing skepticism of a comprehensive sustainability program. Building legitimacy, therefore, can serve as a foundation for operational and competitive success. The conditions of the social contract include granting of legal standing to organizations and offering support to an organization by supplying resources and labor.

A legitimacy gap can arise because of changing organizational performance, changing societal expectations, or a combination of both (Deegan, 2002). Furthermore, legitimacy can be both factual and perceptual. An example of factual legitimacy could be an environmental event (e.g., a catastrophic oil spill, or a fine for non-compliance), which is visible to stakeholders either by their own observation or through widespread media publicity. However, often external stakeholders cannot easily discern environmental performance (Hunter and Bansal, 2007), explaining the importance of perceptions. Legitimacy, as described above and adopted in the framework of this study, is concerned with a status or condition. Hence, it is essential to clarify, from the beginning, the difference between legitimacy (a status or condition) and legitimizing (an act or process aiming at legitimacy. Depending on whether the strategy is to gain, maintain, or repair legitimacy, an organization may engage stakeholders in the decision-making process, redefine its corporate mission statement, establish a separate department dealing with sustainability issues and/or obtain external certifications, contribute to charity, associate itself with other 'legitimate' institutions, and comply with legislation to appear legitimate in the eyes of its relevant publics (Bansal and Roth, 2000). While gaining legitimacy is proactive in nature, repairing legitimacy is generally a reaction to an unforeseen crisis (Suchman, 1995). Moreover, for any legitimizing strategy to be effective, communication between organizations and society is very important (Villiers and van Staden, 2006; Hasseldine et al., 2005; Toms, 2002).

Palazzo and Scherer (2006) proposed a fundamental shift to moral legitimacy, from an output and power-oriented approach to an input related and discursive concept of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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legitimacy. This shift creates a new basis of legitimacy and involves organizations in processes of active justification vis-a-vis society rather than simply responding to the demands of powerful groups. They consider this a step towards the politicization of the corporation and attempt to re-embed the debate on corporate legitimacy into its broader context of political theory, while reflecting the recent turn from a liberal to a deliberative concept of democracy (see also Aerts and Cormier, 2009; Brønn and Vidaver-Cohen, 2009). For such organizations, a symbolic response may only intensify the threat to legitimacy, and consequently, society would be more likely to demand more substantive change in organizational performance (see further Burchell and Cook, 2008). It is argued that a symbolic legitimacy strategy, due to its rhetoric and often manipulative nature, would not give the same positive impact and could possibly further detriment the organizational legitimacy. As a final note, it is important to emphasize that by setting environmental legitimacy as a desired goal, the framework does not simplify the overly complex reality by nullifying other possible motives. Organizations embrace social and environmental responsibility for numerous reasons (e.g., (Okereke, 2007; Solomon and Lewis, 2002; Williamson et al., 2006).

At the global level, researchers were interested in studying the relation between environmental laws and the application of these laws on the real world. They recommended concentrating on environmental protection through public awareness programs. For example, Fernandes Rei (2018) highlighted the southern approaches brought to international governance gets mixed with the addressing of the challenges facing the legal science in harmony with the others sciences to handle with the complex environmental issues of the 21st century and concluded the southern influences suggest a more pragmatic, finalistic international law concerned about the results, the attainment of the targets suggested. Pavoni and Piselli (2016) introduced the implications for global environmental legislation of the maintenance of the continuous development goals, which occurred at the 2015 United Nations Sustainable Development Summit. Kelemen and Knievel (2015) pointed out the United States has lagged the European Union in its approval and implementation of significant multilateral environmental agreements. They argued varying levels of responsibility to substantive environmental management goals at the national level, rather than varying levels of commitment to international law, may best explain US and EU positions concerning international environmental policy.

Wang (2013) reviewed bureaucrats took positive efforts on environmental protection, making major investments in pollution control infrastructure in China. They seek to offer insight into several broader ongoing debates - about environmental regulation in developing countries, accountability and regime survival in authoritarian states, and legal development in China. Sand (2012) recommended a recent focus on techniques for support by the national community in the work and implementation of transnational environmental law. Van Kempen (2012) highlighted European environmental law has become progressively complex and needs the clarity to allow for adequate implementation and therefore effective environmental protection. Howarth (2011) focused the National Audit Office Report, Tackling Diffuse Water Pollution in England, addresses three issues: the Environment Agency’s recognition of the causes of diffuse pollution; whether attention of diffuse pollution is being raised; and whether the Agency is well using its legal the latter because of diffuse pollution.

Yang and Percival (2009) addressed the global growth of public concern about environmental issues over the lost several decades, environmental legal norms have

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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become increasingly internationalized called “global environmental law” for the implementation, practice, and development of environmental law worldwide. Fine and Owen (2005) explored the origins of legal requirements for both public participation and modeling and then considers how models fit within planning processes. They highlighted how planning depends upon models and how model use impedes the public role because of limitations inherent in modeling. Freeman and Farber (2005) proposed framed the enterprise of environmental regulation and resource management as an exercise in designing governance institutions capable of managing multiple and incompatible demands over the long term. This approach departs from the traditional legal framing of such environmental conflicts as shorter-term and zero-sum questions of jurisdiction, authority, entitlement, and prohibition.

The aim of this research was to evaluate the Saudi environmental laws implementation in the construction industry by category. In the next section the methodology will be explained.

Methodology

The methodology involved the data collection regarding Saudi Arabian environmental legislation, population and sample size of respondents, questionnaire, and statistical analysis along with questionnaire analysis using SPSS (Statistical Package for Social Sciences) Version 23.0. *Table 1* highlights Saudi Arabian environmental legislation chronologically by promulgation date.

Table 1. *Saudi Arabian environmental legislation chronologically by promulgation date*

No.	Title	Date of issuance
	General environmental	
1	Environmental Law	2001
2	Arbitration Law	2012
	Air quality/emissions	
3	Ambient Air Standard 2012	2012
4	Standard on Emissions from Mobile Sources 2012	2012
	Water/wastewater	
5	National Ambient Water Quality Standard of 2012	2012
6	Wastewater Discharge Standard of 2012	2012
	Hazardous/non-hazardous waste	
7	Basel Convention on the Control of Trans boundary Movements of Hazardous Wastes and their Disposal	1989
8	Hazardous Waste Disposal Standards 1413-03	1992
9	Regulations and Procedures for Hazardous Waste Control (Document No. 01-2002)	2002
10	Environmental Standards on Material Recovery and Recycling of Waste 2012	2012
	Hazardous/dangerous substances	
11	Technical Guideline of 2012 on the Prevention of Major Accidents	2012

Saudi Arabian environmental legislation

The first comprehensive Saudi Arabian national environmental legislation was enacted on September 24, 2001 in the form of the General Environmental Regulation, Council of Ministers Resolution No. 193. It entered into force on October 31, 2002, and

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its Implementing Rules were published on September 30, 2003. Under the Regulation, the Presidency of Meteorology and Environment (PME), an agency of the Ministry of Defense, is charged with the general supervision of environmental affairs in Saudi Arabia. The legislation sets out wide-ranging prohibitions of pollution and contamination of air, land, and water, with reference to all parties involved in services, industry, or other economic activities. Owners of 'projects' that might influence the environment, are required to comply with existing and future environmental specifications, standards, measurements, and guidelines as promulgated by the PME and set out in the appendices of the Implementing Rules. Moreover, prior to the setting up of a project, an environmental evaluation study must be completed and approved by the PME.

The detail of the legislation is contained in the appendices of the Implementing Rules, as Environmental Protection Standards, Procedures for the Assessment of Environmental Effects of Industrial and Development Projects, Manual of Environmental Qualification Procedures, Rules and Procedures for the Control of Hazardous Waste, National Contingency Plan for Combatting Pollution by Oil and other Harmful Substances of the Marine Environment in Emergency Cases, and Violations and Fines. Apart from the above national environmental legislation, the following specific regulations are also applicable in certain areas of Saudi Arabia:

- The Royal Commission for the industrial cities of Jubail and Yanbu has issued detailed local environmental regulations applicable to facilities located within the Royal Commission areas and contractors operating therein, of which the Jubail Industrial City Royal Commission Environmental Regulations of September 1999 are the most recent.
- Pollution and contamination incidents within ports under the administration of the Saudi Arabian Seaports Authority are governed by the Rules and Regulations for Seaports of the Co-operation Council for the Arab States of the Gulf of 1985, as revised in 2006.
- Saudi Aramco, which administers the oil loading terminals at Ras Tanura, Ju'aymah, and several smaller terminals independently of the of the Seaports Authority, has its own set of rules entitled "Saudi Aramco, Oil Ports & Terminals, Rules, Regulations and General Information."
- Saudi Arabia has ratified the International Convention for the Prevention of Pollution of the Sea by Oil of 1954 (OILPOL 1954, 1958) and its Amendments of 1962, 1969, and 1971, and the International Convention on Civil Liability for Oil Pollution Damage of 1969 (CLC, 1969) and its Protocols of 1976 and 1992.

Table 1 lists the Saudi Arabian environmental legislation chronologically by promulgation date that adopted in this research. *Table 2* lists the questions for the designed questionnaire.

Population and sample size

A survey of construction industry fields was conducted in which the respondent categories included engineers and professionals, authorized contractors, unauthorized contractors, individuals, and officials and owners, along various Saudi Arabian regions. Of the total construction industries (18,573) only 1,000 were incorporated into the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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survey analysis. From the respondent category of engineers and professionals, a greater number of respondents were from the categories individual, maintenance and operation for electrical works, and city cleaning and waste disposal from a total of 202 construction industries. Authorized contractors showed more response from building maintenance, from the total of 29 industries. From unauthorized contractors, a greater number of responses were received from buildings, and from maintenance and operation of electrical works, from a total of 29 industries. The category ‘individual’ got more responses from city cleaning and waste disposal, from among 196. Officials and owners had more responses from catering for medical centers, among 189.

When Saudi regions were compared with construction industries, the eastern region got more responses for water and sanitation, and maintenance roads among 453 districts, regarding 114 industrial fields. The Al Baha region had almost no responses among nine fields, as did the Al-Jouf region among 13 fields and the northern border region among six fields. The Riyadh region responded better in all fields when compared to all other regions as it showed more response towards city cleaning and waste disposal among 443 fields. The remaining regions, like Qassim (62 fields), Madinah (35 fields), Tabuk (12 fields), Jazan (10 fields), Hail (17 fields), Asir (62 fields), and Najran (44 fields) showed less response. The Makkah region responded for catering by individuals from among 173 fields. The details for the sampling plan are shown in *Table 3*.

Questionnaire

After extensive review of the literature and studies related to environmental assessment, it was decided that the questionnaire was the most suitable method for data collection. For this purpose, a questionnaire was designed to meet the objectives of the research and the requirements for answering its questions. These questions consisted of eight sections as general requirements, air quality/emissions, waste water discharges, hazardous waste management, radioactive materials handling and disposal, hazardous/dangerous substance compliance programs, environmental noise, and external emergency planning. The sections include 7, 6, 6, 7, 2, 6, 3, and 12 questions, respectively, as shown in *Table 2*.

Table 2. *Environmental questionnaire questions*

Environmental requirements		Environmental requirements	
F1. General requirments		F4.7	Hazardous wastes spill prevention systems in accordance with any applicable regulations
F1.1	Up-to-date operating license	F5. Radioactive materials handling and disposal	
F1.2	Environmental violations relating to environmental matters approved or signed?	F5.1	Handle the final disposal of radioactive materials, with the procedures the Rules
F1.3	Environmental impact assessment done during the project feasibility stage	F5.2	Storage, treatment, recycling, and transportation of radioactive wastes, in accordance to regulations
F1.4	Making modifications to an existing project, using technologies with lowest level of pollution to the environment	F6. Hazardous/dangerous substance compliance programs	
F1.5	Use of natural resources and apply recycling technologies and reuse of resources	F6.1	Records maintained of dangerous chemicals used and stored
F1.6	Planning for projects and programs, with developing environmental aspects	F6.2	Handling of final disposal of poisonous, hazardous materials, with the procedures
F1.7	Regulations and standards when designing or operating any project or activity	F6.3	Treatment, recycling, and transportation of hazardous waste as per regulation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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F2. Air quality/emissions		F6.4	License and approval to perform disposal of hazardous waste as per regulation
F2.1	Stationary sources of air emissions and recording of sources	F6.5	Responsibility for any incidents of environmental pollution and reporting of any incident
F2.2	Air emission limit details	F6.6	Training programs for the handling, transportation, storage, recycling, disposal of toxic, hazardous, waste
F2.3	Emission frequency and methodology for monitoring, and evaluation	F7. Environmental noise	
F2.4	Emission within allowable limits permitted in the environmental standards	F7.1	Prevention of noise pollution when operating machinery or other equipment or using horns or loudspeakers
F2.5	No leaking or emission of air pollutants into the work place beyond the allowable limits	F7.2	Check noise levels do not exceed allowable environmental standard limits
F2.6	Adequate ventilation system	F7.3	Upgrade technologies and equipment used in existing activities to attain allowable noise levels
F3. Waste water discharges		F8. External emergency planning	
F3.1	Prevention of contamination of surface and ground waters	F8.1	Emergency plans to prevent or alleviate the hazards of such impacts
F3.2	Prohibition of discharge of harmful pollutants	F8.2	Importance to environmental aspects while planning for projects and programs
F3.3	Wastewater discharge to treatment plant or Municipal zone	F8.3	Facility to cope Environmental disaster management plans
F3.4	Discharge of waste water to natural environment with permission	F8.4	Environmental protection from pollution resulting from conducted projects
F3.5	Discharge of wastewater; are any of the compounds, exceeded?	F8.5	Training given with technical emergency environmental protection plans
F3.6	Limitation level in Mixing Zone, exceeded?	F8.6	Emergency plans in case of a pollution emergency incident
F4. Hazardous waste management		F8.7	Coordination to safe employees from environmental damage resulting from emergency pollution incidents
F4.1	License or permit for the handling of hazardous wastes	F8.8	Emergency plans to handle hazards situation
F4.2	Hazardous waste disposal standards (1413-03)?	F8.9	Emergency plans with qualified work force and equipment
F4.3	Permit for transport and/ disposal of hazardous wastes?	F8.10	Emergency plans for projects conducted and prevent or alleviate the risks of potential adverse environmental impacts
F4.4	Record of hazardous wastes and disposal in accordance to regulations?	F8.11	Periodically review of emergency plans that protect the environment from pollution or prevent adverse impacts on the environment
F4.5	Check hazardous wastes are not mixed with other types of waste	F8.12	Conduct periodical trial drills regarding the implementation of emergency plans
F4.6	Hazardous wastes labeled during storage in accordance with regulations		

**Correlation is significant at the 0.01 level (2-tailed)

A Likert scale (*Table 4*) was used to measure the responses of participants to the questionnaire elements. The questions were discussed with 30 reviewers in concerned governmental agencies, and then modified according to reviewer feedback to evaluate their validity. Cronbach’s alpha was used to measure the questionnaire’s internal consistency, which was found to be 0.71 by SPSS, indicating a high level of consistency. *Table 5* shows the Pearson correlation coefficient (ρ) between each questionnaire item and the corresponding dimension. All coefficients were positive

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except F1.2 was negative, as a higher item's score represented a good correlation for the dimension. It shows the significance of items to the corresponding dimension.

Table 3. *Population and sample size details*

Construction industry field (Contractor Classification Agency, Ministry of Municipal and Rural Affairs, 2018)	Respondents category					Saudi Arabia's region													Total (3981)
	Engineers and professionals	Authorized contractors	Not authorized contractors	Individuals	Officials and owners	Eastern region (453)	Al Baha region (34)	Al Jouf region (50)	The northern border region (25)	Riyadh region (1768)	Qassim region (246)	Madinah region (139)	Tabuk region (48)	Jazan region (41)	Hail region (67)	Asir region (246)	Makkah region (687)	Najran region (177)	
CI01: Water & sanitation works (2273)	7	7	9	8	8	10	0	1	0	12	2	1	1	0	1	3	7	1	39
CI02: Electronic works (1025)	5	8	6	6	3	8	0	0	0	8	3	1	1	0	0	2	4	1	28
CI03: Marine works (102)	8	10	8	4	6	2	0	0	0	12	2	2	0	1	1	3	11	2	36
CI04: Industrial works (175)	4	7	11	10	9	2	0	0	0	20	3	1	0	0	0	4	10	1	41
CI05: Electrical works (2397)	10	6	10	4	3	3	0	0	0	9	2	2	1	1	1	3	10	1	33
CI06: Mechanical works (1641)	5	4	11	9	5	3	0	1	0	9	2	1	0	0	1	3	12	2	34
CI07: Dams (671)	3	9	5	5	7	2	0	0	0	18	2	1	0	0	0	2	3	1	29
CI08: Roads (1949)	10	11	6	6	6	3	0	0	0	12	1	2	1	1	1	3	13	2	39
CI09: Buildings (3201)	8	5	14	9	5	3	0	1	0	15	3	1	0	0	0	4	13	1	41
CI10: Slaughterhouses (60)	8	7	5	3	8	3	0	0	0	15	2	1	0	0	1	1	6	2	31
CI11: Catering for individuals (152)	13	12	10	8	12	5	0	1	0	16	2	1	1	1	1	3	23	1	55
CI12: Maintenance health centers (85)	16	4	5	3	7	3	0	0	0	23	2	2	0	0	0	2	2	1	35
CI13: Landscaping (1017)	5	5	8	6	9	2	1	0	0	20	1	1	0	1	1	1	3	2	33
CI14: Communication technology (576)	5	10	11	13	10	6	1	1	1	30	2	1	0	0	0	2	3	2	49
CI15: Well drilling (49)	5	3	5	4	9	8	1	0	0	8	2	1	0	0	0	1	4	1	26
CI16: Maintenance of Landscaping (322)	6	7	11	10	5	7	1	1	0	12	2	1	0	0	1	2	10	2	39
CI17: Maintenance and operation dams (32)	2	3	3	4	0	1	1	1	0	0	2	2	1	0	0	2	0	2	12
CI18: Maintenance roads (330)	9	9	1	12	12	10	1	1	0	15	2	1	0	1	1	1	9	1	43
CI19: Maintenance buildings (513)	6	15	9	8	8	4	1	0	0	24	2	1	1	0	0	2	9	2	46
CI20: Catering for medical centers (429)	6	13	6	6	14	6	1	1	0	23	2	1	0	0	1	2	6	2	45
CI21: Maintenance and operation of water & sanitation works (179)	4	4	1	6	3	2	1	1	1	4	2	1	0	1	0	2	2	1	18
CI22: Maintenance and operation of electronic works (118)	6	6	4	8	5	2	0	0	0	12	3	2	0	0	1	1	6	2	29
CI23: Maintenance and operation of marine works (17)	4	2	2	3	6	2	0	0	0	4	2	1	0	1	1	2	2	2	17
CI24: Maintenance and operation of industrial works (32)	4	5	5	3	1	2	0	1	1	4	2	1	1	1	1	2	1	1	18
CI25: Maintenance and operation of electrical works (272)	13	9	14	9	6	5	0	0	1	35	3	1	1	0	0	2	1	2	51
CI26: Maintenance and operation of mechanical works (178)	4	3	3	2	5	2	0	0	1	5	2	1	1	0	1	2	1	1	17
CI27: Maintenance and operation of slaughterhouses (68)	6	4	6	9	4	2	0	1	0	16	2	1	1	1	1	2	1	1	29
CI28: Maintenance and operation of communication technology (33)	7	6	7	4	7	2	0	0	1	18	3	1	1	0	1	1	1	2	31
CI29: City cleaning & wastes disposal (677)	13	15	8	14	6	4	0	1	0	44	2	1	0	0	0	2	0	2	56
Total (18573)	202	209	204	196	189	114	9	13	6	443	62	35	12	10	17	62	173	44	1000

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 4. *Five-point Likert scale*

Response	Strongly agree	Agree	Neither	Disagree	Strongly disagree
Degree	5	4	3	2	1
The weighted average	4.20-5.00	3.40-4.19	2.60-3.39	1.80-2.59	1.00-1.79

Table 5. *Pearson correlation coefficient (ρ) between each sub-factor with main factors*

Code	ρ	Code	ρ	Code	ρ	Code	ρ	Code	ρ	Code	ρ	Code	ρ
F1.1	0.961**	F2.1	0.790**	F3.2	0.629**	F4.3	0.570**	F6.1	0.592**	F7.2	0.647**	F8.6	0.549**
F1.2	-0.928-**	F2.2	0.902**	F3.3	0.595**	F4.4	0.533**	F6.2	0.645**	F7.3	0.598**	F8.7	0.614**
F1.3	0.710**	F2.3	0.974**	F3.4	0.652**	F4.5	0.568**	F6.3	0.391**	F8.1	0.328**	F8.8	0.638**
F1.4	0.865**	F2.4	0.897**	F3.5	0.664**	F4.6	0.560**	F6.4	0.446**	F8.2	0.185**	F8.9	0.583**
F1.5	0.949**	F2.5	0.934**	F3.6	0.648**	F4.7	0.528**	F6.5	0.330**	F8.3	0.610**	F8.10	0.615**
F1.6	0.924**	F2.6	0.889**	F4.1	0.661**	F5.1	0.687**	F6.6	0.463**	F8.4	0.607**	F8.11	0.587**
F1.7	0.882**	F3.1	0.788**	F4.2	0.240**	F5.2	0.690**	F7.1	0.596**	F8.5	0.443**	F8.12	0.577**

**Correlation is significant at the 0.01 level (2-tailed)

Statistical analysis

For this work, the Statistical Package for the Social Sciences (IBM SPSS Statistics, 2015) was used for data description and analysis. The following techniques were used:

- Descriptive statistics measures to describe the collected data in terms of frequencies, mean, and standard deviation.
- Cronbach's alpha was used to measure the questionnaire’s internal consistency.
- Pearson correlation coefficient (ρ) was used to measure the correlation of each subfactor with the related factor.
- One-way ANOVA was used to evaluate whether the mean of data from one group was different from the others.
- Levene’s test was used to measure homogeneities between sample groups.

The statistical analysis and test results at the 0.05 level of significance are described in the next section. Also, the performance of the law implementation score in addition to the performance for each construction industry category is estimated based on the collected data.

Results and discussion

Table 6 reflects the overall average and average environmental performance of each factor for of the 13 regions. The table highlights that the overall average Likert scale value for Asir, Riyadh, Madinah, Makkah, Eastern, Najran, Al Baha, and Qassim regions are 3.03, 3.03, 3.04, 3.09, 3.01, 3.10, 3.02, and 3.08 respectively. This indicates that the environmental laws implementation is seen more in the southern part of the KSA. The remaining regions perform less than 2.99. Also, the table shows that industry categories get a mean Likert scale value of 3.23, 3.12, 3.10, 3.06, 3.05, 2.95, 2.80, and 2.75 for radioactive materials handling and disposal (F5), hazardous waste management (F4), wastewater discharges (F3), hazardous dangerous substance compliance programs

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

(F6), environmental noise (F7), general environmental requirements (F1), air quality emissions (F2), and external emergency planning (F8). These factors have high exposure of harmful effects on the environment which should be controlled.

Table 7 shows the overall average and average environmental performance of each factor of the 29 construction industry categories KSA. It highlights the average Likert scale score for slaughterhouses, catering for individuals, landscaping, maintenance buildings, maintenance and operation of slaughterhouses, maintenance and operation of communication technology, maintenance and operation of electrical works, city cleaning & wastes disposal, roads, marine works, maintenance and operation of electronic works, maintenance and operation of mechanical works, electrical works, catering for medical centers, buildings, maintenance health centers, industrial works, dams, maintenance roads, and maintenance and operation of marine works are 3.21, 3.20, 3.19, 3.14, 3.13, 3.11, 3.09, 3.09, 3.08, 3.08, 3.07, 3.07, 3.06, 3.06, 3.04, 3.02, 3.01, 3.01, 3.01, and 3.00 respectively highlighted in the table by a green color. Twenty construction industry categories achieve a score greater than 3.00. This indicating that these industries follows to some extend the Saudi environmental laws.

These industries are imposed by strict rules of Saudi government and continuous monitoring done to protect the environments and heavy penalties imposed if disposed in a wrong manner. The environmental monitoring system and procedures in Saudi Arabia is done with perfection to save the environment and maintain the society.

The remaining industries as electronic works, mechanical works, water & sanitation works, maintenance of landscaping, maintenance and operation of industrial works, communication technology, maintenance and operation of water & sanitation works, well drilling, and maintenance and operation dams achieve a total score between 2.80-2.99 with average Likert scores of 2.95, 2.94, 2.93, 2.90, 2.89, 2.86, 2.86, 2.81, and 2.80 respectively, as these construction industries are far away from city dominated areas and does not affect the environment. Also, the performance of the factors is similar to data in *Table 6*.

Table 6. *The overall average and average environmental performance of each factor for of the 13 regions*

Region	Environmental law category (mean Likert scale value)								Overall average	Rank
	F1	F2	F3	F4	F5	F6	F7	F8		
Jazan region	2.66	2.43	2.85	2.89	3.05	3.05	3.07	2.48	2.83	12
The Northern Border region	2.69	2.19	2.89	3.19	3.50	2.86	3.11	2.32	2.88	11
Al Jouf Region	2.78	2.33	2.92	3.16	2.88	2.77	3.23	2.46	2.78	13
Hail region	2.82	2.53	2.88	3.26	3.32	3.10	3.27	2.55	2.98	9
Tabuk region	2.85	2.69	3.14	2.89	2.83	2.90	3.28	2.67	2.90	10
Asir region	2.93	2.93	3.11	3.09	3.23	2.98	3.07	2.78	3.03	6
Riyadh region	2.95	2.82	3.09	3.08	3.27	3.08	3.00	2.76	3.03	5
Madinah region	2.96	2.83	3.13	3.26	3.21	3.14	3.15	2.68	3.04	4
Makkah region	2.99	2.95	3.15	3.19	3.29	3.10	3.07	2.86	3.09	2
Eastern Region	3.00	2.86	3.14	3.09	3.10	3.09	3.03	2.80	3.01	8
Najran region	3.02	2.98	3.13	3.30	3.34	3.06	3.06	2.89	3.10	1
Al Baha region	3.06	2.70	2.83	3.17	3.50	3.06	2.96	2.56	3.02	7
Qassim region	3.09	3.03	3.19	3.18	3.10	3.10	3.18	2.89	3.08	3
Average	2.95	2.80	3.10	3.12	3.23	3.06	3.05	2.75	2.95	
Rank	6	7	3	2	1	4	5	8		

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DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

Table 7. *The overall average and average environmental performance of each factor for the 29 industry*

Construction industry category	Environmental law category (mean Likert scale value)								Overall average	Rank
	F1	F2	F3	F4	F5	F6	F7	F8		
Slaughterhouses	3.09	2.98	3.28	3.50	3.45	3.26	3.18	2.95	3.21	1
Catering for individuals	3.01	3.15	3.25	3.22	3.32	3.00	3.39	3.14	3.20	2
Landscaping	3.12	3.17	3.16	3.17	3.26	3.41	3.18	2.98	3.19	3
Maintenance buildings	3.01	2.97	3.17	3.19	3.42	3.26	3.02	2.80	3.14	4
Maintenance and operation of slaughterhouses	3.14	2.78	3.39	3.25	3.28	2.99	3.44	2.78	3.13	5
Maintenance and operation of comm. Tech.	3.00	2.64	3.18	3.07	3.55	3.08	3.27	2.65	3.11	6
Maintenance and operation of electrical works	3.00	2.84	3.19	3.22	3.32	3.09	3.24	2.76	3.09	8
City cleaning & wastes disposal	3.09	2.91	3.26	3.17	3.20	3.19	3.07	2.83	3.09	7
Roads	2.98	2.92	3.15	3.11	3.27	3.04	3.02	3.00	3.08	10
Marine works	3.10	3.26	3.23	2.94	3.04	3.13	2.92	2.90	3.08	9
Maintenance and operation of electronic works	2.93	2.73	3.28	3.22	3.43	3.06	2.93	2.75	3.07	12
Maintenance and operation of mechanical works	3.00	2.72	3.21	3.07	3.32	2.94	3.29	2.72	3.07	11
Electrical works	2.90	3.05	3.10	3.03	3.39	3.14	2.70	2.79	3.06	14
Catering for medical centers	2.94	2.62	3.13	3.13	3.41	2.99	3.39	2.56	3.06	13
Buildings	2.92	2.78	2.96	3.34	3.38	3.05	3.06	2.82	3.04	15
Maintenance health centers	2.80	2.79	2.87	2.94	3.33	3.03	3.28	2.77	3.02	16
Industrial works	2.93	3.04	2.98	2.98	3.18	3.20	2.89	2.70	3.01	19
Dams	2.92	2.87	3.20	3.25	3.41	2.98	2.63	2.66	3.01	18
Maintenance roads	2.98	2.88	3.02	3.09	3.12	3.16	2.97	2.82	3.01	17
Maintenance and operation of marine works	2.88	2.61	3.19	3.20	3.26	3.08	3.06	2.67	3.00	20
Electronic works	2.97	3.10	2.98	3.05	2.88	2.90	3.10	2.82	2.95	21
Mechanical works	2.82	2.71	2.87	3.14	3.37	3.03	2.71	2.64	2.94	22
Water & sanitation works	3.08	3.00	3.22	3.07	2.71	3.06	2.84	2.84	2.93	23
Maintenance of Landscaping	2.95	2.83	2.88	3.03	3.00	2.92	2.91	2.70	2.90	24
Maintenance and operation of industrial works	2.63	2.21	2.84	3.22	3.42	3.00	3.09	2.52	2.89	25
Communication technology	2.93	2.72	2.94	2.88	2.94	2.98	2.76	2.68	2.86	27
Maintenance and oper. of water & sanitation works	2.76	2.23	3.08	3.17	3.22	2.87	2.94	2.54	2.86	26
Well drilling	2.83	2.47	2.89	3.03	2.90	2.96	3.06	2.47	2.81	28
Maintenance and operation dams	2.75	2.31	2.90	2.80	2.88	2.99	3.22	2.48	2.80	29
Average	2.95	2.80	3.10	3.12	3.23	3.06	3.05	2.75	2.95	
Rank	6	7	3	2	1	4	5	8		

Tables 8–15 shows the count and percentage of respondents in terms of the Likert scale, mean, standard deviation in terms of "mean±standard deviation", priority, and response for all subfactors. The tables show the counts (Count) of all respondents with respect to opinions "strongly disagree, disagree, neither, agree, strongly agree) in the third to seventh columns and respondent percentages (N%). Column 7 calculates the mean while column 8 determine the standard deviation and column 9 highlights priority and finally column 10 attributes the responses.

Table 16 shows the corresponding values for the overall performance for implementation. *Table 8* demonstrates that the mean overall performance in implementing the general requirements of Saudi environmental laws by the construction industry is equal to 2.97 ± 1.31. This indicates that the implementation level of the general requirements is difficult to be defined by practitioners and that the environmental protection concepts were not clear to respondents. The respondents agree with F1.1 that the facility has an up-to-date operating license from the government and agree with F1.2 related to the question "Has the facility been cited for any

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

environmental violations or been issued any notifications relating to environmental matters, and if so, have the conditions or violations been addressed and subsequently approved or signed off by the appropriate authorities”. The response for F1.7 was “Disagree” with a mean 2.36 ± 1.46, indicating that the facility did not comply with all applicable regulations and standards when designing or operating any project or activity. The remaining sub-factors performance was “Neither”, as shown in the tables.

Table 8. *General environmental requirements implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F1.1	Count	193	208	242	197	160	2.92	1.34	4	Neither
	N %	19.3%	20.8%	24.2%	19.7%	16.0%				
F1.2	Count	157	194	248	221	180	3.07	1.32	3	Neither
	N %	15.7%	19.4%	24.8%	22.1%	18.0%				
F1.3	Count	22	240	241	252	245	3.46	1.16	1	Agree
	N %	2.2%	24.0%	24.1%	25.2%	24.5%				
F1.4	Count	5	269	238	263	225	3.43	1.12	2	Agree
	N %	0.5%	26.9%	23.8%	26.3%	22.5%				
F1.5	Count	197	212	265	261	65	2.79	1.21	5	Neither
	N %	19.7%	21.2%	26.5%	26.1%	6.5%				
F1.6	Count	193	238	270	243	56	2.73	1.18	6	Neither
	N %	19.3%	23.8%	27.0%	24.3%	5.6%				
F1.7	Count	401	245	111	83	160	2.36	1.46	7	Disagree
	N %	40.1%	24.5%	11.1%	8.3%	16.0%				
Totals	Count	1168	1606	1615	1520	1091	2.97	1.31		Neither
	N %	16.7%	22.9%	23.1%	21.7%	15.6%				

Table 9 shows the performance of the construction industry in implementing environmental laws, regarding air quality/emissions. The overall performance level was 2.85 ± 1.35, indicating that the response was “Neither”. The performance for all sub-factors was also “Neither”, indicating that the respondents could not decide on the level of implementation. The lowest performance level for F2.4, related to “Does the facility ensure that emission of smoke, gases, vapors, and solid or liquid residues resulting from the burning of any kind of fuel or a similar action are within allowable limits permitted in the environmental standards listed under Attachment A of this document?” was a 2.70 ± 1.37. F2.5 is related to “Does the facility ensure that there is no leaking or emission of air pollutants into the work place beyond the allowable limits of the environmental standards?”. This analysis has priority level of 5 and response result as neither, with a mean of 2.74 ± 1.42 which confirms that the company has no proven records about the leakage of emission and air pollutants beyond allowable standard limits.

The overall performance for implementing regulations related to wastewater discharges from the point of view of respondents is “Neither” with a mean of 3.11 ± 1.30, as shown in *Table 10*. The lowest performance was for F3.1, related to whether the various facilities take the necessary precautions to ensure prevention of direct or indirect contamination of surface, ground, and coastal waters that might be caused by solid or liquid residues; and take actions to ensure that the soil and land were preserved and its deterioration and/or contamination was curbed. “Neither” was the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

response to all sub-factors for implementing wastewater-discharge environmental regulations.

Table 9. *Air quality/emissions implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F2.1	Count	96	258	291	240	115	3.02	1.15	1	Neither
	N %	9.6%	25.8%	29.1%	24.0%	11.5%				
F2.2	Count	198	236	243	153	170	2.86	1.35	4	Neither
	N %	19.8%	23.6%	24.3%	15.3%	17.0%				
F2.3	Count	193	208	242	197	160	2.92	1.34	2	Neither
	N %	19.3%	20.8%	24.2%	19.7%	16.0%				
F2.4	Count	237	266	221	114	162	2.70	1.37	6	Neither
	N %	23.7%	26.6%	22.1%	11.4%	16.2%				
F2.5	Count	258	239	169	175	159	2.74	1.42	5	Neither
	N %	25.8%	23.9%	16.9%	17.5%	15.9%				
F2.6	Count	234	230	130	246	160	2.87	1.42	3	Neither
	N %	23.4%	23.0%	13.0%	24.6%	16.0%				
Totals	Count	1216	1437	1296	1125	926	2.85	1.35		Neither
	N %	20.3%	24.0%	21.6%	18.8%	15.4%				

Table 10. *Wastewater discharges implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F3.1	Count	181	301	202	174	142	2.80	1.31	6	Neither
	N %	18.1%	30.1%	20.2%	17.4%	14.2%				
F3.2	Count	104	201	251	301	143	3.18	1.20	2	Neither
	N %	10.4%	20.1%	25.1%	30.1%	14.3%				
F3.3	Count	109	178	266	288	159	3.21	1.22	1	Neither
	N %	10.9%	17.8%	26.6%	28.8%	15.9%				
F3.4	Count	120	247	222	190	221	3.15	1.33	4	Neither
	N %	12.0%	24.7%	22.2%	19.0%	22.1%				
F3.5	Count	141	205	224	199	231	3.17	1.36	3	Neither
	N %	14.1%	20.5%	22.4%	19.9%	23.1%				
F3.6	Count	139	206	233	232	190	3.13	1.31	5	Neither
	N %	13.9%	20.6%	23.3%	23.2%	19.0%				
Totals	Count	794	1338	1398	1384	1086	3.11	1.30		Neither
	N %	13.2%	22.3%	23.3%	23.1%	18.1%				

Table 11 summarizes the performance of hazardous waste management regulation implementation. It indicates that the overall mean is 3.12 ± 1.26, and that the implementation scale is “Neither”. As shown in the table, the implementing of environmental regulations for this factor ranges from 3.27 for F4.3 to 3.01 ± 0.26 for F4.4 indicates the extreme keenness of the Kingdom’s institutions in the construction industry that deal with hazardous materials.

The performance regarding radioactive materials handling and disposal (shown in *Table 12*) indicates an implementation level mean of 3.23 ± 1.35, and a small range of 0.02 between F5.1 and F5.2, due to the nature of materials that may cause severe chronic diseases and affect the health of individuals and workers. The main reason for the increased percentage of disagree in F5.2 “storage, treatment, recycling, and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

transportation of radioactive waste in accordance to regulations" is due to the nature of the radioactive waste in Saudi Arabia resulting from water treatment with the acceptable levels and controlled completely with the Saudi governmental specialized agencies. The inspection team totally ignore, because used in lower levels and it is within the radioactive acceptable limits zone of disposal standards, but the engineers in and around the company feel that it is disposed with not following the standards.

Table 11. *Hazardous waste management implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F4.1	Count	161	204	185	241	209	3.13	1.38	5	Neither
	N %	16.1%	20.4%	18.5%	24.1%	20.9%				
F4.2	Count	165	156	211	272	196	3.18	1.35	2	Neither
	N %	16.5%	15.6%	21.1%	27.2%	19.6%				
F4.3	Count	86	186	271	289	168	3.27	1.19	1	Neither
	N %	8.6%	18.6%	27.1%	28.9%	16.8%				
F4.4	Count	94	264	314	195	133	3.01	1.17	7	Neither
	N %	9.4%	26.4%	31.4%	19.5%	13.3%				
F4.5	Count	107	231	258	255	149	3.11	1.22	3	Neither
	N %	10.7%	23.1%	25.8%	25.5%	14.9%				
F4.6	Count	137	245	240	180	198	3.06	1.32	6	Neither
	N %	13.7%	24.5%	24.0%	18.0%	19.8%				
F4.7	Count	94	192	344	247	123	3.11	1.13	4	Neither
	N %	9.4%	19.2%	34.4%	24.7%	12.3%				
Totals	Count	844	1478	1823	1679	1176	3.12	1.26		Neither
	N %	12.1%	21.1%	26.0%	24.0%	16.8%				

Table 12. *Radioactive materials handling and disposal implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F5.1	Count	132	199	184	263	222	3.24	1.34	1	Neither
	N %	13.2%	19.9%	18.4%	26.3%	22.2%				
F5.2	Count	130	201	229	200	240	3.22	1.35	2	Neither
	N %	13.0%	20.1%	22.9%	20.0%	24.0%				
Totals	Count	262	400	413	463	462	3.23	1.35		Neither
	N %	13.1%	20.0%	20.7%	23.2%	23.1%				

Table 13 shows the implementation level of hazardous or dangerous substance compliance-program implementation. The performance of implementation had a mean of 3.07 ± 1.30, and the overall performance is "Neither". The lowest implementation level is for F6.1, related to the existence of a listing of all dangerous chemical substances used at the facility on-site (2.75 ± 1.05 and a "Neither" response).

Table 14 demonstrates the extent to which the companies apply environmental noise laws and whether they take the necessary precautions to ensure that noise levels do not exceed permissible/standard environmental limits. The overall average performance measure for this factor was 3.05 ± 1.

Table 15 shows the implementation level of Saudi companies for the emergency planning factor. It shows that the average overall performance is 2.78 ± 1.28. Sub-factor F8.1 represents the opinion of the participants in the field study whether the facility has the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

potential to cause adverse impacts on the environment, and if so, whether the facility has prepared emergency plans to prevent or alleviate the hazards of such impacts, and finally whether the facility has enough means to implement these plans. The average opinion of the participants was 2.80 ± 1.28, representing a "Neither" response. The reason for this evaluation might be explained by the fact that the responsibility to deal with emergency cases was assigned to the Saudi General Directorate of Civil Defense and thus, that the emergency work was not assigned to the industrial organization itself. The lowest performance was assigned to F8.4 (2.59 ± 1.18) with a "Disagree" response in relation to "Has the facility verified plans required for environmental protection from pollution resulting from conducted projects, and periodically reviewed these plans to coordinate efforts for their implementation?"

Table 17 shows the one-way ANOVA for the for environmental law implementation by field of construction industry. It shows the mean, standard deviation, standard error, 95% confidence interval for the mean, test of homogeneity of variances, ANOVA'F and significance, and the statistical significance for each factor. From this table, we conclude that there is no homogeneity between samples of the implementation levels for F1.3, F1.4, F1.6, F1.7, F2.1, F2.2, F2.4, F2.5, F3.1, F4.2, F4.3, F4.4, F4.5, F4.7, F5.1, F6.1, F6.3, F6.4, F6.5, F6.6, F7.2, F7.3, F8.1, F8.3, F8.4, F8.5, F8.6, F8.8, F8.9, F8.10, F8.11, and F8.12, wherein the values of F < 0.05; therefore, the ANOVA test cannot be completed. However, the ANOVA can be completed for the remaining factors. Judgment for non-homogeneity is based on calculating the Levene's test and corresponding significance level of <0.05.

Moreover, the one-way ANOVA test for F3.2, F3.3, and F8.2 demonstrates that homogeneity of samples does exist and consequently, there is a difference between the implementation levels of the environmental laws in all 29 construction industry fields with mean levels of 3.18, 3.12, and 3.06.

The significance value for ANOVA regarding F1.1, F1.2, F1.5, F2.3, F2.6, F3.4, F3.5, F3.6, F4.1, F4.6, F5.2, F6.2, F7.1, and F8.7 was >0.05, indicating that there is no statistical evidence that there is a difference between the implementation levels for the corresponding factors. The main results obtained from this research are supporting the work done in China by Wang (2013) and van Zeben (2014) as they highlighted the importance of monitoring the environmental laws implementation not by enforcement but by societal involvement.

Table 13. *Hazardous/dangerous substance compliance programs implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F6.1	Count	112	302	395	111	80	2.75	1.05	6	Neither
	N %	11.2%	30.2%	39.5%	11.1%	8.0%				
F6.2	Count	193	208	242	356	1	2.76	1.13	5	Neither
	N %	19.3%	20.8%	24.2%	35.6%	.1%				
F6.3	Count	146	186	193	255	220	3.22	1.36	2	Neither
	N %	14.6%	18.6%	19.3%	25.5%	22.0%				
F6.4	Count	173	147	231	201	248	3.20	1.41	3	Neither
	N %	17.3%	14.7%	23.1%	20.1%	24.8%				
F6.5	Count	132	132	266	242	228	3.30	1.31	1	Neither
	N %	13.2%	13.2%	26.6%	24.2%	22.8%				
F6.6	Count	146	187	219	202	246	3.21	1.38	4	Neither
	N %	14.6%	18.7%	21.9%	20.2%	24.6%				
Totals	Count	902	1162	1546	1367	1023	3.07	1.30		Neither
	N %	15.0%	19.4%	25.8%	22.8%	17.1%				

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

Table 14. *Environmental noise implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F7.1	Count	129	204	208	250	209	3.21	1.32	1	Neither
	N %	12.9%	20.4%	20.8%	25.0%	20.9%				
F7.2	Count	151	244	207	192	206	3.06	1.36	2	Neither
	N %	15.1%	24.4%	20.7%	19.2%	20.6%				
F7.3	Count	199	185	262	235	119	2.89	1.29	3	Neither
	N %	19.9%	18.5%	26.2%	23.5%	11.9%				
Totals	Count	479	633	677	677	534	3.05	1.33		Neither
	N %	16.0%	21.1%	22.6%	22.6%	17.8%				

Table 15. *External emergency planning implementation descriptive statistics*

Sub-factor		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
F8.1	Count	209	220	229	246	96	2.80	1.28	6	Neither
	N %	20.9%	22.0%	22.9%	24.6%	9.6%				
F8.2	Count	190	143	237	276	154	3.06	1.33	2	Neither
	N %	19.0%	14.3%	23.7%	27.6%	15.4%				
F8.3	Count	216	280	254	166	84	2.62	1.22	11	Neither
	N %	21.6%	28.0%	25.4%	16.6%	8.4%				
F8.4	Count	210	290	277	151	72	2.59	1.18	12	Disagree
	N %	21.0%	29.0%	27.7%	15.1%	7.2%				
F8_5	Count	172	202	241	167	218	3.06	1.38	1	Neither
	N %	17.2%	20.2%	24.1%	16.7%	21.8%				
F8.6	Count	212	240	214	207	127	2.80	1.32	5	Neither
	N %	21.2%	24.0%	21.4%	20.7%	12.7%				
F8.7	Count	224	274	235	165	102	2.65	1.27	9	Neither
	N %	22.4%	27.4%	23.5%	16.5%	10.2%				
F8.8	Count	216	266	255	193	70	2.64	1.21	10	Neither
	N %	21.6%	26.6%	25.5%	19.3%	7.0%				
F8.9	Count	208	246	247	170	129	2.77	1.30	7	Neither
	N %	20.8%	24.6%	24.7%	17.0%	12.9%				
F8.10	Count	214	277	242	181	86	2.65	1.23	8	Neither
	N %	21.4%	27.7%	24.2%	18.1%	8.6%				
F8.11	Count	159	285	258	180	118	2.81	1.24	4	Neither
	N %	15.9%	28.5%	25.8%	18.0%	11.8%				
F8.12	Count	166	262	235	197	140	2.88	1.29	3	Neither
	N %	16.6%	26.2%	23.5%	19.7%	14.0%				
Totals	Count	2396	2985	2924	2299	1396	2.78	1.28		Neither
	N %	20.0%	24.9%	24.4%	19.2%	11.6%				

Table 16. *Overall environmental laws implementation descriptive statistics*

Overall performance		Strongly disagree	Disagree	Neither	Agree	Strongly agree	Mean	Std. deviation	Priority	Response
Totals	Count	8061	11039	11692	10514	7694	2.95	1.13		Neither
	N %	16.5%	22.5%	23.9%	21.5%	15.7%				

Table 17. *One-way ANOVA for environmental law implementation by field of construction industry*

Environmental law	Mean	Std. deviation	Std. error	95% Confidence interval for mean		Test of homogeneity of variances		ANOVA		Statistical significance
				Lower bound	Upper bound	Levene's statistic	Sig.	F	Sig.	
F1.1	2.92	1.34	0.043	2.84	3.01	1.24	0.176	0.69	0.884	Insignificant
F1.2	3.07	1.32	0.042	2.99	3.16	1.24	0.177	0.83	0.707	Insignificant
F1.3	3.46	1.16	0.037	3.39	3.53	1.90	0.003	--	--	--
F1.4	3.43	1.12	0.036	3.36	3.50	1.77	0.008	--	--	--
F1.5	2.79	1.21	0.038	2.71	2.86	1.15	0.262	0.77	0.791	Insignificant
F1.6	2.73	1.18	0.037	2.66	2.80	1.88	0.004	--	--	--
F1.7	2.36	1.46	0.046	2.26	2.45	3.72	0.000	--	--	--
F2.1	3.02	1.15	0.037	2.95	3.09	2.24	0.000	--	--	--
F2.2	2.86	1.35	0.043	2.78	2.95	2.79	0.000	--	--	--
F2.3	2.92	1.34	0.043	2.84	3.01	1.24	0.176	0.69	0.884	Insignificant
F2.4	2.70	1.37	0.043	2.61	2.78	9.68	0.000	--	--	--
F2.5	2.74	1.42	0.045	2.65	2.83	2.66	0.000	--	--	--
F2.6	2.87	1.42	0.045	2.78	2.96	1.72	0.011	1.33	0.116	Insignificant
F3.1	2.80	1.31	0.042	2.71	2.88	4.99	0.000	--	--	--
F3.2	3.18	1.20	0.038	3.10	3.25	1.37	0.096	2.66	0.000	Significant
F3.3	3.21	1.22	0.039	3.13	3.29	1.47	0.055	1.51	0.042	Significant
F3.4	3.15	1.33	0.042	3.06	3.23	0.60	0.950	1.00	0.466	Insignificant
F3.5	3.17	1.36	0.043	3.09	3.26	1.73	0.010	0.90	0.616	Insignificant
F3.6	3.13	1.31	0.042	3.05	3.21	1.65	0.018	1.00	0.453	Insignificant
F4.1	3.13	1.38	0.044	3.05	3.22	1.17	0.243	0.71	0.859	Insignificant
F4.2	3.18	1.35	0.043	3.09	3.26	2.61	0.000	--	--	--
F4.3	3.27	1.19	0.038	3.19	3.34	2.27	0.000	--	--	--
F4.4	3.01	1.17	0.037	2.94	3.08	2.08	0.001	--	--	--
F4.5	3.11	1.22	0.039	3.03	3.18	2.70	0.000	--	--	--
F4.6	3.06	1.32	0.042	2.97	3.14	1.70	0.013	0.82	0.725	Insignificant
F4.7	3.11	1.13	0.036	3.04	3.18	1.87	0.004	--	--	--
F5.1	3.24	1.34	0.043	3.16	3.33	1.80	0.007	--	--	--
F5.2	3.22	1.35	0.043	3.13	3.30	1.52	0.041	1.29	0.143	Insignificant
F6.1	2.75	1.05	0.033	2.68	2.81	12.08	0.000	--	--	--
F6.2	2.76	1.13	0.036	2.69	2.83	0.97	0.509	0.55	0.970	Insignificant
F6.3	3.22	1.36	0.043	3.13	3.30	3.92	0.000	--	--	--
F6.4	3.20	1.41	0.045	3.12	3.29	2.05	0.001	--	--	--
F6.5	3.30	1.31	0.042	3.22	3.38	2.92	0.000	--	--	--
F6.6	3.22	1.38	0.044	3.13	3.30	2.08	0.001	--	--	--
F7.1	3.21	1.32	0.042	3.12	3.29	1.25	0.174	0.72	0.854	Insignificant
F7.2	3.06	1.36	0.043	2.97	3.14	2.49	0.000	--	--	--
F7.3	2.89	1.29	0.041	2.81	2.97	2.58	0.000	--	--	--
F8.1	2.80	1.28	0.041	2.72	2.88	5.15	0.000	--	--	--
F8.2	3.06	1.33	0.042	2.98	3.14	1.22	0.194	1.95	0.002	Significant
F8.3	2.62	1.22	0.039	2.55	2.70	2.10	0.001	--	--	--
F8.4	2.59	1.18	0.037	2.51	2.66	2.85	0.000	--	--	--
F8.5	3.06	1.38	0.044	2.97	3.14	19.01	0.000	--	--	--
F8.6	2.80	1.32	0.042	2.71	2.88	10.35	0.000	--	--	--
F8.7	2.65	1.27	0.040	2.57	2.73	0.87	0.658	1.01	0.442	Insignificant
F8.8	2.64	1.21	0.038	2.56	2.71	2.03	0.001	--	--	--
F8.9	2.77	1.30	0.041	2.68	2.85	10.03	0.000	--	--	--
F8.10	2.65	1.23	0.039	2.57	2.72	1.78	0.007	--	--	--
F8.11	2.81	1.24	0.039	2.74	2.89	12.74	0.000	--	--	--
F8.12	2.88	1.29	0.041	2.80	2.96	2.24	0.000	--	--	--

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

Conclusions

The aim of this research was to investigate the environmental legitimacy implementation in the Saudi Arabian construction industry. To do this, eight regulation categories, each including a set of sub-laws, were defined: general environmental requirements, radioactive materials handling and disposal, hazardous/dangerous substance compliance programs, air quality/emissions, environmental noise, wastewater discharges, external emergency planning, and hazardous waste management. An analytical statistical approach was used to describe and analyze the data set collected in a newly designed Likert 5-scale questionnaire distributed to 1000 organizations that work in the construction industry in 13 regions in Saudi Arabia. These were filled out (through direct meeting with via email) by engineers and professionals, authorized contractors, unauthorized contractors, individuals, and officials and owners. The analysis was done using measures of descriptive statistics, Cronbach's alpha, Pearson correlation coefficient, ANOVA, and the Levene's test (which was used to measure homogeneities between sample groups). The main purpose was to answer the following questions: "Does the construction facility applies the set of environmental regulations or not?" and "Does the construction facility applies the regulations for each law or not?" The levels of implementation were also indicated.

The main finding of this research was that the mean implementation performance was 2.95, indicating a Likert scale of "Neither". This indicates that the participants had difficulty defining accurate answers regarding environmental legitimacy implementation in the Saudi Arabian construction industry. The implementation levels for all eight sub-regulations were defined as "Neither". The one-way ANOVA test for F3.2, F3.3, and F8.2 demonstrates that homogeneity of samples existed and, consequently, that there was a difference between the implementation levels of the environmental laws in all 29 construction industry fields (mean levels of 3.18, 3.12, and 3.06). The significance value (>0.05) for ANOVA regarding F1.1, F1.2, F1.5, F2.3, F2.6, F3.4, F3.5, F3.6, F4.1, F4.6, F5.2, F6.2, F7.1, and F8.7 indicated that there is no statistical evidence that there is a difference between the implementation levels for the corresponding factors. The remaining sub-regulations do not appear to exhibit sample homogeneities according to the Levene's test, so ANOVA could not be conducted. The means for the eight regulation categories (arranged from highest to lowest) for radioactive materials handling and disposal, hazardous waste management, wastewater discharges, hazardous and dangerous substance compliance programs, environmental noise, general environmental requirements, air quality emissions, and external emergency planning were 3.23, 3.12, 3.10, 3.06, 3.05, 2.95, 2.80, and 2.75, respectively. This performance level could be explained by the existence of multiple systems related to the environment, the conflict of competencies between the government agencies concerned with the environment, and the failure to activate the current environmental legislation to the appropriate extent. The results of this research are in consistence with the work done by Wang (2013) and van Zeben (2014) in China.

The natural expansion of this research would be to evaluate the implementation of environmental accounting audits and to measure the environmental awareness at different levels of employers in the construction industry. Moreover, an investigation is required to evaluate the existence of internal environmental audit teams in industrial organizations.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_37813802

THE INFLUENCE OF WETLAND MEDIA IN IMPROVING THE PERFORMANCE OF POLLUTANT REMOVAL DURING WATER TREATMENT: A REVIEW

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(Received 4th Jan 2019; accepted 18th Feb 2019)

Abstract. Biochar is used for water treatment as a low-cost adsorbent with high efficiency. It has a large pore volume and is environmentally friendly. Recently, biochar has been a popular topic in the field of environmental management and wastewater treatment. There has been a rising interest in biochar due to its low-cost production, environmental stability and potential effects on several ecosystem functions. A high number of studies have shed light on the potential use of biochar as a sorbent as well as the application of biochar to adsorb pollutants in wastewater and enhance the efficiency of constructed wetlands. The removal of heavy metals from aqueous solutions was examined and biochar was found to efficiently remove more heavy metal ions from these solutions than activated carbon. This study has shed light on the role of biochar in water treatments and provided an updated review to produce biochar from different biomass feedstock around the world.
Keywords: *biochar, biomass feedstock, biochar preparation, water treatment, constructed wetlands, potentially toxic elements*

Introduction

A basic necessity of life is clean and safe drinking water. It is documented that water contamination with potentially toxic elements (PTEs) has been a critical problem since the last few decades, which risks the lives of humans, plants, and animals around the globe. Due to increasing amounts of waste and lack of waste management, water bodies have become polluted. In the 1990's, a huge percentage of the world's population was suffering from water scarcity. In 1990, it was reported that more than 2.7 billion people around the world lacked access to improved sanitation services such as garbage collection and wastewater disposal. Raising awareness for common people and waste management authorities brought a mere 7% improvement. In 2015, the number of people who lacked access to sanitation facilities decreased to 2.4 billion (WHO, 2015; Sanjrani et al., 2018). Waste management is a big issue. It is estimated that more than 80% of wastewater in developing countries is released into the environment without treatment. Most problems in regards to wastewater are faced by urban populations. Currently, 54% of the world's population are living in urban areas and the percentage is expected to increase to 66% by 2050 (WHO, 2015). Thus, providing good quality sanitation and wastewater treatment facilities is required. Mining or chemical plants should be documented to deal with sewage, sludge storage and waste treatment, as an attampt to supply safe drinking water to people in affected areas. Several studies are being conducted for solid waste management plans (Sanjrani et al., 2019; WHO, 2015).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

A report has been issued by the United Nations World Water Assessment Programme (UNWWAP, 2017) which stated that in some developing countries, around 80% of sewage effluent and more than 70% of the industrial wastes are being discharged into surface water without proper treatments. This means water bodies contain several potentially toxic elements (PTEs) and the quantities of these pollutants have risen to over the year. These toxic elements include arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb), vanadium (V), selenium (Se), and zinc (Zn) (Sanjrani et al., 2018; Rakotondrabe et al., 2018). Recent studies have recommended that easy and low-cost water treatment techniques should be introduced to the public; especially in the affected areas where health is threatened due to water pollution. Alternative water supply options should be introduced and should consider the social, and economic status of the local population. Several options have been implemented for treatment, but wetlands technology has been found more efficient and low-cost. One of the best and valuable services given by wetlands is that they generally create a system where everything occurs naturally. Constructed wetlands are complex systems with large number of active chemical, physical, and biological processes that mutually influence each other and treat the water properly (Varga and Oirschot, 2017; Sanjrani et al., 2017).

Recently, the use of wetland media “biochar” to increase constructed wetland efficiency for treating wastewater has been demonstrated in several studies. Results from those studies have been highly appreciated by the organizations who work for the improvement of water quality. Biochar was first mainly studied as an amendment of soil but further studies found out it can also acts as a well-adsorbent; later, it was proved to be an easily available option for low-cost wastewater treatment. In addition, after activated carbon, biochars got more attraction for their new techniques as an effective low-cost alternative. Biochar is a generally carbon-rich solid material generated by the pyrolysis of bio-organic things at middle to low temperature approximately (<700_C) under anoxic conditions. The formation methods of biochar are: pyrolysis, hydrothermal carbonization, gasification, and so on. Moreover, some studies concluded that different bio-organic materials, modification methods, and pyrolysis temperatures may show different performances. In addition, many studies also demonstrated that biochar stimulates N transformation, due to its highly porous structure and DOM release (Gupta et al., 2015; Lehmann and Joseph, 2015). The present paper attempts to provide an overview on the role of Biochar in wetlands systems and its effect on removal efficiency. It also provides review about the materials from which biochar is being generated as well as its preparation methods.

Biochar production around the world

The interest in biochar firstly grew in USA, nearly 17 million tons of organic waste was identified in Washington State. This waste mostly came from wood and straw, a situation where pyrolysis is an attractive option to recover energy and produce stable carbon, which can then benefit the soils and climate (Bio, 2018). The Center for Sustaining Agriculture and Natural Resources (CSANR) and the Washington Department of Ecology at Washington State University has produced a series of in-depth reports on biochar production, use, and economics. Later, it was innovated by Australia, China and Japan. Water treatment by biochar and constructed wetlands has been widely studied in temperate countries such as Canada, Belgium (Lesage et al., 2007), the Republic of Czech

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

(Vymazal, 2014), the United States of America (Kadlec et al., 2010) and the United Kingdom (Shutes, 2001). It has also been studied in tropical countries such as Thailand (Kantawanichkul et al., 2009), Kenya (Mburu et al., 2012), Malaysia (Sim et al., 2008) and in subtropical countries such as Australia (Greenway, 2005). Up-to-now, it is being used all around the world, especially; addition of biochar in constructed wetlands is an innovation and adopted by several countries. Recently, For biochar, different materials have been used in different countries, some of them are shown in *Table 2*.

Biochar preparation methods

Recently, the preparation of biochar requires some methods including the cleaning and drying of material, pyrolysis, hydrothermal-carbonization, gasification, as well as some other methods. The preparation of Biochar is not complicated; the basic process is known as pyrolysis. Pyrolysis releases gas as the material is broken down, a process (heating rate up to 1000 °C/s) of a decomposition reaction under high temperature and anoxic-conditions. Before material transferred into high temperature resistance furnace for pre-oxidation, carbonization and activation treatment, the material (mostly less than <2 cm in size) is washed with HCL plus water solution for a few hours and then is baked in a vacuum oven at about 65-105 °C for 12 h depending on the type of material (Li et al., 2018). After pyrolysis, major changes can be found in the material. Finally, the obtained product is immersed in a 1 M hydrochloric acid solution for 2 h to remove impurities, such as ash, from the product, it is washed with deionized water until at a considerable pH level, and then it is baked in an oven (105 °C) for 1-2 h, until the product is ready. It can be further divided into slow-pyrolysis, rapid-pyrolysis, and flash-pyrolysis which are based on temperature, time, and the heating rate of pyrolysis process. Slow-pyrolysis is considered as a main preparation of biochar (Shaheen et al, 2018; Li et al., 2018; Patra et al., 2017; Kambo and Dutta, 2015).

Another method for the preparation of biochar is hydrothermal-carbonization. From studies (Berge et al., 2015; Tan et al., 2015; Kambo and Dutta, 2015) the hydrothermal-carbonization is a process under heating conditions and high pressure, which uses water as the reaction medium. In addition, the reaction of biomass is undertaken in a system (an underwater stagnant system), which has a relatively low temperature (<350 °C) and pressure of 2–6 MPa for 5 min to 16 h. It was concluded in the study (Breulmann et al., 2017), that pyrolysis is more effective than hydrothermal-carbonization. The capacity of cat-ion exchange for sewage sludge was successfully reduced by hydrothermal-carbonization but stayed on a higher-level than after pyrolysis, because it failed to increase the chars' resin-extractable phosphorus contents. In addition, slow pyrolysis is recommended as the comparison was studied, chars were made from same feedstock material (corn, C4) while using hydrothermal-carbonization and slow pyrolysis. Results showed differed chemical properties and decomposition behavior, even different physical appearance. Although both chars were produced from the same feedstock, results by pyrolysis had a higher potential for carbon sequestration than hydrothermal-carbonization. In addition, this method is limited by the preparation conditions; there is a high preparation cost, and a high need for high pressure and high temperature of the expensive reactor (Berge, 2015; Tan et al., 2015; Kambo and Dutta, 2015; Malghani et al., 2013).

Other methods are mainly used to generate gaseous materials or bio-oil, such as gasification, drying, rapid pyrolysis, and "flash" pyrolysis (Tan et al., 2015). Different techniques to prepare biochar require different temperature and time, it is shown in *Table 1.*

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DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

Table 1. *Different techniques to make biochar. (Modified from Tan et al., 2015; Oliveira et al., 2013; Deng et al., 2017)*

Main products	Material size	Mode	Temp °C	Heating rate	Residence time
Oil, gas, char ~1/3 each	1-200 mm	Slow pyrolysis	400-650	Slow 1-20 °C/m	Minutes to days
Bio-oil, 75% Char, 10-20%	<1 mm	Fast pyrolysis	700	Very fast >300 °C/s	Second
Gas, 80% Char, 10-20%	5-20 mm	Gasification	>800	2-100 °C/m	5-30 ins

Characterization of media

Biochar prepared from different biomass, has different physic-chemical properties: the size of particle of the feedstock, the temperature and pyrolysis, the time of pyrolysis, and the conditions of modification. It is recommended to know the characterization of media so one can recognize if it is prepared well or not. Characterizing the biochar is mostly done by scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM/EDX) (Li et al., 2018; Tan et al., 2015). Many factors affect the structure of biochar; in addition, biochar has an abundant surface of functional groups (carbonyl, carboxyl, hydroxyl and methyl). In addition, the high specific surface area, the natural and developed pore structures, and the stable molecular structure, allowbiochar to have good adsorption performance. Hence several studies have concluded that biochar is favorable for waste water treatment (Tan et al., 2015; Li et al., 2018; Berge, 2015; Tan et al., 2015; Kambo and Dutta, 2015; Malghani et al., 2013).

Biochar from different sorbent materials

Different types of sorbent materials were studied and used in the previous studies because media influences numerous processes. Media controls the retention time, rate of water infiltration, provides surface biofilms, provides sorption surfaces so nutrients can absorb heavy metals, filters sediments and particulates, and provides a nutrient source for macrophytes and microbes (bacteria, fungi, protozoan, algae) (Greenway, 2008). Recently, the following sorbent materials are being used: hydrous zirconium oxide (Kumar et al., 2018), clay minerals (Uddin, 2017), brick dust (Allahdin et al., 2017), activated alumina (Millar et al., 2017), nano-materials (Yazdani et al., 2017; Gunti et al., 2018), hematite and feldspar (Yazdani et al., 2017), Zeolite (Visa, 2016), activated carbon (Saleh et al., 2017; Shaheen et al., 2015), and limestone (Iakovleva et al., 2015). Five to ten years ago, coal fly ash (Hizal et al., 2013), Zeolite (Shaheen et al., 2012), and red mud (Gupta and Sharma, 2002) were used in removal of PTEs from waste-water. Though they are effective for water treatment, the most of these sorbents keep some demerits, such as low stability, production of waste products, difficulties in recyclability, high operational cost, use of hazardous chemicals in the synthesis of some sorbents and high operational cost (Gunti et al., 2018; Barakat, 2010). Several studies around the world have different media commonly used in constructed wetlands, which are given in *Table 2*. In addition, studies found that wood-based biochar is more effective with no demerits or less demerits.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 2. *Media commonly used in constructed wetlands around the world*

Composition of media	Study area/location	References
• Combination of turf sand, Krasnozem and coir peat • Combination of turf sand, red mud and coir peat • Combination of turf sand, Water treatment residual and coir peat	Australia Logan, Queensland	Lucas and Greenway, 2011a
• Gravel • Wood mulch • Gravel and mulch	Australia Melbourne	Saeed and Sun, 2011
• Pinewood • Rice husk	China Beijing	Liu and Zhang, 2009
• Wood	China Beijing	Sun et al., 2011
• Cinder 25%, rubble 25% and furnace slag 50%	China Guangzhou	Cui et al., 2015
• Gravel (diameter 10-40 mm) and (30-50 mm)	China Huazhong	Peng et al., 2014
• Macrophytearundodonax	China Shandong	Li et al., 2018
• Zeolite • Caremsite • Quartz granules	China Shanghai	Zhong et al., 2015
• Rice husk • Dairy manure	China Shanghai	Cao et al., 2009
• Corn straw	China Shanghai	Xu et al., 2013
• Luffa	China Qingdao	Zhai et al., 2017
• Gravel	China Wuhan	Chang et al., 2012
• Natural zeolite • Volcanic rocks	China Xiamen	Huang et al., 2013
• Gravel and sand	China Cuihua Xi'an	Wang et al., 2016
• Iron rich soil and gravel	Cuba	Perez et al., 2014
• Crushed rock • Sand	Czech Republic Trebon	Vymazal and Kropfelova, 2011
• Gravel	Egypt Giza	Abou-Elela et al., 2013
• Soil	Egypt Manzala Lake	El-Sheikh et al., 2010
• Light weight aggregate (LWA)	Estonia Paistu	Oovel et al., 2007
• Medium from quarry • Fine gravel from river sand	Greece	Akratos and Tsihrintzis, 2007
• Gravel • Coarse sand	Greece Buyukdolluk, Edirne	Cakir et al., 2015

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• Soil	Greece Pompia	Tsihrintzis et al., 2007
• Soil	Israel Kiryat	Ran et al., 2004
• Combination of marble and gravel • Combination of sand and gravel • Marble chip • Sand	India Nagpur	Kadaverugu et al., 2016
• Gravel and coarse sand	India Patancheru	Datta et al., 2016
• Gravel (diameter 8-16 mm) and river sand	Indonesia Bandung	Kurniadie, 2011
• Fine grain	Iran Isfahan	Haghshenas-Adarmanabadi et al., 2016
• Iraqi Luffa	Iraq	Saleh et al., 2014
• Alum sludge	Ireland Dublin	Babatunde et al., 2010
• Gravel • Sand and gravel	Italy Florence	Masi and Martinuzzi, 2007
• Gravel	Italy Trento	Foladori et al., 2015
• HumicGleyed Andosol	Japan Mito	Abe et al., 2014
• peanut straw • canola straw	Korea	Tong et al., 2011
• Palm kernel shell	Malaysia Sarawak	Jong and Tang, 2015
• Tezontle gravel	Mexico Ocotlan, Jalisco	Zurita et al., 2009
• Sand • Gravel	Nigeria Akure	Akinbile et al., 2016
• Carbonate-silica rock (opoka)	Poland Chrzanow	Jozwiakowski et al., 2017
• Pig manure	Poland Lublin	Kołodyńska et al., 2012
• Gravel	Singapore Nanyang	Zhang et al., 2012
• Sand • Grain • Fine gravel	Spain Barcelona	Avila et al., 2016
• Gravel • Lapilli	Spain Canary Islands	Melian et al., 2010
• Gravel	Spain Galicia, Boimorto	Jacome et al., 2016
• Granitic gravel	Spain Santiago of Compostela	Vazquez et al., 2013
• Gravel • Sand • Iron oxide	Spain Valencia	Martin et al., 2013a

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• Soil	Spain Valencia	Martin et al., 2013b
• Gravel	Srilanka Peradeniya	Weerakoon et al., 2016
• Fine gravel • Medium gravel	Thailand Bangkok	Konnerup et al., 2009
• Soil	Thailand Chiang Mai	Kantawanichkul and Duangjaisak, 2011
• Gravel	Tunisia Joogar	Kouki et al., 2009
• Soil	Turkey Garip	Gunes et al., 2012
• Gravel • Marble stone • Iron slag • Zeolite	Turkey Kocaeli	Ayaz et al., 2012
• Sand	Uganda Kampala	Bateganya et al., 2016
• Rice straw • Corn straw	USA	Chen et al., 2007
• River sand	Vietnam Cao Tho	Trang et al., 2010

Wood-based biochar

Up-to-now, Wood-based biochar has been recommended for the removal of potentially toxic elements in water and wastewater such as such as bioenergy crops (willows, miscanthus, and switchgrass) and forest residues (sawdust, grain crops, and nut shells). Biochar is generally produced from the pyrolysis of different low-cost starting materials. In recent studies (Shaheen et al., 2018; Li et al., 2018; Patra et al., 2017), biochars were originated from different wood feed-stock, i.e. luffa, water-melon, saw dust, pine cone, softwood, hardwood, and bark as they have major chemical and physical properties and their efficiency in removing potentially toxic elements in water. Studies (Shaheen et al., 2018; Li et al., 2018; Patra et al., 2017) demonstrated the biochar and discussed the (i) preparation, characterization and removal efficiency of wood-based biochar; as well as removal mechanisms of potentially toxic elements by wood-based biochar; therefore, it was concluded that wood-based biochar is effective for enhancement of wetlands systems. This particular study (Li et al., 2018) concluded that by-products from forests and agriculture may not be easily available. Furthermore, most of them are seasonal, but the “arundodonax” is a wetland plant which can be easily accessible anytime from many parts of the world. A study from Malaysia (Jong and Tang, 2015) used palm kernel shell and got promising results. Further studies are being conducted to find out the material for biochar which is easily available and effective.

Use of biochar in constructed wetlands for waste water treatment

Use of biochar and wetlands technology has received great interest because they are helpful to treat wastewater at low-cost. The untreated wastewater released in the environment increases the concentrations of suspended solids, nitrogen (N), phosphorus

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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(P), and organic matter (Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD)) in water bodies. These chemicals are easily treated by the combination of wetlands and its media (biochar).

Up to now, only a few studies have focused on integrating biochar into constructed wetlands when treating wastewaters. Combining both of these technologies can play a vital role in pollutant removal performance; when compared in the studies as shown in *Figure 1* (Li et al., 2018; Gupta et al., 2015), it was concluded that the wetlands with biochar were more efficient as compared to the wetland with gravels alone (Li et al., 2018; Gupta et al., 2015). Constructed wetlands (CWs) are an effective option to be applied around the world due to their lower cost. Plants and media are the main factors; they determine the effectiveness of the CWs in removing pollutants. Biochar is a potential media amendment for pollutant removal, which can be used in subsurface CWs (De Rozari, 2017).

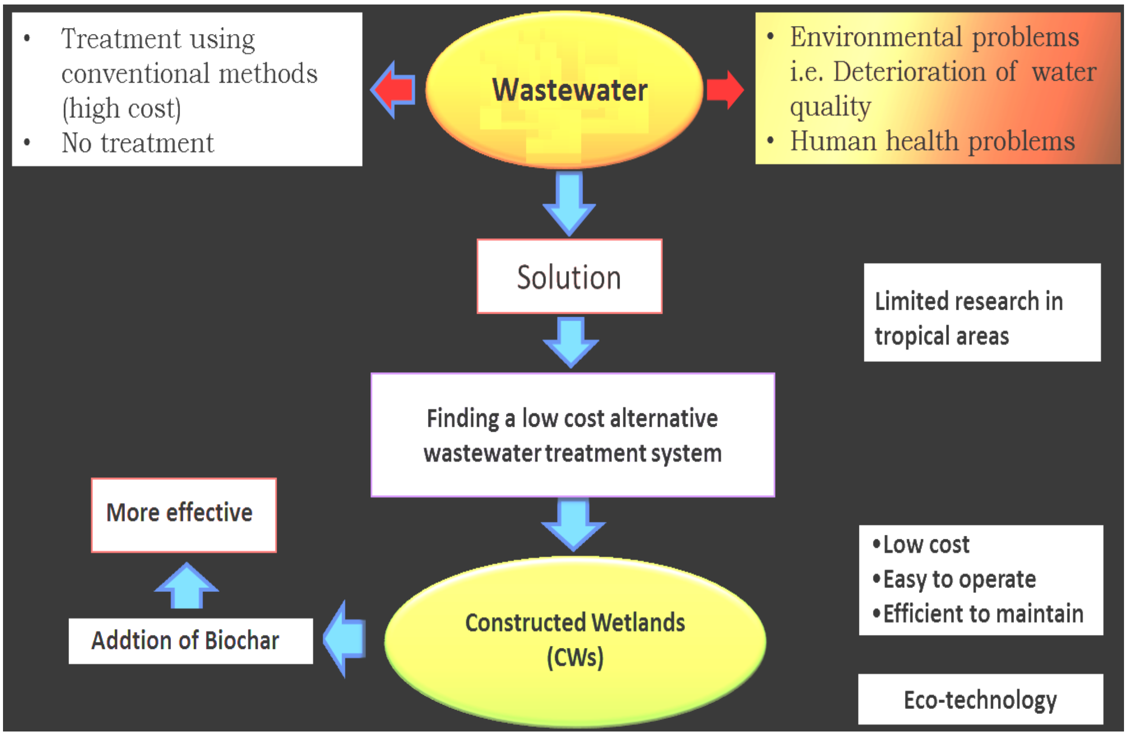

Figure 1. *Combination of constructed wetlands and biochar as an alternative and low-cost technology for wastewater treatment. (Modified from De Rozari, 2017)*

Effect of biochar on contaminants produced from different feedstocks

The removal of the pollutants by biochar depends on various factors such as adsorption, precipitation, filtration, sedimentation, microbial degradation, and plant uptake. The application of biochar produced from different feedstocks and techniques in aqueous solutions has been studied and it has been concluded that biochar isa more effective and better option. For Chromium, the biomass feedstock from Coconut coir goes under slow pyrolysis; the pyrolytic temperature ranges from 250 to 600 °C and has been effective (Shen et al., 2012). For Copper and zinc, the biomass feedstock from Corn straw under slow pyrolysis the pyrolytic temperature 600 °C has been effective (Chen and Chen, 2009; Chen et al., 2007). Comparison of rice husk-and dairy manure-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

derived biochars for simultaneously removing heavy metals (Pb, Cu, Zn, and Cd) from aqueous solutions was studied; biochar was formed from from rice husk and dairy manure under slow pyrolysis the pyrolytic temperature 350 °C (Xu et al., 2013). The biomass feedstock from Sugarcane bagasse was studied for removal of Sulfamethoxazole. Biochar was formed under slow pyrolysis; the pyrolytic temperature (°C) ranges 450 (Subhashini et al., 2013).

Recently, Arundo donax, a wetland plant was used to prepare biochar; biochar was prepared for enhancing the performance in SFCWs nitrogen removal. Studies demonstrated that biochar significantly promoted plant growth in SFCWs (Li et al., 2018). Gupta et al.'s study (2015) was conducted for the removal of phosphorus, nitrogen and organic matter. An oak tree (Quercus sp) was used as a porous media to enhance constructed wetland performances in wastewater reclamation. This study reveals that the wetlands with biochar were more efficient as compared to the wetland with gravels alone with average removal rate of 58.3% TN, 79.5% TP, 91.3% COD, 58.3% NH_3, 92% NO_3-N, and 67.7% PO_4 (Gupta et al., 2015). In SFCWs with 20% (v/v) biochar addition enhanced the average removal efficiencies of TN and NO_3^--N as 85.62% and 81.16%. In the study (Li et al., 2018), it was concluded that the introduction of biochar has played an effective role to strengthen N removal efficiency in SFCWs. As comparisons were made, higher TN removal efficiency was offered by biochar added in SFCWs (Li et al., 2018).

In addition, a study was conducted to examine the treatment of wastewater; an evaluation of the efficiency of twotypes of pyrolysis chars (rice husk biochar and refuse derived fuel char) was done. Results of the study show that the efficiency of biochar in treating wastewater is much better compared to char. Hence, biochar could be the best option as a sorbent for wastewater remediation; moreover, biochar has a strong adsorption effect for organic pollutants such as antibiotics, phenols, herbicides, etc. (Rasheed et al., 2017; Deng et al., 2017). Biochar also has an adsorption effect for pollutants in liquid phase. Tan's (2015) study summarized the applications of biochar for water pollutants adsorption, it was concluded that 39% adsorption of organic-pollutants, 46% adsorption treatment for heavy metal, 13% for the adsorption of phosphorus and nitrogen (Tan et al., 2015). Studies (Zheng et al., 2013) were conducted for biochar preparation from donax to adsorb sulfamethoxazole (hazardous material), an antibiotic which is used for curing acute and chronic urinary tract diseases caused by proteus escherichia coli. Furthermore, the raw material has some inorganic components, which weaken the adsorption capacity of sulfamethoxazole in the high-temperature pyrolysis biochar and enhanced the adsorption capacity of sulfamethoxazolein low-temperature pyrolysis bochar. It varies from material to material; De Rozari et al.'s (2016) was conducted for the preparation of the sand media amended with biochar to develop its effectiveness. Two plant species (Cymbopogoncitratus and Melaleuca quinquenervia) were planted to remove phosphorus from sewage effluent in CWs. Removal efficiencies of TP in the mesocosms loaded with SCW, and septage ranged from 42 to 91%; 30 to 83% were recorded. The results revealed that the sand media performed better than the biochar-amended media (De Rozari et al., 2016). Overall, the application of biochar to enhance water treatment has concluded that biochar produced from different feedstocks and techniques is a more effective and better option.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

Conclusion and recommendation

This review has focused on the effect of biochar in water treatment. A literature survey is conducted on the biochars production from the different feedstocks and pyrolyzed by different processes to reduce water pollution. Several researches confirmed that biochar is a good adsorbent with high efficiency and it has enhanced the efficiency of constructed wetlands for water treatment.

Biochar is efficient, but after the adsorption of heavy metal or organic pollutants, it may lead to secondary pollution if it is not properly treated. There is little research done on biochar recycling or regeneration after adsorption of pollutants. However, more research is needed to understand feedstock material for biochar, preparation parameters, biochar pore structures and the relationship between biochar adsorption performances. Studies for preparation biochar from different materials are recommended as future avenues for research. Experimental and modeling studies need to explore further in depth, especially on heavy metals, nitrogen and phosphorus adsorption, and whether biochar can be used as bio-fertilizers or soil conditioners after adsorption of nitrogen and phosphorus.

Acknowledgements. This research was supported by the Demonstration of Integrated Management of Rocky Desertification and Enhancement of Ecological Service Function in Karst Peak-cluster Depression (grant no.:2016YFC0502400).

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DOI: http://dx.doi.org/10.15666/aeer/1702_38033818

REGIONAL FLOOD FREQUENCY ANALYSIS USING LINEAR MOMENTS AND PARTIAL LINEAR MOMENTS: A CASE STUDY

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(Received 7th Jan 2019; accepted 22nd Feb 2019)

Abstract. One of the major concerns in Flood Frequency Analysis (FFA) is to predict floods of high magnitude for larger return periods. Magnitudes of smaller floods behave as nuisance in the estimation of larger floods. In this study, to avoid the unnecessary effect due to smaller observations, we implemented the technique of left censoring. The primary objective of this study is to see the efficacy of censoring, by comparing Regional Flood Frequency Analysis (RFFA) using Partial Linear Moments (PLM) for censored samples with RFFA using Linear Moments (LM) for uncensored samples of annual peak flows observed at ten stations of Indus Basin in Pakistan. After fulfillment of fundamental assumptions of randomness, independence, homogeneity, and stationarity, a Grubbs-Beck (GB) test for outlier detection is applied to the samples from all stations. For further analysis, Discordancy measure shows that none of the site is discordant and all ten stations would be retained for further investigation. On the basis of geographical closeness, stream hydrology and morphology of Indus basin, a single homogenous region is proposed and testified by the heterogeneity standards. The best fit distribution is selected by implying the Z-statistic (goodness of fit test base on LM) and L-Moments Ratio Diagram (LMRD). Generalized Pareto Distribution (GPA) distribution under PLM while Generalized Normal Distribution (GNO) under LM is selected as the reasonable choice for design flood estimation. Monte Carlo simulation experiment is performed to check the efficacy of PLM over LM through Root Means Square Error (RMSE) and bias. These accuracy measures indicated the outperformance of censored samples under PLM to uncensored samples under LM.
Keywords: *discordancy, heterogeneity, design flood estimation, censored samples, uncensored samples*

Abbreviations: Regional Flood Frequency Analysis (RFFA); Flood Frequency Analysis (FFA); Partial Linear Moments (PLM); Linear Moments (LM); Grubbs-Beck (GB); Generalized Pareto Distribution (GPA); Generalized Normal Distribution (GNO); Generalize Logistic (GLO); Generalize Extreme Value (GEV); Generalize Pearson 3 Type (PE3); Root Means Square Error (RMSE); Pakistan Meteorological Department (PMD); Partial Probability Weighted Moments (PPWM).; Probability Weighted Moments (PWM); Annual Peak Flows (APF); Index Flood Procedure (IFP); Censored (C); Uncensored (UC); L-Moments Ratio Diagram (LMRD); Partial L-Moments Ratio Diagram (PLMRD)

Introduction

Changing climate around the globe is the major challenge of today's world. Global warming leads to many changes in climatic conditions, which ultimately induce to changes in the intensity, spatial extent, frequency, duration and timing of extreme weather and climate events such as bolstering heat waves, lengthening droughts and causing more precipitation and flooding. In Pakistan, climate change has also resulted in extreme

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

weather conditions such as torrential rainfalls, irregular floods, droughts, glacier melting, sea-level rising, etc. (Rasul et al., 2012). Currently, Pakistan is among the top ten countries that are vulnerable to the adverse effects of climate change. Pakistan Meteorological Department (PMD) reported that due to an abrupt change in climate during the past three decades a 100 kilometres spatial shift towards the west as well as the seasonal shift in the overall monsoon distribution pattern is observed in Pakistan. For example, the summer monsoon has moved towards the end of the season, and the winter rains have also driven towards late February and March (Ali, 2013).

In Pakistan, monsoon season is one of the major causes of heavy floods. Monsoon rains and floods usually create havoc in different parts of the country leaving thousands of families affected, a large number of houses destroyed, thousands of acres of ready to harvest crops and fruits orchards damaged, link roads and bridges destroyed. The resulting consequences of floods could be mitigated by improving water resources management. This goal could be achieved by collecting accurate information about the estimation of floods magnitude and frequency of occurrence, which ultimately would be helpful in designing, planning, and management of different hydrological structures such as water reservoirs, spillways, headworks, dams, culverts, bridges and other hydrological related structures. Water resources management could be strengthened by exploring flood frequency analysis and making headworks and stations attuned to water flows (Zin et al., 2009).

To improve above mentioned perspectives, there are two approaches of frequency analysis commonly used in hydrology. First one refers to at-site flood frequency analysis while the second one as regional flood frequency analysis. In at-site frequency analysis, we analyze the results of each site or station individually instead of grouping the information from all sites (Zin et al., 2009; Seckin et al., 2011; Ahmad et al., 2015, 2016a; Afreen and Muhammad, 2012; Rahman et al., 2013; Asikoglu, 2018). At-site frequency analysis is not a promising technique in the presence of small data and non-availability of data at ungauged stations. Due to data scarcity and sampling variability, RFFA is considered as more reliable technique providing information by using multiple sites and grouping them on the basis of similarity of their hydrological and geographical characteristics. RFFA based on Index Flood Procedure (IFP) is more helping and practical technique in providing design flood estimates for different sites especially in the presence of small data or no data at some sites in the region (Yue and Wang, 2014).

This method, with probability weighted moments (PWM) scheme has shown more promising results in the homogeneous regions. PWM was initially introduced by (Greenwood et al., 1979) and further, the L-moments technique was proposed by Hosking (1990). In design flood estimation, our primary goal is the evaluation of larger events on right tail of the frequency distribution. In this situation, practically small flood events are of little relevance and sometimes put unnecessary influence in design flood estimation of larger events. This problem can be tackled with the help of left censoring. We can discard the smaller flood events using some criterion to get rid of the unnecessary effects which they put on the estimation of larger flood events.

Under this motivation, we employed PLM method for censored samples (the samples in which some small values have been discarded) in characterizing the larger events for better water resources management. PLM is an extension of LM and is based on Partial Probability Weighted Moments (PPWM). With the help of PLM, we could mitigate the undesirable influences of small events which they leave on the estimation of higher return period events. RFFA based on method of LM had been successfully applied in many

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

countries across the world, For example: in South Africa (Kjeldsen et al., 2002), Malaysia (Mohd Baki et al., 2015), Turkey (Saf, 2009, 2010), Australia (Zaman et al., 2012; Eslamian, 2014), China (Zhang et al., 2015; Du et al., 2014; Yang et al., 2010a, b), Italy (Noto and La Loggia, 2009; Caporlai et al., 2018), Pakistan (Hussain, 2011; Ahmad et al., 2017, 2016b), Tunisia (Abida and Ellouze, 2007), Canada (Glaves and Waylen, 1997); UK (Fowler and Kilsby, 2003), India (Ngongondo et al., 2011; Bora and Borah, 2017), Iran (Khanmohammadi et al., 2018) and Korea (Sung et al., 2018; Kar et al., 2017).

The initial work on PPWM was proposed by Wang (1996) on censored flood samples by fitting Generalized Extreme Value (GEV) distribution. The idea of PPWM is based on the extension of PWM as by Wang (1990, 1996) and Hosking (1990). Partial L-moments could be derived from PPWM on the same lines as L-moments from PWM. Bhattarai (2004), discussed the matter of various censoring levels and revealed that Partial L moments and L-moments show similar properties up to 30% censoring of the flood samples. Moisello (2007) compared PPWM and PWM and found that in case of unknown parent distribution, PPWM is better than PWM. Kochanek et al. (2008) used PPWM for log Gumble distribution censoring the largest observation in the sample. In Malaysia, some work has been conducted on PLM; see for example (Zakaria et al., 2011, 2012, 2017; Zakaria and Shabri, 2013). In recent years, Khan et al. (2017) used the method of LM and PLM for quantile estimation of extreme precipitation in Pakistan. In this study, we analyzed and compared the performance of the PLM method for censored with method of LM for uncensored samples implementing RFFA of annual peak flows of ten stations on Indus basin in Pakistan. The primary objectives of the study is to see the efficacy of censoring and to compare RFFA under PLM and LM using data of annual peak flows observed at ten stations of Indus Basin in Pakistan. Secondary objective of the study is to estimate the quantiles of upper tail of the distribution and get knowledge about the larger return periods, which would be helpful in designing, planning, and management of different hydrological structures such as water reservoirs, spillways, headworks, dams, culverts, bridges and other hydrological related structures.

Materials and methods

Study area

Irrigation and hydrological structure of Pakistan is world's remarkable irrigation system. Pakistan’s hydrological structure is mainly stretched under the umbrella of Indus basin that comprises of three main reservoirs namely Chashma, Mangla, and Tarbela, 19 stations, 12 linking canals, about 56,000 km of canals, and 110,000 km of watercourses. The climate of the Indus Basin plains varies from semi-arid to arid with temperature ranges from 2 to 49 °C. The Indus River is one of the main transboundary rivers in Asia with nine tributaries (rivers). Among these five tributaries lie on its left bank that are Beas, Chenab, Jhelum, Ravi, and Sutlej rivers. Among these five tributaries, three tributaries as Beas, Ravi, and Sutlej are also transboundary rivers having upper catchments in India. On the other side, right bank tributaries are the Gomal, Kabul, Swat and Kurram rivers. According to the Indus Water Treaty (1960) between Pakistan and India, the water from three rivers (Beas, Sutlej and Ravi) has been allocated to India while of other three rivers (Jhelum, Chenab and Indus) to Pakistan. Based on its morphology and stream hydrology, the Indus River can be divided into three segments: (i) the upstream segment (ii) the midstream segment, (iii) and the downstream segment. The upstream segment ranges from the Singi Khahad spring down to Jinnah Barrage being a

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

hilly catchment area. The midstream segment, prolongs from Jinnah to Guddu stations being an upper floodplains area dominated by an intervening pattern of channels and river inflows. The last one, the downstream segment, ranges from Guddu Barrage to the Arabian Sea, is a lower flood plains area and has a flat topography, a meandering channel pattern and deltas (Ali, 2013). These ten stations are located on the three rivers, the Jhelum, Chenab and Ravi (mid segment) and Indus River/Sindh (lower segment). Stations Location, latitude, longitude, elevation, length and drainage area are given in *Table 1* and geographical locations are depicted in *Figure 1*.

Basic assumptions of the data

First of all, we will check the basic assumptions important for a hydrological analysis such as, homogeneity, randomness/independence and stationarity for the data from all stations.

Assumption of homogeneity

The term homogeneity refers to the notion that all elements of a sample belonging to a single common population. Because the sample of flood data may arise in the result of ordinary rainfall or extraordinary rainfall with high intensities and volumes resulting from extreme hydrometeorological conditions. In these scenarios, we may face non-homogeneous sample of flood data. To check the assumption of homogeneity we apply Mann and Whitney test.

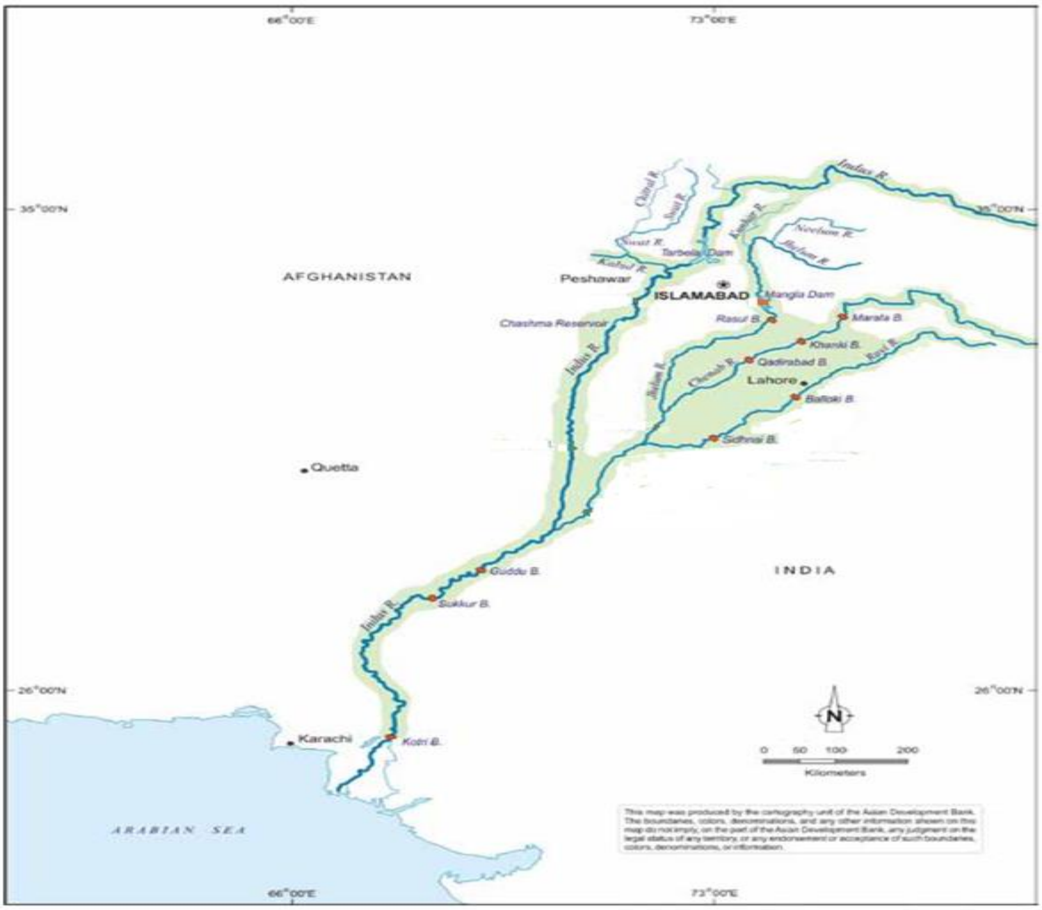
Assumption of independence and randomness

In frequency analysis, the idea of independence postulates that none of the peak flow in the sample will affect the occurrence or non-occurrence of any other value in the same sample. Randomness implies that each element of the sample is independently drawn at random from the population with equal probability. In this study we apply, Wald and Wolfowitz test to check the independence and randomness assumption for the observed data from all stations. The same test has been used in different studies for the same purpose see for example Naghettini (2017) and Hussain (2011).

Table 1. *Basic information of different stations in the study*

Rivers names	Site name	Latitude (degrees north)	Longitude (degrees east)	Altitude	Length of the basin (feet)	Drainage area (km^2)
Jhelum	Mangla	33.15	73.65	774	10,300	20712
	Rasul	32.68	73.5	699	3209	24069
Chenab	Marala	32.68	74.43	702	4472	31148
	Khanki	32.4	73.2	666	4000	29732
	Qadirabad	32.33	73.73	639	3373	2585
Ravi	Balloki	31.22	73.86	600	1644	63712
	Sidhnai	30.58	72.07	426	712	4709
Sindh	Kotri	25.22	68.22	55	3000	25485
	Guddu	28.3	69.5	222	3840	25485
	Sukkur	27.72	68.79	201	4490	42475

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836



Source: Author, modified from an existing map of Pakistan.

Figure 1. *Geographical location of different stations in the study*

Assumption of stationarity

In general, stationarity of hydrological variable implies the invariance over time of some statistical properties of a hydrological variable such as its probability distribution and related parameters (Naghettini, 2017). Usually, we face monotonic trend in hydrology. For measurement of this kind of trend, we can use Spearman's rank correlation coefficient test. It is a nonparametric procedure to test the statistical dependence between two variables. Statistical dependence is not restricted to be linear as in case of simple correlation coefficient test. The basic notion behind this test is that the trend hidden in a flood data evolving over time can be measured with the help degree of association or correlation between ranking orders "m_t" of original series and corresponding time indices T_t.

Outliers detection and Grubbs-Beck (GB) test

In frequency analysis of extreme events like droughts, floods and others, it is very common to have some observation below some threshold value. These values are of little interest, especially, when your interest is in modeling the right tail of the distribution, i.e. estimating the higher return periods of extreme events. In a given sample, a value is treated as an outlier if it shows a significant departure from the overall tendency of other sample values due to any reason. The reason might be measurement error or processing or some other. In any case, these outliers may severely influence parameters and quantiles

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

estimation and in general fitting process of the probability distribution. Once an outlier is detected in the sample, the decision of its removal or not removal needs further investigation. If its removal is conclusive, then it must be censored or removed from the sample. These observations are nuisance in the frequency analysis and problematic for the validity of the end results. In such situation, it is good practice to censor these observations in order to understand the process in a better way. In frequency analysis of annual peak flows or rainfall, the removal of an outlier from the lower side (left censoring) is justified because our main concern is an estimation of upper tail quantile or high return period's events. However, if some outlier is detected from the upper side, it should not be expunged from the sample because it might be due to some natural causes or extraordinary hydrometeorological conditions and helpful in estimating larger return periods events (Naghettini, 2017). Among other tests for outlier detection in statistical hydrology, the Grubbs- Beck (GB) test is the most frequently used test. This test defines two bounds, X_U upper bound and X_L lower bound, as given in *Equations 1* and *2*. Any value below or above these bounds would be treated as an outlier in the ranked sample values, where

$$X_U = \exp\left(\overline{x^*} + K_{n,\alpha} S_X^*\right) \quad \text{(Eq.1)}$$

$$X_L = \exp\left(\overline{x^*} - K_{n,\alpha} S_X^*\right), \quad \text{(Eq.2)}$$

where, $\overline{x^*}$ and S_X^* are the mean and standard deviation of logarithms of original data of annual peak flows. In *Equations 1* and *2,* $K_{n,\alpha}$ shows the critical values of the GB test statistics. The critical values for GB test statistic at 5% level of significance with sample size (n), ranges from 10 to 120, could be found as in *Equation 3*.

$$K_{n,\alpha=0.05} = -5.2269 + 8.768n^{0.025} - 3.8063n^{0.5} + 0.8011\,n^{0.75} - 0.0656n, \quad \text{(Eq.3)}$$

Methods of estimation

Method of linear moments (L-moments)

PWM primarily was defined by Greenwood et al. (1979), and later LM (linear moments) by Hosking (1990). The PWM of order r was defined by Greenwood et al. (1979) as *Equation 4:*

$$\beta_r = \int_0^1 x(F) F^r \, dF, \quad \text{(Eq.4)}$$

where $x(F)$ is quantile function and $F = F(\mathrm{x})$ is the cumulative distribution function. The first four L moments as a linear combination of PWM's are described below:

$\lambda_1 = \beta_0$ (First L-moment as measure of central tendency)

$\lambda_2 = 2\beta_1 - \beta_0$ (Second L-moment as measure of dispersion)

$\lambda_3 = 6\beta_2 - 6\beta_1 + \beta_0$ (Third L-moment as measure of skewness)

$\lambda_4 = 20\beta_3 - 30\beta_2 + 12\beta_1 - \beta_0$ (Fourth L-moment as measure of kurtosis)

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

$\tau_2 = \frac{\lambda_2}{\lambda_1}$ (Measure of coefficient of variation based on L-moments)

$\tau_3 = \frac{\lambda_3}{\lambda_2}$ (Measure of coefficient of skewness based on L-moments)

$\tau_4 = \frac{\lambda_4}{\lambda_2}$ (Measure of coefficient of kurtosis based on L-moments)

In practice, for $r = 0,1,2,3\ldots.n,$ LM could be estimated from sample PWMs by *Equation 5:*

$$b_r = \frac{1}{n}\sum_{i=r+1}^{n}\frac{(i-1)(i-2)\ldots\ldots(i-r)}{(n-1)(n-2)\ldots\ldots(n-r)}x_{i:n} \quad \text{(Eq.5)}$$

The relationship of both sample LM and PWM is determined by *Equation 6:*

$$l_{r+1} = \sum_{k=0}^{r} p_{r,k}^{*} b_k \, r = 0,1,2,\ldots\ldots\ldots(n-1). \quad \text{(Eq. 6)}$$

For $r = 0,1,2,3\ldots.n,$ we obtain:

$$l_1 = b_0$$

$$l_2 = 2b_1 - b_0$$

$$l_3 = 6b_2 - 6b_1 + b_0$$

$$l_4 = 20b_3 - 30b_2 + 12b_1 - b_0$$

Sample version of coefficient of variation, skewness and kurtosis based on LM can be defined by replacing the population values with sample estimates of LM; denoted by $t, t_3, and\ t_4$ respectively.

Method of partial linear moments (PLM)

Wang (1996) extended the idea of probability weighted moments (PWM) and defined PPWM for censored samples as *Equation 7:*

$$\beta_r' = \frac{1}{1-F_0^{r+1}}\int_{F_0}^{1} x(F)F^r\,dF, \quad \text{(Eq.7)}$$

where F_0 is varying point and could be 10%, 20% or 30%; it is also called censoring threshold. Note that, if $F_0 = 0$, the partial L moments becomes simple L moments. The unbiased estimates of β_r' can be defined as *Equation 8:*

$$b_r' = \frac{1}{(1-F_0^{r+1})n}\sum_{i=1}^{n}\frac{(i-1)(i-2)\ldots\ldots\ldots(i-r+1)(i-r)}{(n-1)(n-2)\ldots\ldots(n-r+1)(n-r)}x_{(i)}^{*}, \quad \text{(Eq.8)}$$

where:

$$x_{(i)}^{*} = 0 \quad \text{for}\ x_{(i)} \le x_0$$

and

$$x_{(i)}^{*} = x_{(i)} \quad for\ x_{(i)} > x_0$$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The censoring level tells about the number of sample values to be censored as $F_0 = \frac{n_0}{n}$; where n_0 and n are the number of censored values determined through GB test) and the length of the complete sample respectively. The first four PLM are parallel to first four LMs by replacing PWM by PPWM. In this study, they are denoted by $\xi_1, \xi_2, \xi_3, \xi_4$.

The relationships for "ς_2" (Coefficient of variation based on partial L moments), "ς_3" (Coefficient of skewness based on partial L moments) and "ς_4" (Coefficient of kurtosis based on partial L moments) are defined as *Equation 9:*

$$\varsigma_2 = \frac{\xi_2}{\xi_1},\ \varsigma_3 = \frac{\xi_3}{\xi_2} \text{ and } \varsigma_4 = \frac{\xi_4}{\xi_2} \quad \text{(Eq.9)}$$

Sample version of coefficient of variation, skewness and kurtosis based on PLM can be defined by replacing the population values of PPWMs by their sample counterparts and denoted by $t^{/}, t_3^{/}, and\ t_4^{/}$.

Regional flood frequency analysis (RFFA) using LM and PLM

Hosking and Wallis (1997) provided a comprehensive RFFA procedure using linear moments theory. It consists of mainly four steps.

Data screening

For RFFA, the first step is the initial screening of data. The sites with a trend and showing some gross error are excluded from the rest of sites. Hosking and Wallis (2005) suggested a discordancy measure D_i using LM to distinguish those sites which are discordant from other sites. For $i = 1, \ldots, n$, D_i is given by

$$D_i = \frac{1}{3}(u_i - \bar{u})^T S^{-1}(u_i - \bar{u}), \quad \text{(Eq.10)}$$

where $S = \frac{1}{N-1}\sum_{i=1}^{N}(u_i - \bar{u})(u_i - \bar{u})^T$ is a matrix of sum of squares and cross products. Note that $u_i = [t^{(i)}, t_3^{(i)}, t_4^{(i)}]$ is a vector consisting sample LM ratios, $\bar{u} = N^{-1}\sum_{i=1}^{N} u_i$, and N is the total number of sites

Hosking and Wallis (2005) provided threshold values for D_i. A site is treated as discordant site if calculated value of D_i exceeds the corresponding critical value. Critical values for a different number of sites are given in *Table 2*. We used the same criterion for detection of discordant sites for censored sample under PLM followed by Zakaria and Shabri (2013).

Table 2. *Critical values of discordancy measure for different number of sites*

Number of sites in the region	5	6	7	8	9	10
Critical values	1.333	1.648	1.917	2.140	2.329	2.491

Formation of homogeneous regions

A homogenous region can be formed using geographical convenience strategy, subjective approach, objective approach and cluster analysis for partitioning, e.g. K-means clusters etc. However, the formed region should be confirmed later by the heterogeneity tests. The latter test has been explained by Zakaria and Shabri (2013). In

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our study, we fit four parameter kappa distributions to $1, t, t_3^R, t_4^R$ and estimate the parameters of this distribution analytically. After 500 realizations of artificial regions considering ten sites, each site with the same number of values as real data, we calculate mean and standard deviation denoted by μ_V and σ_V respectively and calculate the heterogeneity statistic given by

$$\boldsymbol{H} = \frac{(V-\mu_V)}{\sigma_V}. \quad \text{(Eq.11)}$$

According to the heterogeneity criterion mentioned in *Equation 11,* if H< 1 the region is acceptably homogeneous. If H is in between 1 and 2, it can be possibly heterogeneous. However, when $H \geq 2$, the region is definitely heterogeneous.

Selection of regional frequency distribution

Selection of the best distribution is possible through different statistical tests and graphical inspections. In this study, we use Z-statistic based on LM and LMRD as suggested by (Zakaria and Shabri, 2013). Regional frequency studies showed that more than one distribution could be marked as best fit. However, the most suitable distribution will be that which also provide robust results. Hosking (1990) marked LMRD as the powerful tool to identify the distribution by the depicting ratio of sample regional skewness and kurtosis. In Z-statistic we assess the quality of fit by evaluating the difference between the L-kurtosis of fitted distribution τ_4^{Dist} and the regional average L-kurtosis τ_4^{R}. To know the significance of this difference, we compare the difference with the sampling variability of t_4^{R}. The statistics is calculated by using

$$Z^{Dist} = \frac{(\tau_4^{Dist} - t_4^R)}{\sigma_4}, \quad \text{(Eq.12)}$$

where σ_4, *is* the standard deviation of t_4^{R} obtained by repeated simulations of the homogeneous region fitted by Kappa distribution and using same record length of the sites as the observed data. More than one distribution can be selected using the criterion given in *Equation 12*. Best fit is claimed on the basis of the lowest value of Z^{Dist}. In literature, $|Z^{Dist}| \leq 1.64$ is the criterion at 90% confidence level. In case of more than one candidature fulfill this criterion, best fit will be declared that one with Z^{Dist} near to zero or equal to zero.

Estimation of selected frequency distribution

In this step, we will estimate the parameters of best fit frequency distribution and regional quantiles (regional growth curve) corresponding to different probabilities of non-exceedance useful in water resources management. Index Flood Procedure (IFP) is a prevailing method to estimate at site quantiles (quantile of every site in the homogeneous region). For a given station, the quantile estimates could be obtained by multiplying the sample estimate of index flood estimate/scaling factor μ_i (which may be an estimate of central tendency of the specific site) with regional quantile function $q(.)$. The latter being a dimensionless quantity is common to all sites in the homogeneous region. The relationship can be defined by *Equation 13:*

$$\hat{Q}_i(F) = l_1^{(i)} \hat{q}(F),\ i = 1,2,3, \dots, N, \quad \text{(Eq.13)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

where $\hat{Q}_i(F)$ refers to at-site quantile function and $l_1^{(i)}$ is the estimate of μ_i. Results obtained from the statistical analysis may contain some inherent errors and hence are uncertain. To make these results more practical and useful, we must assess the uncertainty of these estimates. One of the reasonable choices to assess this uncertainty is Monte Carlo simulation. A simulation algorithm used in this study is known as Regional L-moments algorithm proposed by Hosking and Wallis (2005). For an i^{th} site at m^{th} replication, $\hat{Q}_i^{[m]}(F)$ is quantile estimate for that site while $Q_i(F)$ is the simulated value and F be the probability of non-exceedance. The relative error can be estimated by $\frac{\{\hat{Q}_i^{[m]}(F)-Q_i(F)\}}{Q_i(F)}$. Further, this quantity could be averaged on the total number of M repetitions to derive relative BIAS and relative RMSE respectively given by

$$B_i(F) = \frac{1}{M}\sum_{m=1}^{M}\frac{\{\hat{Q}_i^{[m]}(F)-Q_i(F)\}}{Q_i(F)}, \quad \text{(Eq. 14)}$$

and

$$R_i(F) = [\frac{1}{M}\sum_{m=1}^{M}\{\frac{\{\hat{Q}_i^{[m]}(F)-Q_i(F)\}}{Q_i(F)}\}^2]^{\frac{1}{2}}. \quad \text{(Eq.15)}$$

A summary statistic for accuracy assessment of all sites in the homogeneous region is calculated by regional average relative BIAS and RMSE respectively given by *Equation 16:*

$$B^R(F) = \frac{1}{N}\sum_{i=1}^{N}B_i(F), \quad \text{(Eq.16)}$$

and *Equation 17:*

$$R^R(F) = \frac{1}{N}\sum_{i=1}^{N}R_i(F). \quad \text{(Eq.17)}$$

It is worth noting that the above assessment measures as given in *Equations 14* and *15* deal with the accuracy of quantile estimates of different sites in the region. When our concern is to get the accurate assessment of regional quantile estimates/ growth curve, the quantities $\hat{Q}_i^{[m]}(F)$ *and* $Q_i(F)$ could be replaced with $\hat{q}(F)$ and $q(F)$, respectively.

Results and discussion

Data characteristics

Taking into account the basin's geophysical and hydroclimatic characteristics, the central segment and downstream segments seem more important to assess the severity of floods. We have used Annual peak flows/Annual maximum flows data measured in cusecs (the single maximum value recorded in 24 hours for everyday of the year). This value is the maximum value among 365 values of a year. For every year we have only one value in the sample. In this study, ten stations are selected for RFFA, namely, Mangla (with record length of 87 years), Rasul (with record length of 93 years), Marala (with record length of 90 years), Khanki (with record length of 90 years), Qadirabad (with record length of 45 years), Balloki (with record length of 93 years), Sidhnai (with record length of 90 years), Kotri (with record length of 48 years), Guddu (with record

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

length of 30 years) and Sukhur (with record length of 30 years). The required data has been retrieved from Federal Flood Commission of Pakistan for research purpose. The smallest record length of 30 values for 30 years belongs to Guddu and Sukhur stations while the largest record length of 93 values for 93 years belongs to Balloki and Rasul stations. Some basic characteristics of the original sample without censoring are presented in *Table 3*.

Table 3. *Samples statistics with discordancy measure for uncensored (original) samples*

Site name	n record length of original sample (years)	Mean of original sample (Cusecs)	t (coefficient of variation)	t_3 (coefficient of skewness)	t_4 (coefficient of kurtosis)	D_i (discordancy measure)
Mangla	87	194352	0.4226	0.4544	0.3467	0.77
Rasul	93	202372	0.4551	0.4412	0.2920	1.75
Marala	90	317614	0.3576	0.3659	0.1581	0.79
Khanki	90	360726	0.3520	0.3651	0.1775	0.47
Qadirabad	45	373400	0.3764	0.2526	0.0890	1.58
Balloki	93	90667	0.3421	0.3810	0.2242	1.12
Sidhnai	90	66534	0.4039	0.3871	0.2133	0.42
Kotri	48	382169	0.4048	0.4094	0.3255	1.56
Guddu	30	542060	0.3272	0.2522	0.0284	0.87
Sukkur	30	491594	0.3389	0.2596	0.0743	0.67

Table 3 shows that the value of mean peak flows for Guddu station is maximum as compared to other stations. Coefficient of variation tells us about the relative variation in a data sets. Using the information provided in *Table 3,* it is obvious that there is more variation in the sample values of Rasul station. The station Guddu carries less variation in all of the ten stations. The most skewed and sharp peak sample belongs to Mangla station. On the other side, sample belongs to station Gudu is less skewed and carries small peak among all stations.

Results of the fundamental assumptions show that annual peak series from all sites fulfill the criteria of randomness, independence, homogeneity, and stationarity. All the probability values (P-Values) are less than 5% (level of significance), which implies that the hypotheses of randomness, independence and homogeneity are not rejected, and data are suitable for frequency analysis. Further, using Grubbs-Beck test, we determined the number of outliers need to discard from the lower side as well as from upper side. These numbers of observations are discarded from the original sample in order to avoid their unnecessary influence on design flood estimates for higher return periods using PLM. *Table 4* shows that none of the observation is declared as outlier from upper side. Different number of observation are discarded from lower side for different stations from the original sample in order to avoid their unnecessary influence on design flood estimates for higher return periods using PLM. The Grubbs-Beck test shows that the maximum number of observation discarded from Rasul station are nine (10%) while no observation is discarded from Sukhur sample. Numbers of observations smaller than lower limit given by Grubbs-Beck test are treated as censored observations. Although in our study, the numbers of censored values are not too large, but their presence may pertain an unnecessary effect on design flood estimation. To retain larger observations

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

in the sample is also justified because it might be due to natural causes or extraordinary hydrometeorological conditions and are also helpful in estimating high return periods (Naghettini, 2017). Results are given in *Table 4*.

Table 4. *Results of fundamental assumptions and outlier detection using origional sample*

Site name	Wald-Wolfowitz test		Mann-Whitney test		Spearman rank correlation test		Grubbs-Beck test		
	Statistic	P-value	Statistic	P-value	Statistic	P-value	n (original sample)	Lower outliers	Upper outliers
Mangla	1.081	01398	-0.8918	0.1863	-0.6697	0.2515	87	5	0
Rasul	0.7942	0.2135	-0.5185	0.3021	1.5539	0.0601	93	9	0
Marala	0.7942	0.2135	-0.5185	0.3021	1.5539	0.0601	90	3	0
Khanki	0.8008	0.2116	-0.8918	0.1863	0.5451	0.2928	90	6	0
Qadirabad	07286	02331	-0.8503	0.1976	-0.1569	0.4376	45	2	0
Balloki	0.6079	0.2716	-0.8503	0.1976	-1.0171	0.1545	93	8	0
Sidhnai	0.7925	0.2140	-2.1776	0.0147	2.0187	0.0618	90	7	0
Kotri	1.4308	0.0762	-1.2236	0.1106	-0.3031	0.3909	48	5	0
Guddu	-1.4316	0.0761	-0.3111	0.3779	0.5571	0.2887	30	1	0
Sukkur	-0.4560	0.3242	-0.6014	0.2738	0.1881	0.4254	30	0	0

Regional flood frequency analysis

To know about the discordant site from the group of ten sites, we calculated discordancy measure given in *Equation 10.* Record length, mean, the coefficient of skewness, the coefficient of kurtosis along with discordancy measure for each station are presented for uncensored and censored samples. Results are presented in *Tables 3* and *5* respectively.

The maximum value of D_i is 1.75 for Rasul station, while for all other nine sites, D_i value is comparatively less. According to the criterion of *Table 1*, if the D_i value for a site is greater than 2.491, then that site will be declared as discordant. In present study, none of the sites is exceeding 2.491, none of the site is treated as discordant. Thus, all sites are considered suitable for further procedure. *Table 5* describes the results of discordancy measure and other basic statistics using PLM theory for censored data. *C* denotes discarded values from lower side while *n** is sample size after censoring.

A reduction in discordancy measure using the censored sample is obvious as compared original samples. The reason for this change is censoring of data. Our main focus is on the upper tail of the fitted distribution, so these discarded observations might be the nuisance for further analysis. Initially, a single homogenous region is formed and verified by the heterogeneity standards. Seven stations are located in Punjab province while the three in Sindh province. The stations named Sukhur, Guddu and Kotri constitute the lower segment of Indus River. Under morphology and stream hydrology of the Indus basin, it is justified to make one homogenous region from these ten sites. To verify the homogeneity of the region, the heterogeneity is calculated using LM and PLM (see *Table 6*).

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Table 5. *Samples statistics with discordancy measure for censored samples*

Site name	*n**	C (No. of censored values)	Mean (Cusecs)	t' (coefficient of variation)	t'_3 (coefficient of skewness)	t'_4 (coefficient of kurtosis)	D_1 (discordancy measure)
Mangla	82	5	199414	0.4138	0.4662	0.2070	0.72
Rasul	84	9	208367	0.4421	0.4522	0.1453	1.71
Marala	87	3	317834	0.3576	0.3659	0.0299	0.77
Khanki	84	6	362860	0.3515	0.3618	0.0297	0.43
Qadirabad	43	2	373398	0.3764	0.2526	0.0028	1.56
Balloki	85	8	92263	0.3333	0.3968	0.0910	1.10
Sidhnai	83	7	73269	0.3700	0.4001	0.1128	0.36
Kotri	43	5	382260	0.4048	0.4094	0.2231	1.54
Guddu	29	1	542290	0.3272	0.2522	0.0246	0.85
Sukkur	30	0	491594	0.3389	0.2596	0.0109	0.67

Table 6. *Heterogeneity measure for censored and uncensored samples.*

Heterogeneity measure	H
Uncensored samples	0.98
Censored samples	0.72

As *H* is based on the coefficient of variation and explicates more variation as compared to the coefficient of skewness and coefficient of Kurtosis. For uncensored sample using LM the value of $H = 0.98$ and for the censored sample using PLM the value of $H = 0.72$. Both measures present similar results but heterogeneity measure based on PLM is smaller showing some edge over LM method. It also implies that after discarding some observation from some sites, the heterogeneity measure of the region is likely to decrease. Hosking and Wallis (2005) preferred to use three parameters distributions like generalize Pareto (GPA), generalize logistic (GLO), generalize extreme value (GEV), generalize normal (GNO) and generalize Pearson 3 type (PE-III) based on L-moments. For PLM only GEV, GLO, and GPA are the distributions whose partial L moments are available (Zakaria and Shabri, 2013). The selection of the distribution is made by Z- statistic. According to the criterion, if the absolute value of Z-statistic is less than 1.64, the distribution will be declared as best one. But in case of more than one distribution satisfying this criterion, the closer the absolute value of Z-statistic to zero, better will be the distribution. The simulation procedure for the calculation of Z-statistic is explained in the previous section.

GNO, GPA, PE-III show that the Z-statistic for these distributions is less than the critical value. But the value of this statistic for GNO = 0.71 meets the criterion of the most appropriate distribution for uncensored samples while for GPA = -0.53 for censored samples. Results are presented in *Table 7*. The second best fit distributions using LM and PLM are GPA and GEV respectively.

PLMRD is comparable to LMRD. LMRD and PLMRD are delineated for the described region. Sample regional coefficients of L-skewness/PL-skewness and L-kurtosis/PL-kurtosis are used for LMRD/PLMRD. The ratio of regional sample coefficients of skewness and kurtosis based on LM fall contiguous to the GNO (*Fig. 2a*)

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

while using PLM the ratio falls near to GPA (see *Fig. 2b*). The results found with Z-statistic and ratio diagrams are similar.

Table 7. *Z-Statistic for best fit distribution for censored and uncensored samples*

Z-Statistic Value					
Method	**GNO**	**PE-III**	**GEV**	**GLO**	**GPA**
Uncensored sample	0.71	-1.76	2.14	3.04	-0.83
Censored sample	----	-----	2.29	3.15	-0.53

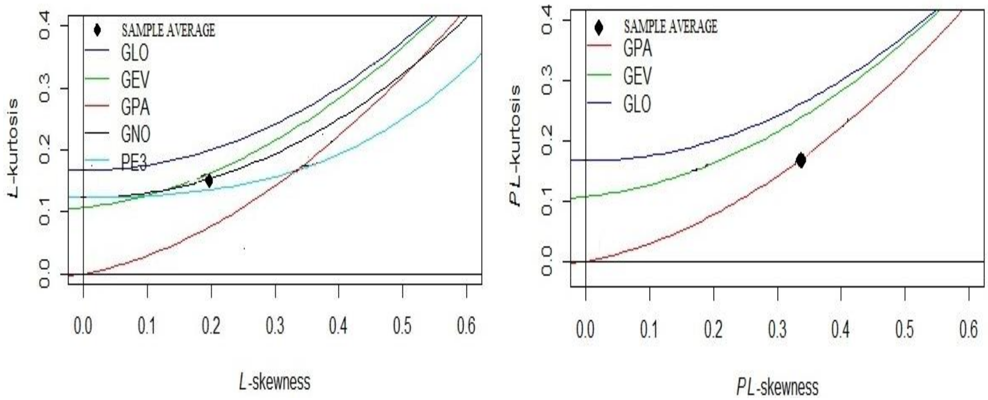

Figure 2. a *L-moments ratio diagram for uncensored data;* ***b*** *partial L-moments ratio diagram for censored samples*

The regional design flood estimates for the prescribed region corresponding to 2, 5, 10, 20, 50, 100 and 500 return periods (average recurrence period of flood events) using LM and PLM are illustrated in *Table 8*.

Table 8*. Quantile estimation for homogenous region for censored and uncensored samples*

Distribution	**Parameters**	**Quantile Estimates**
	b a k	Q_{10} Q_{20} Q_{50} Q_{100} Q_{200} Q_{500} Q_{800} Q_{900}
GNO (uncensored sample)	0.7540 0.5207 -0.8013	0.205 0.230 0.278 0.337 0.435 0.754 1.380
GPA (censored sample)	0.2894 0.6337 -0.1083	0.256 0.302 0.392 0.457 0.541 0.946 1.804

The Monte Carlo simulation procedure based on Regional L-moment algorithm (Hosking and Wallis, 2005) determines the accuracy of estimated quantiles/ growth curves. The repetitions in this process must be larger enough so that the RMSE and bias values are contiguous to the actual RMSE and bias. Therefore, 10000 repetitions are performed (see *Table 9*). Using PLM, Regional Average Relative RMSE and Bias values, i.e. $R^R(F)$ and $B^R(F)$ delivered by GPA under PLM are smaller than GNO under LM for all return periods. But the difference is more pronounced at larger return period events for example 100 years and 500 years.

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DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

The at most decrease in R^R using PLM as compared to LM up to 20 years return period is 0.9%. Similarly, at most decrease in R^R using PLM as compared to LM up to 500 years return period is 4.6%. The at most decrease in B^R using LM as compared to LM up to 20 years return period is 0.12%. Similarly, at most decrease in B^R using PLM as compared to LM upto 500 years return period is 0.22%. This decrease is more rapid and pronounced at higher return periods. It shows that PLM comparatively outperforms than LM especially at larger events. PLM is useful when our concern is higher return period's events and our target is long-term planning in water resources management. Unnecessary bias and errors due to smaller values of flows could be avoided using PLM. As the main objective of the RFFA is to estimate the quantiles for upper tail of the distribution and get knowledge about the larger return periods, PLM would be a more reasonable and effective choice for such type of design flood estimation. This study reinforces the use of PLM using censored samples. Our results support the outcomes of earlier studies (e.g., Bhattarai, 2004; Zakaria et al., 2011, 2012; Zakaria and Shabri, 2013; Khan et al., 2017) where PLM technique using censored samples outperformed than LM for large return periods estimations.

Table 9. *Accuracy assessment for different returns periods using selected distributions*

Method	**Dist.**	**F**	**0.100**	**0.500**	**0.800**	**0.900**	**0.950**	**0.980**	**0.990**	**0.998**
			1	**2**	**5**	**10**	**20**	**50**	**100**	**500**
Uncensored sample	GNO	$R^R(F)$	0.2470	0.0552	0.0319	0.0560	0.0760	0.1070	0.1330	0.2030
		$B^R(F)$	0.0455	0.0054	0.0017	0.0044	0.0016	0.0015	0.0047	0.0043
Censored sample	GPA	$R^R(F)$	0.2465	0.0551	0.0309	0.0540	0.0670	0.0890	0.1080	0.1570
		$B^R(F)$	0.0449	0.0053	0.0015	0.0032	0.0010	0.0011	0.0032	0.0021

Conclusions and recommendations

PLM and LM estimation methods were applied to annual peak flows data of ten stations located at Indus Basin, Pakistan. No station was appeared to be discordant. On the basis of geographical closeness, stream hydrology and basin morphology one homogenous region is formed and verified through the heterogeneity measure. Z-statistics and ratio diagrams depicted the best fit distributions for both methods. Using LM, GNO was more appropriate choice for the homogenous region while GPA under PLM. The accuracy of the estimated design flood estimates (regional quantiles/growth curves estimates) was assessed through, regional average RRMSE and bias, i.e. $R^R(F)$ *and* $B^R(F)$. The decrease in these measures was relatively more at higher return periods under PLM method. Our results support the outcomes of earlier studies (e.g., Bhattarai, 2004; Zakaria et al., 2011, 2012; Zakaria and Shabri, 2013; Khan et al., 2017), where PLM technique using censored samples outperformed than LM for large return periods estimations. During flood frequency analysis, in comparison with LM, PLM describe the data samples in a better way and improve the accuracy of design flood estimates. With the help of PLM, we can mitigate the undesirable influences of small events which they leave on large return period events. Therefore, it is a reasonable and effective method for flood frequency analysis. These estimates would be of great help for designing, planning, and management of different hydrological structures in the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

form of water reservoirs, spillways, headworks, dams, culverts, bridges and other water related structures.

The present study can be extended to other sites of the Indus basin Pakistan, which will help the researchers to make policy implications related to water resources management for the whole country.

Acknowledgements. Authors extend their gratitude to the deanship of scientific research at King Khalid University, Saudi Arabia for providing financial support through research groups program under grant No RGP-1/50/39.

Conflict of interests. Authors have no conflict of interests in publication of this manuscript.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38193836

GERMINATION ABILITY AND BIOCHEMICAL PROPERTIES OF *AJUGA CHAMAEPITYS* SUBSP. *CHIA* VAR. *CHIA* AND *AJUGA ORIENTALIS* CULTIVATED IN CLIMATIC CONDITIONS IN LAKE DISTRICT, TURKEY

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(Received 7th Jan 2019; accepted 20th Feb 2019)

Abstract. The aim of this study is to determine the germination ability and biochemical properties of seedlings of two taxa (*Ajuga chamaepitys* subsp. *chia* var. *chia*, *Ajuga orientalis*) belonging to the *Lamiaceae* family which is naturally distributed in Lakes District-Turkey and cultivated in the Botanic Garden of Süleyman Demirel University in Isparta. These two taxa, which have usage potential as ornamental, medicinal and aromatic plants, were cultivated in 2015 vegetation period and seeds were collected at the end of 2016 vegetation period. During the same vegetation period, seeds were also collected from the areas where these two species were naturally grown. Seed germination tests were performed in 2017 vegetation period on the plants. Seed germination tests and seedling raising tests were carried out in the seedling and phenological properties (plant height, plant diameter, leaf width, leaf length) and SPAD values were also measured. Some macro and micro elements (Total N, P, K, Ca, Mg, Fe, Cu, Mn and Zn) and volatile oil compounds were analysed in the samples taken. As a result of the study, germination was not observed in *Ajuga chamaepitys* subsp. *chia* var. *chia*; however, seedling raising rate was between 33% and 40%. Seedling raising speed was 33.7% in the trial area and 36.41% in the seeds collected from the natural habitat. The germination rate of *Ajuga orientalis* was found to be 18% in the seeds taken from the natural habitat and 21.33% in the seeds taken from cultivars. Seedling raising rate was 43.33% in the seeds taken from the natural habitat and 60% in the seeds obtained from the trial area. Although the germination ability of the seeds from both origins (natural habitat and cultivars) were low, the phenological properties measurements and the biochemical analysis results were in a close relation with each samples.
Keywords: *Lamiaceae, Ajuga sp., cultivated plant, seed, volatile components*

Introduction

Today, the basic material of all designs is nature itself. In the creation and development of design, the use of various structural and plant materials inspired by nature is an indispensable approach. The selection of naturally propagated species from the area or region in which the design will be put, is an important factor both for sustainable design and for lowering costs such as irrigation and maintenance (Bassuk, 2017). In the world, especially in urban areas where water scarcity is felt more prominently in recent years, alternatives to wide grass surfaces are sought, and more sensitive to nature and less water consuming designs come to the fore. In order to put into practice these designs, studies were carried out on the cultivated and development of the natural species belonging to these regions (Çakır and Dönmez, 2018; Gül et al., 2012). However, in order to use natural species in planted design studies, it is necessary to know the breeding and production properties of plant materials. Although successful

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38373848

results can be obtained in some cases in terms of visual characteristics in the cultivation studies, the genetic continuity of the plants can not be achieved and successful results can not be obtained. The most remarkable and critical stages during the life cycle of plants are seed germination and seedling establishment (Copete et al., 2015). Besides, it is necessary to know the germination time and their growth properties in order to be produced both as industrial usage and as ornamental plant.

Lamiaceae family is represented by 400 genera and 3200 species in the world (Kahraman et al., 2009; Bazarragchaa et al., 2012). Members of these families are concentrated mainly in the Mediterranean countries, Australia, South West Asia and South America. Almost in all habitat types and at all heights, *Lamiaceae* family members can grow and there are very few regions in which they can not be seen. *Lamiaceae* family in Turkey is one of the important gene centers. About 546 species are represented in 45 genus. The rate of endemism in Turkey is approximately 44.5%. Most of the members of the *Lamiaceae* family are rich in ornamental leaves, flowers, angular bodies and volatile oil; for this reason, they are very important in landscape architecture, pharmacy, food and cosmetic industries (Castro et al., 2011; Kahraman et al., 2009; Guo et al., 2011). It is known by previous studies that the volatile oil of *Ajuga* species have antibacterial properties thus it can be used as medicinal and aromatic plant (Turkoğlu et al., 2010; Yaldiz, 2012; Delezar et al., 2012; Göder et al., 2015).

"*Ajuga chamaepitys* subsp. *chia* var. *chia*" also known as Ground Pine can be occured in Greece, Crimea, Palestine, North West and West Iran, Northern Iraq. In Turkey; it can be seen in almost all regions, up to 2000 m above sea level (Davis, 1982; Coll and Tandron, 2008). This taxa, which can be sized up to 30 cm in diameter and 10 cm in length, is yellow in color and can be flowered during the vegetation period (*Fig. 1*). This suffrutescent plant can be used either as ground covering or as an element of emphasis (Israili and Lyoussi, 2009; Dönmez et al., 2017; Jakovljević et al., 2015).

Figure 1. *Ajuga chamaepitys subsp. chia var. chia*

"*Ajuga orientalis*" can be grown in Crimea, Sicily, West Syria, Cyprus, Caucasus and North West Iran. In Turkey, it is seen in Mediterranean, Aegean, Black Sea and Central Anatolia regions up to 2500 m above sea level (Davis, 1982). It is a suffrutescent perennial herbaceous plant that can be grown up to 40 cm (Dönmez et al., 2017). The flowers are violet-blue and cream-colored. *Ajuga orientalis* stands out with its dense green texture (*Fig. 2*). It is suitable for use as a background in plant design. It is thought that its use in waterfronts and humid areas will increase the visual appeal.

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DOI: http://dx.doi.org/10.15666/aeer/1702_38373848

Figure 2. *Ajuga orientalis*

These taxas were cultivated for the first time (Dönmez et al., 2018). To the best our knowledge, there are no papers on the germination and seedling growth of these taxas. From this point of view, the aim of the study was to determine the germination abilitiy of *Ajuga chamaepitys* subsp. *chia* var. *chia* and *Ajuga orientalis* taxa belonging to *Lamiaceae* family, which is naturally grown in Lakes District, Turkey and cultivated in Isparta Süleyman Demirel University (SDU) Botanic Garden.

Materials and methods

The plants were cultivated in "SDU Botanic Garden" during the 2015 vegetation period. *Ajuga chamaepitys* subsp. *chia* var. *chia*'s natural habitat plant samples were collected from Asağıgökdere, Isparta (GÜL HERBARIUM No: 14340). *Ajuga orientalis* plants were collected from Kemer, Antalya (GÜL HERBARIUM No: 14341). In May 2015, trial parcels were created in SDU Botanic Garden. A total of 6 parcels were established, three of which with replicates, and each parcel consisting of 25 plants. As the physiographic and climatic factors were considered the same in the trial area where the experimental areas were established, the parceling was done in accordance with the Randomized Complete Block Design. Irrigation and drip was made by irrigation system. It was observed that 55 and 40 plants adapted in trial area, respectively, for *Ajuga chamaepitys* subsp. *chia* var. *chia* and *Ajuga orientalis.* At the end of 2016 (July) vegetation period, seeds were collected from natural habitat and trial area plants. Seeds were stored at 4 °C until germination experiments. Germination experiments were carried out after nine months. At the start of the 2017 (April) vegetation period, they were sowed, measurements and analyzes were carried out on the seedlings.

Germination and seedling raising test

The germination test was carried out in petri dishes (9 cm in diameter) with 3 replicates and 50 seeds were put into each dishes (*Fig. 3*). Sufficient amount (about 5 ml) of pure water was added and seeds were allowed to germinate at 25 °C in the dark. 1 mm length of rootlet is decided to be adequate for germination and germinated seeds were counted every day. This process was continued until the number of germinated seeds is fixed. Germination rate (GR, %) was calculated as percentage of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_38373848

ratio of germinated seeds to total sown seeds. Average Germination Speed (AGS, day) were calculated according to *Equation 1* (Pedersen et al., 1993).

$$AGS = (A_1.D_1 + A_2.D_2 + \ldots\ldots A_n.D_n) / (A_1 + A_2 + \ldots\ldots A_n) \qquad (Eq.1)$$

A: The number of seeds germinated every day,
D: The day required for germination of the seed,
n: Number of the days until the last count.

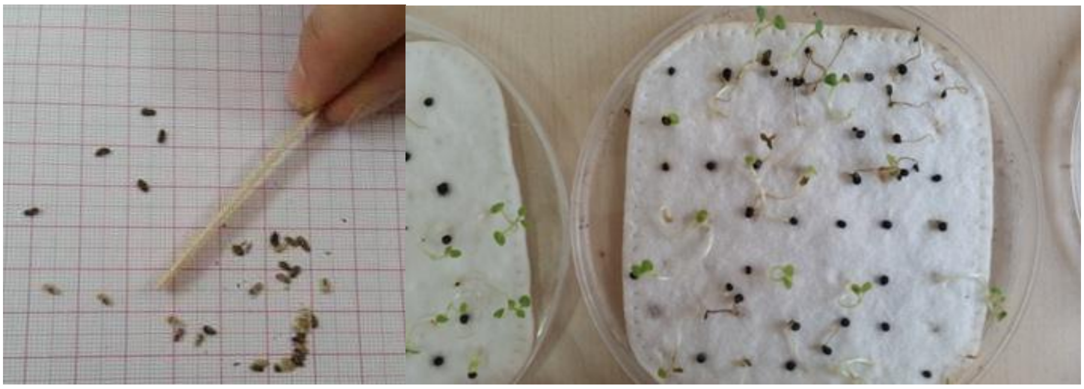

Figure 3. *A view from germination test*

Seeds obtained from the natural habitat and trial area were planted in a viol (4 cm × 4 cm × 6 cm) which includes peat soil and perlite and placed in greenhouse. In 3 replicated experiments, 30 seeds were planted in each replicate for the seedling tests. Appearance of cotyledon leaves was considered as seedling (*Fig. 4*). Each day the seedling was recorded and this process was continued until the number of seedlings was fixed. Seedling raising rate (SRR, %) was calculated as percentage of the ratio of seedlings to total sown seeds. Average Seedling Raising Speed (days, ASRS) were calculated according to *Equation 2*.

$$ASRS = (A_1.D_1 + A_2.D_2 + \ldots\ldots A_n.D_n) / (A_1+A_2 + \ldots\ldots A_n) \qquad (Eq.2)$$

A: The number of seeds raising every day,
D: The day required for germination,
n: Number of the days until the last count.

Determination of phenological properties and chlorophyll (SPAD) value

After germinating, seedlings reached a certain size (65 days after seed sowing), 10 of them were selected and transferred to viols. Flowering was not seen in seedlings. 2017 vegetation period phenological properties such as plant height, plant diameter, leaf width and leaf length were measured. SPAD value of the plants was also determined on the plants (*Fig. 4*). SPAD-502 (Minolta Ltd, Osaka Japan) is hand-held chlorophyll meter equipment based on the measurement of leaf chlorophyll content. It measures the leaf transmittance in red light at 650 nm (at which chlorophyll absorbs) and in near-infrared light at 940 nm (for the correction of leaf thickness). The ratio of these two transmission values is referred to as SPAD reading or SPAD value (Monostori, 2016).

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SPSS (Statistical Programs for Social Science) 13.0 program was used for statistical analyses. The mean values and standard deviation values of the data were given.

Figure 4. *Seedlings on the experimental plots and determination of chlorophyll value*

Biochemical analysis

It is important to ensure the continuity of their biochemical properties as well as their phenological properties especially in medicinal aromatic plants. Biochemical analyzes were performed in the seedlings in 2017 vegetation period and their average values were given. Randomly selected five plants from each plot were cut from the base at each stage and separated into leaf+stem and head. They were oven dried at 65 °C until constant weight was obtained. The oven dried weight of leaf, stem and head were recorded. Total N determination was made according to the Regular Kjeldahl Method (Boț et al., 2017) and Phosphorus (P) amount was determined by Vanadomolybdophosphoric Yellow Color Method (Spectrophotometric) (Rajkumar et al., 2017). Determination of Potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), Copper (Cu), Manganese (Mn) and Zinc (Zn) were determined by wet decomposition-AAS (Atomic Absorption Spectroscopy) method (Tariq et al., 2017).

Besides, after the plant materials collected, flower and leaf samples were subjected to solid phase microextraction (SPME) by GC-MS system. 2 g of samples were placed into a 10 ml vial. After incubation for 30 min at 60 °C, SPME fibre was pushed through the headspace of a sample vial to absorb the volatiles and then inserted directly into the injection port of the GC-MS (Shimadzu 2010 Plus GC-MS with the capillary column, Restek Rxi®-5Sil MS 30 m × 0.25 mm, 0.25 µm) at a temperature of 250 °C for desorption (5 min) of the adsorbed volatile compounds. The constituents were identified using retention times of standard substances by aligning mass spectra with the data given in the Wiley, NIST Tutor, FFNSC library (Özderin et al., 2016).

Results and discussions

Germination ability and seedling raising rate of plant

Seed dormancy varies depending on the temperature and species among *Lamiaceae* family (Copete et al., 2015). Although there is no study in these taxas, the different species belonging to the *Lamiaceae* family are mentioned as non-dormant (Panuccio et al., 2017). In germination experiments, no germination was observed in *Ajuga chamaepitys* subsp. *chia* var. *chia* plants. The germination rate of *Ajuga orientalis* was 18% in the seeds taken from the natural habitat and 21.33% in the seeds taken from the trial area. Although there was no germination for *Ajuga chamaepitys* subsp. *chia* var. *chia* in petri dishes, it was determined that seedlings emerged in peat soil experiments.

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Seedling rising rate increased in seedling trials for both taxa (*Table 1*). In germination experiments in soil, herbal seeds have adaptation advantages, since seeds are protected from environmet (Adams et al., 2005). Avarage seedling raising speed for *Ajuga chamaepitys* subsp. *chia* var. *chia* was 23.67 days. It was observed that germination was seen between the days of 20-30. Avarage seedling raising speed for *Ajuga orientalis* was 20.67 days and germination of these seeds was mostly seen between the days of 20-35 (*Fig. 5*). In different species of the *Lamiaceae* family, the percentage of germination varies from 20 to 50%, while the average germination time can be as long as 30 days (Mattana et al., 2016).

Table 1. *Germination and seedling raising data of plant*

Plant		GR (%)	sig.	AGS (day)	sig.	SRR (%)	sig.	ASRS (day)	sig.
A. chamaepitys subsp. *chia* var. *chia*	Trial	-	-	-	-	33.06	0.02^{ns}	36.43	0.09^{ns}
	Natural	-		-		40.30		33.43	
A. orientalis	Trial	18.00	0.10^{ns}	20.68	0.99^{ns}	43.44	0.06^{ns}	22.38	0.51^{ns}
	Natural	21.36		20.67		48.28		23.57	

^{ns}Not statistically significant (independent sample t test)

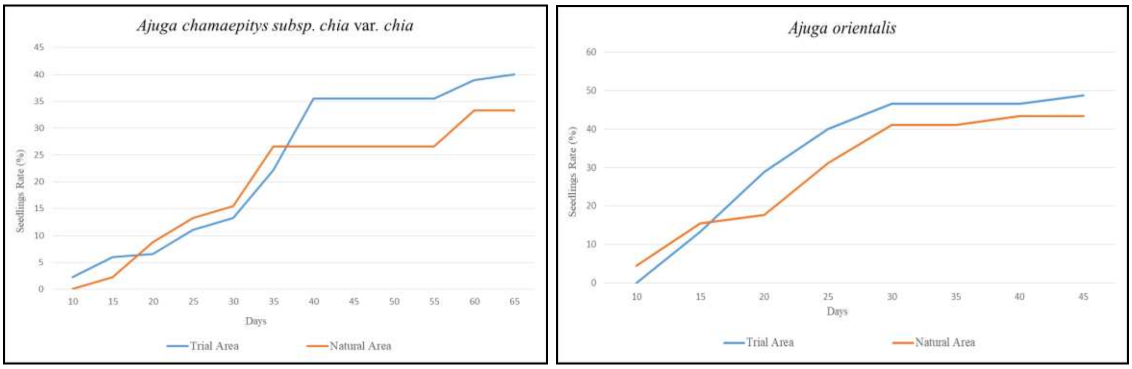

Figure 5. *Distribution of seedling rate by day*

Phenological properties and chlorophyll (SPAD value)

These *Ajuga* taxas are horizantally growing plants and height and diameter values are directly related to each other (Dönmez et al., 2017). According to the data in *Table 2*, no change was observed in plant height and diameter values in the seedlings obtained from seeds taken from natural habitat and trial area.

Chlorophyll is a necessery pigment for photosynthesis. The content of chlorophyll is one of main sources reflecting leaf photosynthesis ability and plant health condition (Jiang et al., 2017). The connection between leaf chlorophyll content determined in vitro and SPAD meter readings (SPAD values) were extensively analysed and usually parameterised by linear relationship (Monostori, 2016). Chlorophyll was measured almost 50.00 SPAD value in both plant species. This rate corresponds to the rate in which the seeds are collected from rootstock plants (Dönmez, 2018). When the phenological properties were compared, no statistically significant difference was found between the seeds collected from natural habitat and trial area. Although studies on

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phenological properties of *Ajuga* seedlings is out of our knowledge, results are compatible with some of the species belonging to *Lamiaceae* family (Rather et al., 2016; Mandal et al., 2008).

Table 2. *Comparison of phenological properties of plants*

Measurements	Group	A. chamaepitys subsp. chia var. chia.		A. orientalis	
		Average	sig.	Average	sig.
Length (cm)	Trial	10.90±0.9	0.79 ns	20.25±1.5	0.37 ns
	Natural	11.00±0.8		20.95±1.8	
Height (cm)	Trial	4.40±0.6	0.33 ns	2.95±0.7	0.88 ns
	Natural	4.10±0.6		2.90±0.7	
Leaf width (cm)	Trial	2.21±0.5	0.36 ns	2.43±0.3	0.22 ns
	Natural	2.45±0.5		2.68±0.4	
Leaf length (cm)	Trial	4.25±0.5	0.43 ns	5.45±0.5	0.46 ns
	Natural	4.05±0.6		5.29±0.4	
Chlorophyll (SPAD value)	Trial	52.00±3.2	0.77ns	50.26±1.2	0.45ns
	Natural	51.57±3.3		49.83±1.2	

nsNot statistically significant (independent sample t test)

Biochemical properties

As a result of biochemical analyzes, the nitrogen amount of *Ajuga chamaepitys* subsp. c*hia* var. *chia* was found to be 2.15% in the seedlings obtained from natural habitat seeds and 2.23% from trial area seeds. There were seen little differences between the other macro and micro-element analyzes, the seedlings of the seeds obtained from the trial area have higher calcium, copper and manganese ratios (*Table 3*). The nitrogen amount of *Ajuga orientalis* was 2.94% in the seedlings obtained from the natural habitat seeds and 3.42% in the seedlings obtained from trial area seeds. In the other macro and micro-element analyzes, the phosphorus, potassium and copper ratios of the seedlings obtained from the trial area seeds were found to be higher. In the literature, there is no study on the macro and micro element content of these plants.

48 different volatile components of *A. chamaepitys* subsp. *chia* var. *chia*, collected from both area, were determined by GC-MS after solid phase micro extraction (SPME). Limonene, which is the highest component, was determined as 28.17% in the natural plants seedlings and 29.24% in the cultivated plant seedlings. The second highest component, β-pinene was found to be 17.71% in the natural plants seedlings and 18.26% in the cultivated plant seedlings (*Table 4*). These results coincide with the studies done by Azizan et al. (2002) and Delezar et al. (2012). In addition, the same components were also seen to be the highest in rootstocks where seeds were collected (Dönmez, 2018). In *A. orientalis* seedligs volatile oil, 41 components were identified from both area samples. 1-Octen-3-ol, which is the highest component, was determined as 23.48% in the natural plants seedlings and 22.89% in the cultivated plant seedlings (*Table 5*). However, germacrene-D and β-cubebene, was previously identified as the highest compounds in this plant (Saijadi and Ghannadi, 2004; Yaldiz, 2012).

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DOI: http://dx.doi.org/10.15666/aeer/1702_38373848

Table 3. *Volatile compounds of A. chamaepitys subsp. chia var. chia seedlings*

Analysis	***A. chamaepitys* subsp. *chia* var. *chia***		***A. orientalis***	
	Natural areas	**Trial areas**	**Natural areas**	**Trial areas**
Nitrogen (N) (%)	2.15	2.23	2.94	3.42
Phosphorus (P) (%)	0.14	0.12	0.25	0.26
Potassium (K)(%)	2.41	2.36	4.04	4.12
Calcium (Ca)(%)	1.64	1.73	2.08	1.93
Magnesium (Mg)(%)	0.94	0.92	0.95	0.86
Iron (Fe)(ppm)	131.02	128.31	154.81	152.61
Copper (Cu) (ppm)	21.64	22.01	18.90	19.31
Manganese (Mn)(ppm)	26.14	27.20	42.86	40.23
Zinc (Zn) (ppm)	62.14	61.79	101.89	98.42

Table 4. *Volatile compounds of A. chamaepitys subsp. chia var. chia seedlings*

Components (%)	**Natural areas**	**Trial areas**
Ethanol	0.33	0.45
Me-acetate	0.35	0.25
2,2-dimethyl-4-ethylhexane	0.36	0.39
Cyclopentanol	0.16	0.13
2-penten-1-ol	0.10	0.12
n-hexanal	0.22	0.27
2-hexanal	0.52	0.51
3-hexen-1-ol	0.07	0.08
2-hexen-1-ol	0.15	0.18
n-hexanol	0.09	0.08
Heptanal	0.09	0.08
α-thujene	0.18	0.16
α-pinene	1.92	1.85
Benzaldehyde	0.09	0.07
Sabinene	0.25	0.22
β-pinene	17.71	18.26
1-octen-3-ol	2.09	2.16
β-myrcene	2.62	2.58
Cymol	2.23	2.10
Limonene	28.17	29.24
Eucalyptol	0.46	0.33
β-ocimene	0.10	0.08
1,4-cyclohexadiene	0.51	0.44
Linalool	1.33	1.28
Trans-limonene oxide	0.44	0.42
4-terpineol	0.19	0.23
α-terpineol	0.21	0.22
p-allylanisole	0.32	0.30
Linalyl acetate	0.85	0.95
α-cubebene	0.71	0.76
Cyclosativene	0.21	0.27
Copaene	3.18	3.39
α-bulnesene	0.36	0.12
β-bourbonene	0.26	0.20
β-cubebene	2.98	2.87

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1-cyclopropazulene	0.48	0.45
3,5-dimethylcyclohex-1-ene-4-carboxyaldehyde	1.62	1.48
Caryophyllene	2.71	2.60
Germacrene-D	12.60	12.79
Epibicyclosesquiphellandrene	0.21	0.33
Γ-cadinene	3.81	4.99
β-ionone	0.40	0.44
Viridiflorene	1.20	1.35
Cyclohexane	0.28	0.24
α-muurolene	0.98	0.82
Δ-cadinene	1.30	1.15
Torreyol	0.15	0.21
Viridiflorol	0.28	0.24

Table 5. *Volatile compounds of A. orientalis seedlings*

Components (%)	Natural areas	Trial areas
Acetaldehyde	1.71	1.34
Ethanol	1.30	1.69
7-hydroxy-5,6,7,8-tetrahydroindoliziaine	0.09	0.07
Me-acetate	1.01	1.21
2-butenal	0.22	0.23
2-pentanone	0.92	0.89
2-pentenal	0.11	0.12
1-pentanol	0.10	0.19
2-penten-1-ol	0.22	0.26
n-hexanal	1.11	1.36
2-hexenal	5.15	5.39
3-hexenyl	0.43	0.27
2-hexen-1-ol	1.12	1.08
n-hexanol	1.41	1.66
Ocimene	0.06	0.09
α-pinene	9.29	9.13
2-heptenal	0.11	0.07
Benzaldehyde	0.10	0.06
Sabinene	0.18	0.23
β-pinene	0.98	1.13
Bicycloheptane	1.70	1.91
1-Octen-3-ol	23.48	22.89
β-myrcene	4.31	4.69
2,4-heptadienal	2.11	2.36
δ3-carene	2.11	2.14
n-octan-3-ol	1.76	1.79
Cymol	0.98	0.69
Limonene	13.02	12.84
1,4-cyclohexadiene	1.43	1.26
α-terpinolene	2.92	2.79
nonanal	1.92	1.61
Linalool	0.76	0.96
Hexyl butanoate	0.66	0.83
Trans-Limonene oxide	1.28	1.25
2-octenal	0.80	0.83

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p-allylanisole	0.66	0.73
Decanal	0.15	0.16
Linalyl acetate	0.93	0.87
Anethole	1.43	1.61
Dodecane	0.66	0.61
2-butenoic acid	3.46	3.22
Pentanoic acid	1.49	1.44
Tridecane	0.98	0.79
Valencene	1.96	1.90
α-farnesene	3.43	3.38

Conclusion

It was seen that the seedling rising rate in production of *Ajuga chamaepitys* subsp. *chia* var. *chia* with seed was about 35% and the rising speed was found to be between 33 and 36 days. Seed experiments were carried out in seeds collected from plants randomly and no improving nursing material was given to the seeds. The phenological properties and biochemical structures of the seedlings had the same characteristics. The seedling rate in production of *Ajuga orientalis* with seed was about 45% and the rising speed was 23 days. There were no differences in germination and seedling characteristics of the seeds. Although germination ratio was seen to be low, the trial area seedlings had the same germination ability with natural plants.

Acknowledgements. This work is a part of a project supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project number 114O345.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38373848

SALINITY TOLERANCE CLASSIFICATION OF SUNFLOWER (*Helianthus annuus* L.) AND SAFFLOWER (*Carthamus tinctorius* L.) BY CLUSTER AND PRINCIPAL COMPONENT ANALYSIS

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(Received 16th Jan 2019; accepted 20th Feb 2019)

Abstract. Germination, emergence and seedling growth are considerably restricted by salinity. The study aimed to compare safflower with sunflower during germination and seedling growth for tolerance to salinity. Four safflower cultivars (Asol, Balcı, Linas and Olas) and sunflower hybrids (Esbella, Oliva, Cartago and C-180) were subjected to various NaCl levels (control, 5, 10 and 15 dS m^{-1}) and their seedling growth and ion concentration were investigated. Classification for salinity tolerance was performed by a combination of Principal Component (PCA) and Cluster Analysis considering germination percentage (GP), mean germination time (MGT), vigor index (VI), Na^+ and Na:K ratio in seedlings. The results elicited that genotypes exhibited varying responses to salinity, MGT significantly prolonged and a dramatic reduction in seedling growth of sunflower was observed. The highest seedling fresh weight was observed in Esbella among sunflower and Asol among safflower and, Na^+ concentration of seedlings increased with increasing salinity levels. The safflower seedlings absorbed a higher Na^+ than that of sunflower. Safflower cv. Balcı had the highest Na^+ concentration in seedling and it maintains its ion balance (Na:K ratio) at all levels of NaCl. Cluster analysis revealed that there were two groups for salinity tolerance, and the safflower cultivars were apparently more salt-tolerant than sunflower hybrids.
Keywords: *Carthamus tinctorius, Heliathus annuus, NaCl, resistance, ion balance*

Introduction

Sunflower (*Helianthus annuus* L.) and safflower (*Carthamus tinctorius* L.) are the most important oil seed crops from *Asteraceae* family. These plants are usually preferred in arid and semi-arid regions under rain-fed conditions, where low rainfall and high evapotranspiration during vegetation periods restricts the growth of crop plants (Kaya et al., 2003). Under these conditions, drought and salinity are the major abiotic stresses that severely inhibit germination, seedling establishment and plant growth; consequently, seed yield decreases (Hussain et al., 2016). The restrictive effects of salinity on plant life are mainly osmotic effect of salts and toxic effects of Na^+, Cl^- and SO_4^{-2} ions (Ashraf and Fatima, 1994; Chinnusamy et al., 2005; Muhammad and Hussain, 2010), but some crop plants or varieties in a species have been more tolerant than the others.

Sunflower and safflower are classified as moderately salt tolerant based on seed yield (Grieve et al., 2012). When the plants were to be classified according to salinity tolerance, they were compared under saline and non-saline conditions with each other in terms of plant growth parameters, yield and quality performance. For the classification, the controlled conditions are commonly preferred because the environmental changes are discarded. A lot of researches on the comparison of genotypes, varieties or species for tolerance to salt stress have been conducted and tried to find a valid selection criteria for the tolerance mechanism (Hussain et al., 2016). Plant biomass and photosynthetic activity by Munns and Tester (2008), Siddiqi et al. (2009) and Farooq et al. (2015), reduced chlorophyll content by Ashraf (2004), leaf water and osmotic potential by Gadallah (1996) and Munns (2002), ion toxicity by Munns et al. (2006) and Rejili et al. (2007) and reduction in seed yield by Francois (1996) and Natarajan et al. (2005) have been considered the suitable parameters for ranking the genotypes for salinity tolerance. On the other hand, these parameters to be used for selection criteria have not been easily investigated and heritable. Recently, several indices have been commonly developed for grouping plants under salt stresses (Nikbakht et al., 2010; Zafar et al., 2015; Krishnamurthy et al., 2016). Saboora and Kiarostami (2006) and Kaya et al. (2012) classified the genotypes using all the parameters they measured by using a combination of Cluster and Principal component analysis (PCA) instead of searching a parameter, and lastly Aslam et al. (2017) ranked lentil germplasm for salinity tolerance using multiple traits by PCA based Biplot. Multivariate analysis has the superiority for genotypic classification because accuracy is considerably increased using multiple traits, and gives a general tolerance level based over mean values derived from each stress level (Zeng et al., 2002). For these reasons, this research focused on determining more tolerant plant to salinity between sunflower and safflower considering germination and seedling growth traits, sodium concentration and sodium / potassium ratio in seedling.

Materials and Methods

This study was conducted at Seed Science Laboratory of Department of Field Crops, Eskişehir Osmangazi University, Turkey. Four native safflower cultivars Asol, Balcı, Linas and Olas registered in Turkey, and four sunflower hybrids Esbella, Oliva, Cartago and Pioneer C-180 were used in the study. The seeds of the respective varieties were subjected to three levels of the electrical conductivity of 5, 10 and 15 dS m^{-1} constituted by NaCl along with distilled water as a control treatment.

Two hundred (4×50) seeds of each genotype were inserted into three layer filter papers irrigated with 7 mL of the saline solutions for each paper. After filter papers with seeds were rolled, they were replaced into a sealed plastic bag to prevent moisture evaporation. Each rolled paper was renewed every 2 days after incubation to avoid the salt accumulation. Germination tests were conducted at 25 ± 1 °C in the dark condition and a seed with 2 mm radicle was counted every 24 h for 10 d as germinated. The speed of germination was evaluated by means of mean germination time (MGT) which was represented by the ISTA (2003) rules. MGT= $\Sigma(Dn)/\Sigma n$, where, n is the seed number germinated on day D, and D is the number of days from the beginning of germination test. Ten seedlings selected from each treatment at the 10^{th} day were sampled for determination of seedling growth traits such as root length (RL), shoot length (SL) and seedling fresh weight (SFW). Vigor index (VI) is calculated by multiplying germination percentage (GP) (%) and seedling length (cm) and the genotypes with the higher vigor index are considered to be more vigorous (Abdul-Baki and Anderson, 1973). Also, percent reduction in shoot length and seedling fresh weight between control and 15 dS m^{-1} were calculated as (control plants-stressed plants / control plants) × 100 described by Shirani Rad and Abbasian (2011). The index didn't give in Tables because they used only for statistical evaluation. To determine Na^+ and K^+ concentration in seedlings, all the seedlings from each treatment were used. After the seedlings were firstly washed with

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DOI: http://dx.doi.org/10.15666/aeer/1702_38493857

distilled water three times, excessive water on seedling surface water was dried with paper towel. The weighed seedlings were dried in an oven at 70°C for 48 hours and then they were grounded. Plant samples were acid digested in microwave oven, and Na^+ and K^+ concentrations were measured using a flame photometric method described by Kacar and Inal (2008).

The experiment was established as two-factor factorial (salinity × genotype) arranged in a completely randomized design (CRD) with 4 replications. Data were statistically analyzed by MSTAT-C program (Michigan State University). The differences between the means were tested by the Least Significant Differences at $P < 0.05$ level. For ranking the varieties for salinity tolerance, Principle Component (PCA) and Cluster Analysis were done by SPSS.

Results and Discussion

A two-way interaction was significant for all characters of safflower varieties ($P < 0.05$; *Table 1*). Similar germination percentages from safflower cultivars were observed while Balcı gave the highest germination percentage at all levels of NaCl. MGT was clearly prolonged by increasing NaCl levels, and NaCl level of 15 dS m^{-1} led to retardation in germination more dramatically compared to lower levels of NaCl. The most rapid time to germination at 15 dS m^{-1} was calculated in Linas (1.96 days). Compared to control, the root length was remarkably depressed at 15 dS m^{-1} and Balcı gave the longest root under NaCl stresses. The shoot length of safflower was the most sensitive parameter adversely affected by salinity. It was drastically diminished and the longest shoot at 15 dS m^{-1} was measured in Asol with 7.00 cm. Similarly, the seedling fresh weight was reduced under salt stresses and Asol exhibited a heavier seedling than the others did. In terms of seedling growth and germination percentage together under salinity, the highest vigor index was obtained from Balcı under all levels of NaCl.

Table 1. *Germination and seedling growth of safflower cultivars affected by NaCl levels*

NaCl (dS m^{-1})	Cultivars	GP %	MGT day	RL cm	SL cm	SFW mg $plant^{-1}$	VI
Control	Asol	92.0^{cde}	1.25^{g}	4.55^{e}	7.68^{b}	4.59^{a}	$1063^{d\text{-}g}$*
	Balcı	95.0^{abc}	1.87^{c}	8.38^{ab}	7.05^{bc}	3.37^{c}	1466^{b}
	Linas	94.5^{bcd}	1.42^{ef}	6.20^{d}	4.35^{g}	2.64^{d}	1003^{efg}
	Olas	98.0^{ab}	1.06^{i}	6.60^{cd}	3.10^{i}	2.08^{f}	951^{fg}
5	Asol	97.0^{abc}	1.11^{hi}	4.50^{e}	7.00^{bc}	4.39^{a}	$1116^{d\text{-}f}$
	Balcı	100.0^{a}	1.67^{d}	9.38^{a}	8.83^{a}	3.26^{c}	1821^{a}
	Linas	97.5^{ab}	1.09^{i}	8.78^{a}	6.80^{cd}	2.51^{de}	1519^{b}
	Olas	98.5^{ab}	1.38^{f}	8.78^{a}	3.88^{gh}	2.05^{fg}	1247^{cd}
10	Asol	98.0^{ab}	1.10^{i}	4.50^{e}	6.88^{cd}	3.78^{b}	1115^{def}
	Balcı	99.0^{ab}	1.75^{d}	8.40^{ab}	7.18^{bc}	3.33^{c}	1542^{b}
	Linas	98.5^{ab}	1.20^{gh}	6.63^{cd}	5.45^{f}	2.11^{f}	1189^{d}
	Olas	98.5^{ab}	1.49^{e}	6.30^{d}	3.70^{ghi}	1.87^{fg}	985^{fg}
15	Asol	98.5^{ab}	1.96^{bc}	4.78^{e}	7.00^{bc}	3.49^{bc}	1160^{de}
	Balcı	98.5^{ab}	1.98^{b}	7.58^{bc}	6.20^{de}	3.24^{c}	1357^{bc}
	Linas	97.0^{abc}	2.13^{a}	6.93^{cd}	5.55^{ef}	2.15^{ef}	1211^{cd}
	Olas	89.5^{de}	2.09^{a}	5.95^{d}	3.20^{hi}	1.68^{g}	819^{g}

*: Means followed by the same letter(s) are not significantly different at $p < 0.05$. GP: germination percentage, MGT: mean germination time, RL: root length, SL: shoot length, SFW: seedling fresh weight, VI: vigor index

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DOI: http://dx.doi.org/10.15666/aeer/1702_38493857

The responses of the investigated sunflower hybrids to salinity stresses are illustrated in *Table 2*. A significant difference was determined for germination percentage of sunflower as affected by the interaction of hybrids × NaCl levels. In general, sunflower hybrids gave similar germination percentage under NaCl levels, but lower germination percentage was observed in C-108 and Cartego at 5 dS m^{-1}. MGTs of sunflower hybrids also showed similarity to each other while it was prominently extended by an increase in NaCl level. Also, an apparent reduction in root length was detected by increasing salinity; however, the root growth of Esbella was not adversely influenced. Esbella and Oliva gave the highest root and shoot length under NaCl stresses. Seedling fresh weight of sunflower hybrids was gradually declined depending on changes in root and shoot growth, but Esbella showed the superiority to other hybrids for seedling growth under salinity. The highest vigor index was observed in Oliva under NaCl levels except for 10 dS m^{-1}.

Table 2. *Germination and seedling growth of sunflower hybrids affected by NaCl levels*

NaCl (dS m^{-1})	Hybrids	GP %	MGT day	RL cm	SL cm	SFW mg plant^{-1}	VI
Control	Esbella	89.0^{cde}	2.67^{bcd}	7.88^{d}	10.43^{bc}	7.72^{a}	1628^{bcd}*
	Oliva	94.0^{abc}	2.77^{bc}	12.52^{a}	12.70^{a}	5.53^{cd}	2369^{a}
	C-108	85.5^{def}	2.90^{b}	10.65^{b}	6.55^{ef}	3.45^{fg}	1471^{cde}
	Cartego	93.0^{a-d}	2.83^{b}	9.73^{c}	3.20^{gh}	2.49^{h-k}	1202^{ef}
5	Esbella	90.0^{b-e}	3.02^{a}	7.98^{d}	9.83^{bc}	7.50^{a}	1599^{bcd}
	Oliva	92.0^{a-e}	2.80^{bc}	8.55^{d}	11.40^{ab}	4.68^{de}	1877^{b}
	C-108	78.0^{fg}	2.69^{bcd}	6.50^{e}	5.88^{f}	3.28^{fgh}	1008^{f}
	Cartego	71.0^{g}	2.77^{bc}	7.88^{d}	2.50^{h}	2.27^{ijk}	712^{gh}
10	Esbella	98.0^{ab}	2.33^{e}	8.63^{d}	10.43^{bc}	6.51^{b}	1867^{b}
	Oliva	100.0^{a}	2.29^{e}	5.40^{f}	11.40^{ab}	3.99^{ef}	1705^{bc}
	C-108	100.0^{a}	2.30^{e}	4.53^{g}	8.00^{de}	2.86^{ghi}	1203^{ef}
	Cartego	96.0^{abc}	2.71^{bc}	1.68^{i}	3.75^{gh}	1.79^{jk}	521^{h}
15	Esbella	84.0^{ef}	2.94^{b}	8.53^{d}	8.90^{cd}	6.28^{bc}	1397de
	Oliva	95.0^{abc}	2.39^{de}	4.85^{fg}	9.93^{bc}	3.05^{ghi}	1431^{cde}
	C-108	89.0^{cde}	2.51^{cde}	3.30^{h}	6.88^{ef}	2.62^{g-j}	928^{c}
	Cartego	93.5^{a-d}	2.78^{bc}	2.33^{i}	4.28^{g}	1.70^{k}	619^{d}

*: Means followed by the same letter(s) are not significantly different at $p<0.05$. GP: germination percentage, MGT: mean germination time, RL: root length, SL: shoot length, SFW: seedling fresh weight, VI: vigor index

Seedling of safflower cultivars accumulated much more Na^+ than did the sunflower hybrids, and the amount of Na^+ was the highest in Balcı at all levels of NaCl (*Figure 1*). Moreover, NaCl level of 15 dS m^{-1}, Asol seedlings absorbed more Na^+ from the medium. Na:K ratio represented that Balcı maintained the ionic balance under salt stresses while Asol become unbalanced at 15 dS m^{-1} (*Figure 2*).

PCA and Cluster Analysis performed by mean values of germination percentage, MGT, vigor index, percent reduction in seedling length and fresh weight, ion concentration in seedlings and Na:K ratio emerged that two main groups were constituted (*Figure 3*). The safflower varieties were found more tolerant than sunflower varieties to NaCl stresses. Among safflower varieties, the superiority of Balcı and Asol appeared under NaCl stresses.

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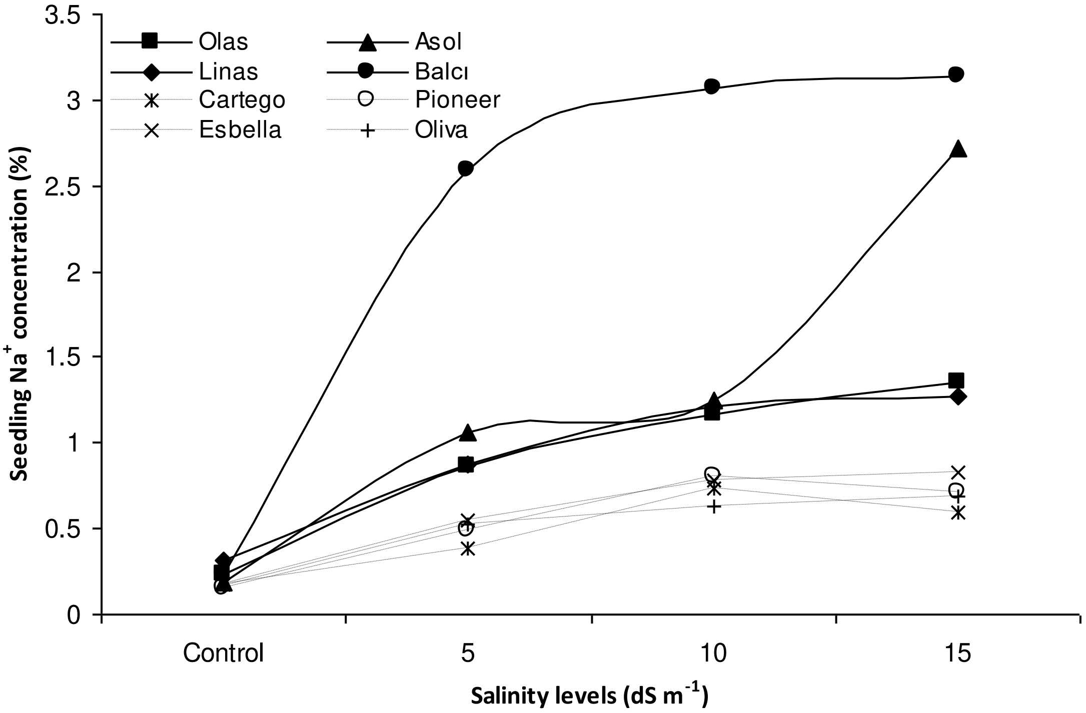

***Figure 1.** Seedling Na^+ concentration of safflower and sunflower genotypes subjected to various levels of NaCl*

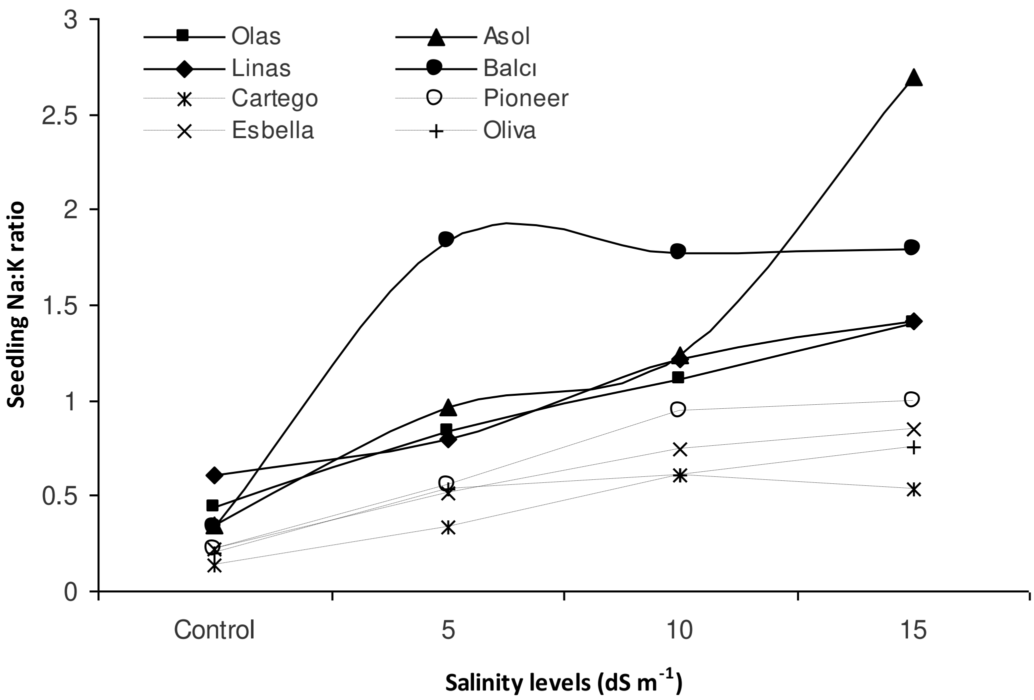

***Figure 2.** Seedling Na:K ratio of safflower and sunflower genotypes subjected to various levels of NaCl*

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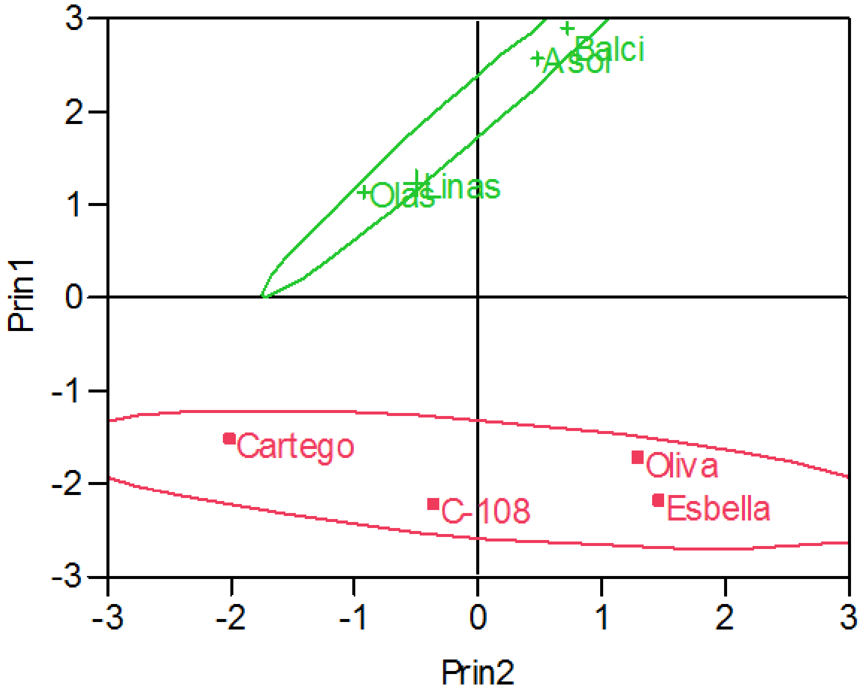

***Figure 3.** Classification of sunflower and safflower genotypes for salinity tolerance using germination percentage, MGT, vigor index, Na^+ and K^+ concentrations of seedling, Na:K ratio, percent reduction in seedling length and fresh weight measured at 10 d old seedlings*

Salt concentration between control and 15 dS m^{-1} didn't lead to a clear increase or decrease in germination percentage of safflower and sunflower genotypes while the statistical differences were determined. Similar findings were reported by Kaya et al. (2006) who found no significant changes in germination with increasing salinity stresses in sunflower. Genotypic variation at 15 dS m^{-1} was significantly appeared and the most rapid germination was recorded in Asol and Oliva. Generally, NaCl levels slightly affected germination of both species, indicating that it should not be used for selection criteria for salinity tolerance. The findings are confirmed by the results of Muhammad and Hussain (2010), who reported that NaCl levels up to 15 dS m^{-1} didn't severely influence germination in flax. Salts constitute osmotic stress or specific ion toxicity, especially Na, responsible for retarding and inhibiting germination of several plants as declared by Chinnusamy et al. (2005), Okcu et al. (2005) in pea, and Atak et al. (2006) in triticale. Nevertheless, seedling growth of both species was severely depressed while sunflower seedling was more sensitive to increasing salinity. The greatest reduction was observed in shoot length of sunflower compared to safflower. Depending on root and shoot growth depletion, seedling fresh weight was severely reduced and this reduction was obvious at 15 dS m^{-1}. These findings have supported the results of previous researches in sunflower by Ashraf and O'Leary (1997), Mohammed et al. (2002), Kaya et al. (2006), Day et al. (2008) and in safflower by Kaya et al. (2003), Siddiqi et al. (2007) and Farhoudi and Motamedi (2010).

Seedling of safflower cultivars accumulated much more Na^+ than did the sunflower hybrids, and the amount of Na^+ was the highest in Balcı at all levels of NaCl (Figure 1).

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DOI: http://dx.doi.org/10.15666/aeer/1702_38493857

Moreover, Na^+ concentration in Asol seedlings clearly improved at 15 dS m^{-1}. This means that Balcı could keep up with higher Na^+ concentration in cells without any significant depression or vitality in seedlings (Chinnusamy et al., 2005). Conversely, NaCl depressed the seedling growth of sunflower hybrids, although they absorbed less Na^+ from the germination medium. It seems that K^+ in the seeds was leaked into the medium and the seedling uptook Na^+ from the medium. Chinnusamy et al. (2005) and Hussain et al. (2016) represented that increasing NaCl levels caused an increase in K^+ leakage from seeds. Atak et al. (2006) found Na^+ concentration in seeds was increased by increasing NaCl levels regardless of K^+ level. In terms of ion balance, Na:K ratio, Balcı was the most stable genotype against increasing NaCl levels, although Asol showed an extraordinary behavior at 15 dS m^{-1} (Figure 2). It is argued that Asol could not maintain the ion balance and ultimately Na^+ was surprisingly peaked at 15 dS m^{-1}.

Principle component analysis (PCA) has been effectively implemented with sorting the genotypes under different environmental conditions, especially drought and salinity stresses (Kaya et al., 2012; Aslam et al., 2017). Cluster analysis and PCA analysis were made on mean data obtained from each genotype subjected to various salinity stresses. Our results showed that two main groups were discovered by Cluster analysis, and PCA1 (67.2%) and PCA2 (13.8%) represented 81.0% of data variability. It is strongly declared that safflower varieties were more tolerant to salinity than sunflower hybrids along with differences among genotypes.

Conclusion

This experimental preliminary study shows that significant differences between safflower and sunflower for salinity tolerance apparently existed at germination and early seedling growth. The superiority of safflower under saline condition was clearly identified, while there were genotypic variations within the species; eliciting that Balcı and Asol among safflower, Oliva and Esbella among sunflower were more tolerant to NaCl stresses. However, the further study is required to evaluate salt tolerance subsequent growth stages of safflower and sunflower under field conditions to confirm their tolerance levels.

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DOI: http://dx.doi.org/10.15666/aeer/1702_38493857

VOLATILE OIL COMPOSITION OF *AJUGA* SPECIES OF NATURAL AND CULTIVATED ORIGIN IN THE LAKE DISTRICT OF TURKEY

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(Received 21st Jan 2019;accepted 20th Feb 2019)

Abstract. Leaves and flowers of *Ajuga chamaepitys* subsp. *chia* var. *chia* and *Ajuga orientalis*, which were taken from both their natural habitat and cultivars, were analyzed by SPME and GC-MS. The amount and the composition of volatile oil of cultivated plants were compared with natural area plants. β-pinene was detected as major component in *A. chamaepitys* in both natural habitat and cultivars. It was seen that its amount was between 17.86-25.21%. Moreover, germacrene-D was determined in significant amount (15.40-21.13%) in both samples of *A. chamaepitys*. 2-hexen-1-al was the most abundant component in the natural area (30.89%) and cultivated plants (23.12%) in the first vegetation period of *A. orientalis* but a decrease of almost 80% was determined in the second vegetation period. The amount of 1-octen-3-ol was found as one of the dominant components in *A. orientalis* and no significant difference was observed in both natural habitat and cultivars, during the vegetation periods. In addition, the amount of limonene in *A. orientalis* volatile oil increased in the second vegetation period compared to the first.
Keywords: *Lamiaceae, SPME, GC-MS, pinene, germacrene, Turkey*

Introduction

Volatile oil mainly consists of volatile components. It is fragrant, naturally occurring, colorless or light yellow and it is obtained from plants, leaves, fruits, bark and root parts, which are liquid and easily crystallizable (Ceylan, 1983). It is known that volatile oil contains a large amount of terpenes and a small amount of alcohols, aldehydes, esters and phenolics (Linskens and Jackson, 1997). There are many ways to reveal the volatile oil components of plants. Although distillation and extraction are quite well-known methods, SPME (Solid Phase Micro Extraction) is used as it is new and easily applicable. It is suitable for both combining the sample preparation, extraction and concentration sections in a single step and for providing significant gains in processing time and cost. SPME is used to identify volatile organic compounds in the samples by combining with GC or GC-MS (Vas and Vekey, 2004).

Research on the volatile oil components of medicinal and aromatic plants has gained a significant place both scientifically and economically. In recent years, the use of volatile oil has also increased with the increase of interest in aromatherapy and phytotherapy, which is a branch of the alternative medicine (Rangahau, 2001).

Ajuga chamaepitys subsp. *chia* var. *chia* and *Ajuga orientalis* are species which belong to the *Lamiaceae* family. The *Lamiaceae* family grows almost everywhere in the Mediterranean climate without considering the plant type and height. *Lamiaceae* family has 400 genus and 3200 species around the world and it is represented by 45 genus and more than 546 species in Turkey. Among the species belonging to the *Lamiaceae* family, *Thymus* and *Origanum* have an important position in food industry, *Pogestamon* and *Lavandula* are considered in the perfume industry, and particularly the volatile oil

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

of the species of *Ajuga* and *Teucrium* is used for therapeutic purposes (Watson and Dallwitz, 1978; Clive and Stace, 1980; Davis, 1982; Baytop, 1991; Ozkan, 2007).

Ajuga chamaepitys subsp. *chia* var. *chia* can grow up to 2000 m altitudes in Turkey, Crimea, Greece, Palestine, Iran and Northern Iraq (Davis, 1982). It is a herbaceous plant with a 20 cm length, yellow flowering and pleasant fragrant perennial suffrutescens (Baytop, 1999). The length of the shoots is 5-30 cm. The whole plant is covered with white feathers and the flowers are yellow and the flowering time is between April and July (Akcan et al., 2006; Jakovljević et al., 2015; Dönmez et al., 2017).

Ajuga orientalis extends from Crimea to Sicily, West Syria, Cyprus, Caucasus and North West Iran. It can grow up to 2500 m from sea level in Mediterranean, Agean, Black Sea and Central Anatolia Regions in Turkey (Tekin, 2007). It is a perennial herbaceous plant with violet-blue and cream-colored flowers that can be sized up to 140 cm (Yazgın, 2010). The leaves are crossed on the trunk and rich in the lower part of the trunk. The flowering time is between April and May (Dönmez et al., 2017).

In this study, it was aimed to determine the composition and the amount of volatile oil and the yield of the same plant species in both natural habitat of the plants and their cultivars after the cultivation of *A. chamaepitys* subsp. *chia* var. *chia* and *A. orientalis*.

Material and method

Material

A. chamaepitys subsp. *chia* var. *chia* and *A. orientalis*, which belong to *Lamiaceae* family, were used for determining volatile oil yield and components. In the vegetation period of 2015, *A. chamaepitys* subsp. *chia* var. *chia* was taken from Aşağıgökdere, Isparta, where the altitude was 324 m (37º32´42´´ N, 30º46´42´´ E) and *A. orientalis* was removed from Kemer, Antalya, from an altitude of 413 m. (36º36´26´´ N, 30º27´53´´ E), with their roots. They were cultivated at Süleyman Demirel University (SDU), Botanical Garden by sowing. Plants, found in natural habitat and cultivars can be seen in *Figure 1*. In the vegetation period of 2016 and 2017, leaves and flowers of these plants, which were taken both from their natural habitat and cultivars at SDU, were used in the analyses of volatile oil production. The study area is located in the transition zone between the Mediterranean and terrestrial climate. According to data of Isparta Meteorological Station, the average annual temperature in the region was 12ºC. While the maximum temperature was 38.7ºC in July, and the minimum was -21ºC in January. The mean annual precipitation amount was 508.3 mm.

Volatile Oil Yield

In order to determine volatile oil yield, hydrodistillation with Clevenger apparatus was used. The material is directly immersed in water and it has direct contact with hot water and heat. For determining volatile oil yield of each species, 100 g ground samples were submitted to hydrodistillation for 5 h using a Clevenger apparatus. Volatile oil yield was calculated as ml/100 g samples.

SPME and GC-MS analyses

The leaves and flower samples that were collected from both natural habitat and cultivars were put into paper packages and transferred to the laboratory in the same day to avoid exposure to sunlight and samples were subjected to solid phase microextraction

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

(SPME). 2 g of samples were placed into a 10 ml vial. After incubation for 30 min at 60°C, SPME fibre was pushed through the headspace of a sample vial to absorb the volatiles and then inserted directly into the injection port of the GC-MS (Shimadzu 2010 Plus GC-MS with the capillary column, Restek Rxi®-5Sil MS 30 m x 0.25 mm, 0.25 µm) at a temperature of 250°C for desorption (5 min) of the adsorbed volatile compounds. The constituents were identified using retention times of standard substances by aligning mass spectra with the data given in the Wiley, NIST Tutor, FFNSC library.

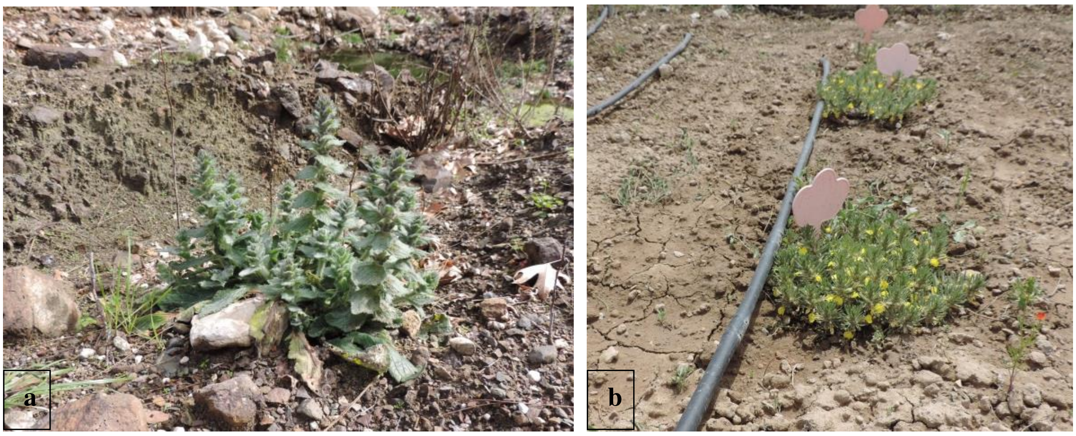

Figure 1. *A view from both natural habitat (a) of A. orientalis and cultivation (b) of A. chamaepitys subsp. chia var. chia*

Results and discussion

Analyses for getting volatile oil and determining volatile oil yield of both *Ajuga* species was performed in two vegetation periods (2016 and 2017) on the samples gathered from their natural habitat and cultivars at the same time. Volatile oil yield of both *Ajuga* species was determined after hydrodistillation. It was found that volatile oil yield of *A. chamaepitys* subsp. *chia* var. *chia* was determined very close in both samples during two vegetation periods. It was determined as 0.10 ml/100 g in natural area plants and 0.15 ml/100 g in field area plants in the first vegetation period. Moreover, it was 0.10 ml/100 g in both samples in the second vegetation period.

The volatile components in the leaves and flowers of *Ajuga* species collected from the sampling plots were identified through gas chromatography mass spectroscopy (GC-MS) after solid phase micro extraction (SPME).

The amount and the composition of volatile oil of *A. chamaepitys* subsp. *chia* var. *chia* can be seen in *Table 1*. 41 components in the samples from natural habitat and 39 components in the samples from cultivars of the first vegetation period were determined. However, 50 and 49 components, respectively, were identified in the second vegetation period. β-pinene was determined as the dominant component in all samples in both vegetation period of *A. chamaepitys* subsp. *chia* var. *chia*. While its amount was 19.15% in natural habitat and 17.86% in the samples from cultivars in the first vegetation period, it was found 22.25% in in natural habitat and 25.21 % in the samples from cultivars in the second vegetation period. Germacrene-D was the second component having highest amount. It was seen that its amount increased in the second vegetation period compared to the first vegetation period.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

Table 1. *The amount and the composition of A. chamaepitys subsp. chia var. chia volatile compounds (%)*

Compounds	1st Vegetation Period		2nd Vegetation Period	
	Amount (%)		Amount (%)	
	Natural Area	Field Area	Natural Area	Field Area
2-ethyl-Furan	0.22	0.43	-	-
Me-acetate	-	-	0.46	0.44
Linalyl acetate	-	-	0.08	0.04
Acetic acid	-	-	0.09	0.13
Me-propyl ketone	-	-	0.04	0.09
Me-heptyl ketone	-	-	0.09	0.12
n-Hexanal	0.48	0.78	-	-
2-Hexen-1-al	4.09	3.39	-	0.08
3-Hexene-1-ol	0.22	0.32	0.45	0.11
2-Hexen-1-ol	-	-	-	0.10
n-Hexanol	-	-	-	0.07
1,4-Cyclohexadiene	-	-	0.54	0.28
2,4-Hexadienal	0.81	0.51	-	-
α-Thujene	0.60	1.93	0.29	0.37
α-pinene	2.37	2.31	2.98	3.45
β-phellandrene	0.31	-	-	-
Benzaldehyde	-	0.20	0.02	0.03
Sabinene	-	0.64	0.29	0.34
β-pinene	19.15	17.86	22.25	25.21
1-Octen-3-ol	4.75	11.69	0.32	0.29
β-Myrcene	10.53	13.33	-	-
Myrcene	-	-	5.15	5.55
Ethyl-hexanol	0.19	1.00	1.52	-
l-Phellandrene	0.37	0.95	-	-
2,4-Heptadienal,	0.37	0.61	-	-
p-cymene	-	1.16	0.98	0.33
Me-cymol	0.14	-	1.23	-
Limonene	4.64	4.36	5.97	6.42
Trans-limonene oxide	-	-	0.41	0.04
Ocimene	0.25	0.23	-	-
β- ocimene	1.83	1.00	1.10	1.37
α-terpinolene	0.22	-	0.05	0.07
1,6-Octadien-3-ol	-	0.75	1.98	-
Nonanal	-	0.26	-	-
Dodecane	-	-	0.12	0.08
Butanoate	-	-	0.02	0.02
l-linalool	0.96	-	0.45	0.56
p-Mentha-1,5,8-triene	0.27	0.27	-	-
2-Pinen-3-one	-	0.22	-	-
2,4,6-octatriene	0.23	-	-	-
Benzoic acid	0.31	-	-	-
Tridecane	0.20	-	0.07	0.07
α-Cubebene	0.53	0.76	0.60	0.69
Copaene	3.71	8.10	-	-
α-Copaene	-	-	10.25	11.56
β-bourbonene	3.10	5.14	4.96	5.03
α-Gurjunene	0.12	-	0.78	0.80
1,2,4,5-tetramethyl-6-methylene-spiroheptane	0.14	-	-	-
β-caryophyllene	-	3.01	1.03	-
α-Amorphene	0.33	-	0.62	-
α-Chamigrene	0.26	-	-	-
p-Allylanisole	-	-	0.02	0.03
Cyclosativene	-	-	0.09	0.08
β-cubebene	-	0.95	0.52	0.15
α-Himachalene	-	0.34	-	-
Caryophyllene	12.17	-	5.82	5.99
Germacrene-D	19.04	15.40	19.63	21.13
β-Farnesene	-	0.48	-	-
Epi-bicyclo sesquiphellandrene	0.69	0.44	-	0.47
bicyclogermacrene	2.57	0.55	0.98	1.08

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DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

Compounds	1st Vegetation Period		2nd Vegetation Period	
	Amount (%)		Amount (%)	
	Natural Area	Field Area	Natural Area	Field Area
α-Guaiene	-	-	0.03	0.04
β-elemene	-	-	0.02	0.02
Torreyol	-	-	0.09	0.07
Eucalyptol	-	-	0.21	0.05
Solanone	-	-	0.03	0.03
α-farnesene	0.50	-	0.63	-
Farnesene	-	-	-	0.19
α-Muurolene	-	0.45	0.22	0.38
Caryophyllene oxide	0.39	0.22	-	-
γ-Cadinene	0.70	0.96	3.12	4.75
1,4-Cadinadiene	-	-	-	0.55
trans-Pinocarveol	-	-	0.14	0.09
δ-cadinene	1.17	1.27	0.48	0.74
α-Patchoulene	-	0.20	-	-
α-humulene	0.84	-	0.34	0.41
Alloaromadendrene	0.22	0.31	0.49	-

The amount of germacrene-D was 19.04% in the samples from natural habitat and 15.40% in the samples from cultivars in the first vegetation period. However, it was detected 19.63% and 21.13%, respectively, in the second vegetation period.

Although β-myrcene was determined only in the first vegetation period in both sampling areas, myrcene was seen only in the second vegetation period samples. Besides, caryophyllene was detected only in natural habitat samples with the amount of 12.17% in the first vegetation period. In the second vegetation period, the amount of caryophyllene decreased almost 50% compared to the first vegetation period samples. It was found 5.82% in the samples from natural habitat whereas it was 5.99% in the samples from cultivars.

Volatile oil of *Ajuga* species were determined by several researches. Azizan et al (2002) determined volatile oil components of *A. chamaepitys* subsp. *chia* var. *chia* by hydrodistillation. Totally 25 components were identified and α-pinene (16.1%), β-pinene (34.38%) and germacrene-D (5.6%) were found as the dominant components. Saijadi and Ghanhadi (2004) found germacrene-D (24.2%), β-cubebene (18.3%), β-caryophyllene (16.9%) and α-cubebene (5.3%) as the highest in *A. chamaepitys* subsp. *chia* var. *chia* volatile oil. It was another study done by Delazar et al (2012) in which α-pinene (23.66%), β-pinene (9.33%), β-phellandrene (8.70%) and germacrene-D (7.92%) were the dominant components of Iranian *A. chamaepitys* subsp. *chia* var. *chia* volatile oil.

Volatile oil yield of *A. orientalis* was 0.10 ml/100 g in the samples from natural habitat and 0.05 ml/100 g in the samples from cultivars in the first vegetation period. In the second vegetation period, volatile oil yield of natural habitat samples was 0.15 ml/100 g while it was the same as the former in the samples from cultivars. The amount and the composition of *A. orientalis* volatile oil determined by SPME and GC-MS can be seen in *Table 2*. In both samples of the first vegetation period, 44 components were identified. However, the identified components were 40 in the samples from natural habitat and 53 in the samples from cultivars in the second vegetation period.

While 2-hexen-1-al had the maximum amount in the first vegetation period in the samples from natural habitat, 1-octen-3-ol was determined as the highest amount component in the samples from cultivars.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

Table 2. *The amount and the composition of A. orientalis volatile compounds (%)*

Compounds	1st Vegetation Period		2nd Vegetation Period	
	Amount (%)		Amount (%)	
	Natural Area	Field Area	Natural Area	Field Area
Acetaldehyde	0.29	0.22	1.51	1.31
Borane-methyl sulfide complex	0.55	0.33	1.97	1.22
Amyl ethyl ketone	-	-	1.53	1.03
2-Butenal	1.32	0.90	0.41	0.26
3-Methylbutanal	0.48	0.65	0.47	0.61
2-Methylbutanal	0.33	0.61	0.47	0.52
4-methylpentanenitrile	0.50	0.50	-	0.33
2-ethyl-Furan	1.14	1.01	-	0.89
2-pentanal	0.54	0.25	-	0.33
2-Penten-1-ol	0.70	0.98	-	0.41
n-Hexanal	3.85	4.22	1.42	1.26
2-Hexen-1-al	30.89	23.12	6.19	7.23
3-Hexene-1-ol	1.42	3.43	-	-
2-Hexen-1-ol	0.87	1.90	-	-
n-Hexanol	2.24	3.54	1.10	0.91
Styrene	-	-	0.94	0.86
4-hepten-1-al	0.29	1.12	1.00	1.23
Heptanal	0.26	0.42	-	0.33
2,4-Hexadienal	0.83	0.99	-	0.67
α-pinene	0.98	1.12	8.29	7.46
Phenylmenthanal	-	-	1.28	0.98
2-heptanal	0.44	0.81	0.40	0.31
Benzaldehyde	1.26	1.22	-	0.83
Sabinene	0.26	0.83	0.33	0.22
1-hexene	0.97	0.56	-	-
β-pinene	-	-	0.65	0.58
Vinyl amyl ketone	-	-	0.52	0.49
1-Octen-3-ol	26.30	28.34	28.16	26.79
β-Myrcene	2.58	3.01	4.84	3.86
2,4-Heptadienal,	3.21	4.56	1.28	1.03
2,2-Pentenylfuran	0.39	0.44	-	-
Ethyl-hexanol	0.85	1.01	-	-
n-octanal	0.57	1.67	-	-
δ3-carene	1.14	1.85	3.42	3.01
n-octan-3-ol	-	-	1.71	1.77
1-hexyl acetate	-	-	1.03	1.01
Hexyl-Ethanoate	2.00	1.12	-	0.98
Cymol	-	-	0.73	0.61
Limonene	3.64	2.56	12.36	11.69
Trans-limonene oxide	-	-	-	1.03
Hyacinthin	-	-	0.32	0.21
2-octenal	0.50	1.32	-	-
3,5-octadien-2-one	0.93	1.22	0.69	0.41
α-terpinolene	0.30	0.10	2.51	1.89
Nonanal	2.76	0.99	0.27	0.64
Dodecane	0.30	0.10	0.59	0.24
n-Decanal	0.48	0.55	0.26	0.63
Benzene	0.75	0.62	-	-
n-Octyl acetate	0.70	0.81	-	0.83
hexyl 2-methylbutanoate	0.51	0.23	-	0.44
l-linalool	0.45	0.12	0.68	0.43
Hexyl butanoate	-	-	0.47	0.44
p-allylanisole	-	-	0.23	0.23
n-octyl 2-methyl butyrate	0.35	0.33	-	0.22
3-Buten-2-one	0.43	0.11	-	0.31
2-Butenoic acid	0.45	0.21	2.81	2.63
Pentanoic acid	-	-	1.09	0.93
Tridecane	-	-	0.64	0.66
Tetradecane	-	-	1.37	1.29
Valencene	-	-	1.45	1.53
α-farnesene	-	-	3.76	3.11

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DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

Moreover, 1-octen-3-ol was also dominant component of the second vegetation period samples with 28.16% in natural habitat and 26.79% in cultivars. Volatile oil composition and antimicrobial activity of *Ajuga orientalis* was presented by Yaldiz (2012). It was seen that germacrene-D, cubebene and caryophyllene were the major components of *A. orientalis* volatile oil.

Conclusions

A. chamaepitys subsp. *chia* var. *chia* and *Ajuga orientalis*, which grow naturally in Lake District in Turkey, were cultivated in 2015. Volatile oil analyses were carried out in 2016 and 2017 by using leaves and flowers of these plant species from both natural habitat and cultivars. It was seen that there were same components and the amount of components were close to each other in both sampling areas. It was concluded that these plants were cultivated without losing their chemical structure. This is a particularly important result for these two plant species belonging to the *Lamiaceae* family, which are known to be used as medicinal and aromatic plants. In this direction, it is thought that these plants can be produced in industrial sense and can also be used in different industries such as pharmaceuticals and cosmetics due to their chemical composition.

Acknowledgements. This work is a part of a project supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project number 114O345. The author thanks to Doc. Dr. İlhami Emrah Dönmez, Department of Forest Products Engineering, ISUBU, Turkey, for his support and discussing on the analyses.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38593866

THE EFFECT OF DIFFERENT CULTIVATION METHODS ON RICE GROWTH AND DEVELOPMENT

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(Received 23rd Jan 2019; accepted 18th Feb 2019)

Abstract. Cultivation is an important part in rice production which could affect rice growth and development significantly. In order to study the effect of different cultivation methods on rice performance, two land preparation ways (plough and rotary) and two rice growing techniques (direct-seed and transplanted rice) were used in present study and four cultivation methods were set as: (PD) the land was puddle twice with plough cultivator and pre-germinated seeds were hill-seeded with direct seeding machine; (RD) the land was puddle twice with rotary cultivator and pre-germinated seeds were hill-seeded with direct seeding machine; (PT) the land was puddle twice with plough cultivator and 15-day-old seedlings were hill-transplanted with transplant machine; (RT) the land was puddle twice with rotary cultivator and 15-day-old seedlings were hill-transplanted with transplant machine. The results showed that the highest yield was recorded in both PT and RT and the lowest yield was recorded in RD. There was no difference among different cultivation methods on net photosynthetic rate but different cultivation affected the transpiration rate and water use efficiency at different stage differently. Furthermore, the highest head rice rate and grain protein and the lowest chalky rice rate was all recorded in PD treatment. Overall, higher yield was recorded in transplanted rice cultivation than direct-seed cultivation and the greatest grain quality was observed under direct seeding rice cultivation and plough tillage conditions.
Keywords: *rice, tillage, cultivation methods, photosynthesis, yield, grain quality*

Introduction

Rice (*Oryza sativa.* L) is one of the main crops in Asia and the rice production system has become the backbone of Chinese food security system for many years (Xing et al., 2019; Wang et al., 2019). In 2017, China's rice planting area was 6.795 billion hectares (Han et al., 2019). However, the availability of arable land has been decreased in recent years and the increasing population has intensified the conflict for land planning between fiber and cereal crops (Fang et al., 2019). Therefore, experts are inevitably striving to find more ways to improve crop productivity.

Normally, land preparation is an important part in rice production because puddling is required to decrease the loss of water and nutrients through excessive percolation and it also is able to reduce weeds and enhance nutrient availability (Hazra et al., 2014; Alam et al., 2018). Previous studies already showed that soil puddling in paddy field benefits rice yield (Datta and Kerim, 1974; Kukal and Aggarwal, 2003). However, the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38673875

effect of puddling is still not clear regarding rice growth and development despite the benefits.

Normally, mechanical puddling is performed twice in Chinese paddy field, ploughing and rotary tillage. There also are two rice system which including direct seeding rice and transplanted-flooded rice in China. Those managements all had their own impacts on rice growth and development. For example, the study of Pan et al. (2017) indicated that direct seeding rice could be a more substitutive rice growing technique to transplanted-flooded rice with less production costs, labor and extra efforts for nursery raising, seedling uprooting, and transplanting. SUN (2017) demonstrated that ploughing tillage could help to improve the soil temperature, improve the environment of soil and promote the root activity and improve the production of tobacco leaf. But there was no much report about the effect of different puddling or growing technique on rice growth, development, yield and quality.

Thus, in order to study the effect of different cultivation methods on rice growth and development, present study was conducted in Guangdong Province, China with two mechanical puddling and two rice growing techniques.

Material and Methods

Plant materials and growing conditions

A fragrant rice cultivar, *Meixiangzhan-2*, which having a growth period of 111-114 days and widely planted in South China, was used in the experiment and planted at late season of 2017 in Zengcheng (23°13’ N, 113°81’ E, altitude 11 m), Guangdong. The experimental site enjoyed a subtropical monsoon climate with mean annual air temperatures of 21.3ºC and mean annual precipitation of 2660.9 mm. Before sowing, the seeds were soaked in water for 24 h, germinated in manual climatic boxes for another 12 h and shade-dried. Some of the germinated seeds were sown in polyvinyl chloride trays for nursery raising. The experimental soil in Zengcheng was sandy loam containing organic matter 10.04 g kg^{-1}, total N 0.50 g kg^{-1}, total P 0.26 g kg^{-1}, and total K 15.10 g kg^{-1}.

Treatment description and plant sampling

Two land preparations, ploughing(P) and rotary(R) were adopted with two growing techniques. The treatment description is as below:

- PD: Before planting, the land was puddle twice with plough cultivator. Pre-germinated seeds were hill-seeded with direct seeding machine at a space of 25×15 cm while each hill was planted with 4–6 seeds.
- RD: Before planting, the land was puddle twice with rotary cultivator. Pre-germinated seeds were hill-seeded with direct seeding machine at a space of 25×15 cm while each hill was planted with 4–6 seeds.
- PT: Before planting, the land was puddle twice with plough cultivator. 15-day-old seedlings were hill-transplanted with transplant machine at a space of 25 × 15 cm while each hill was transplanted with 4–6 seedlings.
- RT: Before planting, the land was puddle twice with rotary cultivator. 15-day-old seedlings were hill-transplanted with transplant machine at a space of 25 × 15 cm while each hill was transplanted with 4–6 seedlings.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The treatments were arranged in randomized complete block design (RCBD) in triplicate in each year with net plot size of 120 m^2. At tillering stage, heading stage and maturity, ten random rice plants were collected from each plot, for estimation of dry matter accumulation.

Photosynthesis

Portable photosynthesis system (CIRAS 2; PP systems, Amesbury, MA, USA) was used to determine net photosynthetic rate and gas exchange attribute i.e., stomatal conductance, intercellular carbon dioxide concentration, transpiration rate, water use efficiency and vapor pressure deficit at 09:00–10:30 a.m.

Grain quality

After sun drying, grains were stored at room temperature for at least a month to determine grain quality components. About 1.0 kg rice grains from each treatment was taken from storage and brown rice rate was estimated using a rice huller (Jiangsu, China) while milled rice and head rice recovery rates were calculated by using a Jingmi testing rice grader (Zhejiang, China). Grains with chalkiness and chalkiness degree were estimated by using an SDE-A light box (Guangzhou, China) while an Infratec-1241 grain analyzer (FOSS-TECATOR) was used to determine the grain amylose and protein contents.

Yield and yield related traits

At maturity stage, the rice grains were harvested from seven-unit sampling area (1 m^2) in each plot and then threshed by machine. The harvested grains were sun-dried and weighted in order to determinate the grain yield. Twenty hills of rice from different locations in each plot were sampled for estimate the average effective panicles number per hill. Then, eight hills representative plants were taken for estimation of the yield related traits.

Statistical analysis

Data were analyzed on Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) while differences among means were separated by using least significant difference (LSD) test at 5% probability level. Graphical representation was conducted via Sigma Plot 14.0 (Systat Software Inc., California, USA).

Result

Yield and yield related trails

As shown in *Table 1*, different cultivation methods affected rice yield and its related trails significantly. The highest yield was recorded in both PT and RT while there was no significant difference between RT and PT. The lowest yield was recorded in RD. The difference in rice yield among different cultivation could be explained by the yield related trails. For panicle number, the panicle number per hill in RT and PT was significantly higher than PD and RD; For grains number per panicle, the highest grains number was recorded in PD and grains number in RD and PT was remarkably number

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38673875

than PD; For seed-setting rate, the highest rate was recorded in PT; For grain weight, the 1000-grain weight in PD was significantly higher than RD, PT and RT.

Table 1. *The effect of different cultivation methods on rice yield and its related trails*

Treatment	Panicle number per hill	Grains number per panicle	Seed-setting rate (%)	1000-grain weight (g)	Yield (t ha^{-2})
PD	328.74±2.37b	117.87±4.22a	74.29±1.77b	22.08±0.31a	6.63±0.14b
RD	329.38±1.01b	107.33±4.15b	74.05±4.15b	20.74±0.27b	5.61±0.38c
PT	385.06±4.65a	106.80±6.99b	82.41±6.99a	20.84±0.05b	7.11±0.29ab
RT	395.73±18.51a	111.87±3.25ab	77.77±3.23ab	21.16±0.28b	7.43±0.29a

Values sharing a common letter within a column don’t differ significantly at (P≤ 0.05) according to least significant difference (LSD) test for both the years. The same as below

Grain quality

As shown in *Table 2*, different cultivation methods affected grain quality significantly. There was no remarkable difference among different cultivation methods in both brown rice rate and milled rice rate. However, the head rice rate in PD and RD was significantly higher than PT and RT. The moisture in PD and RD was significantly higher than PT and RT. The trend of crude protein content was recorded as: PD > RD > RT > PT. The lowest amylose content was recorded in RT while there was no significant difference among PD, RD and PT. Moreover, there was no remarkable difference among Akali and chalkiness whilst the trend of chalky rice rate was recorded as: RT > PT > RD = PD.

Table 2. *The effect of different cultivation methods on grain quality*

Treatment	Brown rice rate (%)	Milled rice rate (%)	Head rice rate (%)	Moisture (%)	Crude protein content (%)	Amylose content (%)	Akali	Chalky rice rate (%)	Chalkiness (%)
PD	77.12±0.87a	69.86±0.29a	33.39±0.87a	12.90±0.01a	8.87±0.07a	17.63±0.09a	6.37±0.03a	8.33±1.20c	14.17±1.17a
RD	77.50±0.28a	70.33±0.41a	36.45±0.45a	12.67±0.03b	8.50±0.01b	17.80±0.06a	6.47±0.09a	7.33±1.45c	11.33±0.88a
PT	78.20±0.22a	70.38±0.74a	24.21±1.21b	12.97±0.03a	7.87±0.07d	17.73±0.09a	6.30±0.01a	18.00±0.58b	13.00±1.32a
RT	77.50±0.41a	70.19±0.21a	22.60±0.51b	12.77±0.07b	8.10±0.01c	17.17±0.09b	6.37±0.03a	24.67±3.18a	10.67±1.09a

Plant height

As shown in *Figure 1*, the plant height of rice under different cultivation methods increased with the growth until 70 days after the sowing and then it gradually stabilized and remained unchanged. The trend of plant height was recorded as: PT = RT > PD > RD since 42 days after the sowing.

Photosynthesis and gas exchange

As shown in *Figure 2*, there was no remarkable difference in net photosynthetic rate among different cultivation methods at tillering stage, heading stage and maturity. Similar conditions were also observed in both intercellular carbon dioxide concentration and stomatal conductance. However, at tillering stage, the transpiration rate in RT was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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significantly higher than PD while the both the lowest vapor pressure deficit and water use efficiency were recorded in RT. Moreover, at maturity, both the highest transpiration rate and the lowest vapor pressure deficit were recorded in RD treatment.

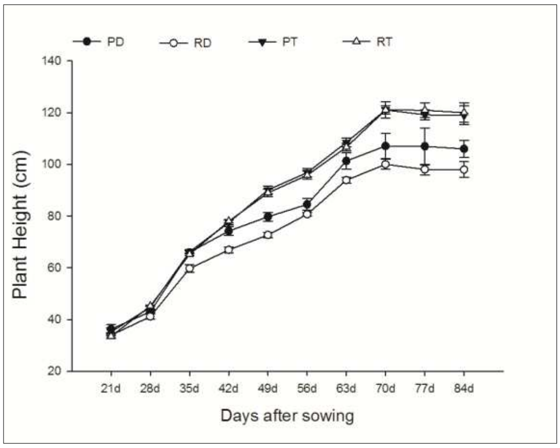

Figure 1. *The effect of different cultivation methods on rice plant height*

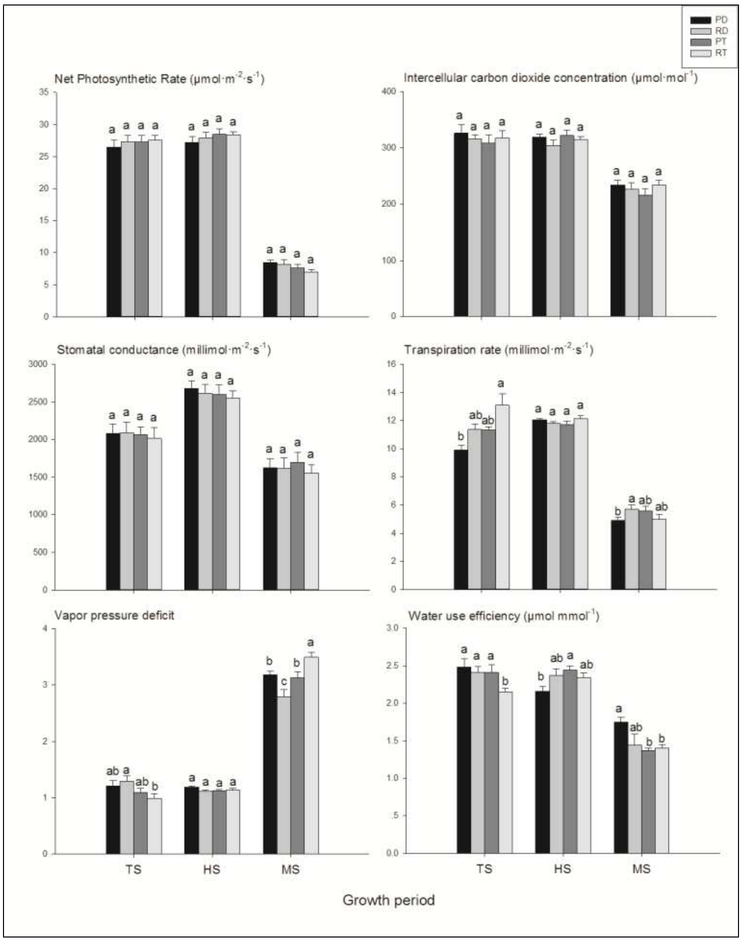

Figure 2. *The effect of different cultivation methods on rice photosynthesis and gas exchange. Means sharing a common letter don't differ significantly at (P≤ 0.05) according to least significant difference (LSD) test*

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DOI: http://dx.doi.org/10.15666/aeer/1702_38673875

Dry matter accumulation

Different cultivation methods affected dry matter weight differently (*Figure 3*). At heading stage, the dry matter weight in PT and RT were significantly higher than PD and RD. At maturity, the trend of dry matter weight was recorded as: PT = RT > PD > RD.

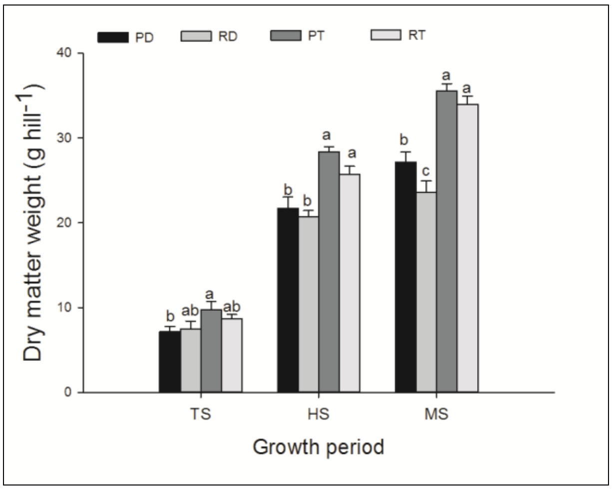

Figure 3. *The effect of different cultivation methods on dry matter accumulation of rice*

Discussion

Cultivation is a very important part in rice production. Some researchers already investigated the effects of some cultivation methods on rice performance and paddy fields. For example, Zhang et al. (2008) indicated that non-flooded mulching cultivation not only could increase root oxidation activity and photosynthesis, but also enhanced the activities of key enzymes in sucrose-to-starch conversion at filling stage. The study of Bouwman (1991) revealed that different cultivation methods could affect the methane emissions in paddy field by influencing the oxido-reduction capacity of the soil. Moreover, the study of Qin et al. (2006) showed that non-flooded rice cultivation was able to mediate rice growth, decrease the evapotranspiration and increase water use efficiency. In our study, there were also some differences among different cultivation methods in rice yield and yield related trails. The highest yield was recorded in both PT and RT while the rice yield in PT was significantly higher than PD just like RT was higher than RD. This discovery indicated that the transplanted-flooded rice cultivation had higher yield compared to direct seeding rice. The increment of yield in transplanted-flooded rice cultivation could be explained by the higher panicle number and seed-setting rate. The further reason might be the transplanted rice cultivation had the nursery raising part for seedling growth which direct seeding rice culitivation didn't. This result was consistant with previous studies which showed that nursery practice was benefit to early establishment for rice and could improve the rice yield potential (Sarangi et al., 2015; Zhang et al., 2017). Moreover, there was no signifcant difference in yield between PT and RT whilst the grain number, grain weight and yield in PD were signifcantly higher than RD. This discovery might indicated that the land prepation of plough tillage would be more suitable for direct seeding rice system.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38673875

Present study also showed that different cultivation methods also affected the grain quality significantly. Compared to PT and RT, PD and RD had higher head rice rates and crude protein contents. The lower chalky rice rates were also recorded in PD and RD than PT and RT. Grain quality is the main factor which depends the rice price in market (Ahmad et al., 2017; Norton et al., 2017). In our study, the result showed that the grain quality in direct seeding rice was higher than transplanted-flooded rice. Moreover, the highest grain protein content was recorded in PD and it might indicate that the environment of direct seeding rice cultivation and plough tillage would be the most suitable for protein content biosynthesis in grains. The reasons could be PD condition enhanced the activity of key enzymes involved in protein biosynthesis or improved the nitrogen absorption, utilization and transportation in rice (TsukaguchiNitta and Matsuno, 2016; Luo et al., 2018; Wang et al., 2018).

In our study, there was no significant difference among different cultivation methods in net photosynthetic rate. But interestingly, we found that different cultivation methods could alter the vapor pressure deficit in paddy field by affecting the transpiration rate of rice. The values of water use efficiency were also different under different cultivation methods. Those differences might be caused by plant-soil systems which were different because of the different land preparations and rice growing techniques (Pan et al., 2017; Wang et al., 2017).

Conclusion

Different cultivation methods affected rice yield and quality differently. Present study showed that the higher yield was recorded in transplanted rice cultivation than direct-seed cultivation and the greatest grain quality was observed under direct seeding rice cultivation and plough tillage conditions. In order to reveal the mechanism of how cultivation affected rice performance, more investigation should be couducted at the field trials or at molecular level before the real application.

Acknowledgements. This study was supported by the National Key Research and Development Program of China (2016YFD0700301), National Natural Science Foundation of China (31601225), Science and Technology Planning Project of Guangzhou, China (No. 201610010071), Science and Technology Planning Project of Yunfu, China (201702-2) and Special Funds for Scientific and Technological Innovation and Cultivation of University Students Guangdong (pdjh2019a0075).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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APPENDIX

Photos of the experiment

THE EFFECT OF PLANT GROWTH-PROMOTING BACTERIA ON THE DEVELOPMENT, YIELD AND YIELD COMPONENTS OF BREAD (*TRITICUM AESTIVUM* L.) AND DURUM (*TRITICUM DURUM*) WHEATS

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(Received 26th Jan 2019; accepted 27th Feb 2019)

Abstract. This study was carried out with 3 replications and 24 parcels according to randomized complete blocks trial design between 2017 and 2018 as two separate experiments. The study was planned to determine the effects of previously identified *Stenotrophomonas maltophilia* TV14B (phosphate solubilizing), *Bacillus atrophaeus* TV83D (nitrogen fixation), *Bacillus*-GC group TV119E (phosphate solubilizing), *Cellulomonas turbata* TV54A (nitrogen fixation) and combined effects of *Bacillus atrophaeus* TV83D and *Bacillus*-GC group TV119E on the development of Ceyhan-99 and Fırat-93 bread wheat cultivars. According to the results, the highest grain yield for Fırat-93 durum wheat cultivar was obtained under full dose chemical fertilizer application (288.89 kg/da); while the highest grain yield (283.33 kg/da) in the case of bacteria applications was observed with *Bacillus atrophaeus* TV83D, nitrogen-fixation bacteria. The difference between these two was not significant, and these bacteria can be seen as an alternative to chemical fertilization. The highest yield among bacterial applications was obtained under treatment with *Bacillus atrophaeus* TV83D, a nitrogen-fixation bacterium with 319.45 kg/da, and the difference between them was statistically significant. At the end of the study it was concluded that *Bacillus atrophaeus* TV83D bacterial strain can be used as a microbial fertilizer because of its ability to increase grain yield significantly in both genotypes.
Keywords: *biofertilizer, diazotrophs, PGP activities, P-solubilization, sustainable production, wheat cultivars*

Introduction

Cereals is a large group consisting of economically important species like the *Graminea* (wheats = corbels) family, cool climate cereals like wheat (*Triticum*), barley (*Hordeum*), oats (*Avena*), rye (*Secale*) and triticale, and warm climate cereals like corn (*Zea*), paddy (*Oryza*), maize (*Sorghum*), flint corn (*Panicum*) and avian (*Phalaris*) (Elçi et al., 1994). Wheat, corn and rice generate 85% of the world's grain production. Wheat is the most cultivated grain among these agricultural products providing high energy with grains containing plenty of starch. Wheat (*Triticum* sp.) is the most highly composite among cereals. Wheat is an indispensable plant used as a source of carbohydrates, providing basic input to livestock and industry. It is also the main food source of most countries with the largest cultivation area in the world.

Wheat constitutes approximately 29-30% of the world total grain production and is the most important plant-based protein source of humankind (Gustafson et al., 2009). Scientists consider its genetic complexity as a miracle, and which blends perfectly with changing environmental conditions. Wheat cultivars are the more superior in comparison with other grains and their nutritive values (Zohary and Hopf, 2000).

Global efforts to increase wheat production in parallel with population growth have been partially successful. Although this has also increased the yield of wheat, it is not sufficient to meet the needs of the growing population.

As the aim in crop production is to increase the dry matter yield per unit area, intensive input use increases in parallel. Fertilizer is one of the most important items among these inputs. It is determined that approximately 15% of the total chemical fertilizer used in the world is in wheat farming. There is an increasing trend towards the use of organic fertilizers worldwide to avoid the negative effects of chemical fertilizer use. In these organic fertilizers, Plant Growth-Promoting Bacteria (PGPB) is an alternative source of microbial fertilizers. In researches, especially *Pseudomonas* ssp., *Azospirillum* sp. and *Pantoea* sp. bacteria groups were found to increase plant growth and nitrogen intake in wheat and maize grown in temperate climates (Meena and Rai, 2017).

In recent years, the commercial formulations of these bacteria have been used in the cultivation of many plants such as corn, wheat, potatoes and tomatoes and it has been determined that these bacteria have a significant effect on plant height, root development and increase in dry matter. In addition, the use of these bacteria is predicted to reduce the use, cost and environmental pollution of chemical fertilizers and pesticides (Mehnaz et al., 2001, 2010; Tozlu et al., 2014). It has been reported that bacterial activity varies according to soil properties, soil organic matter content, plant and bacteria variety and plant growing conditions (Çakmakçı et al., 2006). In this respect, further studies are needed on the effects of bacteria on plant growth. In this study, the effect of Plant Growth Inhibiting Bacteria on the development, yield and yield components of wheat were investigated. It has been determined that the effect of the bacteria, which are revealed by field and greenhouse studies especially on wheat, has not been investigated at an adequate level before. In this study, the bacteria, which are isolated from Van Lake Basin and known to promote plant growth, are inoculated in bread and durum wheat varieties. Afterwards, their effects on wheat yield, yield and yield components were investigated.

One of the aims of this study was to quantify the productivity of dryland farmed bread and durum wheat cultivars that was seed inoculated. In addition, the effect of these bacteria on reducing the use of inorganic fertilizers was also examined.

Literature research

In many countries, the effect of Plant Growth-Promoting Bacteria (PGPB) on durability against biotic and abiotic stress on yield increase in cereals has been extensively researched and will continue to be researched more. Studies conducted in different countries and ecologies show that PGPB significantly increased wheat yield parameters in single applications (Naveed et al., 2008; Zabihi et al., 2011; Hussain et al., 2016; Mukhtar et al., 2017; Sood et al., 2018; Tahir et al., 2018). In addition, it has been reported that biomass, grain yield, macro and micronutrient intake increased significantly compared to the control in studies where different bacteria were administered in multiple combination (Zabihi et al., 2011; Sood et al., 2018). In addition to the yield parameters of wheat, PGPB has been reported to increase the number of germination, increase in germination speed and germination rate (Rana et al., 2011; Laloo et al., 2017), increase the amount of capillary root (Dal Cortivo et al., 2017), increase the root length (Afzal and Bano, 2008; Abbasi et al., 2011; Dal Cortivo et al.,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

2017), increase the height of the plant (Afzal and Bano, 2008; Abbasi et al., 2011; Mukhtar et al., 2017) and increase the root and body dry weight (Mukhtar et al., 2017). In addition, plant growth-promoting bacteria containing 1-Aminocyclopropane-1-Carboxylate Deaminase (ACCD), which provides resistance to plant stress conditions, contribute to the growth and development of plants normally by reducing plant ethylene levels under various environmental stress conditions (Çakmakçı, 2009). In the study conducted with Indol Acetic Acid (IAA), siderefor, Hydrocyanic Acid (HCN) and ACC deaminase producing bacteria, germination rate and speed of the bacterium producing siderophore (Rana et al., 2011) was observed to increase. In addition, it has been reported that the bacteria with ACC deaminase activity have a significant increase in the yield parameters (Naveed et al., 2008).

It is aimed to reduce the amount of chemical fertilizers by using PGPB since chemical fertilizers cause significant environmental problems during and after their application to agriculture areas and also due to the use of intensive fossil fuels in their production. For this purpose, it was revealed in the studies conducted on wheat that the use of chemical fertilizers could be reduced by 20% to 50% with the usage PGPBs (Naveed et al., 2008; Zabihi et al., 2011; Abbasi et al., 2011; Hussain et al., 2016; Sood et al., 2018). In a study, it has been reported that PGPBs increase the yield of grain by 30-40% according to chemical phosphorous fertilization (Afzal and Bano, 2008).

Materials and methods

Materials

Wheat species

In this study, Fırat-93 durum and Ceyhan-99 bread wheat varieties were used. Seeds were obtained from Seed Sales Office of General Directorate of Agricultural Enterprises. General characteristics of varieties are below.

Fırat-93: Medium plant height, very waxy and strong stems; narrow, long and steep leaf structure. Spike is medium length, white in color and has black stringiness. It has a fuller, hard and glassy grain and its thousand grain weight is between 45-50 g. It is suitable for summer, middle early and drought tolerant and has good tillering characteristics. It is recommended for rainy, irrigable areas and coastal areas of Southeastern Anatolia. It shows a tolerant reaction to yellow rust disease which can be seen from time to time in the region (Anonymous, 2018a).

Ceyhan-99: Plant height is between 90 and 100 cm. The spikes are white and lengths are medium, and they are upright. It is resistant to bending. Grain color is white, and it has a hard structure. The weight of thousand grains varies between 41 and 45 g. This wheat variety reacts well to fertilizers. Drought and winter resistances are moderate. It is resistant to yellow rust and *Septoria* diseases and it is half resistant to brown rust. Harvest-blending ability is high. The strings are not spilled at harvest time (Anonymous, 2018b).

Bacteria strains

The bacteria were isolated from the Van Lake basin with the TOVAG 108O147 TÜBİTAK project and their PGPB activity was detected (Erman et al., 2010). These bacteria were diagnosed with the microbial identification system (MIS) they were used as double combinations. These bacteria are: *Stenotrophomonas maltophilia* TV14B (phosphate solubilizing), *Bacillus atrophaeus* TV83D (nitrogen fixation), *Bacillus*-GC group TV119E (phosphate solubilizing), *Cellulomonas turbata* TV54A (nitrogen fixation) and *Bacillus atrophaeus* + *Bacillus*-GC group (TV83D + TV119E)

Methods

Climate and soil properties of the research site

The research was carried out in the laboratory of Field Crops Department of Agricultural Faculty of Siirt University in the period of 2017-2018 and in the trial area of Field Crops Department in dry agricultural conditions. The field trial was established at 37°58'2.18"K and 41°51'13.10"D coordinate point and 538 meters altitude.

In Siirt, terrestrial climate is dominant in general. Summers are hot and dry. The highest air temperature measured is 43.3 °C and the lowest air temperature is –19.5 °C. The average temperature, precipitation and relative humidity values of Siirt, where the research was conducted are presented in *Table 1*. These values cover the long years (1938-2017) as well as the period between October-June 2017-2018. As can be seen from *Table 1*, the 2017-2018 production season was generally dry and the rate of rainfall in March and April decreased. However, the rainfall in the month of May has been 84.6 mm higher than the average of many past years (Anonymous, 2018c). This situation affected the wheat yield negatively.

Table 1. *Average climate values of Siirt for long term averages in the past and for 2017-2018 October-June period*

Months	Temperature (°C)		Precipitation (mm)		Humidity (%)	
	LTA*	2017-18	LTA*	2017-18	LTA*	2017-18
October	17.9	18.4	49.1	5.4	50.3	34.6
November	10.4	11.2	81.4	85.0	64	64.4
December	4.8	8.0	94.4	48.6	72.4	65.2
January	2.6	5.7	96.8	56.4	72	70.5
February	4.2	8.2	97.5	74.2	66.6	67.7
March	8.3	13.7	111.1	47.6	61.3	55.9
April	13.7	16.8	104.7	61.6	58.2	47.6
May	19.3	19.8	62.0	146.6	49.9	58.9
Jun	26.0	27.4	9.2	3.0	34.1	31.3
Average	11.91	14.36	--	--	58.76	55.12
Total	--	--	706.2	528.4	--	--

*LTA = long-term average (1938-2017)

The soil structure of the trial area where the research is carried out is clayey, has a neutral pH, its lime content is very low, it is salt-free, its K content is sufficient, in terms of organic matter and phosphorus it is poor (*Table 2*).

Table 2. *Soil analysis values of the experiment area*

pH	Ec s/cm	Lime $CaCO_3$ %	Organic matter %	Phosphorus P_2O_5 kg/da	Potassium K_2O kg/da	Fe ppm	Cu ppm	Zn ppm	Mn ppm	Sand %	Clay %	Silt %	Texture
6.87	602	0.64	0.90	1.67	114	13.01	1.78	0.60	21.89	41.64	51.32	7.04	L

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Trial pattern

The trial consisted of 48 parcels in two separate trials consisting of 24 parcels of 1 × 5 m size with 3 replications, with bread and durum wheat according to the randomized block design.

Fertilizer application

Fertilization has been done to the bacteria untreated parcels as full fertilization with values of 9 kg N/da and 7 kg P_2O_5/da; half of these values were applied in 50% fertilization. In addition, 50% and 100% chemical fertilizers were applied without control and fertilization (0% fertilizer). No bacteria were applied to the parcels where the controls were located. The activity of the bacteria was determined according to the controls. All of the phosphorus and 1/3 of the nitrogen were given during planting and diammonium phosphate (18-46-0) was used. The remaining part of the nitrogen was given by urea fertilizer (46% N) during the period of tillering and stalking.

Bacteria application

In the experiment, within the scope of the TÜBİTAK project numbered TOVAG 108O147, nutrient agar (Merck-VM71680604), which was isolated from Van Lake basin and was previously diagnosed with MIS system, was used as a feed-lot for the multiplication of bacteria, PGPB activity of which was exposed to greenhouse and field conditions. 20 g of nutrient agar was added to one liter of distilled water, adjusted to pH 7.0, and the mixture was sterilized by autoclave for 15 min at 121 °C. After sterilization, the feed-lots were cooled to 50 °C, then transferred to petri plates and allowed to solidify. The stock cultures of the bacteria were planted in nutrient agar medium with the help of the needle and incubated at 26 ± 2 °C for 24 h (*Fig. 1*).

Figure 1. *Development of bacteria in nutrient agar and nutrient broth fed-lot*

The nutrient broth (Merck-VM775843711) was used as the liquid feed-lot. 8 g of nutrient broth feed-lot was added to one liter of distilled water and pH was adjusted to 7.0. The mixture was sterilized by autoclave for 15 min at 121 °C and then allowed to cool. A single colony was taken from the bacteria developed in nutrient agar feed-lot and was transferred to nutrient broth feed-lot in aseptic conditions. The bacteria transferred to the liquid feed-lot were incubated at 26 ± 2 °C for 24 h and at 120 rpm in

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the horizontal shaker. After incubation, the bacteria concentrations were turbidimetrically adjusted to ~ 10^8 cfu/ml (*Fig. 1*). In the final stage, the bacteria were inoculated into the seed. Surface sterilization of the seeds was made with 5% (v/v) sodium hypochlorite for 20 min and washed 3 times with distilled water. Bacteria were applied to the surface sterilized seed for 3 h in order to allow the swelling of the seed (Clark, 1965). Seeds were laid on drying sheets and allowed to dry. Dried seeds were planted on the field one day later. Sterile nutrient broth feed-lot was used as control.

Planting, care, harvest and threshing

The experiment was established on the experimental area of Agricultural Faculty of Siirt University in the 2017-2018 production season with three replications according to the randomized block design (*Fig. 2*).

The size of the parcels is 5 m, the width is 1 m (5 rows and between each row there is 20 cm) and each parcel area is 5 m^2. Seeds were planted in parcels as 500 seeds per m^2. In each parcel, gloves were used to prevent the bacteria from smearing on the seed surface. Experiment was established on 21.11.2017, and the plants sprouted on 01.12.2017 (*Fig. 3*). Weed control was done by hand. No disease or harmful situation has been observed in the trial area which could cause economic damage.

Figure 2. *Land parcelling and planting*

Figure 3. *Trial and parcel views*

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With the planting process, diammonium phosphate (DAP) was given as 7 kg phosphorus and 3 kg nitrogen per decare. According to the results of soil analysis, to meet the nutrients needed by plants; in the period of tillering and bolting, urea was applied in the form of upper fertilization with 3 kg of pure nitrogen. 9 kg pure nitrogen and 7 kg phosphorus were given to the plants during planting, tillering and bolting periods. One row on each side of the parcels and 50 cm of space at the beginning and end of the parcel were left and the remaining 2.4 m^2 area was harvested with the sickle on 01.07.2018. Then it was taken to the warehouse and blended with the machine.

Obtaining data

The experiment was continued during one growing season. Plant height (cm), spike length (cm), number of tillerings (pcs), number of spikes per square meter (pcs/m^2), number of spikelets per spike (number/spike), number of grains per spike (pieces/spike), weight of thousand grams (grams), grain yield (kg/da), biological yield (kg/da), hectoliter weight (kg/100 l), harvest index (%) values were determined based on the methods applied in the studies of Tosun et al. (1971) and Ünver (1995).

Statistical evaluation of the results

The mean values obtained from the data were analyzed using the Statix 10 package program. Grouping of means was done according to Tukey's HSD test.

Results

Plant height (cm)

In terms of applications, the highest plant height of Fırat-93 durum wheat was obtained from *Stenotrophomonas maltophilia* (TV14B) with 74.13 cm. and the lowest plant height was obtained from non-inoculated and non-fertilized control with 68.23 cm. Plant sizes in other applications are among these two values (*Table 3*).

Table 3. *Average values of plant height in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	68.23	71.67^b
50% fertilized control	70.76	78.20^a
100% fertilized control	72.43	77.33^a
Stenotrophomonas maltophilia (TV14B)	74.13	80.07^a
Bacillus atrophaeus (TV83D)	73.36	78.60^a
Bacillus-GC Group (TV119E)	73.71	79.47^a
Cellulomonas turbata (TV54A)	72.80	77.50^a
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	72.33	78.40^a
Average	72.22	77.65
P	**0.1002**	**0.0003**
Tukey's HSD	6.4455	4.3347

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The highest plant height of the Ceyhan-99 bread wheat was obtained from the application of *Stenotrophomonas maltophilia* (TV14B) with 80.07 cm. and the lowest plant height was obtained as 71.67 cm. from non-inoculated and non-fertilized control. Plant sizes in other applications are among these two values. Plant height in TV14B bacterial application was observed to be 11% higher than non-inoculated and non-fertilized control. However, in all bacterial applications, the height of the plant was observed to be higher than non-inoculated and non-fertilized control (*Table 3*).

Spike length

The highest spike length of the Fırat-93 durum wheat was obtained from *Bacillus*-GC Group (TV119E) bacteria with 5.92 cm. and the lowest spike length was obtained from non-inoculated and non-fertilized control as 5.23 cm. Spike length in other applications is among these two values. In addition, it was observed that other bacterial applications had a positive and significant effect on spike size compared to non-inoculated and non-fertilized control (*Table 4*).

Table 4. *Average values of spike size in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	5.23^{b}	6.45^{c}
50% fertilized control	5.38^{ab}	6.94^{bc}
100% fertilized control	5.42^{ab}	6.98^{bc}
Stenotrophomonas maltophilia (TV14B)	5.69^{ab}	7.90^{a}
Bacillus atrophaeus (TV83D)	5.84^{ab}	7.22^{ab}
Bacillus-GC Group (TV119E)	5.92^{a}	7.92^{a}
Cellulomonas turbata (TV54A)	5.86^{ab}	7.63^{ab}
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	5.77^{ab}	7.37^{ab}
Average	5.64	7.30
P	**0.0126**	**0.0001**
Tukey's HSD	0.6471	0.7668

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

The highest spike length of the Ceyhan-99 bread wheat was obtained from the *Bacillus*-GC Group (TV119E) bacteria with 7.92 cm. The lowest spike length was obtained from non-inoculated and non-fertilized control with 6.45 cm. Spike length in other applications is among these two values. It has been observed that bacterial applications have a positive and statistically significant effect on the increase of spike length compared to all three controls (*Table 4*).

Tillering count

In terms of applications, the highest number of tillerings of Fırat-93 durum wheat was obtained from 2.93 to 50% fertilized control application, while the lowest number of tillerings was obtained by *Cellulomonas turbata* (TV54A) with 2.52. The number of tillerings in other applications was among these two values. Only *Bacillus atrophaeus*

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(TV83D) (2.73 count) bacterial application increased in terms of the number of tillerings compared to non-inoculated and non-fertilized control (2.63 count) and other bacterial applications remained below this value (*Table 5*).

Table 5. *Average values of number of tillerings in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	**Fırat-93 durum wheat**	**Ceyhan-99 bread wheat**
Non-inoculated and non-fertilized control	2.63	2.07
50% fertilized control	2.93	2.30
100% fertilized control	2.76	3.27
Stenotrophomonas maltophilia (TV14B)	2.57	2.07
Bacillus atrophaeus (TV83D)	2.73	2.23
Bacillus-GC Group (TV119E)	2.60	2.50
Cellulomonas turbata (TV54A)	2.52	2.53
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	2.53	2.27
Average	2.66	2.40
P	**0.722**	**0.2748**
Tukey's HSD	0.8756	1.6299

The highest number of tillerings of Ceyhan-99 bread wheat was obtained from the application of 100% fertilized control with 3.7. The lowest number of tillerings was obtained with 2.07 from non-inoculated and non-fertilized control and *Stenotrophomonas maltophilia* application (TV14B). The number of tillerings in other applications was among these two values. With the exception of *Stenotrophomonas maltophilia* (TV14B), there were positive increases in other bacterial applications compared to non-inoculated and non-fertilized control (*Table 5*).

Number of spikes per square meter

In terms of applications, the highest number of spikes per square meter that belongs to Fırat-93 durum wheat was obtained as 450 from 50% fertilized control application. The lowest number of spike number for square meter was obtained from *Cellulomonas turbata* (TV54A) bacteria application with 291.67. In other applications, the number of spikes per square meter was between these two values. It has been found that bacterial applications generally do not contribute to the increase in the number of spikes per square meter compared to the controls, and that the number of spikes per square meter in all bacterial applications is lower than the general average (369.79) (*Table 6*).

The highest number of spikes per square meter of Ceyhan-99 bread wheat was obtained as 535.33 from 100% fertilized control application, while the lowest number of spikes were obtained as 370.00 from non-inoculated and non-fertilized control bacterial application. In other applications, the number of spikes per square meter was between these two values. It has been observed that bacterial applications have more spike numbers per square meters compared to non-inoculated and non-fertilized control. According to the results of the study, the highest number of spikes per square meter was obtained in chemical fertilizer applications (*Table 6*).

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Table 6. *Average values of spike number per square meter in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	360.00[ab]	370.00
50% fertilized control	450.00[a]	483.33
100% fertilized control	401.00[ab]	535.33
Stenotrophomonas maltophilia (TV14B)	365.00[ab]	421.67
Bacillus atrophaeus (TV83D)	391.67[ab]	456.67
Bacillus-GC Group (TV119E)	305.00[ab]	396.67
Cellulomonas turbata (TV54A)	291.67[b]	386.67
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	393.33[ab]	373.33
Average	369.79	427.96
P	**0.0331**	**0.1554**
Tukey's HSD	146.67	217.04

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

Spikelet count of the spike

In terms of applications, the highest number of spikelets that belong to Fırat-93 durum wheat was obtained from *Bacillus*-GC Group (TV119E) bacteria application with 18.10 and the lowest number of spikelets was obtained as 15.93% with 50% Fertilized Control application. In other applications, the number of spikelets was between these two values. However, it was observed that *Stenotrophomonas maltophilia* (TV14B) and *Bacillus atrophaeus* (TV83D) bacterial applications did not affect the increase in the number of spikelets (*Table 7*).

Table 7. *Average values of the number of spikelets per spike in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	16.90[ab]	14.43[b]
50% fertilized control	15.93[b]	15.70[ab]
100% fertilized control	16.77[b]	16.97[ab]
Stenotrophomonas maltophilia (TV14B)	16.53[b]	16.83[ab]
Bacillus atrophaeus (TV83D)	16.83[b]	16.93[ab]
Bacillus-GC Group (TV119E)	18.10[a]	16.87[ab]
Cellulomonas turbata (TV54A)	17.07[ab]	17.57[a]
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	16.47[b]	17.23[ab]
Average	16.82	16.56
P	**0.0025**	**0.0356**
Tukey's HSD	1.2782	2.8926

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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In terms of applications, the highest number of spikelets of Ceyhan-99 bread wheat was obtained from *Cellulomonas turbata* (TV54A) as 17.57 and the lowest number of spikelets was obtained from non-inoculated and non-fertilized control as 14.43. In other applications, the number of spikelets in the spike was between these two values (*Table 7*).

Grain count of the spike

In terms of the applications, the highest number of grains per spike for Fırat-93 durum wheat is 36.40 with *Bacillus*-GC Group (TV119E) bacterial application, the lowest number of grains per spike obtained as 27.93 with 50% Fertilized Control application. The number of grains per spike in other applications was between these two values (*Table 8*).

Table 8. *Average values of the number of grains per spike in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	**Fırat-93 durum wheat**	**Ceyhan-99 bread wheat**
Non-inoculated and non-fertilized control	28.70^{b}	25.63
50% fertilized control	27.93^{b}	29.90
100% fertilized control	29.13^{b}	31.67
Stenotrophomonas maltophilia (TV14B)	29.10^{b}	31.06
Bacillus atrophaeus (TV83D)	29.77^{ab}	33.03
Bacillus-GC Group (TV119E)	36.40^{a}	33.63
Cellulomonas turbata (TV54A)	30.87^{ab}	33.33
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	30.47^{ab}	34.53
Average	30.29	31.60
P	**0.0264**	**0.1686**
Tukey's HSD	7.2193	10.615

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

The highest number of grains per spike of Ceyhan-99 bread wheat is observed to be 34.54 with *Bacillus atrophaeus* + *Bacillus*-GC Group (TV83D + TV119E) bacterial application and the lowest grain number per spike was obtained as 25.63 with non-inoculated and non-fertilized control. The number of grains per spike in other applications was between these two values. The data obtained from all bacterial applications were found to be higher on average than the non-fertilized and non-inoculated and 50% fertilized control applications (*Table 8*).

Thousand grain weight

In terms of applications, the highest thousand grain weight of Fırat-93 durum wheat is obtained from 100% fertilized control application as 47.99 g. while the lowest thousand grain weight is obtained from *Bacillus atrophaeus* (TV83D) application as 43.88 g. The weight of one thousand grain in other applications was between these two values (*Table 9*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

Table 9. *Average values of thousand grain weight in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	46.30	38.45
50% fertilized control	46.17	38.81
100% fertilized control	47.99	38.45
Stenotrophomonas maltophilia (TV14B)	45.54	40.03
Bacillus atrophaeus (TV83D)	43.88	38.28
Bacillus-GC Group (TV119E)	45.87	39.30
Cellulomonas turbata (TV54A)	46.46	39.42
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	46.80	39.60
Average	46.13	39.00
P	**0.0706**	**0.0806**
Tukey's HSD	3.7015	2.268

The highest thousand grain weight of the Ceyhan-99 bread wheat was obtained from the application of *Stenotrophomonas maltophilia* (TV14B) application as 40.03 g. and the lowest thousand grain weight was obtained as 38.28 g. with *Bacillus atrophaeus* (TV83D) application. One thousand grain weight in other applications is among these two values (*Table 9*).

Grain yield

In terms of applications, the highest grain yield of Fırat-93 durum wheat was obtained from 100% fertilized control application as 288.89 kg/da. The lowest grain yield was obtained from *Bacillus atrophaeus* + *Bacillus*-GC Group (TV83D + TV119E) bacteria application as 218.75 kg/ha.

Table 10. *Average values of grain yield in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	243.06	256.95^{c}
50% fertilized control	256.25	354.17^{ab}
100% fertilized control	288.89	360.00^{a}
Stenotrophomonas maltophilia (TV14B)	275.69	304.08^{abc}
Bacillus atrophaeus (TV83D)	283.33	319.45^{abc}
Bacillus-GC Group (TV119E)	229.17	285.41^{abc}
Cellulomonas turbata (TV54A)	244.44	273.61^{bc}
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	218.75	274.30^{bc}
Average	254.95	303.50
P	**0.1174**	**0.0052**
Tukey's HSD	89.116	85.447

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DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

The grain yield in other applications was between these two values. In addition, *Bacillus atrophaeus* + *Bacillus*-GC Group (TV83D + TV119E) and *Bacillus*-GC Group (TV119E) bacterial applications were found to be scarcer and less effective on grain yield compared to the non-inoculated and non-fertilized control. However, yield in *Bacillus atrophaeus* (TV83D) (283.33 kg/da) and *Stenotrophomonas maltophilia* bacterial applications (TV14B) (275.69 kg/da) was found to be close to the full dose fertilizer application (288.89) (*Table 10*).

The highest grain yield of Ceyhan-99 bread wheat was obtained from 100% fertilized control as 360.00 kg/da and the lowest grain yield was obtained from non-inoculated and non-fertilized control application as 256.95 kg/da. The grain yield in other applications was between these two values. Bacterial applications were found to contribute to grain yield compared to non-inoculated and non-fertilized control, but the yield was 50% and 100% lower than fertilized control. It was observed that *Bacillus atrophaeus* (TV83D) bacterial application showed a 24% increase in yield compared to non-inoculated and non-fertilized control (*Table 10*).

Biological yield

In terms of applications, the highest biological yield of Fırat-93 durum wheat was obtained from *Stenotrophomonas maltophilia* (TV14B) bacteria as 756.95 kg/da. The lowest biological yield was obtained from *Bacillus atrophaeus* + *Bacillus* -GC Group (TV83D + TV119E) bacterial application as 565.97 kg/da. Biological yields in other applications are among these two values. *Bacillus*-GC Group (TV119E) and *Cellulomonas turbata* (TV54A) bacterial applications were found to have a negative effect on biological yield. It was observed that *Stenotrophomonas maltophilia* (TV14B) application had the highest biological yield (756.95 kg/da) and was found to be more efficient than the yield in full dose fertilizer application (752.78 kg/da) (*Table 11*).

Table 11. *Average values of biological yield in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	745.14^{a}	615.28^{c}
50% fertilized control	704.17^{ab}	850.00^{a}
100% fertilized control	752.78^{a}	794.00^{ab}
Stenotrophomonas maltophilia (TV14B)	756.95^{a}	672.92^{bc}
Bacillus atrophaeus (TV83D)	699.66^{ab}	704.17^{abc}
Bacillus-GC Group (TV119E)	577.78^{b}	653.47^{bc}
Cellulomonasturbata (TV54A)	627.78^{ab}	655.56^{bc}
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	565.97^{b}	654.86^{bc}
Average	678.78	700.09
p	**0.0017**	**0.0014**
Tukey's HSD	154.21	156.68

Mean values in columns shown with different letters are significant at $p < 0.05$ level according to Tukey's HSD test

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DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

The highest biological yield of Ceyhan-99 bread wheat was determined as 850.00 kg/da from 50% fertilized control application and the lowest biological yield was observed as 615.28 kg/da from non-inoculated and non-fertilized control application. Biological yields in other applications are among these two values. Bacterial applications were found to contribute to the increase in biological yield compared to non-inoculated and non-fertilized control (*Table 11*).

Hectoliter weight

In terms of applications, the highest hectoliter weight of Fırat-93 durum wheat was obtained as 77.60 kg with 50% fertilized control application, the lowest hectoliter weight was obtained as 74.13 kg from *Bacillus atrophaeus* + *Bacillus* -GC Group (TV83D + TV119E) bacterial application, hectoliter weight in other applications were among these two values. At the same time, according to the full dose fertilizer application (76.13 kg), *Stenotrophomonas maltophilia* (TV14B) bacterial application (77.60 kg) was found to be higher but not statistically significant (*Table 12*).

Table 12. *Average values of hectoliter weight in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains*

Application	**Fırat-93 durum wheat**	**Ceyhan-99 bread wheat**
Non-inoculated and NON-FERTILIZED CONTROL	76.80	75.33
50% fertilized control	76.00	77.47
100% fertilized control	76.13	74.73
Stenotrophomonas maltophilia (TV14B)	77.60	76.13
Bacillus atrophaeus (TV83D)	75.47	76.53
Bacillus-GC Group (TV119E)	74.13	76.00
Cellulomonas turbata (TV54A)	75.93	76.00
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	74.27	77.73
Average	75.79	76.24
P	**0.1276**	**0.2728**
Tukey's HSD	4.14	4.1987

The highest hectoliter weight of the Ceyhan-99 bread wheat was obtained as 77.73 g. with *Bacillus atrophaeus* + *Bacillus* -GC Group (TV83D + TV119E) bacterial application and the lowest hectoliter weight was obtained as 74.13 g. with *Stenotrophomonas maltophilia* (TV14B) bacterial application. The weight of hectoliter in other applications was between these two values. It was observed that the bacterial application with the highest values, *Bacillus atrophaeus* + *Bacillus* -GC Group (TV83D + TV119E) (77.73 kg), was higher than chemical fertilizer applications. In terms of hectoliter weight, which is an important quality parameter, *Bacillus atrophaeus* + *Bacillus*-GC Group (TV83D + TV119E) bacterial application having the highest value suggests that bacterial preparations may be an alternative to chemical fertilizer (*Table 12*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

Harvest index

In terms of applications, the highest harvest index of Fırat-93 durum wheat was obtained from *Bacillus atrophaeus* (TV83D) bacterial application as 40.16 and the lowest harvest index was obtained from non-inoculated and non-fertilized control application as 32.91. The harvest index in other applications is among these two values. In general, it was observed that all of the bacterial applications caused a statistically positive and significant increase in the harvest index compared to chemical fertilization (*Table 13*).

Table 13. *Average values of harvest index in Fırat-93 durum and Ceyhan-99 bread wheat varieties inoculated with different bacterial strains (%)*

Application	Fırat-93 durum wheat	Ceyhan-99 bread wheat
Non-inoculated and non-fertilized control	32.91	41.70
50% fertilized control	36.54	41.79
100% fertilized control	38.48	45.08
Stenotrophomonas maltophilia (TV14B)	36.60	45.02
Bacillus atrophaeus (TV83D)	40.16	45.34
Bacillus-GC Group (TV119E)	39.69	43.66
Cellulomonas turbata (TV54A)	39.25	41.94
Bacillus atrophaeus + *Bacillus* -GC Group (TV83D+TV119E)	38.71	41.95
Average	37.79	43.30
P	**0.1503**	**0.2919**
Tukey's HSD	8.65	7.0067

The highest harvest index of the Ceyhan-99 bread wheat was obtained from *Bacillus atrophaeus* (TV83D) bacterial application as 45.34% and the lowest harvest index was obtained from non-inoculated and non-fertilized control application as 41.70%. The harvest index in other applications is among these two values. The average of bacterial applications was generally higher than non-inoculated and non-fertilized control (*Table 13*).

Discussion

Considering the environmental hazards of chemical fertilizers, the fact that plant height values, which are known to increase with nitrogenous fertilizers, do not show a significant difference between the bacterial applications, indicates that the bacterial applications show promising effect. In addition to the fact that no bacterial application studies were performed on wheat before, it was stated that bacterial inoculation related to bacterial growth promotes the nitrogen content and vegetative development of the plant and this increases the plant height. (Şahin et al., 2004; Mukhtar et al., 2017; Kumar et al., 2014; Abbasi et al., 2011; Sultana et al., 2016; Arshadullah et al., 2017). Zafar-ul-Hye et al. (2017) reported that the bacterial applications increased the spike size.

In previous studies, it has been shown that inoculating the seeds in the form of double combination application results in significant increases in yield and yield

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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components compared to control (Darmwal and Gaur, 1988; Rai and Gaur, 1988; Şahin et al., 2004). Sood et al. (2018) reported in their study that bacterial application increased the number of tillerings by 28.3%. As a result of our study, it is thought that differentiation of bacterial strains, wheat varieties, climate and soil characteristics may cause in obtaining different findings. According to the results of our research, the highest number of spikes per square meter was obtained in chemical fertilizer applications. It has also been reported by the researchers that nitrogen contributes to healthy plant and spike formation by increasing vegetative growth in the plant and therefore increases the number of spikes per square meter (Millet and Feldman, 1984; Davidson and Chevalier, 1990; Öztürk et al., 2003; Salantur, 2003). Öztürk et al. (2003) investigated the effect of plant growth-promoting bacteria inoculation on wheat and barley yield at various nitrogen fertilization levels. They found that inoculation with *Bacillus* sp. OSU-142 significantly affected the number of spikelets in wheat spikes but did not have a significant effect on other characters.

It has been reported by many researchers that bacterial inoculation increases the number of grains per spike compared to non-inoculated and non-fertilized control (Öztürk et al., 2003; Salantur, 2003; Sood et al., 2018). In addition, it is said that double and single bacterial inoculation increases the number of grains in the spike (Dokuyucu et al., 1997; Afzal and Bano, 2008; Nain et al., 2010). Bhattarai and Hess (1993) and Das and Saha (2005) reported in their field study that bacterial applications were effective in increasing the thousand grain weight and combined inoculation was more effective than single inoculants. Although there is no similarity between the findings of the cited studies and the findings of the durum wheat variety, some researchers have reported that bacterial applications have a positive effect on the thousand grain weight (Zahir et al., 2009; Mukhtar et al., 2017; Sood et al., 2018). It is thought that this situation is caused by working with different bacteria and varieties.

Many researchers have reported that bacterial inoculation increases grain yield (Barazani and Friedman, 1999; Şahin et al., 2004; Çakmakçı et al., 2005; Çakmakçı et al., 2007; Behera and Rautaray, 2010). In this respect, the results obtained are similar to the previous studies. In previous studies, it has been reported by many researchers that chemical fertilization and bacterial inoculation provide increases in biological yields according to control (Kumar and Ahlawat, 2006; Sultana et al., 2016; Dos Santos et al., 2017; MeCarty et al., 2017; Inwati et al., 2018; Sood et al., 2018). Çığ (2011) reported that bacterial applications do not affect hectoliter. The researcher also stated that the two-bacterial applications cause an increase in the harvest index, but the single bacterial application showed the lowest value.

In terms of the effect of bacteria on varieties; *Bacillus*-GC groupTV119E bacteria application in durum wheat on spike size, spikelet count, the grain count on the spike has been found to have a statistically significant positive effect. In bread wheat, it was observed that it has a significant positive effect on spike size. *Stenotrophomonas maltophilia* TV14B (phosphate solubilizing) bacterial application statistically significantly increased the biological yield of durum wheat and plant height in bread wheat varieties. *Bacillus atrophaeus* TV83D (nitrogen-fixation) bacterial application has been found to have a statistically significant effect on the increase of the biological yield of bread wheat. None of the bacterial applications had statistically significant effect on the number of tillerings, weight of thousand grains, hectoliter weight and harvest index in both varieties.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

All bacterial applications in the bread wheat varieties increased the grain yield statistically significantly compared to the non-inoculated and non-fertilized control. In particular, bacterial application of *Bacillus atrophaeus* (TV83D) increased yield by 24% compared to non-inoculated and non-fertilized control. This bacterial strain also yielded an increase on yield in durum wheat, but it was not found to be statistically significant compared to other applications. The fact that durum wheat grain yield (283.33 kg/da) is close to 100% chemical fertilization (288.89 kg/da) shows that this bacterium significantly contributes to yield. In addition, the yield of durum wheat varieties in 100% chemical fertilizer (288.89 kg/da) was less than the bread wheat (360.00 kg/da). It is believed that this situation may be caused by the fact that the water demand for durum wheat is higher than that of bread wheat and the drought in 2018 spring months has a negative effect on the development of durum wheat.

As a result, bacterial applications significantly increased the yield in bread wheat compared to non-inoculated and non-fertilized control. In addition, it has been determined that bacterial applications show different reactions according to varieties and different bacteria have different effects on plant yield. The highest grain yield increase among microorganism applications was obtained from *Bacillus atrophaeus* (TV83D) bacterial application. In this respect, it is thought that this bacterial strain can be used as a bio-fertilizer material.

Conclusion

At the end of the study, mainly *Bacillus atrophaeus* (TV83D) which has a positive effect on all parameters, Bacillus-GC group TV119E and *Stenotrophomonas maltophilia* TV14B bacteria strains were found to be promising. However, in order to determine the effectiveness of the bacteria used in the study, it is thought that field trials should be established on different plant species and under different ecological conditions, at least for two years. In addition, in later studies, it is thought that testing of bacterial applications and other organic fertilizers (barn, worm etc.) will contribute to revealing the potential of these bacteria.

Acknowledgements. This study is Ms. thesis and is supported with the project no 2018- SİÜFEB-007 by Siirt University Coordinator of Scientific Research and Projects.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_38773896

LEAD CONTAMINATION AND BIOACCUMULATION IN A LOTIC ECOSYSTEM AROUND AN ABANDONED MINING AREA OF KHLI TI CREEK, KANCHANABURI PROVINCE, THAILAND

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(Received 16th Apr 2018; accepted 26th Feb 2019)

Abstract. This research investigated the lead contents of the water, sediment and freshwater organisms around an abandoned mining area in Kanchanaburi province, western Thailand. Samples were collected in 2017 during the monsoon season from three zones (upstream or control, middle-stream or inferred source and downstream or impacted zone) of Khli Ti creek. We found that the lead concentration of the water was the highest (0.0549 mg/l) on average in the downstream zone, and slightly exceeded the national standard level. In the sediment, the lead content was the highest (31,937.30 mg/kg) in the middle-stream zone and was approximately 25 times higher than the national standard level. The means of other selected heavy metal concentrations in the sediment were the following in descending order: Fe>Zn>As>Cd>Hg. The results also revealed lead accumulation in freshwater flora and fauna (plankton, macroinvertebrates, fish and macrophytes). Macroinvertebrates, in the middle-stream zone in particular, had the highest lead contents. Our study also showed the potential biomagnification of lead through the food chain. High contents of lead in organisms may cause both acute and chronic effects on stream dwellers on which further detailed research is needed. Lastly, the consumption of freshwater organisms by the local community should be avoided to reduce any health risks.
Keywords: *environment, heavy metals, invertebrate, stream, water quality*

Introduction

Mining is important for the development of the country since minerals and metal ores such as lead, coal and potash can be used as valuable raw materials for various industries. However, mining operations such as mineral dressing and smelting processes can be a major cause of heavy metal contamination in freshwater bodies because heavy metals are non-biodegradable and can persist in the environment for a very long time (Horowitz et al., 1993; Mazej et al., 2010; Thorslund et al., 2012).

Long term accumulation of heavy metals such as lead, copper and cadmium can reach elevated toxic levels even after the mine has closed, thus creating negative environmental problems (Dudka and Adriano, 1995). Several studies have shown that the water and sediment in lakes and rivers are contaminated by heavy metals. For example, in India, downstream sites in the Yamuna River had high concentrations of cadmium, chromium, iron, nickel and these values exceeded the standard maximum permissible limits prescribed for drinking water (Kaushik et al., 2009). In the Tigris River, Turkey, contamination of arsenic, cadmium, chromium, copper, manganese, nickel and zinc derived from anthropogenic sources was found in sediments (Varol, 2011). In Thailand, manganese (40-382 µg/l) and arsenic (0.68-8.79 µg/l) concentrations in Phayao Lake, northern Thailand, were close to the maxima for

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DOI: http://dx.doi.org/10.15666/aeer/1702_38973908

minimum drinking water standards proposed by USEPA and WHO (Tupwongse et al., 2007).

Heavy metal contamination can also impact biotic components through bioaccumulation and biomagnification (Miller et al., 1992; Goodyear and McNeill, 1999; Mazej et al., 2010). Producers, consumers and predators can be contaminated by heavy metals (Nagayama et al., 2010) through 1) consumption of food and water and 2) from direct exposure via gills and tissue surfaces (Newman and McIntosh, 1991; Goodyear and McNeill, 1999; Lee et al., 2012). The study of Moiseenko and Kudryavtseva (2001) revealed that fish in Russian lakes were contaminated with high concentrations of nickel and copper and showed kidney pathologies. In Thailand, several species of fish (*Rasbora tornieri* and *Systomus rubripinnis*) and frog (*Kaloula pulvhra* and *Microhyla heymonsi*) have taken up metals such as copper and manganese from the environment around a gold mine area in Loei province (Intamat et al., 2016). Furthermore, heavy metals can biomagnify in aquatic food webs when the heavy metal concentration increases through the trophic levels (Chen et al., 2000; Barwick and Maher, 2003). For example, in the newly-formed wetlands of the Yellow River Delta of China, heavy metals such as cadmium and zinc increased with the trophic level (Cui et al., 2011).

In Thailand, it is known that Kanchanaburi province is a major important source of lead. Lead mining operations in Khli Ti village started in the mid 1960s and then in 1998 the mining was ordered to close due to leakages of toxicants from tailing ponds to Khli Ti creek and surrounding environment (Department of Pollution Control, 2009; Greenpeace Thailand, 2012). Lead and other associate heavy metals from mining are toxic and could cause adverse impacts to the environment and human health if present in large quantities. Therefore, the objectives of this study were to assess the present state of spatial distribution of lead contents in the water and sediment environment of Khli Ti creek and to determine any lead contamination in terms of bioaccumulation and biomagnification in freshwater flora and fauna. The results will be useful to understand lead accumulation in the environment and freshwater organisms and to evaluate potential lead transfer pathways from producers to consumers in a lotic food web.

Materials and methods

Study site

Khli Ti creek is located in Chalae sub-district, Thong Pha Phum district, Kanchanaburi province, western Thailand. Its total length is 22 km and the water flows from north to south ending in the Srinakharind Dam. Sampling locations were divided into three zones: 1) sampling point I in the upstream zone or reference site (14.962309, 98.909397); 2) sampling point II in the middle-stream zone or source of contamination in an old abandoned lead mining area (14.955716, 98.921583); and 3) sampling point III in the downstream zone or impacted site (14.878604, 98.948969) (*Figs. 1, 2*). There were five sub-sampling points in each zone and therefore in total there were 15 sub-sampling points along the creek. Data were collected only one time in the rainy season of 2017 due to high water level and suitable time for sampling.

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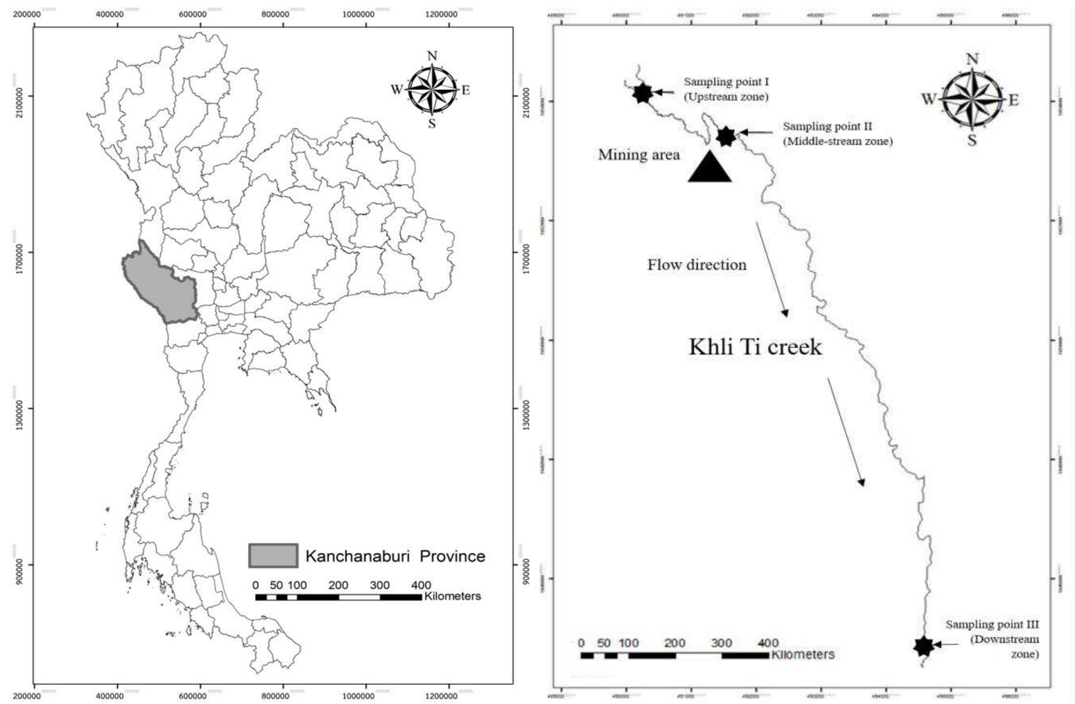

Figure 1. *Map of Khli Ti creek (left) and sampling locations in up, middle- and downstream zones (right)*

Figure 2. *Sampling points around old mining area*

Water and sediment sampling

At each sampling point, we used a multi-parameter analyzer (Consort 663) to measure the water temperature, pH, dissolved oxygen (mg/l), total dissolved solid (mg/l) and conductivity (µs/cm). Transparency was measured using a Secchi disk and 2 L of water were also collected in plastic bottles from each point and then nitric acid was added as preservative and the samples were kept in a cool container for further analysis of total nitrogen (mg/l) and total phosphorus (mg/l) at the Department of Soil Science, Kasetsart University, Bangkok, Thailand. Another 1 L water sample was taken and preserved with nitric acid for lead (mg/l) analysis at the Central Laboratory,

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Bangkok using an in-house method TE-CH-126 based on standard methods for the examination of water and wastewater (APHA, AWWA, WEF 21st edition 2012 Part 3030E) (APHA, 2005) and AOAC (2016) using an inductively coupled plasma mass spectrometer (ICP-MS). Contents of chlorophyll a (mg/l) were determined using acetone attraction at the Department of Environmental Technology and Management, Faculty of Environment, Kasetsart University, Bangkok, Thailand. All analyses were done in triplicate.

Sediment sampling was conducted at the same water sampling points. Approx. 1 kg of sediment was collected and transferred to a plastic bag. Sediment samples were air dried, ground into fine powder and sieved through a 0.2-mm sieve. Prepared sediment samples were then analyzed for pH, electrical conductivity (EC, µs/cm) using an electrical conductivity meter, organic matter content (%) using Walkley and Black titration (Walkley and Black, 1934) and soil texture using the pipette method at the Department of Environmental Technology and Management. Sediment samples (20 g from each sampling point) were also sent to the Central Laboratory for analysis of heavy metals (lead (Pb), iron (Fe), arsenic (As), mercury (Hg), and zinc (Zn)) using in-house method TE-CH-329 based on the Environmental Protection Agency (EPA) 3052 standard.

Biotic sampling

We collected biotic samples and measured their lead contents. Plankton (both phytoplankton and zooplankton) were collected by pouring 50 L of water through a plankton net with a mesh size of 20 µm and 67 µm. Subsequently, the water was filtered again through Whatman GF/C and kept in a cool plastic container. Benthic macroinvertebrates (mainly shrimp, mollusc, dragonfly nymph and crab) were taken from the sediment using an Ekman grab and invertebrates associated with plants were collected using a hand net (1 min per sampling). Plant and other materials collected from sampling were separated and removed by hand and animals were sorted and kept in plastic bags in an ice box. Fish specimens were collected using gill nets left overnight in the creek and the following day fish samples were retrieved. Samples of fish were kept in an ice container. Freshwater plants present in the creek (mainly taro) were sampled from a 1×1 m quadrat and whole plant specimens were collected in plastic bags. Plants were identified to the Family level by the Forest Herbarium, Bangkok, Thailand. All biotic samples were sent to the Central Laboratory for analysis of lead contents using in-house method TE-CH-134 based on AOAC (2016) 999.10 and the ICP-MS. Then bioaccumulation factors (BAF) were calculated based on (USEPA, 2001) by using the lead content in aquatic animals (mg/kg) divided by the lead concentration in water (mg/l). In addition, biotransference factors (BTF) or biomagnification factors were obtained from the calculation of the lead content in selected consumers or predators divided by that of the selected producer or prey (mg/kg) according to Laskowski (1991).

Data analysis

Values were reported as mean±standard deviation (n=5) throughout. Data distribution was tested by using Test of Homogeneity of Variance and Robust Tests of Equality of Mean (Welch) and then Post Hoc Multiple Comparison (Turkey) was applied to analyze which parameters or variables have different statistical significance among the upstream, middle-stream and downstream zones.

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Results

Water and sediment properties

The results of water quality analysis are shown in *Table 1*. The water temperature was rather low. Transparency was in the range 0.27-0.66 m and corresponded well with the total suspended solids. The pH values were neutral and tended to be higher in the downstream zone. The dissolved oxygen contents were relatively high from the upstream to downstream zones. The total nitrogen concentration was high in the upstream zone and gradually decreased toward the downstream zone whereas the total phosphorus concentration was comparable among zones. Significant differences in water quality parameters (temperature, transparency, pH, conductivity, TDS and TSS) were found among zones.

Table 1. *Water quality analysis in Khli Ti creek (n=5)*

Parameter	Upstream zone	Middle-stream zone	Downstream zone
Temperature (C)	$25.9±0.6^{a}$	$24.9±0.7^{b}$	$26.3±0.4^{a}$
Transparency (m)	$0.5±0.2^{a}$	$0.7±0.3^{a}$	$0.3±0.1^{ab}$
pH	$7.96±0.03^{a}$	$8.04±0.06^{b}$	$8.15±0.05^{c}$
Conductivity (µs/cm)	$378.2±10.8^{a}$	$409.0±9.7^{b}$	$428.8±3.3^{c}$
Dissolved oxygen (mg/l)	$12.28±0.65^{a}$	$12.72±0.62^{a}$	$12.32±0.38^{a}$
Total dissolved solid (mg/l)	$204.2±8.7^{a}$	$220.0±3.5^{b}$	$230.4±2.1^{c}$
Total suspended solid (mg/l)	$0.71±0.35^{a}$	$0.89±0.34^{a}$	$4.04±0.38^{b}$
Total nitrogen (mg/l)	$21.00±14.64^{a}$	$14.00±11.74^{a}$	$5.25±4.79^{a}$
Total phosphorus (mg/l)	$1.51±0.90^{a}$	$1.57±0.77^{a}$	$2.31±1.52^{a}$
Chlorophyll a (mg/l)	$0.51±0.46^{a}$	$0.40±0.06^{a}$	$0.33±0.0^{a}$

Mean values followed by different superscript letters are significantly different ($p < 0.05$)

Analysis of the sediment properties revealed that the pH values were neutral (6.9-7.2) (*Table 2*). Electrical conductivity was highest in the middle-stream zone and significantly different from that in the upstream and downstream zones. The organic contents of the upstream and middle-stream zones were significantly higher than in the downstream zone. The results of the sediment texture analysis disclosed that the bottom sediment was sandy loam. In particular, the sediment texture of the downstream zone had a lower percentage of sand and a higher percentage of clay and silt compared with the upstream and middle-stream zones.

Table 2. *Sediment quality analysis in Khli Ti creek (n=5)*

Parameter	Upstream zone	Middle-stream zone	Downstream zone
pH	$6.93±0.25^{a}$	$7.19±0.22^{a}$	$7.00±0.14^{a}$
EC (µs/cm)	$370.03±172.25^{a}$	$519.80±102.95^{ab}$	$259.33±55.55^{a}$
Organic content (%)	$76.59±8.46^{a}$	$74.60±5.42^{ab}$	$55.12±6.81^{a}$

Mean values followed by different superscript letters are significantly different ($p < 0.05$)

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The result of the lead content in the water showed that they were significantly different among zones. The lead content was lowest in the upstream zone (0.0037-0.0075 mg/l) and increased in the middle-stream zone (0.0132-0.0184 mg/l) being highest in the downstream zone (0.0476-0.0549 mg/l). Furthermore, in the sediment, the lead content was also highest in the middle-stream zone (2,175.04-31,937.30 mg/kg) and lower in the upstream (92.76-184.97 mg/kg) and downstream (504.02-1,514.99 mg/kg) zones. *Figure 3* shows the lead contents in the sediment among zones. The contents of cadmium, mercury and zinc were highest in the middle-stream zone, whereas the arsenic content was highest in the downstream zone (*Table 3*). The iron contents were saturated in all zones.

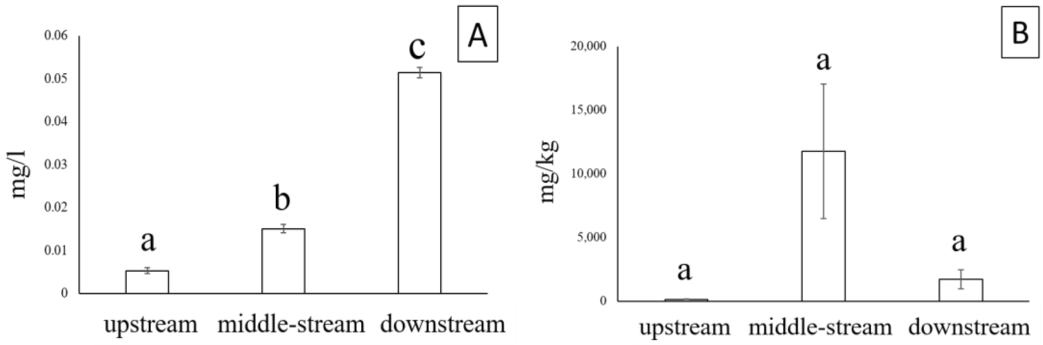

Figure 3. *Lead contents with standard error bars in water (A) and sediment (B) of Khli Ti creek*

Table 3. *Accumulation (mean±S.D.) and standard levels (mg/kg) of other heavy metals in sediment of Khli Ti creek (n=5)*

Heavy metal	Upstream zone	Middle-stream zone	Downstream zone	Standard level*
Arsenic (As)	15.44±5.16	20.28±1.82	35.36±4.06	3.9
Cadmium (Cd)	0.25±0.06	0.36±0.10	0.33±0.11	37
Zinc (Zn)	48.59±5.65	64.77±11.39	51.182±24.81	-
Mercury (Hg)	nd	0.1±0.08	0.05±0.03	23
Iron (Fe)	Saturated	Saturated	Saturated	-

*Department of Pollution Control (2004) and nd is non detectable

Lead contents in biotic samples

We found lead contamination in biotic samples and the degree of contamination in organisms increased in the downstream zone. In contrast, the lowest values foe the lead content in organisms were detected in the upstream zone. The highest lead contents in plankton and plants were found in the downstream zone whereas the lead contents in macroinvertebrates and fish appeared to be high in the middle-stream zone (*Table 4*).

Bioaccumulation and biomagnification

Table 5 presents the bioaccumulation and biomagnification results for lead in freshwater organisms. It shows that the highest bioaccumulation values were found in the middle-stream zone. Furthermore, on average, macrophytes had the highest bioaccumulation value followed by macroinvertebrates, plankton and fish, respectively. The biomagnification values increased from the lower trophic to higher trophic levels. In particular, the highest biomagnification value was in the middle-stream zone.

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Table 4. *Lead contents (mean±S.D., mg/kg) in biotic samples in Khli Ti creek (n=15)*

Parameter	Upstream zone	Middle-stream zone	Downstream zone	Standard level*
Plankton	6.93±0.25 [a]	7.19±0.22 [a]	7.00±0.14 [a]	-
Macroinvertebrates	370.03±172.25 [a]	519.80±102.95 [ab]	259.33±55.55 [a]	0.5
Fish	76.59±8.46 [a]	74.60±5.42 [ab]	55.12±6.81 [a]	0.2
Macrophytes	4.51±3.20 [a]	106.77±173.32 [a]	155.89±211.20 [a]	-

Mean values followed by different letters are significantly different ($p < 0.05$). *Department of Public Health (1986)

Table 5. *Bioaccumulation and biomagnification (mean±S.D. mg/kg) of lead in freshwater organisms along Khli Ti creek*

Factor	Upstream zone	Middle-stream zone	Downstream zone
Bioaccumulation			
Plankton	1,112.95±466.26	1,667.74±1,452.49	852.11±245.41
Macroinvertebrates	2,923.90±3,593.24	2,935.33±2215.91	635.64±595.77
Fish	329.02±153.35	238.68±99.08	76.43±33.56
Macrophytes	830.36±509.34	8,581.21±10,539.43	3,181.14±4,487.72
Biotransference			
Plankton- shrimp	0.5	1.91	0.1
Shrimp-dragonfly nymph	-	1.98	13.2
Dragonfly nymph-minnow	0.28	0.02	0.02
Minnow-needlefish	0.31	1.78	1.9

Discussion

Overall, the water quality values met the standards for inland surface water set by the Department of Pollution Control. The water temperature was rather low due to shading from forest cover. Water was also turbid as indicated by the low transparency and high suspended solid contents. This was likely due to erosion from the high discharge during the rainy season. The nitrogen content was relatively high in the upstream zone compared to the other zones, suggesting fertilizer runoff from agricultural areas. In contrast, the phosphorus content was high in the downstream zone. The chlorophyll a content did not reflect nutrient concentrations in the Creek and this could have been the result of the fast flow and suspended solids that inhibited phytoplankton growth. This was similar to the assertion by Mayer and Likens (1987) that algal production had a small portion of energy input in a shaded headwater stream.

The lead content results in the water in the downstream zone exceeded the water quality standard level (0.05 mg/l) of Department of Pollution Control (1992). This was rather consistent with the lead contents in the stream sediment that were relatively lower in the upstream zone and below the standard level for soil quality. However, in the middle-stream and downstream zones, the lead contents in the water were exceptionally higher than the standard levels and increased along with the distance from the inferred source indicating its accumulation.

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Contamination of lead in the water and environment reflects the impact from abandoned old mining at the inferred source (middle-stream) and in the downstream (impacted) area. Our study also revealed that the lead contents in the sediment were very high, similar to previous studies (Department of Pollution Control, 2009). In particular, the highest lead content was detected around the old abandoned mining area in the middle-stream zone, similar to Gomes Ribeiro et al. (2017). The detected level of lead in the middle-stream zone was approximately 25 times higher than the standard level (400 mg/kg) for soil quality set by Department of Pollution Control (2004) for Thailand and much higher than in other studies (Beane et al., 2016; Pavlowsky et al., 2017). The lead content in the sediment of the downstream zone also exceeded the standard level reflecting the fact that contaminated sediment can be carried downstream from the inferred source by stream flow. This is consistent with the results for the lead concentration in the water that was highest in the downstream zone as mentioned earlier. A similar case was reported for metal-polymetallic tin mining of the Potosí deposits in Southern Bolivia which began operation in 1945 and has led to severe contamination of the Pilcomayo River's water and sediments for at least 200 km downstream of the mines (Miller et al., 2004). The study of Pavlowsky et al. (2017) also showed that the lead concentrations in the channel and floodplain sediments were above the background levels from mine sources for up to 171 km downstream.

The analyzed sediment data also revealed other heavy metal contents which were high in the middle-stream and downstream zones. In particular, the arsenic and zinc contents were higher than for the other metals and this could have been due to these metal forms usually being associated with mining (Davies, 1987; Ferreira da Silva et al., 2004). Iron contents are usually abundant (>10 g/kg) in this area.

This study revealed the accumulation of lead in organisms from producers to consumers that were higher than in previous studies (*Table 6*). We found the lead content in plankton was relatively lower than in other organisms. However, it should be noted that the lead content from plankton could be a combination of plankton and suspended sediment since some sediment could not be separated through filtration.

Table 6. *Lead contamination ranges in the environment and freshwater organisms in Khli Ti creek from previous years*

Lead content	Year						
	1998	**1999**	**2000-2003**	**2004-2007**	**2008**	**2009**	**2017***
Water (mg/l)	0.005-0.55	0.003-0.08	0.001-0.256	0.005-0.287	0.005-0.06	0.007-0.896	0.0037-0.0549
Sediment (mg/kg)	402-65,771	228-68,920	152-118,872	115-143,097	145-105,943	141-117,000	92.76-31,937.30
Fish (mg/kg)	5.8-81.8	-	-	1.65-8.85	0.4-8.48	0.08-16.3	55.12-76.59
Crab (mg/kg)	222.8-451.8	-	-	13.4-52	10.8	13.6-82.6	11.16-52.77
Shrimp (mg/kg)	130.17	-	-	42.5-164	4.14-22.9	0.11-4.27	1.23-16.16
Mollusc (mg/kg)	125.6	-	-	42.3-112	59.8	186-471	3.17-91.42

Source: Department of Pollution Control (2009) and data of 2017 are from this study

Freshwater macrophytes had also a high content of lead due to their assimilation through root uptake (Pourrut et al., 2011) from the contaminated sediment. Among freshwater organisms, macroinvertebrates appeared to be the most affected by lead

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contamination (Beane et al., 2016) because they dwell in the benthic zone and thus have a greater chance of exposure to lead accumulation through feeding and direct contact (Yoo-iam et al., 2014). The highest lead content in macroinvertebrates was detected in the middle-stream zone. In a higher trophic level, fish had a much lower lead content than macroinvertebrates. Therefore, it could be stated that the contents of lead in the sediment were somewhat paralleled by their content in benthic macroinvertebrates as was also observed in water (Kiffney and Clements, 1993). The BAF values in macrophytes were high due to direct uptake through roots since the higher heavy metal concentration in the soil is, the higher will be the concentration in plants (Davies, 1987). In addition, lead accumulating in organisms is of concern, as all organisms had higher lead contents than the recommended standard levels for food set by Department of Public Health (1986) and by the World Health Organisation (WHO) of 1 mg/kg and 25 µg/g for fish, respectively. Therefore, it would be wise to avoid any consumption of freshwater flora and fauna from the affected area since lead is one the major threats for heavy metal contamination of food chain (Hezbullah et al., 2016).

The results of bioaccumulation identified the bioaccumulation potential of the organisms and the metal concentrations in the water. The BAF values of all organisms were higher than 100 meaning that the organisms had potential to accumulate heavy metals (Yu et al., 2012). Our results showed marked lead accumulation when compared to other studies. For example, in Loei province, Thailand, the BAF values of lead in fish around a gold mining area were in the range 44.30-133.90 mg/kg (Intamat et al., 2016). *Table 5* indicates the trophic transfer of lead in the stream food web as lead increased with the trophic level (from producer to primary consumer) in the middle-stream zone around the old mining area because biomagnification of heavy metals occurs when the values of the biotransference factors of heavy metals between prey and predator are greater than one (Gray, 2002; Misztal-Szkudlinska et al., 2011) and when an increase in trace metal concentration occurs through at least two trophic levels in a food chain (Barwick and Maher, 2003). On the contrary, biomagnification in the upstream zone did not occur due to lower concentrations of lead there in the environment and organisms.

Conclusion

This study highlighted the lead contamination from old mining activity and its effects on the water, sediment and freshwater organisms in Khli Ti creek, Kanchanaburi province, western Thailand. The middle-stream zone around the mine area had highest lead content in the sediment whereas the downstream zone had the highest concentrations of lead in the water. Lead accumulation in plankton, macroinvertebrates, fish and macrophytes was also detected. Furthermore, lead can be passed through the food web as indicated by the biomagnification factor from producers to consumers in the most contaminated zone around the mining area. Based on our results, the lead contents detected in the environment and the food web may pose a potential risk to human health and may also cause acute and chronic impacts on freshwater organisms.

Acknowledgements. This research was funded by the Kasetsart University Research and Development Institute (KURDI) during 2016-2017. We are also grateful to the staff of the Department of Pollution Control for useful information regarding sampling sites and field work planning. Lastly, we thank the Headman of Khli Ti village for background information, site navigation and fieldwork assistance.

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DOI: http://dx.doi.org/10.15666/aeer/1702_38973908

REMOTE ESTIMATION OF WHEAT YIELD BASED ON VEGETATION INDICES DERIVED FROM TIME SERIES DATA OF LANDSAT 8 IMAGERY

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(Received 8th Aug 2018; accepted 31st Oct 2018)

Abstract. Crop yield estimation prior to harvest is an important for planning and taking various policy decisions. The recent development in satellite remote sensing technologies with their increased spatial and temporal resolution enabled their enormous application for many users with low cost. The current study was planned based on time series Landsat 8 remote sensing data for real time estimation of wheat (*Triticum aestivum* L.) yield based on vegetation indices and ground-truthing wheat yield data for growing season 2015-16 in district Chakwal, Pakistan. Wheat yield data were collected from an area of 10 m × 10 m at 43 sites along with GPS positions at farmer's field from district Chakwal to develop regressional model. Different indices like, Green Normalized Difference Vegetation Index (GNDVI), Normalized Difference Vegetation Index (NDVI), Wide Dynamic Ratio Vegetation Index (WDRVI) and Enhanced Vegetation Index (EVI) were derived from time series Landsat 8 imagery throughout the growing season (2015-16). Linear regression models fitting were developed between all the indices and ground truthing wheat yield data were analyzed based on Coefficient of Determination (R^2) and Root Mean Square Error (RMSE). The results revealed that EVI index showed higher values for the month of March 2016, compared with other months. This showed that crop was at the booting and anthesis stage in these months. The EVI and GNDVI indices showed better accuracy and precision with coefficient of determination (R^2) 0.89 and 0.82 values with RMSE value of 203.83 and 224.67 respectively for the month of March-2016. This indicated that Landsat 8 imagery can be used for reliable estimation of wheat yield prior to harvest which can be useful for planning and maintaining national food security stock timely.
Keywords: *remote sensing, wheat yield, time series, Landsat 8, vegetation indices*

Introduction

Wheat (*Triticum aestivum* L.) is an important food cereal for more than 33% of the world population. Wheat yield was recorded as 754.1 million tons with production of 3.07 metric tons/ha globally which makes wheat the third most grown cereal after maize and rice (World Wheat Production, 2016). By 2050, to provide food for 900 million to 1 billion people seems to be a big challenge (Smith, 2013). Yield assessment before actual production helps in making numerous policies in agricultural production system due to high demand of food grain in the world (Sawasawa, 2003). In various countries, conventional techniques of data collection based on field visits and reports are used for crop inventory. All the rural development plans which are based on such unreliable crop acreage and production estimates become unrealistic leading to faulty decisions and

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actions and subsequent uncertainties in agricultural sector (Al-Gaadi, 2010). Remote sensing is a key component of precision agricultural practices. It is used to examine the crop growth and production and in provision rapid and nondestructive estimation of plant biomass, leaf area index (LAI), nitrogen (N) content and grain yield (Aparicio et al., 2000; Tahir et al., 2013). Remote sensing provides a better option for precision agriculture like providing low cost data, high spectral, spatial and temporal resolution. Remote sensing works collectively with technologies of Geographic Information System (GIS), Global Positioning system (GPS) and through integration their strengths can be fully utilized (Tayari et al., 2015).

Landsat Multispectral Scanner System (MSS), Landsat Thematic Mapper (TM), and Satellite Pour l'Observation de la Terre (SPOT) are the major satellite systems. Other systems are National Oceanic and Atmospheric Administration (NOAA), Advanced Very High Resolution Radiometer (AVHRR). Early work with these satellite imagery used visual interpretation for classification (Ozesmi and Bauer, 2002). Landsat 8 Operational Land Imager (OLI) sensor has improved abilities to investigate land surface observations by adding new spectral bands of blue and cirrus cloud-detection spectrum, two improved thermal bands which includes narrower near-infrared waveband, higher signal-to-noise ratio (SNR), and better radiometric sensitivity (Roy et al., 2014; Ke et al., 2015). Spectral vegetation indices are numeric transformations of mathematical models by using different spectral bands which measures seasonal variations of vegetation, but across space as well. Hence, these are appropriate in detecting within-field spatial variations (Viña et al., 2011). However, most vegetation indices tend to be species specific. They can recognize and provide material on spatial variation and allow more proficiency and accuracy in field investigation (Schuler, 2002). Remote sensing data in the visible, near and thermal infrared spectral bands had been used in monitoring phenology and infer biophysical variables of canopy (Rodriguez et al., 2004).

Previous researches demonstrated the utility of remote sensing but there are many areas of possible application which are still unexplored. This system is quite reliable and comprehensive and also cost effectiveness. Improvements in the accuracy and timeliness of crop production statistics are still in business. There is still lack of accurate, rapid and need practical methodologies to estimate yield of ground area. Therefore, the current study was planned to evaluate different spectral vegetation indices for accurately estimation of yield based on time series LANDSAT8 imagery data in Chakwal district. The main objectives of study were to: 1) real time (non-destructive) estimation of wheat yield based on spectral vegetation indices derived for time series Landsat 8 imagery, 2) compare the vegetation indices derived from Landsat 8 imagery with ground truthing data for developing regressional model and validation to improve the prediction accuracy of wheat yield in district Chakwal.

Review of literature

Hooda et al. (2006) observed that increasing the sample segments and decreasing the size of segments improved the area and production estimates by gradually decreasing the size of various districts. From last 15 years, the satellite data's spatial resolution has gradually improved. Moreover, improvement in the spatial, spectral and temporal resolution has extremely enhanced the capabilities to discriminate of crop from other vegetation. Since all the era the accuracy and the precision of estimation enhanced its efficiency. However, better accuracy and precision has been shown in large areas of the

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crop as compared to the areas with numerous mixed cropping systems. Presently remote sensing technique with 95% efficiency and precision enabled the acreage and yield estimation at district level.

There are many other indices that could assist in vegetation sensitivity rather than frequently used VIs in archeological investigation i.e. NDVI, SR etc. Results proved the efficiency of hyperspectral VIs by maximizing differences among archaeological and non-archaeological areas. These differences can be maximized by using narrowband indices up to 20% than broadband indices. In this study SARVI, SR and PVI are reported as most auspicious vegetation indices of broadband. SR-MTCI, SR-SR and VOG are reported as narrowband indices. Each phenological stage has different vegetation index to enhance the archaeological area than non-archaeological area (Agapiou et al., 2012).

NDVI, SM, ST and RF reported as the key factors or variables that control normal crop production. This approach might be utilized for estimation the gaps in crop produce by bringing empirical equations in use. Factors such as pests, diseases and human activities observed as the main factors of subsistence variabilities of yield estimation, which act as limiting factor in estimation techniques. Though, if NDVI is added to model which may help in determining yield losses caused by diseases or pests. The model can be calibrated by gaining more historical data of growth for the better prediction (Prasad et al., 2006).

Dempewolf et al. (2014) in his experiment evaluated four different indices, among them more consistent and more accurate yield prediction was observed by WDRVI as compared to the NDVI, EVI2 and SANDVI. While using the six years' data at district level RMSE values were found to be very low (within 0.2% and 11.5%) among the forecast and the recorded yield. Results reported that deviations in wheat area and yield prediction were slightly higher than the previous year's moving average values. The reason behind is that 250-m MODIS data is not able to provide the sufficient spatial resolution for improved wheat area and the yield estimation.

Castaldi et al. (2015) in his introductory investigation reported vegetation variables which were gained from the SPOT data (LAI, Fcover, FAPAR, and Cab) had accurate simple and the spatial significance related to final yield of wheat on stem elongation stage. The model for the yield estimation expressed the maximum RMSE when CR models were used. Moreover, when wheat crop production mapped it aided farmers in obtaining the estimate for the crop yield and identifying variability in the field as well. This approach is best suitable to several crops as well as for several regions. It could also be observed a valued practice particularly in the conditions where limited mapping techniques of yield are present for farmers. These tools can also be utilized for the environmental changes and have ability to give the significant information for the ecological remote sensing.

Materials and methods

Study area and ground truthing data collection

The proposed study was carried out in the Chakwal district of Pakistan. District Chakwal lies at 33° 40' North and 72° 51' East with elevation range from 500 to 1200 m. Total area of Chakwal is 6,524 km^2. District Chakwal has population 1083725 and about 70% people's livelihood depends on agriculture directly and indirectly. The district Chakwal has temperate climate with semi-arid area and annual rainfall ranged

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from 400 mm to 600 mm. It has hot summer and cold winter season. Total area depends on rain fall for agriculture. No irrigation system is present in this Area. Wheat crop usually planted in mid-November to end of November in districts Chakwal and Rawalpindi. The wheat crop has main distinct growth stages, like tillering, jointing, booting, anthesis, grain filling and physiological maturity. The March was the peak growth month of wheat crop. Wheat crop is usually harvested in end of April to mid-May. Chakwal district comprises of four Tehsil and total 68 union councils. We collected the data from different locations in district Chakwal t (*Fig. 1*). We collected the information about crop history and then collected the wheat yield samples from farmer's field during 2015-16 growing season. Wheat yield data was collected manually from an area of 10 × 10 m in the farmer's field, then expressed this yield in Kg/ha. We also took the GPS data with the help of GPS meter from the same place of wheat yield samples collected at selected sites in district Chakwal.

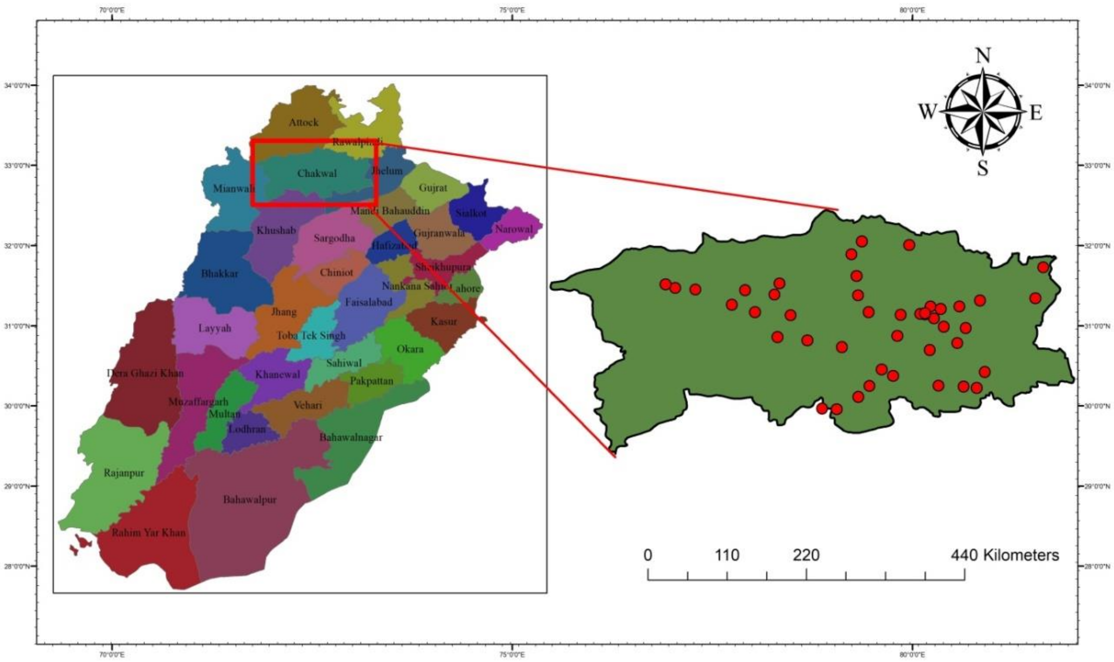

Figure 1. *Map of study area of Chakwal*

Remote sensing data and digital image processing and analysis

The remote sensing data was downloaded from USGS website (http://earthexplorer.usgs.gov/). We used the time series LANDSAT 8 satellite images for remote sensing data for the growing season 2015-16. Landsat 8 has 11 bands. Landsat 8 has operational land imager (OLI) and thermal infrared sensors (TIRS) with nine spectral bands with a spatial resolution of 30 m. The bands 1 to 7 having different wavelengths with red, green and blue sensors are combined to produce true-color image. The band 8 has 15-m resolution. The New band 9 (ultra-blue) is useful for cloud detection and also useful for costal and aerosol studies. Band 10 and 11 are thermal bands which are useful in providing more accurate surface temperature with resolution of 100 m.

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After collecting time series images from available sources, the images were passed through different analysis processes. ERDAS IMAGINE (2014) and Arc GIS 10.2 software were used for processing and analysis of remote sensing data. The images of the study area pertaining to specific orbit, path and row were downloaded and opened into the viewer of ERDAS IMAGINE (2014). Later, geometric correction of the images was performed. Magnification of the original images was done to obtain the detailed information about the spectral reflectance of the area of interest. Images were atmospherically and geo-corrected using these ground control positions (GCPs). Stacking was performed by using Arc GIS 10.2 software. We performed the unsupervised classification by using ERDAS Imagine software. Following vegetation indices were performed on the processed images for the estimation of wheat yield by using ERDAS IMAGINE (2014) and Arc GIS 10.2 software (*Fig. 2*).

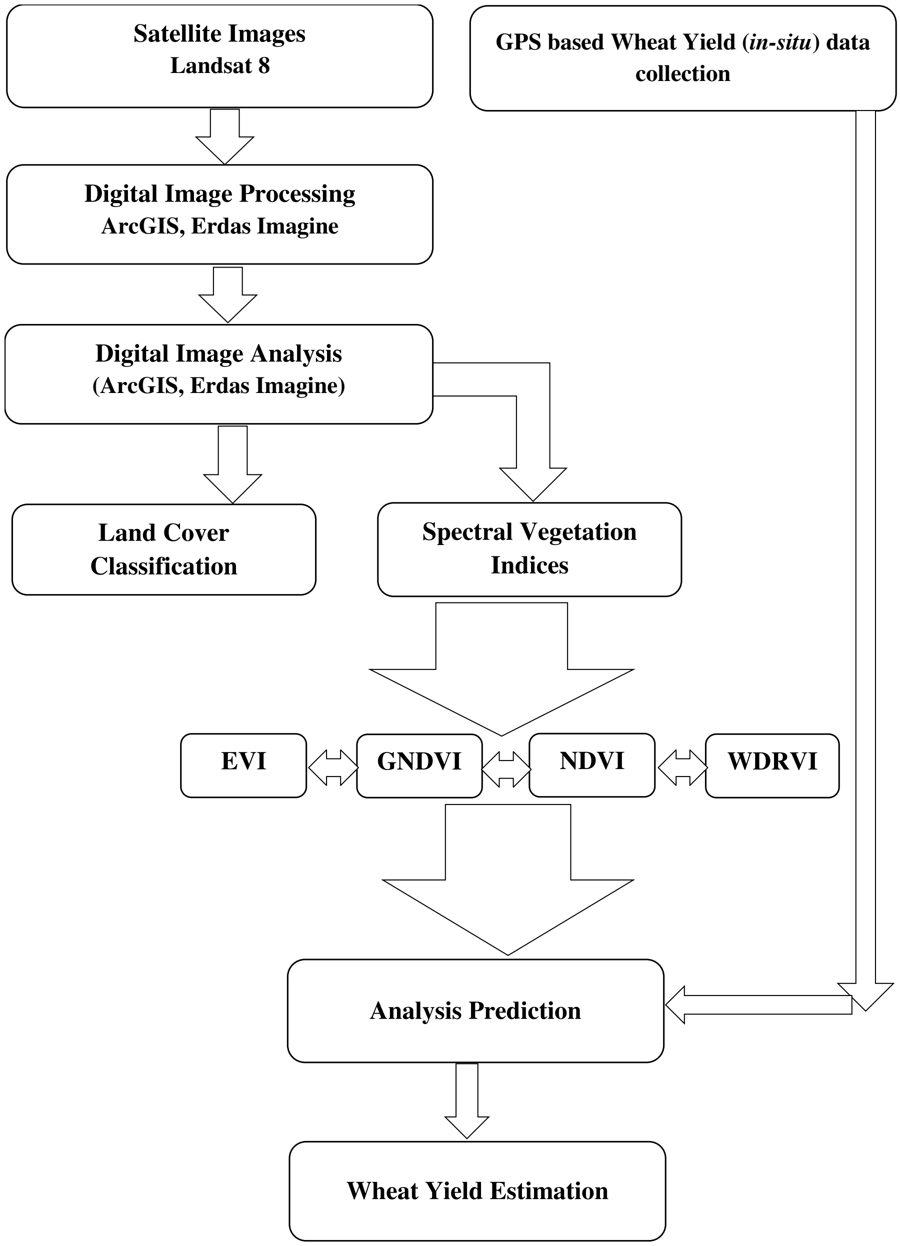

Figure 2. *Flow diagram of remote estimation of wheat yield*

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Spectral vegetation indices

The spectral vegetation indices are presented in *Table 1.*

Table 1. *Spectral vegetation indices*

Sr.#	Vegetation indices	Formula	References
1	Green normalized difference vegetation index (GNDVI)	$GNDVI = \frac{(NIR - GREEN)}{(NIR + GREEN)}$	Huete et al. (2002), Wang et al. (2007)
2	Normalized difference vegetation index (NDVI)	$NDVI = \frac{NIR - R}{NIR + R}$	Ferencz et al. (2004)
3	Enhanced vegetation index (EVI)	$EVI=2.5\frac{NIR-RED}{(NIR+6RED-7.5BLUE)+1}$	Glenn et al. (2008)
4	Wide dynamic range vegetation index (WDRVI)	$WDRVI = \frac{0.1NIR-RED}{0.1NIR+RED}$	Henebry et al. (2004)

Statistical analysis

Regression was used to describe the effect of independent variables on dependent variable by representing the latter as a function of a former. Linear regression model were performed between satellite derived spectral vegetation indices and ground truthing yield data of wheat crop in Chakwal district. The performance of the model was estimated by comparing the differences in coefficient of determination (R^2) and root mean square error (RMSE) in prediction. The higher the R^2 and the lower the RMSE value and the higher will be the precision and accuracy of model for prediction. The RMSE was calculated using following equation:

$$RMSE = [\frac{1}{n}\sum_{i=1}^{n}(Si - Mi)^2]^{\frac{1}{2}}$$

Results

Vegetation indices

Following full processing of the LANDSAT 8 time series imagery for different vegetation indices were calculated using the mean values of the reflectance in green, red and NIR portion of the electromagnetic spectrum. The derived vegetation indices; GNDVI, NDVI, WDRVI and EVI proposed band ratios demonstrated the feasibility of estimating the wheat yield throughout the growing season of 2015-16. All vegetation indices for each month were used for the simple regression analysis which was performed on the field yield data to calculate equations for predicting wheat production.

GNDVI yield maps derived from time series data of Landsat 8 (2015-16)

Green normalized difference vegetation index is used for measurement of greenness in crops. The progressive increased in the GNDVI value was shown in the maps as the change in vegetation was occurred over time (*Fig. 3*). The GNDVI maps showed the

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variations across the Chakwal district with minimum values (-0.1888–0.2154) in the month of November-2015 when crop was at early stage or in some areas at two to three leaf stage while the highest values (-0.0806–0.36672) were shown in the month of March-2016 (*Fig. 3*). Legends indicated in maps of GNDVI represented with low to high crop vegetation in these areas.

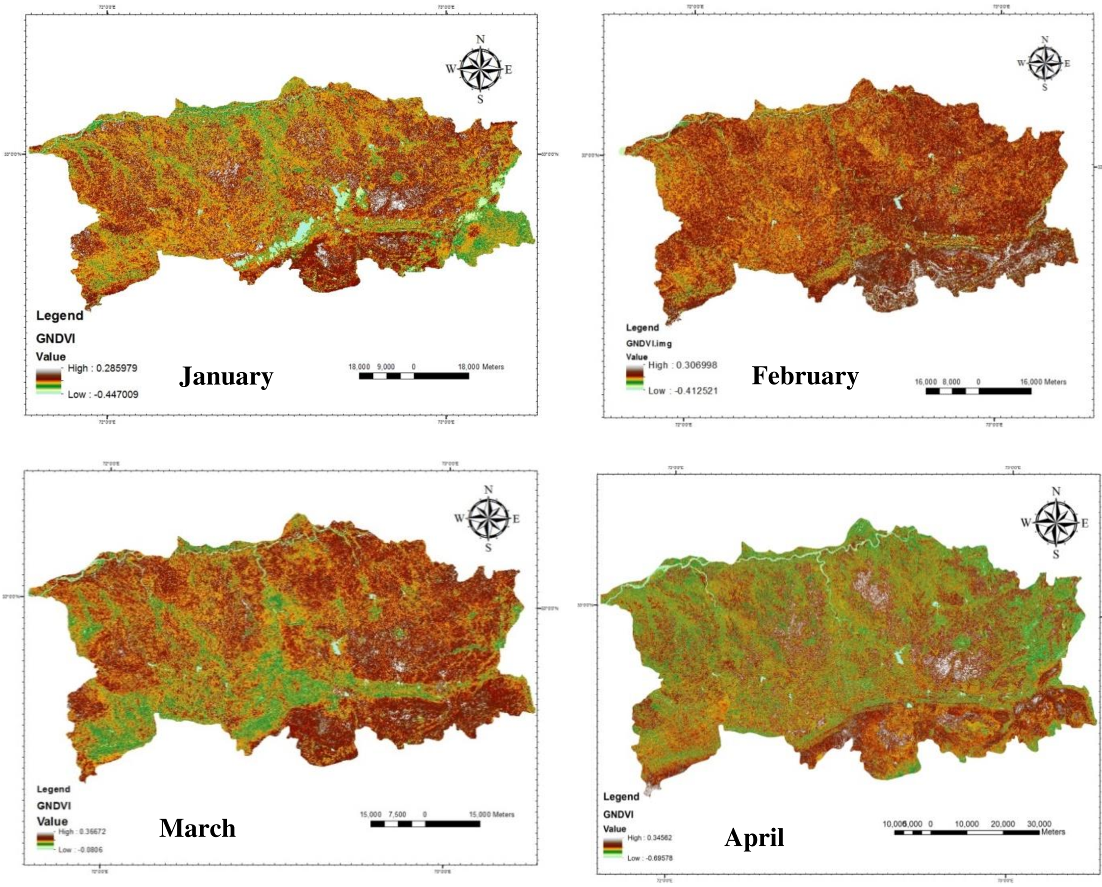

Figure 3. *GNDVI map of district Chakwal during wheat growing season of 2015-16*

Relationship between GNDVI derived from time series Landsat 8 with ground truthing wheat yield data

Figure 4 of wheat crop for growing season 2015-16 showed positive and strong relationship between GNDVI and wheat yield. The regressional model for each month expressed the relationship between GNDVI and wheat yield over time. A significant linear and positive relationship was observed between GNDVI and wheat yield for the month of March-2016 with coefficient of determination $R^2 = 0.82$ value as compared to other months throughout the growing season which showed the highest vegetation content/cover of the wheat crop in this month. GNDVI showed great variation from November-2015 to April-2016 with coefficient determination values (R^2) 0.37, 0.48, 0.61, 0.72, 0.82 and 0.55 respectively. GNDVI showed the higher accuracy for predicting wheat yield accurately with the value of $R^2 = 0.82$ and RMSE = 224.67 for the month of March-2016.

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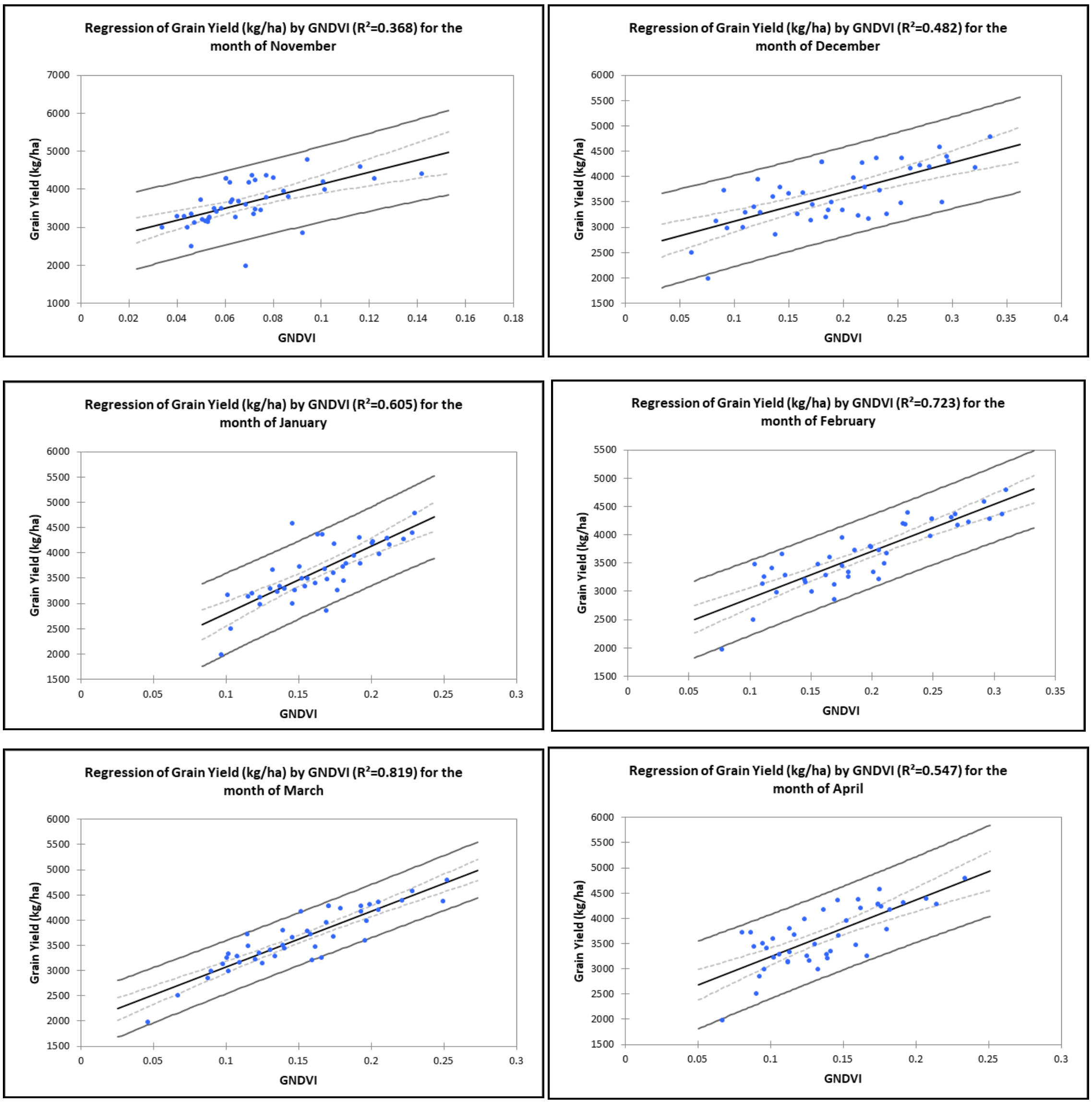

Figure 4. *Relationship between GNDVI and ground truthing wheat yield throughout the growing season*

NDVI yield maps derived from time series data of Landsat 8 (2015-16)

The NDVI map showed the status of whole growing season of wheat crop in district Chakwal. NDVI value ranged between -0.2538 to 0.40236 for the month of March-2016 while in the month of November-2015, NDVI value ranged between -0.1885 to 0.23224 (*Fig. 5*). This showed that highest crop vegetation was present in month of March-2016 and the lowest value was present in the month of November-2015. This showed that the vegetation was sparsely covered the ground during the month of November-2015. Water bodies and build area have negative value where as barren and rocks have zero value. Where as strong positive value represented only high dense green vegetative area.

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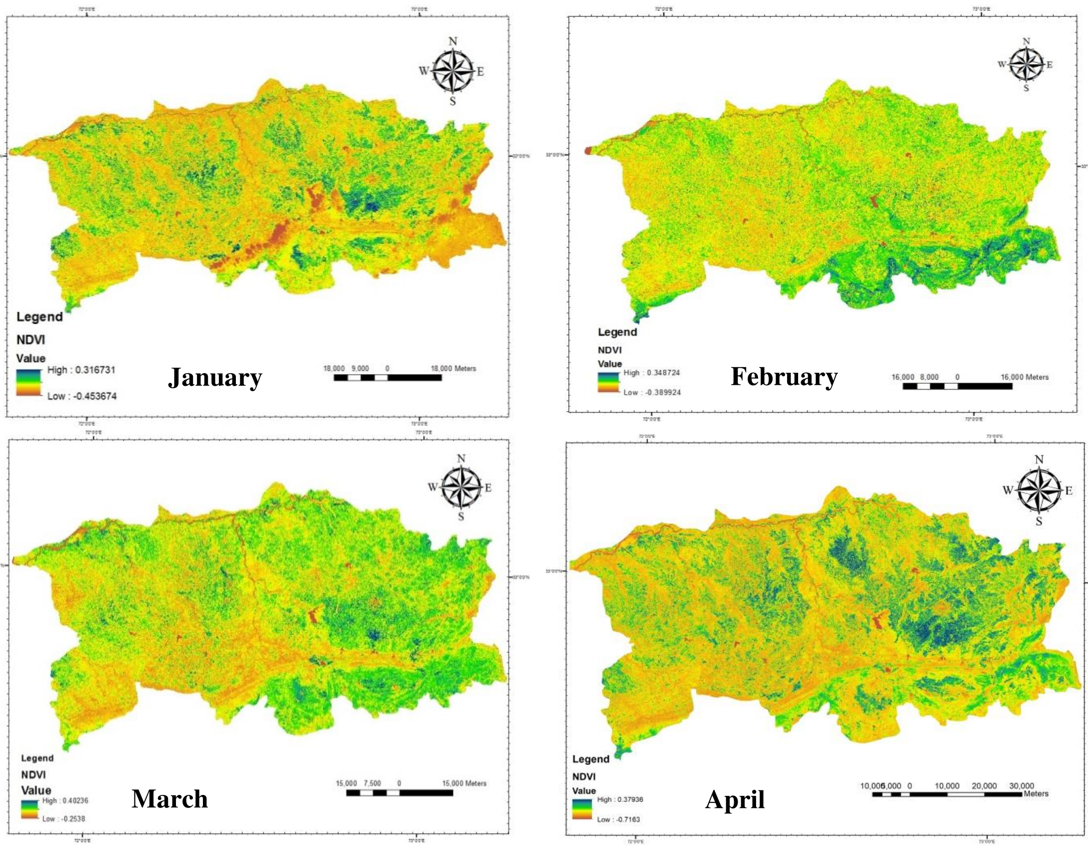

***Figure 5.** NDVI map of district Chakwal during wheat growing season of 2015-16*

Relationship between NDVI derived from time series Landsat 8 with ground truthing wheat yield data

Figure 6 showed the significant relationship between NDVI and wheat yield throughout the growing season 2015-16. There was positive and linear relationship between NDVI and wheat yield and the regression accounted for 75% of the variation in the data (R^2 = 0.75) for month of March-2016. The higher value of R^2 between the NDVI and wheat yield explained the vegetation conditions. *Figure 8* showed the relationship between NDVI and high vegetation.

If α = 1, then the WDRVI is equivalent to the NDVI. By calculating the value in current study α was also equal to 1. Maps of WDRVI are shown in *Figure 7*. The value of WDRVI was same as NDVI for all the months. So the NDVI and WDRVI produced exactly the same results for March-2016. WDRVI showed higher value of R^2 = 0.75 for month of March-2016, while November showed the weak relationship with R^2 = 0.36 (*Fig. 8*).

EVI yield maps derived from time series data of Landsat 8 (2015-16)

The EVI maps showed the variations across the district Chakwal. EVI wheat yield map showed great variations throughout the growing season. The highest value was shown in the month of March-2016 with the value ranged from -0.913381 to 0.903439 while the lowest value was shown in the month of November-2015 (-0.08294–0.27622). The highest value showed the highest greenness content of wheat crop (Booting and

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anthesis stage) in this month. The lowest value showed that crop was at 2-3 leaves stage and ground cover was not fully covered with the crop at this stage (*Fig. 9*). Legends indicated in maps of EVI represented with low to high value of crop vegetation in these areas.

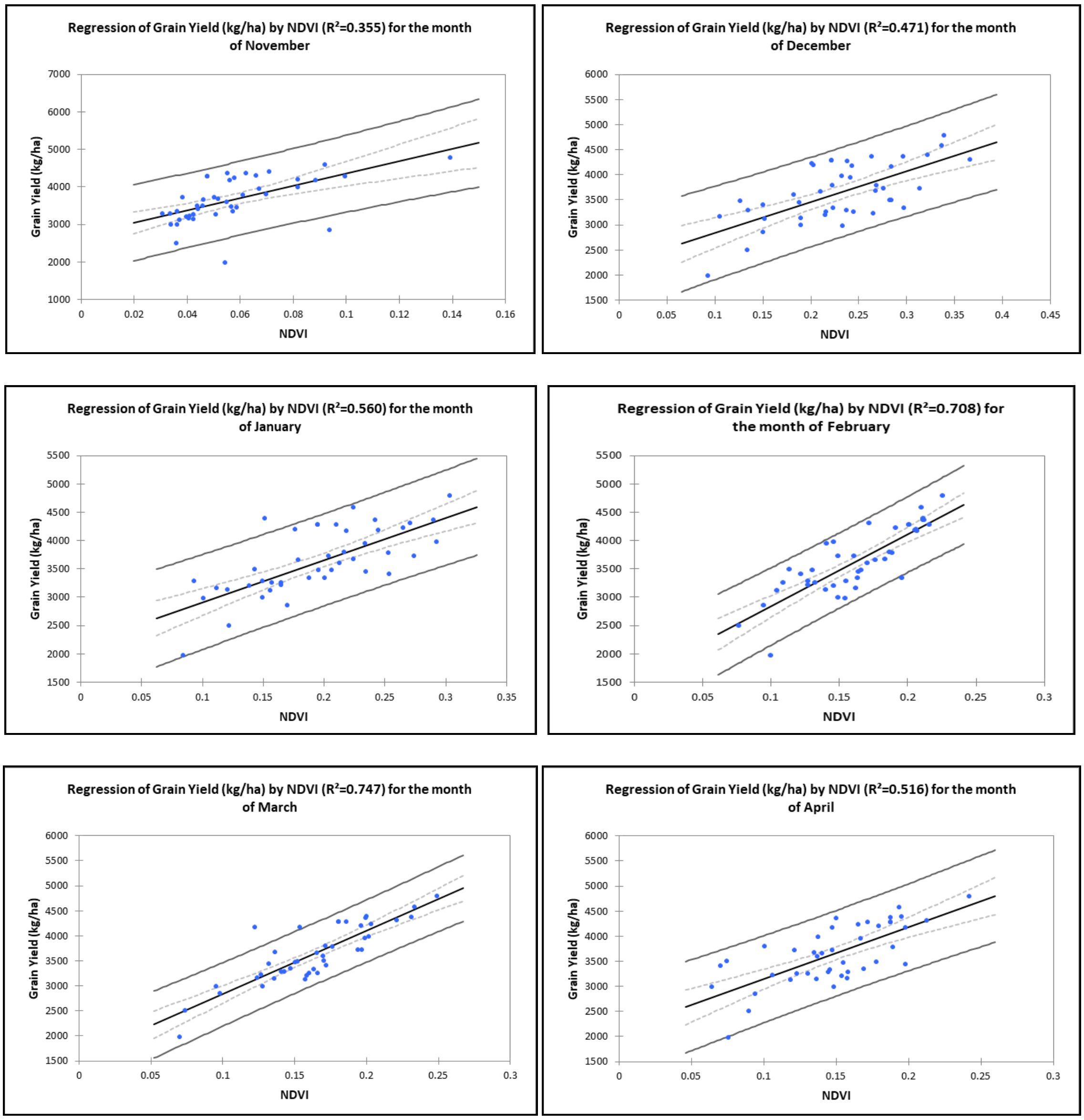

Figure 6. *Relationship between NDVI and ground truthing wheat yield throughout the growing season*

Relationship between EVI derived from time series Landsat 8 with ground truthing wheat yield data

Figure 10 showed very significant interactions between EVI and wheat yield. The regressional model for each month interprets the relationship between EVI and wheat yield production (*Fig. 10*). There was a positive and strong linear relationship between EVI and wheat yield in the month of March-2016 as compared to other months throughout the growing season of wheat crop. Minimum value of coefficient of

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DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

determination was R^2 = 0.39 in the month of November when crop was at emergence stage or in some areas at two or three leaf stage while the maximum value of R^2 = 0.89 was in the month of March where highest vegetation was present. As soon as the crop turned to yellow in most areas the EVI value also reduced to R^2 = 0.65 in the month of April-2016. The EVI showed the higher accuracy for predicting wheat yield for month of March-2016 with value of R^2 = 0.89 and RMSE 203.83. Results revealed that EVI has highest efficiency to predict wheat yield than other indices used in the current study.

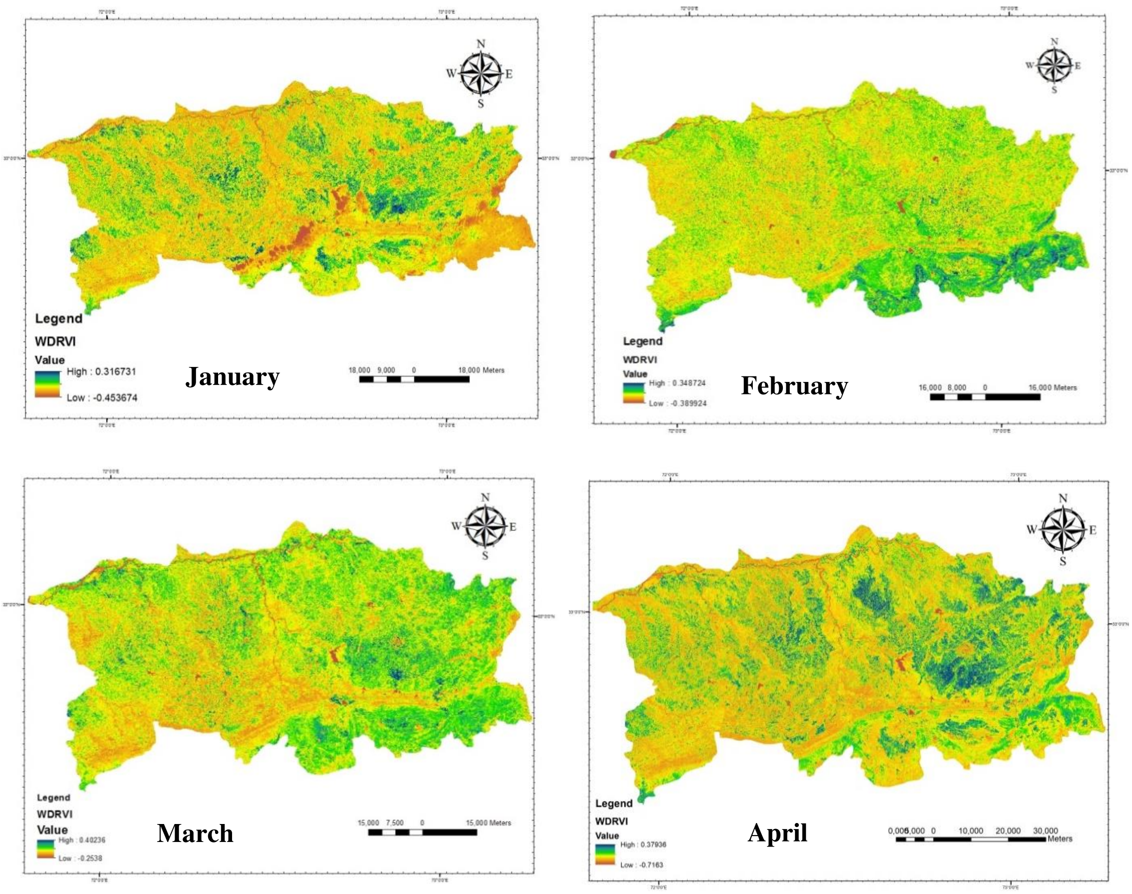
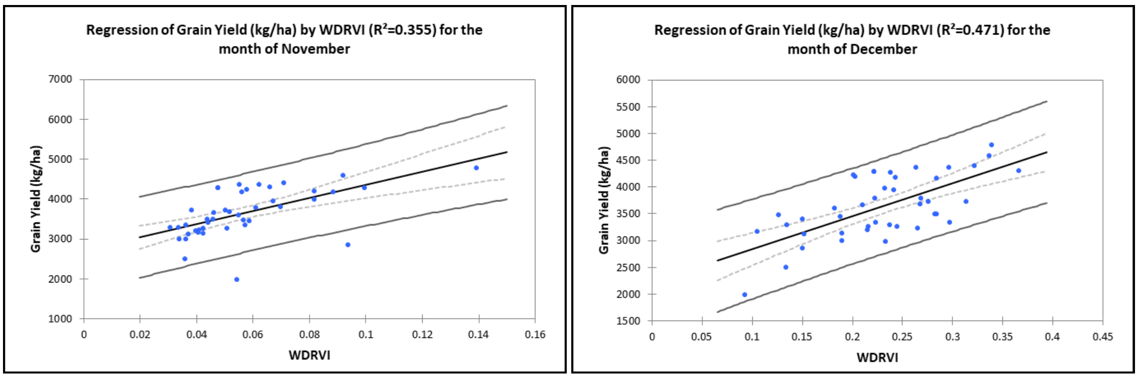


Figure 7. WDRVI map of district Chakwal during wheat growing season of 2015-16



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DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

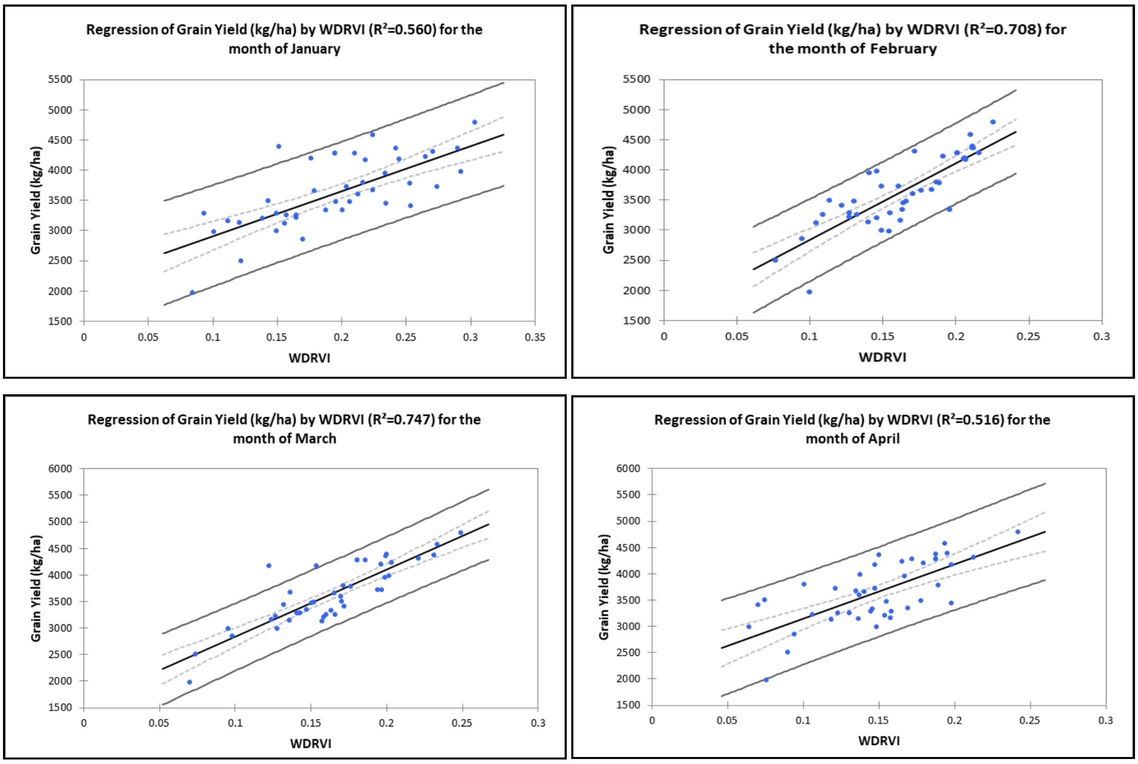

Figure 8. *Relationship between WDRVI and ground truthing wheat yield throughout the growing season*

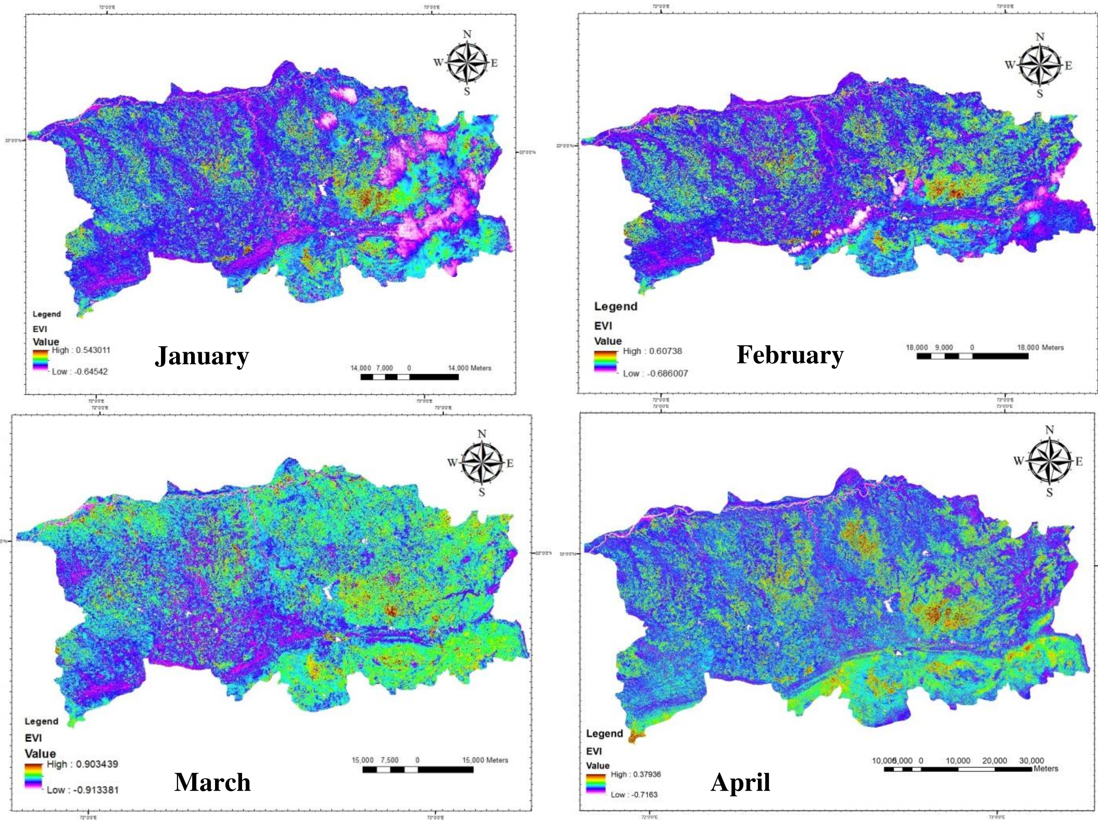

Figure 9. *EVI map of district Chakwal during wheat growing season of 2015-16*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

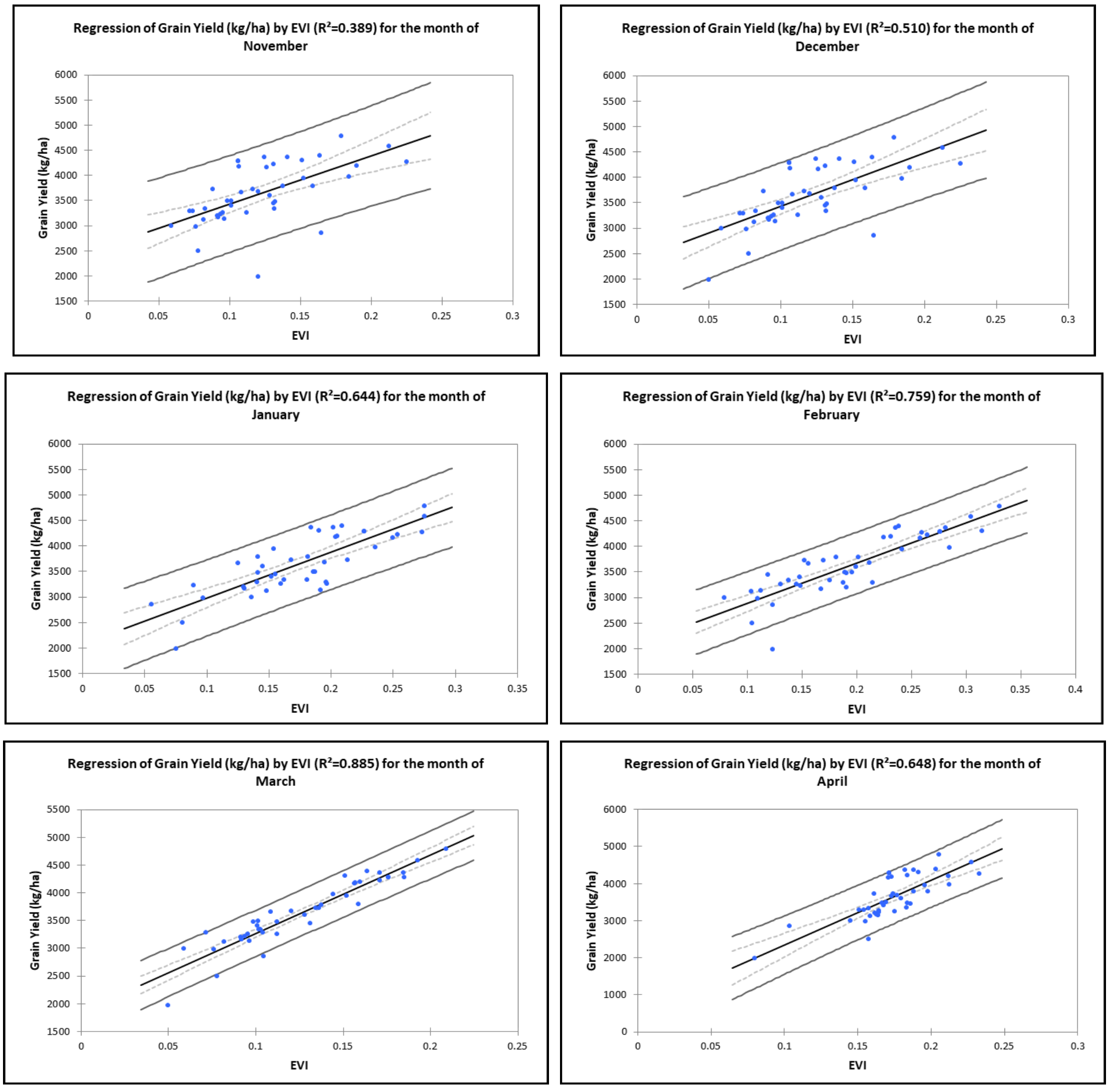

Figure 10. *Relationship between EVI and ground truthing wheat yield throughout the growing season*

Probability map of yield

The Probability Map of wheat yield showed the status of yield in the district Chakwal. The probability map of wheat yield was developed based on the satellite derived yield to predict the yield for the future year 2017-18. The values of probability map ranged from 1981 to 4778 Kg/ha. Some areas showed with white color in map had low wheat yield with the lower value of 1981 Kg/ha in district Chakwal. When more values lied near to 4778 Kg/ha showed higher wheat yield in that part of the map as represented in blue color in the map. From map, trend of future wheat yield is shown approximately from lower to high wheat yield (*Fig. 11*).

Discussion

Due to advancement in satellite remote sensing technologies reduced the cost of the imageries with improved temporal and spatial resolution which improved the crop area

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

and yield estimation reliable. The study was planned based on Landsat 8 time series remote sensing data to derive vegetation indices and compared with observed wheat yield data using regressional model to estimate wheat yield accurately. All vegetation indices GNDVI, NDVI, WDRVI, and EVI were derived from November 2015 to April 2016 during the whole wheat growth season. Map of all indices developed using ERADAS 14.0. February and March 2016 months were the peak wheat growing months. The Map of all indices showed higher value for the month of February and March 2016 compared with other months. This explained that crop was at booting/anthesis stages. In district Chakwal, the weather remained mild and cold till the end of March. Therefore, all indices showed higher value for the month of March 2016. Lower values of all vegetation indices showed that wheat crop was at early stage or ground cover was not fully cover by the wheat crop (Viña et al., 2011). Wheat crop was harvested from selected sites in district Chakwal during mid-April to end of May 2016.

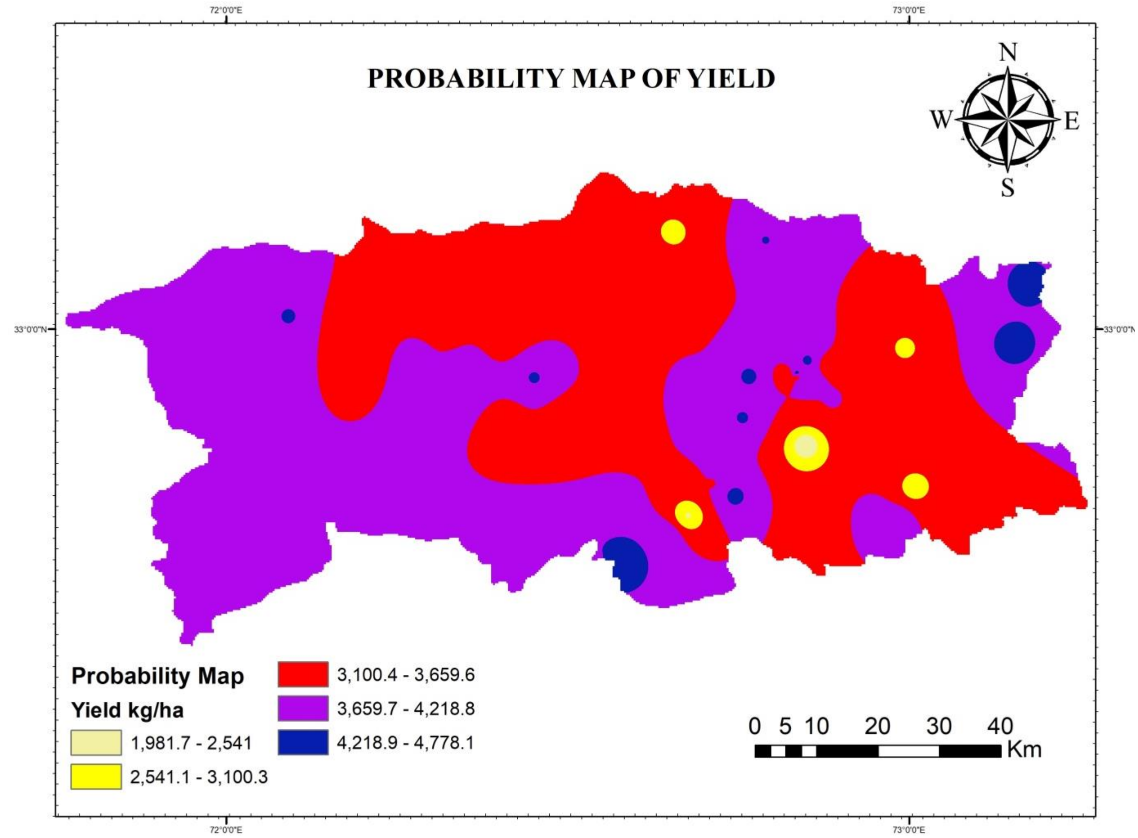

Figure 11. *Probability map of district Chakwal of wheat yield*

Regressional model were developed between Landsat 8 derived vegetation indices and observed wheat yield in district Chakwal for predicting wheat yield accurately. EVI and GNDVI proved more robust indices compared with NDVI and WDRVI. EVI showed positive and strong relationship with coefficient of determination value 0.89 for the month of March 2016. This is also explained that crop at booting /anthesis stage. This is the most important stage of wheat which directly related with increase of decrease crop production. The study proved that vegetation indices are robust in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

estimating wheat yield accurately. Similar results were also reported by Agapiou et al. (2012) and Shen et al. (2009, 2010).

Future wheat crop prediction was developed based on the probability analysis in ArcGIS 10.2. Remote sensing derived wheat yield were plot in district Chakwal which showed the same trend as the observed yield data collected at different sites. Results showed the improved accuracy for estimating wheat yield compared with previous studies. The current study can be helpful in future prediction of wheat yield well before time which can be useful to managing food security issues and taking better decisions for policy making.

Conclusion

Wheat yield can be achieved in real time accurately by using remote sensing data prior harvesting. Different vegetation indices were evaluated derived from time series data of Landsat 8 imagery for the growing season 2015-16. Regressional model were developed between the vegetation indices and wheat yield data for predicting wheat yield accurately. Study results showed that EVI index showed higher value for the month of February-March 2016 compared with the month of November 2015. This showed that crop was at peak vegetation stages (booting/anthesis). EVI and GNDVI proved more robust indices and showed better accuracy compared with other indices. EVI showed the higher accuracy with $R^2 = 0.89$ value and RMSE value of 203.83 for determining wheat yield accurately. The current study can be helpful in future prediction of wheat yield well before time which can be useful to managing food security issues and taking better decisions for policy making.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39093925

MOLECULAR ANALYSIS OF GRAPEVINE GERMPLASM BY SSR (SIMPLE SEQUENCE REPEATS) IN DIYARBAKIR PROVINCE, TURKEY

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(Received 14th Oct 2018; accepted 2nd Jan 2019)

Abstract. This study was aimed to identify the grape cultivars growing in Diyarbakır. A total of 45 genotypes were analyzed using 7 microsatellite loci. The numbers of alleles per locus ranged between 7 and 10, whereby VVS2 and VrZAG47 had the highest and VVMD7, VrZAG62, and VrZAG79 had the lowest number of alleles. The expected and observed heterozygosity were 0.77 and 0.73, respectively. Clustering analysis was performed using the UPGMA method (Unweighted Pair-Group Method using Arithmetic means). A dendrogram was constructed based on the genetic similarity among the genotypes, which indicated 5 distinct groups, with each group involving numerous subgroups. Two additional genotypes that are used as reference genotypes around the world, Cabernet Sauvignon and Merlot, were also analyzed and were classified into a separate subgroup and differentiated from the other 43 genotypes. In conclusion, synonyms and homonyms were detected among some of the genotypes analyzed in the study.
Keywords: *Vitis vinifera L., molecular analysis, genotype, similarity index, homonym, synonym*

Introduction

Grape (*Vitis vinifera* L.) is one of the most important fruit in the world. Turkey has a long history of grape cultivation. Diyarbakir province, located in the Southeastern Anatolian Region in Turkey, has an important position in Turkey with regard to its plant diversity. Grapevine, in particular, is a leading perennial garden plant well adapted to this region. The region also features various forms of grapes including table grapes, wine grapes, and grapes for drying. Kaplan (1994) conducted an ampelographic study using the classic method for naming the grapes growing in this region. The researcher demonstrated the rich genotypic diversity in the region and also noted that the naming of grapes is complicated mainly due to the complexity of the synonyms used for grapes. Accordingly, it is commonly known that the use of different names for plants based on regional variation leads to significant problems and confusion in terms of the correspondence of these names to those used in studies and to those mentioned in each phase of production. These problems can only be resolved by the use of molecular markers of polymorphism.

Microsatellite, a highly powerful type of DNA markers, provides a unique genetic profile for every cultivar, permitting unambiguous identification that is not affected by environment, disease, or farming methods (Meredith, 2001). Since the first grape microsatellites were reported by Thomas and Scott (1993), many more microsatellites have been developed for characterization of *Vitis* germplasm (Bowes et al., 1996, 1999; Sefc et al., 1999; Dı Gaspero et al., 2000; Scott et al., 2000; Dı Gaspero et al., 2005).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39273939

Microsatellite markers have been extensively used for genotyping and the determination of synonyms and homonyms of grape genotypes (Costantini et al., 2005; Karaağaç, 2006; Cipriani et al., 2010; Emanuelli et al., 2013; Alifragkis et al., 2015; Maletic et al., 2015 Maul et al., 2015; Li et al., 2017; Zequim Maia et al., 2018; Van Heerden et al., 2018) and for pedigree analysis and for investigating the parentage of cultivars and genome mapping (Meredith et al., 1996; Sefc et al., 1998; Grando et al., 2003; Vouıllamoz et al., 2004; Adam-Blondon et al., 2004; Akkak, 2007; Huber, 2016; Dong et al., 2018). These markers have also been used for the identification of chimaeras of grapes (Franks et al., 2002; Riaz et al., 2002; Hocquigny et al., 2004; Boz et al., 2011). Moreover, these SSR markers have recently been successfully used for the protection of the germplasms of wild grapevine (*Vitis vinifera* ssp. silvestris) and the elucidation of the historical development of grapevine (Schneider et al., 2015; Zdunić et al., 2017; Butorac et al., 2018).

The aim of this study was to identify the grape cultivars growing in Diyarbakır based on the DNA profiles of the grape cultivars transplanted to the Tekirdağ National Germplasm Repository Vineyard in order to protect the germplasms of these grapes and to provide an accurate genetic identification for these cultivars. With the aims stated above, we performed genetic identification of 43 grape cultivars growing in Diyarbakır Province, where gene potential is remarkably high.

Materials and methods

Materials

The study was conducted at Ankara University Agriculture Faculty Horticulture Department. The materials used in the study consisted of 36 genotype samples that were collected from Diyarbakir Province and its districts (D) and all the 7 genotype samples of the cultivars that had been transplanted to the Tekirdağ National Germplasm Repository Vineyard several years earlier (TD). One-year-old seedlings with 3-5 buds were obtained from each genotype and were planted in polyethylene tubes filled with a 2:2:1 mixture of perlite, turf, and powder and then germinated in greenhouse conditions until the buds were rooted. In addition to D and TD genotypes, two additional genotypes that are used as reference genotypes around the world, Cabernet Sauvignon and Merlot, were also analyzed.

Table 1 presents the microsatellites used for the characterization of the genotypes analyzed in the study.

Methods

DNA was extracted from the young leaves of cultivars collected during the summer season, using the method proposed by Lodhi et al. (1994). The DNA concentration was adjusted to 30 ng/µl for polymerase chain reaction (PCR) amplification.

A total of 7 SSR primers were used in the study including the 6 microsatellite loci, VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79, which were used in a previous GENRES 081 European Union (EU) research project and are currently accepted as minimum standards around the world, and another primer, VrZAG47, which was used in our previous studies and was proven to be a polymorphic primer. Forward primers of each primer pair were labeled with fluorescent dyes including Fam (blue), Vic (green), and Ned (yellow) (*Table 1*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_39273939

Table 1. *Primers used for the study*

Primer	5´-3´	Base sequences of primers		Reference
VVS2	F	VIC-CAG CCC GTA AAT GTA TCC ATC	Vic	Thomas and Scott (1993)
	R	AAA TTC AAA ATT CTA ATT CAA CTG G		
VVMD5	F	6-FAM-CTA GAG CTA CGC CAA TCC AA	Fam	Bowers et al. (1996, 1999)
	R	TAT ACC AAA AAT CAT ATT CCT AAA		
VVMD7	F	NED-AGA GTT GCG GAG AAC AGG AT	Ned	
	R	CGA ACC TTC ACA CGC TTG AT		
VVMD27	F	NED-GTA CCA GAT CTG AAT ACA TCC GTA AGT	Ned	
	R	ACG GGT ATA GAG CAA ACG GTG T		
VrZAG47	F	VIC-GGTCTGAATACATCCGTAAGTATAT	Vic	Sefc et al. (1999)
	R	ACGGTGTGCTCTCATTGTCATTGAC		
VrZAG62	F	6-FAM-GGT GAA ATG GGC ACC GAA CAC ACG C	Fam	
	R	CCA TGT CTC TCC TCA GCT TCT CAG C		
VrZAG79	F	6-FAM-AGA TTG TGG AGG AGG GAA CAA ACC G	Fam	
	R	TGC CCC CAT TTT CAA ACT CCC TTC C		

DNA amplification was performed using GeneAmp PCR System 9700 with EU-Applied Biossystems, and PCR optimization was achieved for each cultivar. PCR amplification was performed in a reaction volume of 20 µl containing 5 µl of DNA, 2 µl of 10X Buffer, 1.2 µl of $MgCl_2$, 0.6 µl of dNTP, 1 µl of primer 1, 1 µl of primer 2, 0.2 µl of GoldTaq (0.5 U), and 9 of µl distilled water. Touchdown PCR was performed using the following cycling conditions: 95 °C for 10 min, 94 °C for 30 s, and 52 °C for VVS2, VVMD5, and VVMD7, 58 °C for VVMD27, 55 °C for VrZAG47, and 62 °C for VrZAG62 and VrZAG79 for 30 s, depending on the rate of primer annealing, with a decrease of 0.2 °C/cycle. After 25 cycles, 15 additional cycles were performed with a reduction of 5 °C from the primer annealing temperature, finally followed by holding at 72 °C for 40 min.

To screen for amplification of the fragments in the loci analyzed, a minimum of 10 samples representing each locus were separated on agarose gel, and the amplified fragments were subjected to sequencing using ABI Prism 3730 automated DNA sequencer with GeneScan™ 500 LIZ™ dye Size Standard. The resulting data were analyzed, visualized, and processed using GeneMapper v 3.7 software. The allele size per locus was represented as peak levels.

A total of 45 grapevine genotypes were analyzed in 7 SSR loci and the genetic similarity between the genotypes was calculated using the Microsat software (Minch et al., 1995) and the genetic parameters (number of alleles per locus [n], allele frequency, expected heterozygosity [H_e], observed heterozygosity [H_o], parentage, null allele frequency (r), and probability of identity [PI]) were calculated using the IDENTITY 1.0 software (Wagner and Sefc, 1999). The dendrograms were constructed and visualized using the NTSys software (version 2.02g, Exeter Software, Setauket, NY). Clustering analysis was performed using the UPGMA method (Unweighted Pair-Group Method using Arithmetic means).

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DOI: http://dx.doi.org/10.15666/aeer/1702_39273939

Results

The analysis of 45 genotypes (including 43 grapevine genotypes collected from Diyarbakir Province and 2 reference genotypes) characterized by 7 microsatellite markers using the Gene Mapper v. 3.7 software, including peak levels, allele size, and basepair per locus (*Table 2*).

Table 2. *Allele sizes of the genotypes characterized by 7 microsatellite loci*

No	Genotype	VVS2		VVMD5		VVMD7		VVMD27		VrZAG47		VrZAG62		VrZAG79	
1	*D* Mikeri	121	141	230	232	246	246	181	191	155	157	186	198	244	248
2	*D* Balcani	141	149	222	228	244	244	177	191	155	169	190	202	240	246
3	*D* Hatunparmağı (white)	139	143	232	242	236	244	191	191	169	169	186	202	244	248
4	*D* Vilki	131	133	230	232	240	244	181	181	159	159	186	190	246	246
5	*D* Şitu	133	149	222	230	236	244	179	183	157	161	190	194	248	248
6	*D* Kızılbanki	131	141	232	236	240	246	181	191	159	169	188	188	244	246
7	*D* Kohar	139	149	224	230	244	246	183	183	157	161	186	202	248	248
8	*D* Hasani	131	141	232	236	246	246	181	191	159	169	188	198	244	248
9	*D* Asuri	141	149	222	228	244	244	177	191	155	169	190	202	240	246
10	*D* Zerik	133	153	230	232	244	244	191	191	157	169	190	198	248	254
11	*D* Ağek	141	149	228	242	244	252	179	191	157	169	202	202	236	244
12	*D* Gençmehmet	131	141	230	232	244	244	181	191	159	169	190	202	244	246
13	*D* Şaraplık	149	155	228	234	246	252	177	191	155	169	192	202	244	246
14	*D* Iskıcuna	141	155	230	242	244	246	177	191	155	169	186	192	246	248
15	*D* Merir	133	139	230	236	244	244	181	181	159	169	186	190	246	254
16	*D* Abderi	131	133	230	232	240	244	181	181	159	159	186	190	246	246
17	*D* Tahannebi	131	131	232	232	244	246	177	191	155	169	198	202	244	246
18	*D* Morek	149	155	228	234	246	252	177	191	155	169	192	202	244	246
19	*D* Vanki	131	141	232	236	246	246	181	191	159	169	188	188	244	248
20	*D* Şamuzli	131	155	230	234	244	244	191	191	169	169	190	202	240	254
21	*D* Şirelik	131	131	232	232	244	246	177	191	155	169	198	202	244	244
22	*D* Karik	133	155	222	232	244	246	191	191	169	169	190	198	244	248
23	*D* Mazrumi	131	131	232	232	244	246	191	191	155	169	198	202	244	246
24	*D* Belelük	139	155	226	228	246	246	177	177	155	155	192	192	246	248
25	*D* İm küçük	121	143	228	242	246	246	183	183	155	161	198	198	246	248
26	*D* Siyahgıldun	143	155	228	232	246	246	183	183	155	161	198	198	246	248
27	*D* İm büyük	141	149	222	228	244	244	177	191	155	169	190	202	240	246
28	*D* Beyazgıldun	133	143	222	232	246	246	183	191	161	169	198	198	246	248
29	*D* Amorku	133	149	222	242	236	246	177	191	155	169	194	198	244	246
30	*D* Avkenek	139	149	228	234	244	244	191	191	169	169	190	202	254	254
31	*D* İstanbullu	131	131	232	232	244	246	177	191	155	169	198	202	244	246
32	*D* Şekeri	131	131	222	222	244	262	181	181	155	159	186	198	240	254
33	*D* Şarabi	149	155	232	234	244	246	183	191	161	169	190	198	248	256
34	*D* Hatunparmağı (black)	121	121	232	242	236	252	185	191	163	169	202	202	244	246
35	*D* Kışgıldun	133	149	222	230	236	244	179	183	157	161	190	194	248	248
36	*D* Kabarcık	131	141	230	230	244	250	175	177	153	155	190	202	244	244
37	*TD* Siyahüzüm	133	155	222	232	244	246	181	191	159	169	190	198	244	246
38	*TD* Şarabi	141	149	228	234	244	252	177	191	155	169	202	202	244	246
39	*TD* İsimsiz	133	133	228	236	244	246	191	191	169	169	198	202	240	254
40	*TD* Vanki	139	149	228	234	244	244	191	191	169	169	190	202	254	254
41	*TD* Tahannebi	131	155	230	234	244	244	191	191	169	169	190	202	254	254
42	*TD* Abderi	131	133	230	232	240	244	181	181	159	159	186	190	246	246
43	*TD* Abdullah	131	133	230	232	240	244	181	181	159	159	186	190	246	246
44	Cabernet Sauvignon	137	149	228	236	236	236	171	185	151	165	186	192	244	244
45	Merlot	137	149	222	232	236	244	185	187	165	167	192	192	256	256

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Genetic parameters of 45 genotypes (i.e. number of alleles per locus [n], allele frequency, expected heterozygosity [H_e], observed heterozygosity [H_o], parentage, null allele frequency (r), and PI) were calculated for each of the 7 loci using the IDENTITY 1.0 software (Wagner and Sefc, 1999). Genetic diversity was calculated based on the following formula: the expected heterozygosity (H_e) = $1-\Sigma p_i^2$, where p_i refers to the frequency of individual alleles (Nei, 1987). The observed heterozygosity was considered as the ratio of the number of heterozygous genotypes to the total number of genotypes analyzed. The frequency of null alleles was calculated based on the following formula: $(H_e - H_o)/(1 + H_e)$ (Brookfield, 1996). PI was defined as the probability that two randomly selected samples have the same SSR profile and was calculated based on the following formula: $\Sigma p_i^4 + \Sigma\ \Sigma(2p_ip_j)^2$, where p_i and p_j indicate the frequencies of alleles *i* and *j*, respectively (Paetkau et al., 1995).

In total, 59 alleles were identified in 7 loci, whereby the total number of alleles per locus ranged between 7 and 10 and the mean number of allele per locus was 8.43. The highest numbers of alleles were detected in VVS2 and VrZAG47 and the lowest were detected in VVMD7, VrZAG62, and VrZAG79. The expected and observed heterozygosity were 0.77 and 0.73, respectively. The PI value per locus was higher than 0.05, the value proposed by Sefc et al. (2001). The PI value detected in our study implicates that the microsatellite markers used in this study are highly polymorphic for genotypic analysis of grapevine.

Genetic similarity between the genotypes was calculated using the Microsat software based on the following formula: genetic distance (D), D = 1-(proportion of shared alleles) (dissimilarity). The resulting value was then converted to genetic similarity index. Genotypes with a value of 1.000 had the highest similarity index and were considered as synonym cultivars, some other genotypes had a value of 0.929 and were considered to be genetically similar, and the remaining samples genotypes that had no shared alleles or no genetic similarity in any locus were considered to be genetically dissimilar.

Dendrograms were constructed and visualized using the NTSys software (version 2.02g, Exeter Software, Setauket, NY). Clustering analysis was performed using the UPGMA method. The genotypes of the samples collected from Diyarbakır were grouped based on the proportion of shared alleles. The dendrogram of the 43 genotypes indicated 5 distinct groups, with each group involving numerous subgroups. The 2 reference genotypes were classified into a separate subgroup and were differentiated from the other 43 genotypes. Genotypes with a similarity index of 1.000 were classified as synonym cultivars on the dendrogram. Overall, the dendrogram indicated that the genotypes of the samples collected from Diyarbakır were not remarkably dissimilar and even showed close relationship with each other. Moreover, numerous synonyms and homonyms were detected among the accessions in the dendrogram (*Figure 1*).

Discussion

In the present study, a total of 59 alleles were identified in the 45 genotypes characterized by 7 loci, whereby the total number of alleles per locus ranged between 7 (VVMD5, VrZAG62, and VrZAG79) and 10 (VVS2 and VrZAG47) and the mean number of alleles per locus was 8.43. *Table 4* presents the number of alleles per locus reported by previous studies. In contrast, the studies by Crespan and Milani (2001), Dangl et al. (2001) and Hvarleva et al. (2004) were conducted with lower numbers of

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DOI: http://dx.doi.org/10.15666/aeer/1702_39273939

genotypes and thus detected lower mean numbers of alleles compared to our study. In our study, the number of alleles per locus ranged between 7 and 10 (*Table 3*), which are higher than the numbers reported by Dong et al. (2018) and Zequim Maia et al. (2018). Dong et al. (2018) analyzed 34 grape genotypes using 15 SSR markers and found that the number of alleles per locus ranged between 1 and 8. Zequim Maia et al. (2018) analyzed 69 grape genotypes and reported that the number of alleles per locus ranged between 1.94 and 2. This difference is associated with the large number of low-frequency alleles in large-scale sample sets (Laiadi et al., 2009). However, our study was similar to those reported by Akkak et al. (2005), Costantini et al. (2005), Vouillamoz et al. (2006), and Li et al. (2017) with regard to the number of alleles per locus (*Table 4*).

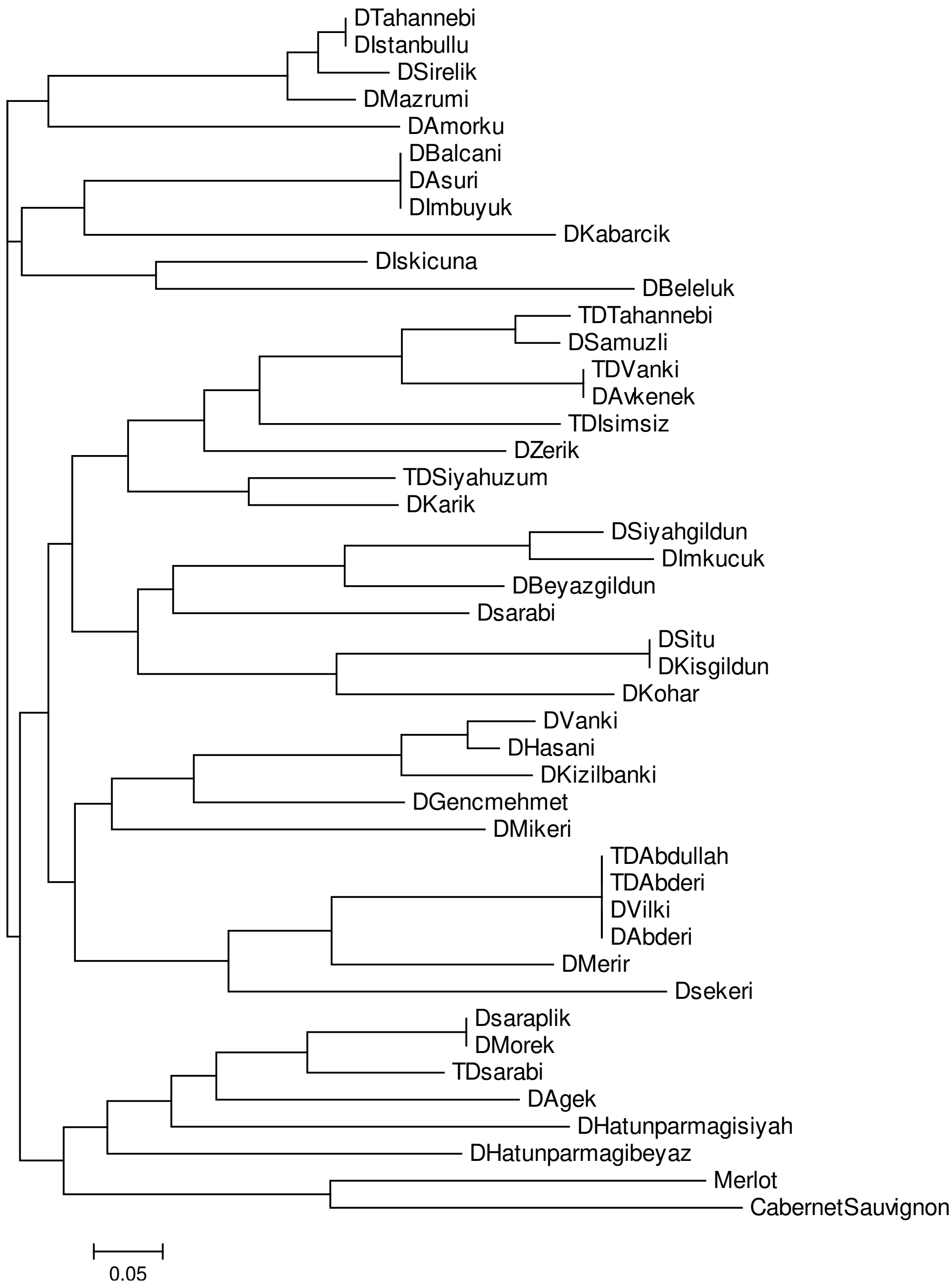

Figure 1. *Dendogram of 45 grape cultivars based on similarity index from SSR data*

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Table 3. *Number of alleles per locus, He, Ho, and PI*

Marker	Number of alleles	He	Ho	PI
VVS2	10	0.85	0.84	0.83
VVMD5	9	0.83	0.87	0.80
VVMD7	7	0.67	0.60	0.62
VVMD27	9	0.73	0.58	0.70
VrZAG47	10	0.75	0.73	0.71
VrZAG62	7	0.81	0.78	0.78
VrZAG79	7	0.78	0.69	0.74
Mean	8.43	0.77	0.73	0.74

Table 4. *Number of alleles per locus reported by previous studies*

Number of genotypes	Number of microsatellite locus	Alleles range	Mean number of alleles	Reference
406	8	4-16	9.60	Borrego et al. (2001)
64	25	3-11	6.58	Crespan and Milani (2001)
41	11	4-11	8.00	Dangl et al. (2001)
62	9	4-16	9.60	Fatahi et al. (2003)
111	13	4-16	9.85	Ibáñez et al. (2003b)
176	6	9-13	11.00	Martín et al. (2003)
74	9	4-10	8.10	Hvarleva et al. (2004)
60	12	7-12	9.10	Akkak et al. (2005)
69	8	6-9	8.00	Costantini et al. (2005)
116	12	6-16	11.90	Vouillamoz et al. (2006)
94	9	5.01-8.57	12.78	Li et al. (2017)
69	17	1.94-2.0	2.0	Zequim Maia et al. (2018)
34	15	1-8	3.6	Dong et al. (2018)

Of the loci used for the analysis of 43 genotypes in our study, VVS2 and VrZAG47 were detected with the highest number of alleles (n = 10 each). In previous studies, VVS2 has also been reported to have the highest number of alleles by Lopes et al. (1999), Borrego et al. (2001), Lefort and Roubelakis-Angelakis (2001), Fatahi et al. (2003), Martín et al. (2003) and Núñez et al. (2004). This marker was followed by VVMD5 and VVMD27 (n = 9) and VVMD7, VrZAG62, and VrZAG79 (n = 7).

The PI values varied from 0.62 to 0.83 with a mean value of 0.74 (*Table 3*). The mean PI value was similar to the value reported by Ramezani et al. (2009) (0.77). The most informative locus was VVS2 (0.83) (*Table 3*). This finding was consistent with the finding reported by Li et al. (2017) that indicated that VVS2 showed the highest level of polymorphism with a value of 0.815.

The PI value per locus in our study was higher than 0.05, the value proposed by Sefc et al. (2001). This finding implicates that the microsatellite markers used in this study are highly polymorphic for genotypic analysis of grapevine. With the highest PI values, VVMD5 (0.80) and VVS2 (0.83) were the most informative markers in our study, also revealing maximum differentiation among the genotypes. In other words, these two markers showed the highest level of differentiation compared to other markers. In other

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studies, the most informative markers have been shown to be VVMD5 (Lefort and Roubelakis-Angelakis, 2001; Ibáñez et al., 2003b; Martín et al., 2003) VVS5 (Borrego et al., 2001), and VVMD14, VVMD28, and VVMD36 (Crespan and Milani, 2001). Meaningfully, VVMD5 has been shown to be an informative marker in numerous studies, as seen in our study.

The expected and observed heterozygosity in our study were 0.77 and 0.73, respectively (*Table 3*). Accordingly, the observed heterozygosity was lower than the expected heterozygosity, which could be attributed to the existence of null alleles. However, the absence of a significant difference between these two rates further confirms that our cultivars were heterozygous. Moreover, the observed heterozygosity in our study was lower than those reported by other studies including Sefc et al. (2000), Dangl et al. (2001), Aradhya et al. (2003), Fatahi et al. (2003), Costantini et al. (2005) and Vouillamoz et al. (2006).

Based on the proportion of shared alleles among the 45 genotypes characterized by 7 microsatellite loci, 15 synonyms and 8 homonyms were detected among the genotypes (*Table 5*).

The reference genotypes used in our study, Cabernet Sauvignon and Merlot, have also been studies by other researchers (Bowers et al., 1999; This et al., 2004). The researchers found similar differences to those of our study, between the two alleles in a single loci for each of the 7 loci.

Table 5. *Synonyms and homonyms detected in the genotypes of the samples collected from Diyarbakır that were characterized by 7 microsatellite markers*

<u>Synonyms</u>
D Vilki, *D* Abderi, *TD* Abderi, *TD* Abdullah
D Balcani, *D* Asuri, *D* İm Büyük
D Şaraplık, *D* Morek
D Tahannebi, *D* İstanbullu
D Avkenek, *TD* Vanki
D Şitu, *D* Kışgıldun
<u>Homonyms</u>
D Hatunparmağı black, *D* Hatunparmağı white
D Vanki, *TD* Vanki
D Tahannebi, *TD* Tahannebi
D Kızılbanki, *Ş* Kızılbanki

Conclusion

In conclusion, we obtained a very high allelic polymorphism among the genotypes that were expected to be different or between the genotypes that were supposed to have the same variety name. Moreover, 15 synonyms and 8 homonyms were detected among the 45 genotypes characterized by 7 microsatellite loci, based on the proportion of shared alleles among the genotypes. However, in the genotypes detected with homonyms, it was not clear as to which homonym represented the real name of each genotype. A total of 30 distinct cultivars were detected among the 45 genotypes characterized by 7 microsatellite markers, suggesting that Diyarbakır Province is rich in genetic diversity of grapevine and that the gene sources of these cultivars should be protected.

We consider that the differentiation in the names of homonymous cultivars could be attributed to erroneous naming of the cultivars or to the variations (e.g. clone, type) that

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have emerged within the same cultivar over time. Considering the naming of synonymous cultivars, it appears that a single genotype might have been erroneously given several different names.

Our results indicated that the microsatellite markers used in our study were highly suitable for the genetic identification of grape cultivars and the determination of synonyms and homonyms. Notably, VVS2 and VVMD5 were the most informative markers among the others and successfully differentiated the 45 genotypes.

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ANALYSIS OF DOUBLE-PEAK SEASONALITY IN THE AETIOLOGY OF PERINATAL MORTALITY AND CHILDHOOD ACUTE LYMPHOBLASTIC LEUKAEMIA USING THE WALTER–ELWOOD METHOD

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(Received 25th Oct 2018; accepted 7th Jan 2019)

Abstract. Our study demonstrates the use of the Walter–Elwood method in double-peak seasonal variation. The concept of the geometrical model for analysing cyclic variation is described. Monte Carlo simulation procedures are used to compare the performance of the Walter–Elwood and negative binomial regression methods with double-peak seasonality, in both a comparison between the two methods and a power analysis. The results of 10,000 independent Monte Carlo simulations showed that the Walter–Elwood method and the negative binomial regression analysis identified the same peak in 9,956 samples, indicating that the power of both methods is similar in analysing double-peak cyclic trends. Additionally, two epidemiological applications of double-peak seasonality are presented, which were analysed using the Walter–Elwood method. Further, this is the first study to describe the power of the Walter-Elwood method for double peak seasonality. In conclusion, double-peak seasonality could be investigated with the Walter–Elwood method in ecological studies when only the population at risk is available and there is no other variable.
Keywords: *environmental effect, geometrical model, cyclic variation, Monte-Carlo simulations, power analyses, population at risk, perinatal mortality, childhood leukaemia*

Introduction

Seasonal variation is a feature of many diseases with a potential infectious component and contributes to a better understanding of aetiology. There are a number of methods for analysing cyclic trends, and this type of patterning can be studied in several different ways (Hewitt et al., 1971; Roger, 1977; Marrero, 1983; Jones et al., 1988). In 1961, Edwards introduced a geometrical model, which only used the number of observations (Edwards, 1961). Walter and Elwood (1975) extended Edwards' idea by including the population at risk. Stolwijk et al. (1999) described an application of logistic regression. The methodology developed by Walter and Elwood is widely used in single-peak analyses but not in double-peak analyses. However, the method is entirely suited to analysing double-peak seasonal variation.

The most frequent numbers of deaths occur in infants aged under 1 year, and a relatively high proportion of children aged between 0 and 14 years die from cancer. Acute lymphoid leukaemia (ALL) is the most common type of cancer found in children.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

Environmental hazards including infections around the time of birth have long been suspected as a possible factor in the aetiology of childhood ALL and in perinatal mortality (McNally and Eden, 2004).

The aim of our study was to demonstrate the use of the Walter–Elwood method in double-peak seasonal variation when the investigated event repeats in six-monthly cycles. Two datasets are used to illustrate the use of the Walter–Elwood method for analysing seasonality in the aetiology of ALL in children under 5 years of age and in perinatal mortality, respectively.

Methods

We describe the concept of the geometrical model for analysing cyclic variation. Seasonal fluctuations of an event (e.g. infant death) which occurs on a fixed date every year might be described using cyclic patterns over a period of time (e.g. two decades).

We suppose that within a certain time span (e.g. a year) there are k sectors (e.g. 12 months) and that in sector i there are n_i events from a population at risk of size m_i (e.g. total births during that month). The total number of events is $N=\sum n_i$ and the total size of the population at risk is $M=\sum m_i$.

H_0: The expected number of events in a sector is proportional to the population at risk in that sector, i.e. $E(n_i)=Nm_i/M$.

The basic idea of the Walter–Elwood method is that the monthly number of observed events and the population at risk are grouped by month over the study period yielding data for all 12 months for both events and population at risk. Then the weighted monthly numbers in the aggregated data are placed on the circumference of a circle with unit radius using the polar co-ordinate system. In the case of single-peak seasonality, this circle represents the year, and the sectors correspond to 12 months.

The data by weights $\sqrt{n_i}$ are placed around a unit circle at points corresponding to the sector midpoints at angles θ_i to an arbitrary diameter (e.g. the diameter through 1 January: January is $0°$).

Average observed frequencies are:

$$\bar{x}=\frac{\sum_{i=1}^{k}\sqrt{n_i}\cos\theta_i}{\sum_{i=1}^{k}\sqrt{n_i}} \quad \bar{y}=\frac{\sum_{i=1}^{k}\sqrt{n_i}\sin\theta_i}{\sum_{i=1}^{k}\sqrt{n_i}} \qquad \text{(Eq.1)}$$

From (*Eq. 1*) we calculate the expected values of x and y:

$$\mu_{\bar{x}}=\frac{\sum_{i=1}^{k}\sqrt{Nm_i/M}\cos\theta_i}{\sum_{i=1}^{k}\sqrt{Nm_i/M}}=\frac{\sum_{i=1}^{k}\sqrt{m_i}\cos\theta_i}{\sum_{i=1}^{k}\sqrt{m_i}} \quad \mu_{\bar{y}}=\frac{\sum_{i=1}^{k}\sqrt{m_i}\sin\theta_i}{\sum_{i=1}^{k}\sqrt{m_i}} \qquad \text{(Eq.2)}$$

The variances of the coordinates of the sample centre of gravity are:

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

$$\sigma^2_{\bar{x}} = \frac{\sum_{i=1}^{k} \frac{1}{4} \cos^2 \theta_i}{[\sum_{i=1}^{k} \sqrt{Nm_i / M}]^2} \quad \sigma^2_{\bar{y}} = \frac{\sum_{i=1}^{k} \frac{1}{4} \sin^2 \theta_i}{[\sum_{i=1}^{k} \sqrt{Nm_i / M}]^2} \tag{Eq.3}$$

The test statistic (*Eq. 4*) is distributed on the null hypothesis as χ^2 with 2 degrees of freedom:

$$\left(\frac{\bar{x} - \mu_{\bar{x}}}{\sigma_{\bar{x}}} \right)^2 + \left(\frac{\bar{y} - \mu_{\bar{y}}}{\sigma_{\bar{y}}} \right)^2 \tag{Eq.4}$$

The distance *d* of the sample centre of gravity from its null expectation is provided by

$$d^2 = (\bar{x} - \mu_{\bar{x}})^2 + (\bar{y} - \mu_{\bar{y}})^2 \tag{Eq.5}$$

If the null hypothesis is true (no seasonal trend or equal underlying rates in each month), then the centre of gravity has an expected value of zero on both the *x* and *y* axes. This hypothesis is tested using chi-square statistics with 2 degrees of freedom. If the test statistic is greater than 5.99, then a simple harmonic trend should be fit to the data (*Fig. 1*). We may suppose that the expected frequency in sector *i* is proportional to

$$c_i = m_i \left[1 + \alpha \cos(\theta_i - \theta^*) \right] \tag{Eq.6}$$

where amplitude $\alpha = 4d$ is a measure of the cyclic variation, which is calculated using the formula developed by Edwards (*Eq. 6*), and where θ^* (*Eq. 7*) provides the discretion of the maximum rate, which may be estimated as

$$\theta^* = Tan^{-1} \left(\frac{\bar{y} - \mu_{\bar{y}}}{\bar{x} - \mu_{\bar{x}}} \right) \tag{Eq.7}$$

The adequacy of the description of the data afforded by a simple harmonic curve may be evaluated by a goodness-of-fit test using a further χ^2 statistic. The expected frequencies n_i' are calculated by

$$n_i' = Nc_i / \sum_{i=1}^{k} c_i$$

and the goodness-of-fit test statistic (*Eq. 8*) is:

$$\sum_{i=1}^{k} \left(n_i - n_i' \right)^2 / n_i' \tag{Eq.8}$$

which has a χ^2 distribution with (*k-1*) degrees of freedom.

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DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

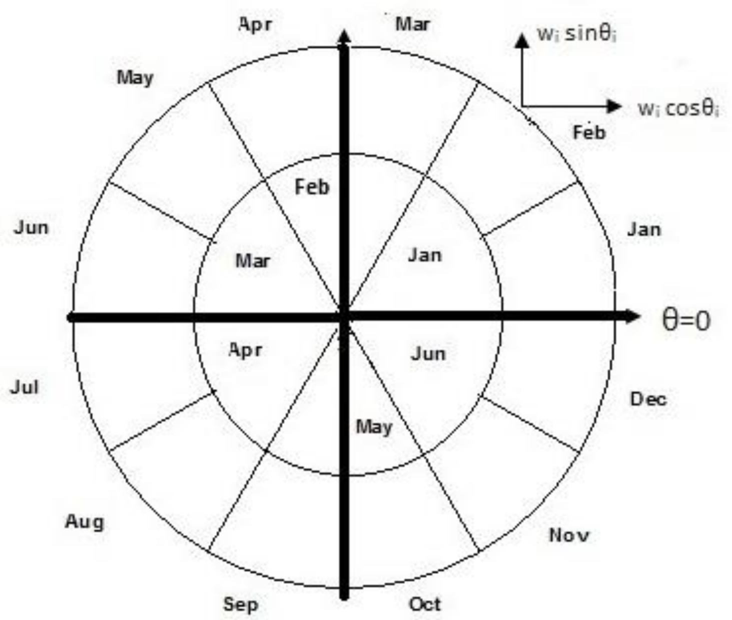

Figure 1. *Sectors for single-peak (outside circle) and double-peak (inside circle) analysis*

The use of the Walter–Elwood method to analyse double-peak seasonality with six sectors is based on the assumption that the observed events occur six months apart. Thus, the monthly data are aggregated using the months of the first half year and adding to them the corresponding data from six months later (e.g. January and July). Additionally, the first angle of maximum variation is detected by this test and the second maximum is at $\theta^*+\pi$ (*Fig. 1*). The test statistic remains unchanged, that is a chi-square statistic with 2 degrees of freedom. Similarly, the goodness-of-fit test remains the same for both single-peak and double-peak analyses. Nevertheless, Monte Carlo simulation procedures were used to compare the performance of the Walter–Elwood and negative binomial regression methods. 10,000 random samples from a Poisson distribution were generated with double-peak seasonality and used in both a comparison between the two methods and a power analysis. A p value of less than 0.05 was considered to indicate a significant effect.

Two datasets are used to illustrate the use of the Walter–Elwood method for analysing seasonality.

Acute lymphoblastic leukaemia study

We have investigated seasonal trends in the incidence of ALL around the time of birth in children aged 0-4 years. Children born between 1981 and 1997 in south-eastern Hungary were considered. The monthly number of cases and births in the 1981-1997 period are displayed in *Table 1*.

Perinatal mortality study

In this study, cyclic changes in early perinatal mortality rates were investigated during the 34-year interval between 1 January 1980 and 31 December 2014 in Hungary. The data on the cause of perinatal deaths were published annually by the Central Statistical Office (KSH, 1980-2014).

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DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

Results

Monte Carlo simulation

The results of the Monte Carlo simulations showed that out of 10,000 independent data sets, the Walter–Elwood method indicated significant double-peak seasonality in 8,416 cases, while the negative binomial regression analysis did so in 8,672 cases. Moreover, the two methods identified the same peaks in 9,956 samples, indicating that the power of the Walter–Elwood method is similar to the negative binomial regression in analysing double-peak cyclic trends.

Acute lymphoblastic leukaemia study

Table 1. *The monthly number of acute lymphoblastic leukaemia (ALL) cases and births in the period 1981-1997*

Month	Number of live births	Single peak		Double peak	
		Observed number of ALL	Expected number of ALL	Observed number of ALL	Expected number of ALL
1	23631	9	11.537	12	11.476
2	21894	10	9.721	16	14.808
3	23862	6	9.515	12	14.905
4	21922	3	8.038	12	9.945
5	22953	2	8.023	6	6.234
6	23274	3	8.204	6	6.632
7	25236	3	9.696		
8	24631	6	10.384		
9	23872	6	11.299		
10	22724	9	11.535		
11	21799	4	11.428		
12	22358	3	11.615		

The calculated expected frequencies in the analysis using a single-peak seasonal pattern are also shown in *Table 1*. The χ^2 test statistic is 2.45, which indicates no significant (p = 0.293) cyclic pattern. However, with the Walter–Elwood method using a double-peak seasonal pattern, the χ^2 test statistic is 6.11, which indicates a significant (p = 0.047) cyclic pattern. The amplitude and the angle of maximum rate were 0.43 and 116.5, respectively. Thus, peak rates occurred in children born in February and August. The goodness-of-fit test statistic was 1.18 (p = 0.946). These findings with the Walter–Elwood method are similar to our published results from southern Hungary (Nyári et al., 2008).

Perinatal mortality study

The monthly number of deaths and births in the study period are summarised in *Table 2*. The calculated expected frequencies in the analysis using a single-peak seasonal pattern are also shown in *Table 2*. The χ^2 test statistic is 1.114, which indicates no significant (p = 0.57) seasonality.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

Table 2. *The monthly number of perinatal mortality and births in the period 1980-2014*

Month	Number of live births	Single peak		Double peak	
		Observed number of perinatal mortality	Expected number of perinatal mortality	Observed number of perinatal mortality	Expected number of perinatal mortality
1	164527	691	702.11	1404	1429.44
2	148237	569	627.27	1292	1266.50
3	159762	663	671.50	1315	1334.36
4	151516	680	634.84	1373	1362.08
5	156203	720	655.32	1399	1406.96
6	159247	711	671.70	1441	1424.63
7	175431	713	745.89		
8	169170	723	725.35		
9	169792	652	732.83		
10	163604	693	708.29		
11	153903	679	665.46		
12	158881	730	683.38		

If we employ the Walter–Elwood method using a double-peak seasonal pattern, the χ^2 test statistic is 18.74, which indicates a significant ($p < 0.001$) cyclic pattern. The amplitude and angle of maximum rate were 0.068 and -71.8, respectively. Maximum rates occurred in children born in May and November in the simple harmonic model for perinatal mortality. The goodness-of-fit test statistic was 1.567 ($p = 0.905$). These findings using the Walter–Elwood method confirm our published results that significant double-peak seasonality was found in children born in May and November for perinatal mortality (Nyári et al., 2015).

Discussion

We have presented detailed descriptions of the use of the Walter–Elwood method for double-peak seasonality and shown the power of this method using Monte Carlo simulations. These simulations have revealed that the power of the Walter–Elwood method is very close to the negative binomial regression method in analyses of double-peak seasonality.

Using the Walter–Elwood method seasonal variability for ALL related to date of birth of all children diagnosed under age five years was detected. We found evidence of seasonality related to month of birth with peaks in February and August. Both peaks correspond to previous findings and could reflect the seasonality of infectious diseases.

Significant double peak model was fitted to characterize the seasonal variation of perinatal mortality using the Walter-Elwood method. The peaks were observed in May and November and we might assume that perinatal mortality may have been related to respiratory infections (Borchers et al., 2013). Formerly, we have detected double peak of seasonality with generalized linear models. This study demonstrates that the application of the Walter -Elwood method for double peak of seasonality gives similar

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

results with high power. Further, this is the first study to describe the power of the Walter-Elwood method for double peak seasonality.

Many health conditions and exposures (or risk factors) involve some seasonal element (Marrero, 1983; Jones et al., 1988). The harmonic model is one of the simplest. It may be adopted to represent oscillating rates. However, the main limitation of using a fitted sine curve is the inability to adjust for covariates. Furthermore, the fitted harmonic trend yields a poor goodness-of-fit test statistic when the variation of the observed data is high.

The choice among analytical approaches should ideally reflect the research question of interest, and simple methods are compelling in many cases. There is a free programmed spreadsheet known as Episheet to apply Edwards' geometrical model (Rothman, 2008). However, this program only detects single-peak seasonality. Our study demonstrates that the Walter–Elwood method is easy to calculate using a spreadsheet program for double-peak seasonality.

Walter (1977) published a power calculator for the Edwards method for a single-peak cyclic pattern but not for a double peak. Stolwijk and his colleagues (1999) criticized the Walter–Elwood method for analysing double-peak seasonality. Similarly, Barnett and Dobson (2010) described a poor power for the use of the Walter–Elwood method in analysing double-peak seasonality. In contrast, our findings have revealed a similar power for the Walter–Elwood method, which characterizes the negative binomial regression method in analyses for double-peak seasonality.

Christiansen and his colleagues (2012) reviewed methods for analysing seasonal variation. They suggested using the peak-to-trough ratio. Cave and Freedman (1975) presented a method for double-peak seasonality analysis of Crohn's disease using an extension of the Edwards test in methodology. Similarly, our study found that the Walter–Elwood method is a suitable method to analyse double-peak seasonal variation. Furthermore, this method allows the inclusion of the population at risk, and it is simple to calculate both for the test statistics and the fitted expected frequencies.

In conclusion, double-peak seasonality could be investigated with the Walter–Elwood method in ecological studies when only population at risk is available and there is no confounding variable. Since cyclic trends in the aetiology of a disease reveal some effect of environmental factors we might assume that both childhood acute lympoid leukaemia under age five years and perinatal mortality may have been related to respiratory infections. These findings could prove useful in preventive strategies, but further cohort studies need to investigate this hypothesis.

Acknowledgements. This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund under EFOP-3.6.1-16-2016-00008.

Ethical approval. No ethical approval was necessary based on the Hungarian Law on the use of statistical data (Act XLVI 1993, Sec. 10).

Conflict of interests. The authors declare that they have no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39413948

ADSORPTION OF METHYLENE BLUE BY DIFFERENT TYPES OF STRAW BIOCHAR WITH PERFORMANCE COMPARISON

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(Received 1st Nov 2018; accepted 25th Jan 2019)

Abstract. In order to study the adsorption property of straw biochar of methylene blue dye, five kinds of biochar (rice straw, corn straw, wheat straw, peanut straw and reed straw) were prepared at 500°C through the method of oxygen limiting and temperature control. The microstructure, functional group and elemental composition of biochar were characterized with a scanning electron microscope, Fourier transform infrared spectroscopy and X-ray diffraction. The adsorption experiments of the methylene blue solution by five kinds of straw biochar were carried out, and the properties of the biochars after the adsorption of the methylene blue solution were compared and analyzed. The result revealed that S500, X500 and L500 have round hole microporous structure, and Y500 and H500 have parallel wall microporous structure. The FTIR spectra of five kinds of straw biochar contained polar groups and aromatic structures. Their adsorptive capacities were as follows: X500 > S500 > L500 > Y500 > H500. Among the five kinds of straw biological charcoals, X500 had good surface characteristics and the ability to adsorb methylene blue. It is the preferred material for adsorbing organic contaminants. Moreover, the surface characteristics of the five kinds of straw biological charcoal also affected their ability to adsorb the methylene blue solution.
Keywords: *charcoal, surface features, adsorb-ability*

Introduction

Biochar is a carbon rich material (Warnock et al., 2007) cracking under both the presence of oxygen and oxygen free conditions, has rich surface functional groups, high carbon content and developed pore structure, and can be used as a new adsorbent of organic pollutants (Yang and Sheng, 2003). Methylene blue ($C_{16}H_{18}ClN_3S$) is widely used in the field of chemical indicators, dyes, biological dyestuffs, drugs and so on (Zhou, 2010). In the printing and dyeing industry, a large amount of organic dye wastewater is produced, which holds great environmental risks. Using a low-cost material to control environmental pollution has become a popular topic of researches (Yang et al., 2006; Warnock et al., 2007). A new technology of biomass carbonization and a resource for agricultural products has been gradually conceived (Jian et al., 2016). Therefore, biochar plays an important role in industrial waste treatment and the remediation of environmental pollution.

Through studying three kinds of improved corn straws, Ci and Chen (2016) discovered that the improved corn straw adsorption capacity of methylene blue increased. Huang et al. (2018) found that the adsorption of methylene blue on activated carbon from rice straw was mainly affected by pore size. Fragiskos et al. (2008) found that the untreated wheat straw had strong ability to adsorb methylene blue and could be used as a low-cost adsorbent. Hammes et al. (2008) explored the changes in the elemental composition of black carbon after pyrolysis, indicating that the hydrogen and oxygen elements in biochar continued to remove hydrophobicity with increasing temperature. Chen and Chen (2009) studied the surface functional groups of wood biochar and found that they were adsorbed by π-π conjugated structure and filled with pores. Lu et al. (2013) compared rice straw biochar and corn straw biomass, found that corn straw biochar degree of aromatization was higher, thus

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DOI: http://dx.doi.org/10.15666/aeer/1702_39493958

enhancing organic solution adsorption. Zhang et al. (2016) studies showed that the biochar adsorption capacity of reed straw pyrolyzed at 500°C was the strongest. Ji et al. (2016) found that the adsorption of methylene blue on cationic dyes by rice straw biochar was mainly mediated by ion exchange. From the above, the surface morphology, functional groups and chemical composition of straw biochar have great influence on the adsorption properties of methylene blue organic solution. The biological charcoal of different straw has different structures and components. It is of great significance to study the physicochemical characteristics and adsorb-ability of straw biological charcoal for the treatment of organic dyes.

In this paper, five kinds of straw biomass were selected at 500°C for slow oxygen slow thermal cracking and carbonization, the structure characterization of five kinds of biological charcoal and the adsorption experiment on methylene blue solution. The physical and chemical properties of five kinds of biological charcoal were characterized by electron microscope scanning, Fourier transform infrared spectrum analysis and modern analysis of X-ray diffraction. The physicochemical properties of five kinds of biological charcoal were compared.

Materials and Methods

Preparation of biological carbon from straw

Five kinds of straw biomass were selected as the raw materials for preparing biochar. Rice straw and corn straw were collected from farm field in Pu Kou District of Nanjing, China. Wheat straw and peanut straw were collected from a farm field in Yang Zhou, and reed straw was collected from Nanjing Bagua. Each straw was collected at 1 kg. The straw was washed, dried, cut in 1-2 cm pieces, put into a grinding machine, smashed in 60 mesh, and stored in 90°C drying oven.

The biological carbon was prepared by the slow speed hot cracking method. The straw treated with powder was placed in a quartz boat and placed in a muffle furnace and passed into nitrogen for 10 min to discharge the air in the tube. The heating rate was set at 5°C /min, and the temperature was raised to 300°C for 1 h. Then the temperature was kept at the highest temperature of 500°C for 2 h, and then cooled to room temperature after the pyrolysis was completed, and the biological charcoal was dried. The biochar samples of rice straw, wheat straw, corn straw, peanut straw and reed straw were signed as S500, X500, Y500, H500 and L500.

Properties and characterization of biological carbon

Carbon yield

The quality of five kinds of straw before and after pyrolysis was weighed. The carbon yield of biological charcoal was referred to "*Wood Charcoal and Charcoal Test Method (GB/T17664 - 1999)*" (Wood charcoal and test method of wood charcoal), and the mass ratio of straw before and after was the carbon yield. The carbon yield of different straw biochar was calculated according to *Formula (1)*.

$$\eta = \frac{m_2}{m_1} \times 100\% \qquad \text{(Eq.1)}$$

where η is carbon yield and m_1 and m_2 represent the mass /g before and after carbonization.

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Microstructure of biological carbon from straw

Field emission scanning electron microscopy (SEM) (*US FEI Inspect F50*) was used to observe the microstructure of the straw bio carbon. The instrument voltage of the scanning electron microscope was 20 KV, and the magnification was 1000 and 3000, and their SEM images were recorded.

Fourier transform infrared spectroscopy

Five kinds of biological charcoal were called each 0.002 g, and the KBr particles of 0.2 g were mixed (1:100), lapped, pressed (Lu et al., 2013) via using *Nicolet 380 Fourier Transform Microscopic infrared spectrometer*, and the sample was scanned on the spectrometer sample frame.

X- ray diffraction

The crystal structure characteristics of biochar were analyzed by *Brook D8 ADVANCE*. 1.5 g biochar samples were collected and placed on the sample stage for equipment diffraction. CuK was used as the transmitter source, and the tube voltage and tube current were 40 kV and 30 mA, respectively. The scanning angle was 10-60°, the step length was 0.02°, and the scanning speed was 7° /min (Kim et al., 2012).

Adsorption test of methylene blue by biological carbon

Five kinds of biochar 0.8 g were added and standard methylene blue solution with 20 mL of $100\,mg \cdot L^{-1}$ (BZ) was added. Then it was put in constant temperature cradle for shocking at 25 ℃ and $150\,r \cdot \min^{-1}$ for 2 h, then is was placed statically for 24 h. The adsorbed methylene blue solution was filtered through the 0.45 μm polyethersulfone filter membrane through a needle filter, and the color image of the filtrate was recorded. The filtrate was used to measure the absorbance of the solution at 665 nm with the UV visible spectrophotometer, and compared with the 5 g/L$CuSO_4$ solution ($CuSO_4$).

The absorbance and concentration of methylene blue solution after adsorption were measured by ultraviolet visible spectrophotometer after absorption of methylene blue standard solution by five kinds of biochar. The adsorption rate of 5g/L$CuSO_4$ solution for standard methylene blue solution is 100%, and the setting value is 100, and the standard methylene blue solution corresponds to the standard methylene blue adsorption rate of 0% and the setting value of 0.

Results and discussion

Analysis of carbon yield

Five kinds of straw biochar were calculated by *Formula (1)*. *Figure 1* was pyrolytic carbon yield of five kinds of straw materials at 500°C. From the *Figure. 1*, the yield of H500 is 46.96% and the yield of Y500 is 37.01%. The yields of S500 and X500 are 38.83% and 38.93%, respectively, and their yields are similar. The yield of L500 is 39.93%. The decomposition of cellulose, hemicellulose and lignin in the straw component at 500℃ shows that the carbon yield will decrease slowly with the increase of pyrolysis temperature, so the biochar has a good thermal stability (Yang et al., 2017). The carbon yield of H500 is high. On the one hand, the ash content of H500 is high and the component of pyrolysis is not easily affected by high temperature. At 500℃, the heat stabilization

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ability of H500 is stronger than other straw. On the other hand, the lignin content in H500 is higher than that of other straw. Lignin is a kind of high aromatic substance. The temperature range of the molecular compound is broad and the thermal stability is strong. The yield of S500 is similar X500 (Yang et al., 2017; Wei, 2017). It is found that the composition of metal salts in Y500 is low, and the composition of metal salts can catalyze the pyrolysis of biomass (Raveendran et al., 1995), resulting in low carbon yield of Y500.

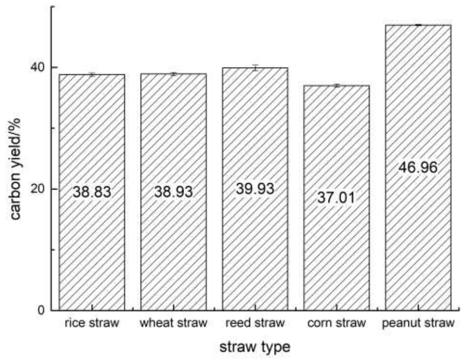

Figure 1. *Yield of biological coals*

Morphology analysis

Figure 2 is a scanning electron microscope (SEM) of five kinds of straw biomass at 500°C carbonization. *Figure 2* shows, there are pore structures on the surface of five kinds of straw biochar.

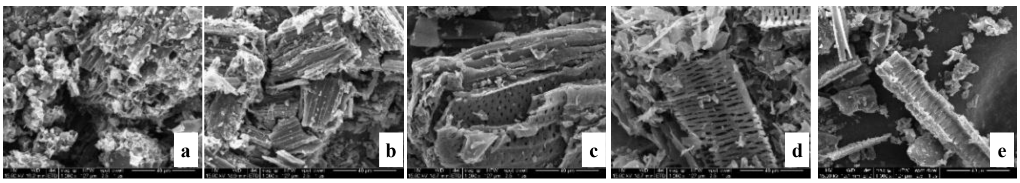

Figure 2.1 *×1000 SEM*

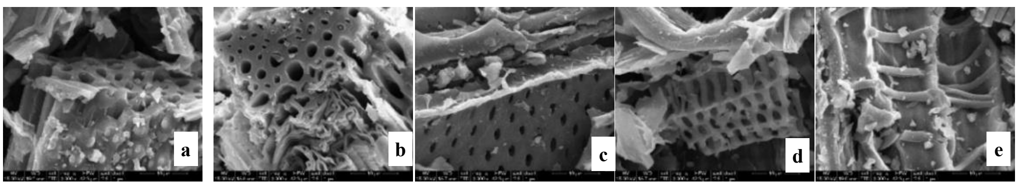

Figure 2.2 *×3000 SEM*

Figure 2. *SEM of biochar (a. S500; b. X500; c. L500; d. Y500; e. H500)*

The pore shape of S500, X500 and L500 show a circular pore shape, with smooth surface and different pore structure and specific shape. The pore distribution of S500 is uniform, the pore size is the same, the pore distribution of X500 is uneven, the pore size is gradually reduced, and the pore size of L500 is smaller. The space is large and the hole is evenly distributed. The pore shape of Y500 and H500 appear as parallel wall type, the surface is rough, the arrangement of pore structure and specific shape are different.

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Compared with the first three kinds of pore size, the maize straw bio carbon hole arrangement is dense, H500 arrangement is compact, the pore distribution is uneven, and the aperture is not shaped. Under the same amplification conditions, the number of holes in S500, X500 and L500 is more than that in Y500 and H500. Although there is obvious pore structure on the surface of S500 and H500, there is no lamination structure of X500, L500 and Y500, which may be related to the temperature and time of carbonization, the structure breaking of pore structure as well as the surface structure of its own straw. The volatile components in biomass can be precipitated in large quantities and thus gradually form porous structures. The number of pores and pore size pores will affect the adsorption capacity of biological carbon. The pore structure of the straw bio carbon is rich in pore structure and the pore size is obvious. It can be used as an efficient adsorbent to remove methylene blue from the waste-water (Xu et al., 2012).

FTIR analysis

Figure 3 is a Fourier transform infrared spectrum analysis of straw biochar. The changes in the functional groups of the five kinds of biological carbon are basically the same.

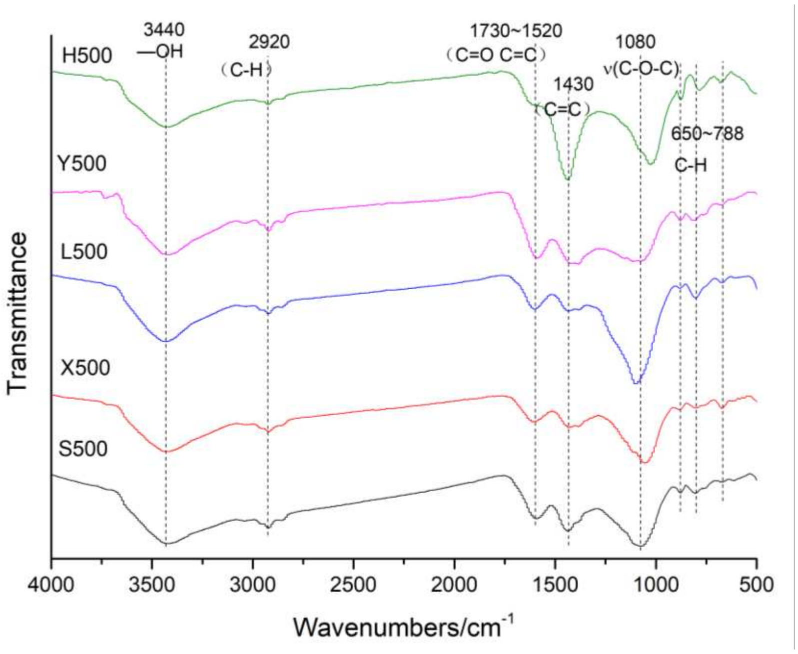

Figure 3. *FTIR of five types of straw biochar*

There is a peak of phenol hydroxyl absorption at 3440 cm^{-1}, and a higher methylene absorption peak of the aliphatic (C-H) is found at 2920 cm^{-1} (Zhang et al., 2016). Because of the bending vibration of aromatic ring C=O, the absorption peak appears between 1730~1520 cm^{-1}. 1080 cm^{-1} appeared C-O-C antisymmetric stretching vibration in Six-element dioxygen ring ether (Yang et al., 2010). The absorption peak at 1522 cm^{-1} and 1430 cm^{-1} is aromatic ring C=C, and there are many aromatic (C-H) flexural vibration peaks between 650~788 cm^{-1}. It shows that straw biochar contains hydroxyl, carboxyl, carbonyl, ester, aldehyde groups and other oxygen-containing functional groups. Therefore, five kinds of biochar mainly exist hydroxyl and other polar groups, and they

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DOI: http://dx.doi.org/10.15666/aeer/1702_39493958

have aromatic structure at the same time. It shows that the adsorption of methyl blue on methylene blue solution is due to the effect of polar group, and the aromatic structure of biological carbon and aromatic methylene blue can form π-π conjugated structure, and there is a kind of π-π interaction (Ji et al., 2016).

The content of surface functional groups on the surface of biochar affects its adsorption capacity. From *Figure 3*, in the vicinity of 1520~1730 cm^{-1} it can be seen that the expansion vibration intensity of aromatic ring functional groups on the surface of S500, X500 and L500 is greater than that of H500 and Y500, among which H500 is used. The vibration intensity of straw biochar is the weakest. It can be concluded that the content of π-π conjugated structure of H500 is the lowest in the process of adsorbing methylene blue.

XRD analysis

Figure 4 is a X- ray diffraction pattern of five kinds of straw biochar. It can be seen from the *Figure 4* that the main peaks of S500, X500 and Y500 appear in 2θ=23.5°, L500 has a wide peak of 22.5°, and H500 has no obvious main peak. This kind of peak corresponds to the diffraction peak of the amorphous structure. The diffraction peak of the amorphous structure on the surface is mainly related to the crystalline structure of cellulose in biomass (Keiluweit et al., 2010; Kim et al., 2012).

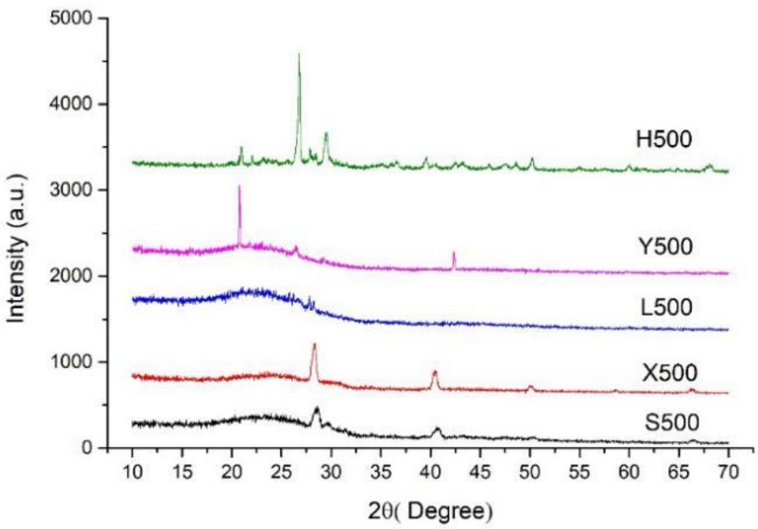

Figure 4. *XRD of five types of biochar*

The main peak is not found in H500. Most of the cellulose is completely pyrolyzed at 500℃. The other four kinds of biological carbon cellulose diffraction peak still exist at 500℃, which may have the good thermal stability of these crystalline cellulose. Without complete pyrolysis, it can be concluded that the crystallisation of H500 is not high, the high crystal stability is good, and the adsorption effect is good (Lin et al., 2016). In this experiment, there are potash mineral components in S500 and X500. The mineral diffraction peak of SiO_2, $CaCO_3$ is found in Y500 and H500. It is found that L500 has a amorphous structure but no mineral diffraction peak, but it is L500. The content of the components of the mineral salt is very small, and the content of K is only 0.22%, and the content of P is 0.16%. Compared with the C element with the content of 46.8%, the

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DOI: http://dx.doi.org/10.15666/aeer/1702_39493958

content is not obvious (Meng et al., 2015). On the other hand, it might be related to the source of L500 and the content of local environmental elements, which has a low impact.

Experimental analysis of adsorption of methylene blue standard solution by biochar

The absorbance and concentration of methylene blue solution after adsorption were measured by ultraviolet visible spectrophotometer after the absorption of methylene blue standard solution by five kinds of biochar. The adsorption rate of 5 g/L$CuSO_4$ solution for standard methylene blue solution is 100%, and the set value is 100, and the standard methylene blue solution corresponds to the standard methylene blue adsorption rate of 0% and the set value of 0. Absorbance and adsorption rate of biochar after adsorption of standard methylene blue solution are in *Figure 5*. The lower the absorbance value, the higher the transmittance of the solution (is) and the better its adsorption effect. With the absorbance 0.1068Abs of 5 g/L$CuSO_4$ solution as the transparent index of solution, the absorbance of X500 is lowest 0.0571Abs, less than the absorbance of $CuSO_4$ solution. It is considered that the adsorption rate reaches the maximum value of this experiment, which is 100% adsorption, and the absorbance of S500 is 0.1091Abs, its adsorption rate is 97.4%. The absorbance of L500 is 0.3356Abs, its adsorption rate is 94.6%. The absorbance of Y500 is 0.6865Abs, its adsorption rate is 83.9%, and the absorption rate of H500 is 2.1945Abs, and its adsorption rate is 51.5%. The biological charcoal with higher adsorption rate than 90% believes that the adsorption effect is good, and the adsorption efficiency is less than 50%. The adsorption effect of S500, X500 and L500 on methylene blue solution is better, the adsorption rate of H500 is near 50%, and the adsorption effect of H500 is not good (Fu et al., 2012).

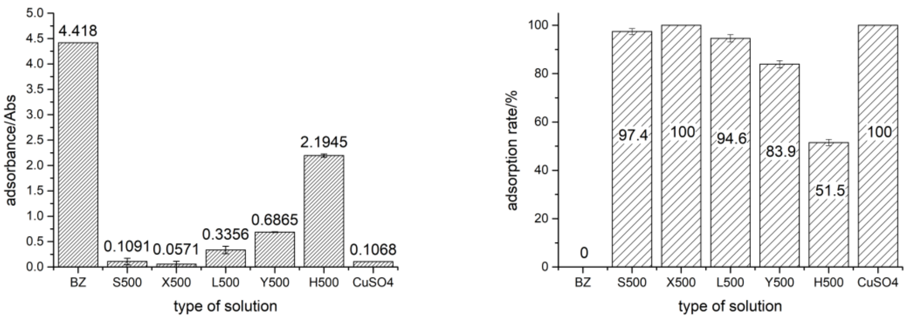

Figure 5. *Absorbance and adsorption rate of five kinds of straw biochar adsorption solution, methylene blue standard solution and 5g/L$CuSO_4$ solution*

Figure 6 is the effect of five kinds of biological carbon adsorbed on the standard methylene blue solution. The effect of the adsorbed solution is: X500 > S500 > L500 > Y500 > H500. Compared with the effect of five kinds of adsorbed methylene blue solution and standard methylene blue solution, the adsorption solution of X500 becomes transparent and clear due to the pore shape of the microstructure of X500. Besides that, it is also because the number of pores is more, the texture structure is obvious, the polar and aromatic structure content of X500 is more than that of X500, and it has an amorphous structure. The pore size of S500 and L500 is different, and the content of functional group is less than that of X500, so the adsorption effect of methylene blue solution is second only to that of X500, which can be determined as the main factor affecting the adsorption

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capacity of methylene blue solution. The pore shape of H500 microstructure is parallel wall shape. The pore structure is damaged by high temperature, large pore size, less pore number and is has no obvious bedding structure. Most of the cellulose pyrolysis after the carbonization of H500 has a large number of SiO_2, $CaCO_3$, and the peak expansion vibration peak of polar group is weak, and the number of aromatic structure is obviously less. In other biochar, the adsorption capacity of ion exchange and π-π interaction are weakened, which is the reason why the adsorption effect of H500 is not good. The biological charcoal of Y500 also has the shape of parallel wall, and its polar and aromatic structure content is less, it contains more SiO_2, $CaCO_3$ mineral diffraction peak, and the adsorption effect of Y500 to methylene blue solution is not good.

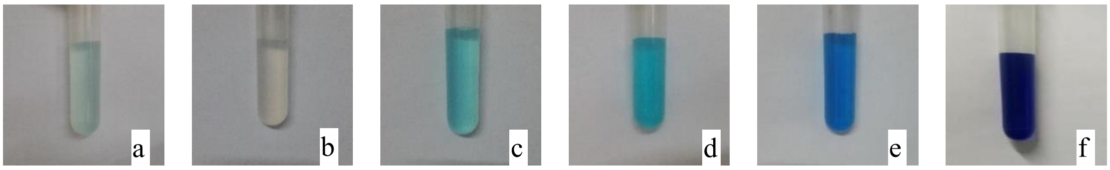

Figure 6. *Comparison of adsorption effect between five kinds of biochar and standard methylene blue solution (a. S500; b. X500; c. L500; d. Y500; e. H500)*

Conclusion

The microstructure, elements and functional groups of five kinds of straw biochar, also the adsorption properties of methylene blue solution were analyzed. The conclusions are as follows:

1) The pore shape of S500, X500 and L500 is round, and the hole shape of Y500 and H500 is parallel wall shape, and the number of pore number is more than that of the latter. Pore shape, pore number and pore size affect the adsorption capability of biochar to methylene.
2) There are mainly hydroxyl group and aromatic structure in the straw biological carbon. Additionally, the higher content of the polar and aromatic structural functional groups of the straw bio carbon, the better effect of the adsorption of methylene blue.
3) The adsorption capacity of five biochar to methylene blue: X500 > S500 > L500 > Y500 > H500. The adsorption rate of X500 reaches 100%. The results show that X500 has the best adsorption capacity for methylene blue and it is suitable for the application of organic dye pollution control.

Acknowledgements. The authors acknowledge the National Students' platform for innovation and entrepreneurship training program(Grant:201710307098).

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DOI: http://dx.doi.org/10.15666/aeer/1702_39493958

GENOTOXICITY ASSESSMENT OF THE GROUNDWATER QUALITY IN THE TEBOULBA REGION-TUNISIA USING THE *VICIA FABA* MICRONUCLEUS TEST

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(Received 2nd Nov 2018; accepted 28th Jan 2019)

Abstract. The groundwater of the Teboulba region in Tunisia suffers from overexploitation and from the degradation of its quality due to salt water intrusion from the Mediterranean sea as well as from the sebkha of Moknine. The actual groundwater quality was assessed using the *Vicia faba* root meristem micronucleus assay. The exposure of *V. faba* to irrigation water from 3 wells showed no spectacular changes in the root growth, fresh and dry matter and the membrane integrity compared to the control plants. However, the plants exhibited decreases in their mitotic index and high inductions of micronuclei and chromosomal aberrations including stickiness, bridges, fragments, vagrant chromosomes and laggards. Consequently, and despite the fact that the morphological parameters and the membrane integrity did not show noticeable variation, the micronucleus test has proved to be an early bioassay allowing the detection of the Teboulba groundwater genotoxicity, which may probably result from contamination of the groundwater by agricultural and industrial activities in/or surrounding the region.
Keywords: *aberrations, contamination, membrane integrity, micronucleus, mitotic index, morphological parameters, Teboulba groundwater*

Introduction

The groundwater is considered as the primary resource for water. It is widely used for agriculture and industry especially in regions with limited annual precipitation. However, shallow groundwater is vulnerable to various types of pollution. In fact, in coastal regions, the overuse of groundwater wells located near the shoreline is the major cause of the salt water intrusion in the groundwater (Bouri and Ben Dhia, 2010). This phenomenon is considered as the primary cause of the salinization of groundwater (Bear et al., 1999). Moreover, groundwater contamination with anthropogenic materials is possible, harming both the groundwater quality and quantity. Such materials dissolve in the liquid phase, and may penetrate into the soil and affect the groundwater quality via percolation (Bear and Cheng, 2010). The groundwater quality may also suffer from other contamination like septic tanks and wastewater effluents. There are different types of pollutants that can be found in the groundwater such as salt, nitrate, heavy metals and new contaminants like pharmaceuticals and personal care products (Zghibi et al., 2013; Ameur et al., 2016; Ouessar et al., 2017).

In Tunisia, numerous irrigation regions are located near the coast where extensive areas have evolved into an advanced agricultural production zone with considerable economic importance. However, the agricultural practices in these regions have already led to the overexploitation of the groundwater resources and an increased salinity by seawater or saline water intrusion. Additional groundwater contamination was caused

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39593971

by industrial activity, applications of artificial fertilizers, manures, pesticides and irrigation with treated wastewater in many Tunisian coastal regions (Saidi et al., 2013; Fries et al., 2016; Dahmouni et al., 2017). 750 sources of contamination of urban, industrial and agricultural origins were identified. They cause significant damage to the Tunisian water resources according to the national inventory established in 2004 by the Ministry of the Environment and Sustainable Development (Besbes et al., 2013). These sources include alimentary, textile and plastic industry sectors and domestic and industrial treated wastewater, the quality of which frequently exceeds the Tunisian standard.

Teboulba is a Tunisian coastal region with a long tradition of highly profitable irrigated agriculture. Like most coastal zones with shallow groundwater, the Teboulba region suffers from overexploitation caused by intensive pumping that led both to a decrease of the water level and to an increase of salinity. It caused salt water intrusion from the Mediterranean sea as well as from the sebkha of Moknine, an endorheic and salty depression resulting from Quaternary tectonic events (Chairi et al., 2010) which was used as a discharge site for many industrial activities (Wali et al., 2015). There is not much known about the current state of the groundwater, only measurements of water and salinity levels during and a few years after the artificial recharge from the overspill of the Nebhana dam and its injection into a limited number of wells (Bouri and Ben Dhia, 2010). Even if there is no recent information about the quality of the Teboulba groundwater, there are increasing complaints about the poor quality of the irrigation water. Therefore, a short-term laboratory study has been carried out with the root meristem of *Vicia faba* plants to evaluate the genotoxicity potential as an indicator of the groundwater quality. The *V. faba* test has widely been used to detect and evaluate toxic elements and has commonly been regarded as an excellent genetic model for toxicity monitoring since it offers a detection of the DNA damage and/or DNA abnormalities in cell division. The fast and sensitive *V. faba* test enables to assess the mitotic index, the micronucleus formation, and the chromosome and the nuclear aberrations. This test was used to assess the genotoxicity of waters collected from 3 wells used for irrigation when water from Nebhana dam is unavailable. The aim of this paper is therefore to evaluate the genotoxicity of the irrigation water and this get an overview of potential quality problems.

Materials and methods

Study area

The experiment was carried out in the Teboulba region situated in the Monastir governorate (Tunisia), some tens of kilometers southeast of the city of Monastir (*Fig. 1*). The Teboulba region belongs to the Nebhana irrigation district where water scarcity remains an important issue. This region has a semiarid to arid climate with mild, wet winters and dry, hot summers. The mean annual rainfall is approximately of 375 mm and the mean annual temperature is about 20°C. The coastal Teboulba aquifer is confined between the sea in the north and the salinized sebkha of Moknine in the south. It is threatened by overexploitation causing a decreasing water level and increasing salinization due to the seawater and/or sebkha intrusions. The aquifer basin of Teboulba covers approximately 35 km^2 and represents an average hydrological recharge potential of 0.65 $Mm^3\ y^{-1}$ with an exploited volume of 1 $Mm^3\ y^{-1}$. This heavy deficit of 0.35 $Mm^3\ y^{-1}$ is compensated not only by reserves but also by an inflow of

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saltwater from the sea and/or the sebkha as well as by a return flow of the irrigation water from the neighboring regions (Beni-Akhy, 1998).

The total agricultural area in Teboulba is 1914 ha, of which 600 ha are irrigated. In the governorate of Monastir, 39% (572 ha) of the total land area are reserved for greenhouses, almost one third of this lies in the Teboulba region (CRDA, 2004). The agricultural sector is dominated by rain-fed olive plantations for olive oil production and horticulture in non-warmed greenhouses. The main water source for irrigation is the Nebhana dam. Each farm in the irrigation perimeter was equipped by a storage tank. Irrigation water prices in Teboulba are among the highest in Tunisia (Chebil et al., 2010). To minimize the water cost, farmers mixed the water from the dam with water pumped from their wells.

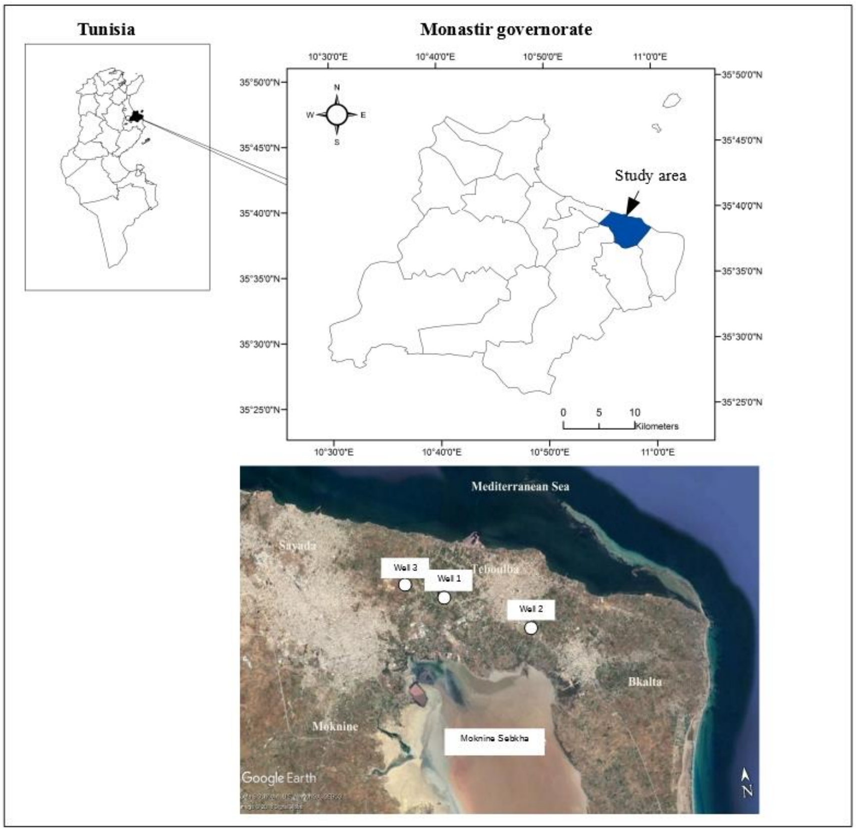

Figure 1. *Study area and location of the 3 sampling wells*

The present study was carried out in 2017 and with the most frequently used well of 3 farms from the irrigation perimeter with poor water quality in their wells (*Fig. 1*). The coordinates of the 3 wells (in Degrees Minutes Seconds) are: 35°38'49.94"N and 10°56'30.64"E for Well 1; 35°38'4.38"N and 10°58'35.72"E for Well 2, and 35°38'49.88"N and 10°55'37.52"E for Well 3. The well depths were measured and water samples were collected, pH and electrical conductivity (EC) were measured with a pH meter (Lutron pH-211) and a conductivity meter (Cond./TDS, AZ 8361), respectively. Mineral composition was determined using the following methods: sodium (Na^+) and potassium (K^+) with flame emission spectrophotometry (Jenway, PFP7), calcium (Ca^{2+}) and magnesium (Mg^{2+}) with complexometric titration using a

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standardized solution of ethylenediaminetetraacetic acid (EDTA), chloride (Cl^-) and bicarbonate (HCO_3^-) by titrating with silver nitrate ($AgNO_3$) and sulfuric acid (H_2SO_4), respectively, and sulphate (SO_4^{2-}) with UV-Visible spectrophotometer (Up Lab, UV-Vis-Auto). The 3 wells extracted the shallow groundwater at depths ranging between 27.5 and 44 m (*Table 1*). The water used for irrigation had a pH between 7.93 and 8.01 and a considerable salt content. The highest salinity was 7.26 dS m^{-1}, found in Well 1 and the lowest salinity was 3.60 dS m^{-1}, recorded in Well 3. Cl^- and Na^+ were the dominating ions at all salinity levels.

Table 1. *Characterization of water collected from the 3 wells in the region of Teboulba*

	Well 1	**Well 2**	**Well 3**
Depth (m)	27.5	29	44
pH	7.97 ±0.06	7.93 ±0.02	8.01 ±0.04
EC (dS m^{-1})	7.26 ±0.08	5.82 ±0.19	3.60 ±0.15
Na^+ (me l^{-1})	37.75 ±4.26	31.78 ±4.01	25.06 ±0.97
Ca^{2+} (me l^{-1})	21.33 ±3.88	15.66 ±0.57	8.83 ±1.44
Mg^{2+} (me l^{-1})	15.16 ±4.64	11.00 ±2.00	4.66 ±2.51
K^+ (me l^{-1})	0.31 ±0.01	0.24 ±0.00	0.16 ±0.00
Cl^- (me l^{-1})	34.78 ±0.81	29.61 ±2.44	19.74 ±7.05
HCO_3^- (me l^{-1})	4.66 ±0.76	7.00 ±0.50	6.01 ±0.00
SO_4^{2-} (me l^{-1})	30.39 ±9.40	17.63 ±6.18	8.56 ±2.09

Plant treatments

The *V. faba* seeds (Tunisian Chahbi variety) were surface sterilized with 10% sodium hypochlorite, rinsed several times with water and placed on moistened filter paper at 25°C for 3 to 4 days. Subsequently, the roots were introduced in both distilled water (control) and 3 samples of water collected from the 3 wells. For the genotoxicity assessment, a positive control was added. After 24 hours of exposure, the seedlings were rinsed a few times with distilled water and divided into three groups: the first was used for the growth measurement; the second to determine the membrane integrity and the third for the micronucleus test.

Growth parameters

Control and treated seedlings were separated into roots and epicotyls for root length, fresh and dry matter measurement. Root length was determined by measuring the length of the whole root after the treatments and dry matter was determined after drying the plant parts at 65°C. At least 6 seedlings were used for the measurement of the growth parameters.

Assessment of the plasma membrane integrity loss

The loss of plasma membrane integrity was evaluated with three root tips by a spectrophotometric test using Evans blue (an indicator for the measure of the plasma membrane integrity) as described by Souguir et al. (2011). The first centimeter of the roots was incubated in 0.025% Evans blue (m/v) for 30 min, rinsed for 15 min and squashed in a 800 µl solution of 50% of MeOH (v/v) and 1% of sodium dodecyl sulphate (SDS). The root extracts were then incubated for 15 min at 50°C. The homogenate was centrifuged at 14,000 rpm for 15 min. The optical density of the supernatant was determined spectrophotometrically at 600 nm.

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Genotoxicity assessment

The genotoxicity of the water from the 3 wells and the control was assessed with the micronucleus test applied on the meristematic roots of *V. faba* plant. A positive control with the hydrazide maleic (HM, 4 10^{-3} M) was added since the HM is well-known for its induction of numerous and very easily observable micronuclei (Souguir et al., 2008; Cotelle et al., 2015). The root tips were cut, placed overnight in a Carnoy's fixation solution containing ethanol and glacial acetic acid (3/1), and stored in the dark in 70% of ethanol before being hydrolyzed with 1N HCl as described by Souguir et al. (2008). The root meristematic tissues were stained with orcein, squashed between a slide and a coverslip, and finally examined under a research microscope (Leica DM2500) with 40 or 100 times magnification.

Three slides were prepared for each of the three replicates and a total of 9,000 cells were observed. The mitotic index (MI) was expressed as the number of dividing cells in 100 observed cells. Micronuclei were observed in both the interphasic and the mitotic cells. Only micronuclei observed in interphases were counted and expressed in terms of micronuclei per 1,000 interphase cells.

Chromosomal abnormalities were photographed with a digital camera (Canon EOS 1100) attached to the microscope.

Statistical analysis

The results were presented as the mean ± standard deviation (SD) obtained from three replicates. Significant differences were determined by Tukey's test at the 0.05% confidence level with the SPSS software (IBM SPSS statistics, v20).

Results

Morphological parameters and membrane integrity

The morphological parameters, root growth and fresh and dry matters of the different *Vicia* organs (root and epicotyl), are illustrated in *Table 2*. In general, the plants exposed to the water from wells and control did not show any significant difference in terms of root length. The average length varied between 3.92 and 5.22 cm. Fresh and dry matter of the plants irrigated with the water from the wells did not differ from the control plants.

The root treatment with Evans blue indicator also did not show a change of membrane integrity loss between the control and the roots (*Fig. 2*).

Genotoxicity data

Figure 3 lists the genotoxicity data of the 3 wells. The MI related to the number of cells undergoing division was higher in the control cells (exposed to distilled water) and reached 37%, whereas the HM as a positive control decreased severely the mitotic activity. This decline also occurred in the roots exposed to all kinds of irrigation waters. Values oscillated between 7% in well 3 and 14% in Well 1. In addition, cells supporting a micronucleus were counted. Low micronucleus frequencies (0.2 micronucleated cells per 1,000 counted cells) were recorded in the root tips of the control plants. As expected, the HM treatment sharply increased the frequency of micronuclei (21.8 micronucleated cells per 1,000 counted cells) compared to the control. The well water significantly increased the frequency of micronucleus formation in the root cells

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of *V. faba*. The highest frequency of micronucleus (7.15 micronucleated cells per 1,000 scored cells) was detected in Well 1, while the lowest level (5.75 micronucleated cells per 1,000 scored cells) was noticed in Well 3.

Table 2. *Growth parameters of the V. faba exposed to water collected from the 3 wells*

	Control	Well 1	Well 2	Well 3
Root length (cm)	4.560 ±0.996 a	3.928 ±1.071 a	4.925 ±0.869 a	5.225 ±1.252 a
Fresh matter (g)				
Root	0.234 ±0.051 a	0.272 ±0.073 ab	0.367 ±0.079 b	0.248 ±0.069 ab
Epicotyl	0.301 ±0.082 a	0.366 ±0.110 ab	0.488 ±0.088 b	0.326 ±0.065 ab
Dry matter (g)				
Root	0.016 ±0.003 a	0.018 ±0.008 a	0.026 ±0.005 a	0.018 ±0.005 a
Epicotyl	0.026 ±0.006 a	0.033 ±0.009 ab	0.045 ±0.008 b	0.029 ±0.006 a

Values are means ± SD. Different letters indicate significant differences at *P < 0.05* according to Tukey's test

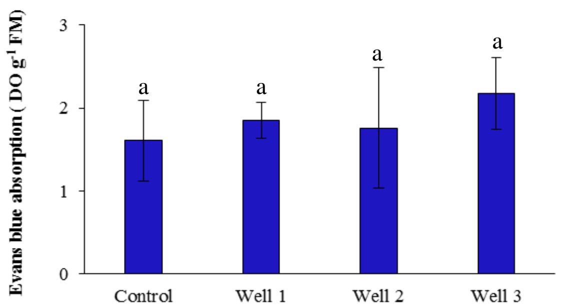

Figure 2. *Plasma membrane integrity as determined by Evans blue absorption of V. faba root extracts while exposed to water collected from the wells. Values are means ± SD. Same letters indicate no significant differences at P < 0.05 according to Tukey's test*

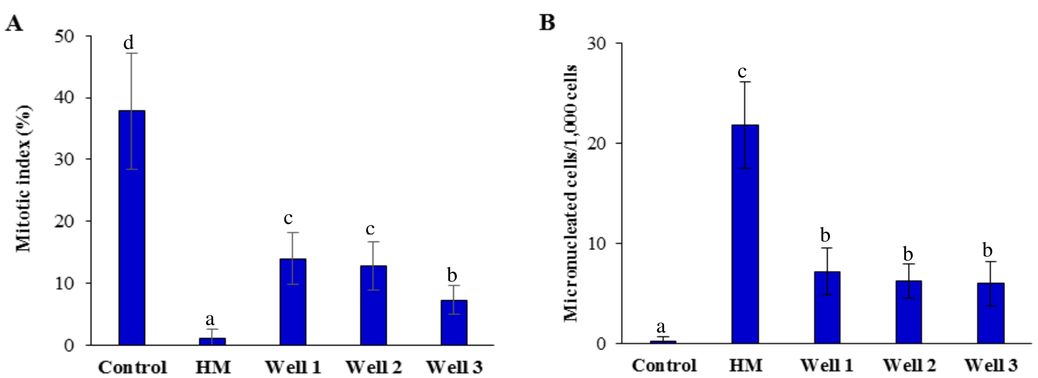

Figure 3. *Mitotic index (%) (A) and interphasic cells showing micronuclei (B) induced under exposure to different qualities of water collected from the studied wells. Control: negative control with distilled water; HM: positive control with Hydrazide maleic. Values are means ± SD. Different letters indicate significant differences at P < 0.05 according to Tukey's test*

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Chromosomal abnormalities

The root cells of *V. faba* treated with the 3 water qualities showed various types of chromosomal aberrations (*Fig. 4*). The cytological aberrations recorded in the bean root tips were stickiness at the prophase, bridges at the anaphase, fragments at the anaphase and the telophase, and finally vagrant chromosomes and laggards at the anaphase and the telophase.

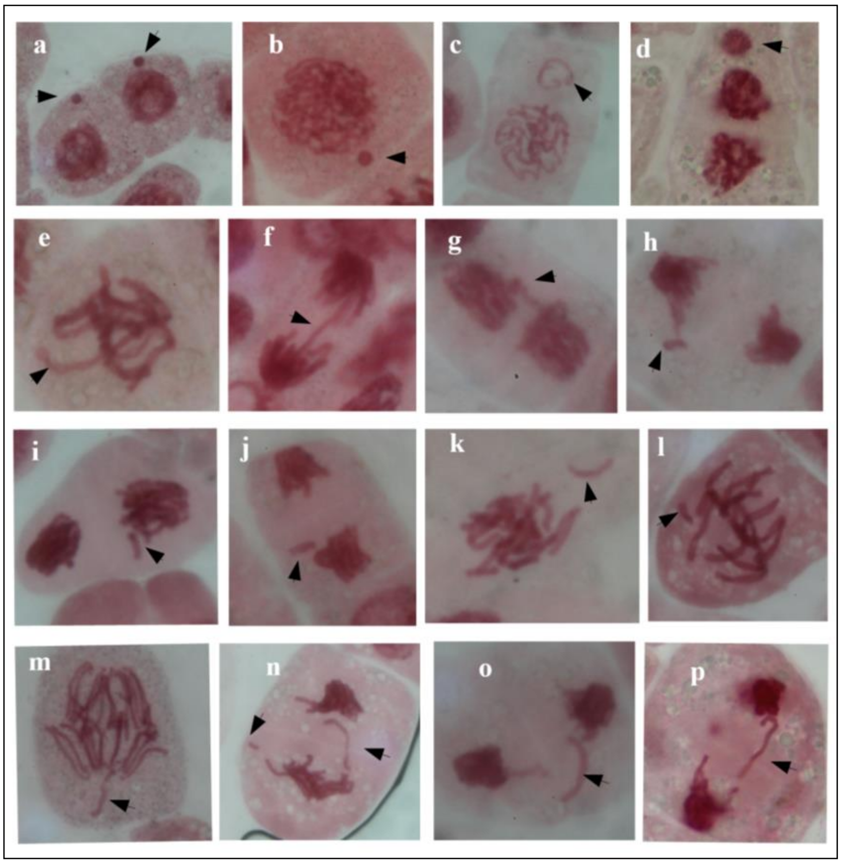

Figure 4. *Chromosomal aberrations detected in the root-meristematic zone of V. faba exposed to the water collected from the studied wells.* ***a-*** *interphasic cells with a micronucleus;* ***b****- prophase with a non-synchronized micronucleus;* ***c****- prophase with a synchronized micronucleus;* ***d****- telophase with a micronucleus;* ***e-*** *stickiness;* ***f-g****- bridge;* ***h-j-*** *fragment;* ***k-l****- vagrant chromosome;* ***m****- abnormal anaphase;* ***n****- chromosome laggard (1) and fragment (2);* ***p-q****- chromosome laggard; (magnification: 1000x)*

Discussion

The water quality used to irrigate the Teboulba parcels was evaluated by analyzing root morphology, membrane integrity and by screening genotoxicity. *V. faba* showed no changes in their morphological parameters (root growth, fresh and dry matters) between control plants and those exposed to the tested well water. The same results were obtained for membrane integrity measured by the Evans blue uptake. However, several effects were observed in the *Vicia* meristematic root tips on genotoxic parameters, estimated by MI, a micronucleus induction band, and some chromosome abnormalities.

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A significant retardation in the MI was remarked for all treatments compared to the control, which indicates the presence of cytotoxic substances in the irrigation water causing inhibition of the mitotic activity (Fernandes et al., 2007). The cell division inhibition could result from an arrest of the DNA synthesis or a blocking in the G2 phase of the cell cycle, preventing the cell from entering mitosis (Sudhakar et al., 2001). It could also be caused by an impaired nucleoprotein synthesis and a reduced level of ATP to provide energy for spindle assembly and chromosomal movements (Majewsk et al., 2003).

Micronuclei were considered as an indication of a true mutation effect (Auerbach, 1962). Thus, the high percentage of micronuclei induced may indicate the mutagenic effect of contaminants in the tested samples. Micronuclei detected in interphasic cells may originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of the telophase during mitosis (Fenech, 2011). Micronuclei may also be generated by the elimination of exceeding genetic material from the main nucleus (Fernandes et al., 2007). In addition to micronucleus appearance, many chromosomal aberrations in different mitotic phases including bridges, fragments, stickiness, vagrants and laggards were noticed in cells exposed to the irrigation waters. The induction of such aberrations in the bean root tip meristems indicates that the well water probably contains genotoxic chemical constituents with clastogenic and aneugenic impacts: The clastogenic compounds may induce a disruption or a breakage of chromosomes, whereas the aneugenic ones may result in a tubule formation or a chromosome segregation errors (Parry et al., 2002; Bignold et al., 2009).

The high salinity in the well water (3.60 - 7.26 dS m^{-1}) may lead to such mutagenic/genotoxic effects. The effect of salt on micronucleus induction and on chromosome changes was recently assessed in the *Vicia* root tips treated with 25 dS m^{-1} of NaCl (Souguir et al., 2018). The salt treatment caused micronuclei and changed the organization and the morphology of chromosomes and nuclei. It was shown for salinity values close to those in the Teboulba wells (Teerarak et al., 2009) that the cytotoxicity could be attributed to salinity.

In addition to salt-induced DNA damages, the genotoxicity may be caused by contaminants released from the sebkha of Moknine into the Teboulba groundwater. The sebkha of Moknine, a salty coastal depression, is considered as a hotspot of the industrial activities in the region. One of the most significant sectors in the Monastir region is the textile industry with about 917 factories. This industry is considered to be the most polluting sector of all industries (Garg and Kaushik, 2007; Sarker et al., 2015). About 24 tanneries discharge their effluents on the open land of the sebkha of Moknine and into the surrounding water bodies. Other industries such as blue jeans dyeing, printing industries and washing companies, also release their sewage directly and/or indirectly into the sebkha (Wali et al., 2015). The extensive use of dyes in textile industries and the release of effluents containing toxic compounds can harm soil, water and the living systems including human beings (Minhaz et al., 2018; Vega-Negron et al., 2018). Using *Allium cepa,* known as sensitive as *V. faba*, Sudhakar et al. (2001) showed a marked lowering of the mitotic index (from 9 to 2.2%) when germinating onion bulbs were immersed in 25, 50, 75, 100% of silk industry dye effluents for 24 hours. Silk dyeing effluents were shown to be genotoxic due to presence of mitotic inhibitors like tannins and alkaloids (Rath and Sethi, 2017).

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The contribution of the textile industries as well as that of the potteries and the brick industry to the high pollution of the sebkha of Moknine has been previously pointed out through a sebkha sediment analysis (Wali et al., 2015). Moreover, the sebkha receives treated wastewaters from the treatment plants of Moknine, which treat domestic (50%) and pretreated industrial (50%) wastewater using naturally aerated lagoons as secondary treatment process (Rafraf et al., 2016). Effluents from textile industries, brick kilns as well as industrial and household wastewater treatment plants contain a high amount of heavy metals (Achakzai et al., 2015). These contaminants were found in the sebkha of Moknine and lead to groundwater contamination (Malarkodi et al., 2007).

Soil leaching may also contribute to the contamination of groundwater with heavy metals through the overuse of fertilizers, which are usually not completely removed during the production. Being an agricultural region providing nearly a third of the Monastir governorate production (CRDA Monastir, 2004), the use of fertilizers in order to improve soil fertility and plant production is a common agricultural practice in Teboulba. For instance, the cadmium (Cd) contained in P fertilizers may cause high contents in the soil (McLaughlin et al., 1996). Like other metals, Cd might be either absorbed by plants or leached to the groundwater. Its behavior in the soil, accumulation by crops or groundwater leaching depend on numerous factors such as pH, organic matter, salinity, crop species and cultivar, etc. As a non-essential element, this metallic element is well-known for its genotoxic potential and many authors focused on its ability to decrease the MI value and to induce micronuclei and chromosomal aberrations. Souguir et al. (2011) investigated the cyto-mutagenic defects in *V. faba* root tip cells exposed to $CdCl_2$ (10, 100 and 200 μM) for 24 hours. They found increased micronucleus incidences in exposed root cells and with severe mitotic activity decreases. Such effects were also reported by Ünyayar et al. (2006) with $Cd(NO_3)_2$ but for much longer exposure (48 hours) and by Beraud et al. (2007) under lower concentrations of $CdCl_2$ (0.075 - 0.5 μM). Likewise, the genotoxic potential of many other heavy metals has been previously determined. *V. faba* has also been used to analyse mitotic activity decrease, micronuclei and chromosomal abnormality induction, which appeared when roots exposed to 0.5 - 1 mM of CrO_3 for 24 hours (Duan et al., 2013), 0.01 - 10 mM of $AlCl_3$ for 12 hours (Yi et al., 2009), and 0.005 - 0.01 mM of $(Pb(NO_3)_2$ for only 6 hours (Pourrut et al., 2011). Souguir et al. (2008) found that high concentrations of Cu (2.5 - 50 mM) were needed to increase significantly the micronuclei frequency in *V. faba* after 42 hours of exposure to $CuSO_4$, while Steinkellner et al. (1998) found that neither $CuSO_4$ nor $ZnCl_2$ induced any significant changes of genotoxic parameters in *V. faba* root tips. Heavy metals as well as salinity are usually associated with oxidative stress and generation of reactive oxygen species (ROS), which may also be behind the genotoxicity observed in the water collected from the tested wells. ROS production could increase DNA alterations and DNA-repair inhibition at the origin of micronucleus (Valverde et al., 2001).

Excessive fertilization may also cause the high levels of nitrate detected in the groundwater of Grombalia, Korba and Sminja in the North- East of Tunisia, Bekalta and Mahdia-Ksour Essaf in the Center-East of Tunisia, and Sidi-Aïch Gafsa oases and Djerba in the South of Tunisia (Wakida and Lerner, 2005; Einschlag, 2011; Zghibi et al., 2013; Hamed et al., 2013; Ameur et al., 2016; Souid et al., 2017). Nitrate concentrations can be caused by many factors such as the over use of nitrogen fertilizers in agriculture, the leaching of natural soil nitrate by the recharged water, nitrate-rich irrigation-return flow and septic tanks allowing the infiltration of the major part of the

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liquid phase. The potential genotoxicity of nitrates in the drinking water was previously investigated in the peripheral blood lymphocytes of children exposed to nitrate concentrations exceeding 70.5 mg l^{-1} (Tsezou et al., 2010).

Groundwater may also be contaminated by pesticides reaching groundwater through runoff and leaching. They can be harmful for the environment and human health. Pesticides including a wide variety of compounds belonging to different chemical classes, have been considered as inciters of gene mutation and micronuclei and chromosomal alteration (Bolognesi and Morasso, 2000; Sta et al., 2012; Goujon et al., 2014).

The sensitivity of the biomass endpoint was less than cell division and micronucleus induction. The cyto-mutagenicity/genotoxicity was not accompanied by notable changes in morphological and physiological parameters. Such sensitivity was previously pointed out in the *Vicia* roots exposed to Cd stress (Souguir et al., 2011) where cell division inhibition and micronucleus induction occurred soon after Cd exposure (12 h), prior to plasma membrane integrity and lipid peroxidation. Other authors thought that contaminants may disrupt microtubule arrays at a concentration lower than that causing root growth inhibition (Eun et al., 2000).

Conclusion

The analysis of the Teboulba groundwater revealed signs of genotoxicity in terms of mitotic index inhibition, micronucleus and abnormality induction. The micronuleus test using *V. faba* can be recommended as a fast, practical and reliable genotoxic assay for groundwater assessments. Genotoxic effects appeared while no changes of growth parameters (root elongation and plant biomass) and membrane integrity were found in treated plants compared to control plants, thus confirming the early sensitivity of the *Vicia* test to toxicity. The micronucleus test gave an idea about the effect of the whole contaminants present in the Teboulba groundwater. A more detailed study based on physico-chemical analyses of waters collected from different wells in the region should be carried out. Such analyses would allow to identifiy the harmful contaminants and to locate the anthropogenic sources. This could help the regional environmental services, local authorities, communities and industries to act responsibly against Teboulba groundwater contamination and to avoid further pollution.

Acknowledgements. The work was carried out under the PRM-IRESA project «Gestion Intégrée et Durable des Systèmes de Cultures Légumières Protégées (CléProD)». We would like to thank the Regional Commissariat for Agricultural Development of Monastir for the information support and the ease of access to farmers.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39593971

GEOGRAPHIC SCENARIO OF DRINKING WATER QUALITY OF LAHORE METROPOLITAN, PAKISTAN, IN RESPONSE TO URBANIZATION AND WATER DEMAND: A GIS PERSPECTIVE

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(Received 9th Nov 2018; accepted 8th Feb 2019)

Abstract. This study is intended to evaluate and segregate potential zones of groundwater quality over residential area of Lahore, Pakistan, where population growth and modernization of urban infrastructure are exerting pressure on finite groundwater resources. The study is based on the groundwater quality data of the year of 2016, with physicochemical quality parameters of total dissolved solids (TDS) potential hydrogen (pH), calcium (Ca), chlorine (Cl), magnesium (Mg) and bicarbonates (HCO_3). Optimal spatial interpolation methods are applied to generate continuous information of all quality parameters using geospatial techniques. Each thematic map of quality parameter has been classified using standard laid down by World Health Organization (WHO). A brief spatial analysis of given quality parameters has been made with reference to their possible source and underground movement. Then, all parameters are overlaid to generate a single indicator of water quality in form of water quality index (WQI) that has been used for segregation of groundwater quality zones. An insight analysis of the spatial pattern of WQI has shown that urban expansion in the southeastern region of study area that has been transformed from agricultural land to impervious surface is the reason behind increased concentration of contaminants in local groundwater. Other prominent zones of contaminated groundwater are found near regions of Anarkali and Gulberg.
Keywords: *groundwater quality, geographic information system, spatial interpolation, spatial analysis, water quality index*

Introduction

Water is one of the primary natural resources to all existing living bodies and ecological systems on earth. Today, preservation of pure water has become a serious issue to maintain healthy environment and economic growth on earth (Ojo et., 2007; Postel et al., 1996). Domestic water demand is increasing day by day with the evaluation in the modern living culture commonly in most of the densely populated urban regions of the world. Over 2.5 billion people in the world have no access to proper sanitation and quality drinking water that costs in deaths of over 1.8 million people, annually (Shrivastava et al., 2015; WHO and UNICEF, 2013a; WHO, 2002). In general, a challenging situation has been driven out due to uncertain atmospheric conditions and lack of decision making by the world leading powers (Interpress, 1999; Shrivastava et al., 2015). In developing countries, including Pakistan, decreasing precipitation rates are ultimately affecting the conservation of freshwater resources (Azizullah et al., 2011). United Nations International Children's Emergency (UNICEFF) and Environmental Protection Agency (EPA) have reported an alarming

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

situation in the main cities of Punjab where high proportions of toxic arsenic have been observed in drinking water (WWF, 2007). In Lahore city, provincial capital of Punjab, aquifer recharge has been reduced due to high abstraction rates of groundwater (Muhammad and Zhonghua, 2014). Water is a good solvent, having strong hydrogen bonding and distinctive nature of polarization it can dissolve many bacterial and natural components. Therefore, naturally occurring water always get polluted through anthropogenic as well as biological activities over and under the surface (Chitmanat and Traichaiyaporn, 2010; Khattak et al., 2012; Mendie, 2005). Today most of the studies regarding groundwater quality assessment are being conducted to highlight sources of contamination behind groundwater pollution in a region. Usually, natural processes of rock-water interactions, precipitation rates and anthropogenic activities like extensive use of fertilizers, disposal of wastes and intrusion of salts are general causes of groundwater contamination (Kumar et al., 2011). Nature of groundwater pollution sources can be categorized in point sources and non-point sources. Point sources are such distinct spots which spread pollutants directly like septic container etc. Whereas non-point sources are diffused sources of contamination that could be in form of fertilizers used over a wide spread agricultural land. As, groundwater flows from recharged regions to discharge areas because of gravity. So, high rates of groundwater withdrawal from certain regions tends towards the aquifer depletion of whole of the area (Siebert et al., 2010). TDS is key standard of drinking water quality and it is used to determine saline properties of groundwater. Presence of salts and slight number of organic components in water can change the groundwater quality. Such constitutes of salts either organic or inorganic are soluble in water in form of magnesium, calcium, sodium, potassium cations and their carbonates (WHO, 2003). Weathering of rocks is another cause of rising concentration of bicarbonates in the groundwater that could be either physical, chemical or biological (Mohsin et al., 2013). Successive natural weathering of rocks, decomposition of multiple salt rocks and percolation through soil significantly rise the chlorine concentration level in groundwater (Sivakumar et al., 2000). Calcium is insoluble in water, but its high concentrations in the presence of magnesium can cause hardness of the water (WHO, 2009). Alkaline or acidic nature of groundwater can be computed by pH, which is one of common indicator to describe the pH level. Values ranges from 7–1 reflects acidic nature and alkaline nature of groundwater is taken more than 7 (WHO, 2007). Ultimately, intrusion of pollutants into the steadily flowing water generate a downslope plume. Such plume elongates through its point source up to the several kilometres and contaminate the water body or groundwater recharge source (Montgomery, 2011). A preview of studies suggests that multivariate spatial techniques are useful to interpret spatial variability of different environmental variables (Mahmood et al., 2011; Sharaf and Sabyani, 2011). Geographic Information System (GIS) offers a complete set of tools to identify the spatial extent of groundwater variability and interpretation of groundwater quality over concerned area. It also helps to evaluate potential regions through variety of operational tools to examine the consequences behind the variability (Mahmood et al., 2016). Spatial interpolation is an artistry tool commonly used to analyze the variability by creating a continuous surface of estimated values from limited known sample data values. Different models and complex mathematical functions are operated to generate optimal output surfaces under the GIS environment (Batool et al., 2015). It also helpful to generate water quality index (WQI) for an overall groundwater quality assessment by using single number or grade based upon locational data of different water quality

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

parameters. WQI converts complex data values into the user-friendly information for the public awareness and policy makers to make better decisions regarding water resource management (Shabbir and Ahmad, 2015). Asadi et al. (2007) have concluded the use of GIS as effective toolset for calculating groundwater quality index (GWQI). Overall results show that most of the study area is in permissible limits for drinking water, as defined by WHO. Nas and Berktay (2010) investigated the groundwater conditions in the area of Konya city using the same handy toolset of GIS. In the study thematic maps of different physical and chemical water quality parameters were critically analyzed to elaborate the decreasing trend of groundwater quality from south to north of the city. Adnan and Iqbal (2014) have performed physicochemical analysis for groundwater quality assessment to explore the spatial distribution of water quality parameters over the region of Peshawar, Pakistan. They have obtained spatial surfaces using Inverse Distance Weighted (IDW), as the interpolating function without checking accuracy with other available options. A comparison of different deterministic and stochastic interpolation techniques was explained by Gunarathna et al. (2016), for mapping pH concentrations in the region of Malwathu Oya cascade-I, Sri Lanka. It was justified that stochastic methods give more precise results for the spatial analysis of pH values in groundwater of the study area (Gunarathna et al., 2016). It has highlighted the importance of exploring all the interpolation techniques to generate most reliable prediction surfaces. Current study is designed to analyze groundwater quality trends in the potential zones of Lahore with an emphasis that how rapid urbanization and growing economic development effect on groundwater quality and cause water pollution.

Materials and methods

Study area

Lahore is the most dynamic metropolitan of the province Punjab, Pakistan. Geographically it is extended 31°15′ to 31°45′ in north and 74°01′ to 74°39′ in east over Asian plate. Lahore is surrounded by district Sheikhupura in north and easterly bordered with India (Muhammad and Zhonghua, 2014). At northwest edge, a prominent river course exists named as River Ravi which acts like administrative boundary between Sheikhupura and Lahore district, as given in *Figure 1*. Fresh water demand has become a big concern of the study area due to overpopulation, modern urban infrastructure and accelerated economic transformation rate (Shah and Abbas, 2017). Latest Census-report of Pakistan has shown that population of Lahore district has been reached to 11.13 million with an average annual growth rate of 4.07% (Pakistan Bureau of Statistics, 2017). Seasonal variations in temperature patterns and precipitation rates have decreased rainfall in Lahore. In hot summer, temperature rises from 40 °C, whereas in winter it reaches up to 5 °C. Monsoon period contributes almost up to 40 mm in groundwater recharge with approximately 75% precipitation per year (Dogar, 2008; Muhammad and Zhonghua, 2014). Various studies have revealed that unconciliated alluvial deposits have made Lahore’s aquifer unconfined, highly permissible and more substantial (Kanwal et al., 2015). Although, River Ravi accomplishes the water demand of the whole city but its condition is going worse day by day. With decreasing rain water runoff, it receives approximately 47% of the total municipal and industrial waste of the city which have been voided into it without any treatment (Akhtar et al., 2014). Extensive digging for the construction of deep surface infrastructure like underground bus systems and pillar bases for overhead bridges has

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

been observed to provide a temporarily increased groundwater recharge, showing importance of local recharge in sustainability of this precious source of drinking water (Mahmood and Tariq, 2017; Wateraid and BGS, 2009). Ultimately, degradation of groundwater quality has caused serious health related issues in nearby areas (Basharat and Rizvi, 2011). Domestic and industrial needs of water are being fulfilled by pumping out groundwater either by the private societies and industries or government authorities. More than 500 production wells have been installed by Water and Sanitation Agency (WASA), Lahore to supply water to 531,336 connections in the mega city. WASA, Lahore also monitors groundwater quality by examining its chemical, physical and bacterial properties to ensure the availability of pure water (Akram and Gabriel, 2007; Basharat and Rizvi, 2011).

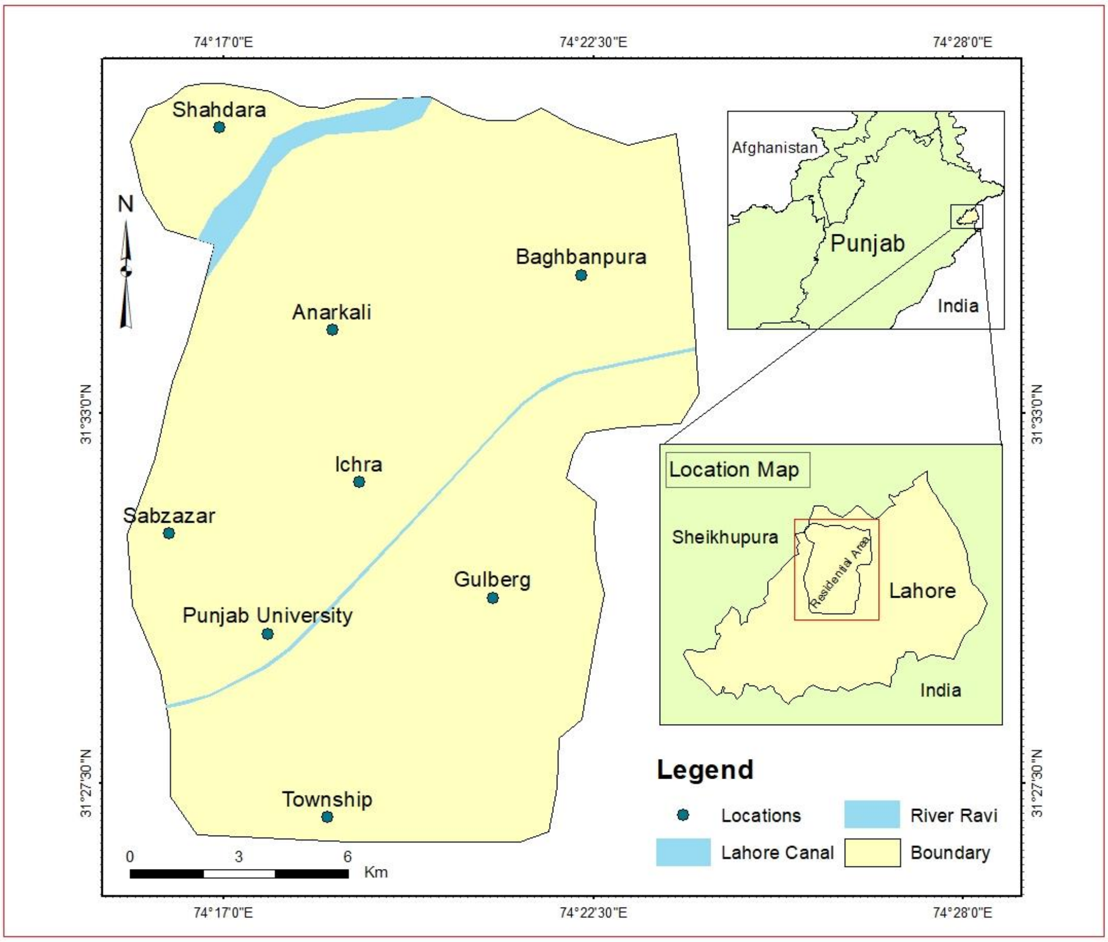

Figure 1. *Study area*

Data collection

Groundwater quality assessment of a region can be conducted by using multiple analytic approaches. In the present study, 200 water samples have been collected from installed tub wells by WASA in different regions of the district Lahore in the year of 2016, as presented in *Figure 2*. Sample data consists of limited quality parameters, Calcium (Ca), Chlorine (Cl), Bicarbonates (HCO_3), Magnesium (Mg), pH and Total

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

Dissolved Solids (TDS) which were chemically analyzed in Water Quality Laboratory WASA, Lahore. All the collected samples have been chemically examined through different titration techniques recommended by American Public Health Association (APHA) and World Health Organization (WHO). pH values are calibrated through buffer solution of 7, 8 and 9 and alkalinity has been measured through acid-base titration method due to the presence of calcium and magnesium components. Geographic coordinate data of each sample location were collected through a GPSmap-76Sx, with an accuracy of approximately 3 m by field survey. All the sample data values and their location data have been combined in excel sheet. Further geographic coordinates have been assigned to assembled data and displayed in the ArcGIS 10.1 to generate a geographic layer. General statistical characteristics of each Groundwater quality parameters have been mentioned in *Table 1*. Different spatial interpolation techniques (Deterministic and Stochastic Methods) have been assessed to evaluate the spatial variability of water quality parameters over the study area.

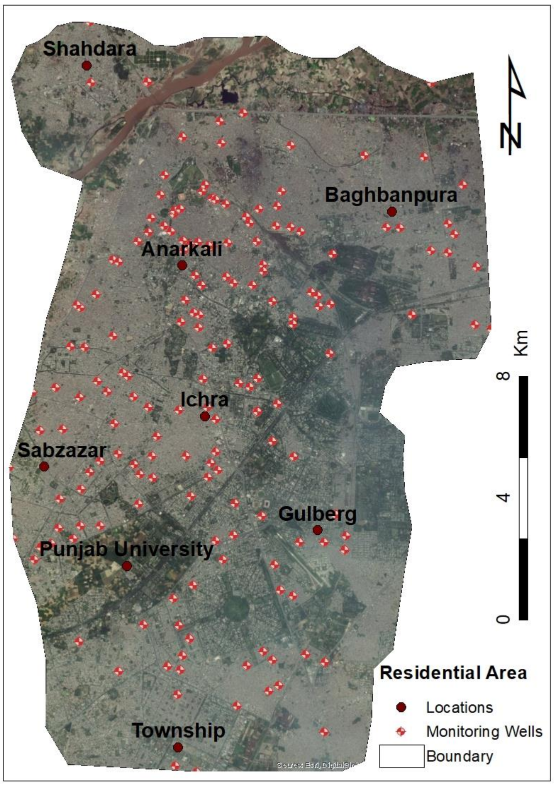

Figure 2. *Production wells in residential area of Lahore*

Table 1. *General statistics of selected groundwater quality parameters*

Water quality parameters	Mean	Median	Maximum	Minimum	Std.
Calcium (mg/L)	32.56	29.20	87.20	05	15.60
Chlorine (mg/L)	47.82	37	260	15	33.69
HCO3 (mg/L)	232.83	214	506	94	79.68
Magnesium (mg/L)	19.79	16.80	65.30	5.60	11.16
pH (Value)	7.87	7.80	8.30	7.80	0.11
TDS (mg/L)	424.16	381.10	988.40	173.20	190.8

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

Spatial interpolation

Geo-statistical techniques help to analyze spatial continuity and therefore have been used by most of groundwater studies, especially those which have used geographically unevenly distributed sample data. Spatial interpolation techniques are based on the first law of Geography which states that closer objects are more similar than those that are far apart (Batool et al., 2015). Inverse Distance Weighted (IDW) is being used since a long time in many studies which now has been replaced by advance methods of Kriging (Mahmood et al., 2016). IDW method interpolates through assigning weights, depending upon mutual distances between sample data values using a power function. Optimized power values define the influence of each quality parameters over the region in generating a continuous surface. On the other hand, kriging family get the real surfaces by using probabilistic function like semivariogram and its different models. Kriging have three main types, i.e. Ordinary Kriging, Simple Kriging and Universal Kriging that are based on the complex functions (Javari, 2017). Each spatial interpolation technique has its own significance; therefore, each of them has been applied to the current dataset to optimize the best predicted results. Accuracy of each method is measured and assessed using Root Mean Square Error (RMSE) which has been mentioned in *Table 2.*

Table 1. *Comparison of different interpolation methods by using RMSE values*

Water quality parameter optimal interpolation methods	Calcium (mg/L)	Chlorine (mg/L)	HCO_3 (mg/L)	Magnesium (mg/L)	pH	TDS (mg/L)
IDW	14.590	28.782	70.644	10.117	0.102	153.668
Global polynomial	14.877	32.392	65.464	10.058	0.105	169.060
Local polynomial	14.513	29.602	65.511	9.815	0.101	152.692
Radial basis function	14.430	28.627	68.883	9.982	0.102	151.286
Ordinary kriging	14.085	**27.609**	**64.779**	**9.545**	0.099	146.145
Simple kriging	**14.065**	27.703	65.167	9.555	**0.098**	146.474
Universal kriging	14.126	27.625	64.900	9.576	0.099	**145.245**

Calculation of water quality index (WQI)

Groundwater quality status is quite significant to evaluate the potential regions of good or poor water quality. Spatial distribution of each water quality parameter provides a pictorial view of measured and estimated sample data values. However, WQI indicates permissible limits of drinking water quality standards at a general scale for an absolute assessment of groundwater quality scenario in a region. Commonly weighted overlay analysis is being used to calculate water quality index in most of current studies (Acharya et al., 2018; Kumar et al., 2013; Mahmood et al., 2016). In weighted overlay method weights assign to each groundwater quality parameter as per their relative importance over each other. It is prerequisite that sum of weighted values of contributing groundwater quality parameters should be equal to 1 (in numbers) or 100 in percentage to generate WQI. According to the Mahmood et al. (2016), WQI for a certain region can be determined through a series of different steps. Input weights of each quality parameter have been computed by using the following expression given in *Equation 1:*

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

$$wi = \left\{ Wi / \sum_{i=1}^{n} Wi \right\} \times 100 \qquad \text{(Eq.1)}$$

where w_i and W_i are input and relative weights respectively, and n is the number of water quality parameters considered. Quality rate of each parameter was computed using *Equation 2,* by comparing measured and suitable values in the spatial analyst extension using raster calculator tool. The mathematical expression for the development of quality rating is:

$$Qi = \left(\frac{Ci}{Si} \right) \times 100 \qquad \text{(Eq.2)}$$

Since Qi stands for quality rating of ith parameter, Whereas C_i is concentration of ith parameter in an individual sample and S_i is the standard value of drinking water for ith parameter.

Further quality rates and weights of each water quality parameter have been taken into account according to *Table 3*, to calculate water quality index as follows:

$$WQI = \sum_{i=1}^{n} (wi \times Qi) \qquad \text{(Eq.3)}$$

Table 3. *Weight and standard values for selected groundwater quality parameters*

Water quality parameter	Ca (mg/L)	Cl (mg/L)	HCO_3 (mg/L)	Mg (mg/L)	pH	TDS (mg/L)
Relative weight (Wi)	2	3	3	2	4	6
Input weight (wi) %	10	15	15	10	20	30
WHO standard (Si)	75 mg/L	250 mg/L	120 mg/L	50 mg/L	8.5	500 mg/L

Results of calculated water quality index by using *Equation 3* represents a range from good to poor water quality status of Lahore district. The range is classified in five equal interval classes according to the standards defined by WHO, which are given below in the *Table 4.*

Table 4. *Recommended equal interval classes for each groundwater quality parameter*

No. of classes	Class-1	Class-2	Class-3	Class-4	Class-5
WQI Values	< 70	70–84	84–97	97–110	>110
Category	Good	Moderately good	Marginally good	Moderate	Poor

Results and discussion

Spatial analysis of water quality parameters

Continuous spatial surfaces of each groundwater quality parameter generated by different interpolation techniques, demonstrate some high and low potential areas. As per *Table 2*, kriging method with its different empirical models demonstrate optimized surfaces with least RMSE values. However, optimization of any method is completely depended on

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

dataset in hand and may vary for a different set of sampling points. Final maps of each water quality parameters have been classified in five classes according to the drinking water quality standards provided by WHO. A brief analysis of measured and interpolated concentration levels of each groundwater quality parameters has been discussed below.

Calcium

Calcium is an essential component to regulate heart rhythms and control over blood clotting in human (WHO, 2009). In present scenario, the measured values of calcium ranges from 05 mg/L to 87.20 mg/L in the study area. Whereas the least values of less than 34 mg/L have been observed at garden town near Punjab University. A comparison of different spatial interpolation methods for Ca suggests that simple kriging is producing the most reliable prediction surface with RMSE of 14.065. Spatial distribution of Ca shows that most of the study area lies within permissible and safe limits of the element's concentration in drinking water, i.e. approximately less than 34 mg/L. However, a continuous central spot of values ranges from 45 to 57 mg/L can be observed near the Anarkali region as shown in *Figure 3*. This central spot has also been highlighted as a high concentration zone by previous studies done by Mahmood et al. (2016) and Mahmood and Tariq (2017). Maximum concentration values were measured at Napier road near Anarkali. A continuous spatial trend of estimated values ranges from 34 mg/L to 45 mg/L, is emerging from northeastern edge of study area. A set of past studies refers to this trend as an influence of landfill site existing in the region (Butt and Ghaffar, 2012; Mahmood et al., 2016). Presence of municipal solid waste near water body increases the rate of leachate production and percolation through underlying sediments that ultimately contaminates groundwater (Mahmood et al., 2013; Mahmood et al., 2017). Present discussions manifest that this trend may lead towards subsurface lateral flow of contamination from eastern side of the aquifer.

Chlorine

Natural water contains an excess amount of chloride ions due to its high polarizable nature. Highly chlorinated water causes different infections like asthma and other dermatitis infections and may increase the risk of bladder cancer (NHMRC, 2011). Comparison of deterministic and stochastic spatial interpolation methods suggests that ordinary kriging provides precise spatial distribution of Cl than radial basis function with RMSE value equals to 27.609. In present case, minimum values of Cl concentration level have been found at the upper region of Baghbanpura. Whereas high concentration of Cl (approximately 260 mg/L) found at Mozang nearer to Anarkali region. In general increase in concentrations of Cl in the region is due to increase of mineral content (Nas, 2009). Long distance lateral flow of groundwater towards the deepest aquifer near Anarkali allows subsurface materials to get mixed in the water and causes groundwater contamination (Mahmood et al., 2016). In general, spatial distribution of Cl shows that the concentration of Cl in the groundwater lies in acceptable range. A regular expansion towards north and south eastern domains of study area can be assessed through geographical representation of Cl as given in *Figure 3.*

Bicarbonates

Bicarbonates are water soluble in form of calcium and magnesium constituents, but their excessive quantities make water hard. Definite permissible guideline values have not been specified by WHO for bicarbonates but their concentrations more than 500 mg/l is usually

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

not appreciated (Mohsin et al., 2013). In the study area the concentration level of bicarbonates has been observed from 94 mg/L to the maximum value 506 mg/L in 2016. The comparison of deterministic and stochastic spatial interpolation methods for HCO_3 suggests that ordinary kriging produces the more precise prediction surface with RMSE of 64.779. An overriding trend has been observed in the spatial distribution of bicarbonates over the study area. Spatial analysis of HCO_3 indicates a peculiar profile of descending order of concentration levels from south eastern region (Gulberg) to north western region (Shahdara). Maximum concentration levels are estimated at Gulberg that lies in the south eastern region of Lahore. However, least quality measures have been evaluated near the areas of river Ravi and Shahdara region. In current scenario, unusual spatial behaviour of bicarbonates tends towards high rate of underground weathering of rocks. As geological conditions explain that study area is composed of bicarbonates which dissolves in moving water. Due to longer path distance, ratio of dissolving sub-surface materials becomes greater causing alkalinity rise in the source.

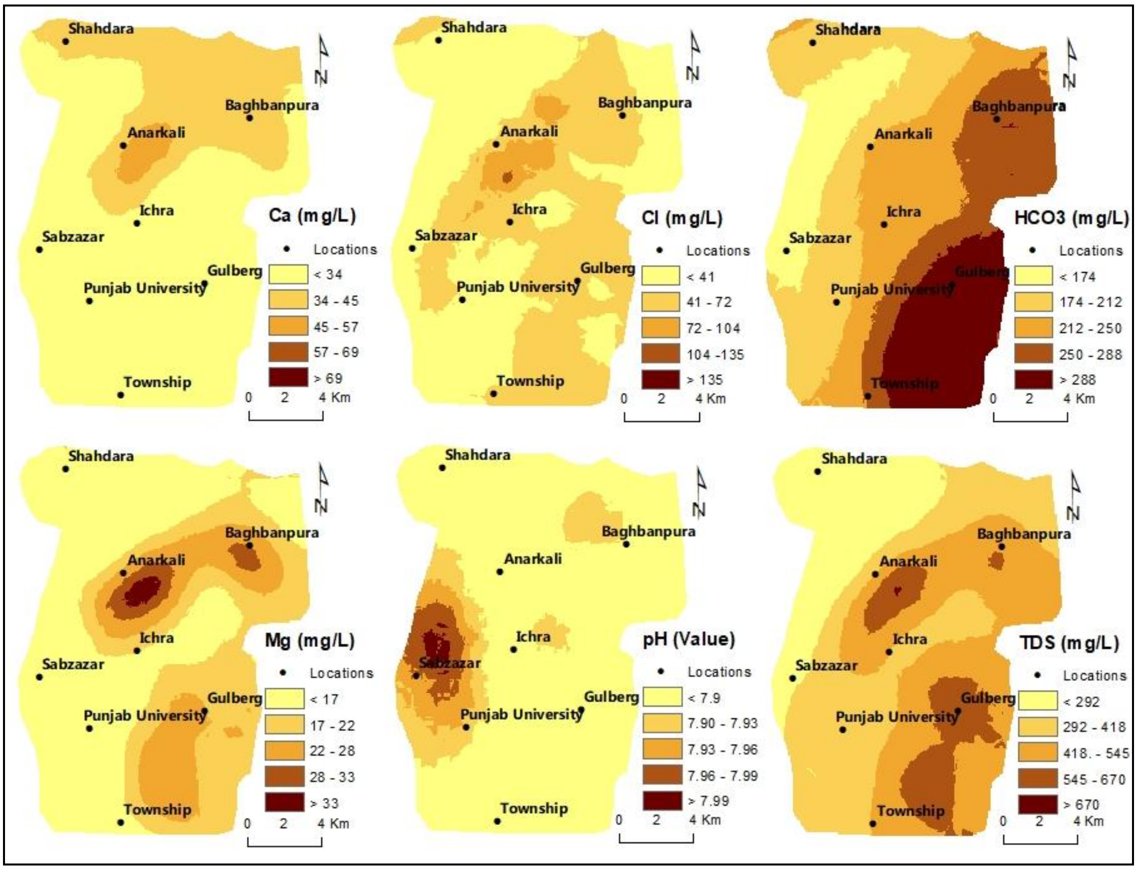

Figure 3. *Spatial analysis of considered water quality parameters*

Magnesium

Magnesium is most common component of natural water and usually exists in form of Mg^+ ion (WHO, 2009). Drinking water quality standards of Pakistan has specified approximate concentration of Mg in drinking water that should not exceeds from 50 mg/L. Quantitative measures show that the concentration of Mg over the study areas varies from 5.60 mg/L and 65.3 mg/L in the year of 2016. A comparison of different

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spatial interpolation methods for Mg suggests that ordinary kriging is producing the most reliable prediction surface with RMSE of 9.545. Least values were measured at Garhi Shahu, which located in the south of Baghbanpura, Lahore. However, spatial distribution of classified concentration data values shows that minimal values of Mg exist over the region near Shahdara. Again, a prominent central spot has been observed at Anarkali region with high concentration of Mg, approximately up to 65.30 mg/L. Mahmood et al. (2016) identified the same trend for Ca and Mg in temporal analysis of groundwater quality from 2010 to 2013. Moreover, Mahmood et al. (2016) and Mahmood and Tariq (2017) have also reported this spot near Anarkali region as a permanent source of contamination. The same lowering of water table at centre of the study area is responsible for high concentrations of Mg, Ca and HCO_3 (Mahmood and Asim, 2017; Mahmood et al., 2016). In case of Mg, a trend can be seen in the northeastern region near Baghbanpura, which illustrates a persistent range of concentration values from 22 to 28 mg/L. Local influencing parameters like least permeable surfaces causes weathering of rocks. Usually untreated waste from different industries is disposed of into the river without any purification, which consequently raises magnesium concentration in running river water. As most of the studies conclude that the presence of both Ca and Mg cause temporary and permanent hardness in water.

pH

It is one of physical parameter which controls the presence of hydrogen ion in water. Low values of pH make water soft and high values tends to make water hard (WHO, 2007). An evident trend of pH values exists in the given dataset that ranges from 7.80 to 8.30 respectively. Comparison of different spatial interpolators shows that simple kriging provides more reliable surface for pH with RMSE of 0.098. Classification of spatial data reveals that pH concentration values below 7.9 have covered a large portion of the study area. Whereas, a continuous convergence of high concentrations of pH, ranges from 7.99 to 8.30, towards the western centre of the study area, near Sabzazar has been observed, reflecting alkaline nature of groundwater as shown in *Figure 3*. This emerging spot is geographically located at the region where major drains (Babu Sabu and Gulshan-e-Ravi) of the city fall into the river Ravi. It has been asserted by Hamid et al. (2013) that inclusion of drain water into the river Ravi without any treatment is ultimately rising alkaline properties of river water. Baqar et al. (2014) has also investigated the impact of falling drains into river Ravi by using geospatial technologies with the conclusion that mean concentrations of total suspended solids were greater at the region closer Gulshan-e-Ravi outfall and region closer to it (Baqar et al., 2014). However, this impact is still at the closer regions of river Ravi. But in future it may expand towards the eastern areas of the study area. A range of 7.93 to 7.96 of pH levels have been observed near the regions of Baghbanpura and Ichra, reflecting presence of some temporary alkaline source across this region. Overall, pH values lie within their permissible limits suggested by WHO and PSQCA in the study area. But it should be noted that measured pH values range just below from the standard authorized values.

Total dissolved solids (TDS)

Natural water consists of different ionic constitutes in their specific amounts to made it saline (Atekwana et al., 2004). In Pakistan, the surface area is highly permissible, therefore concentrations of total dissolve solids in water is comparatively high.

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Although all the samples have been found within the safe limit suggested by WHO for TDS but a considerable range of 173.2 mg/L to 988.40 mg/L exists in drinking water of the study area. A comparison of different spatial interpolation methods for TDS suggests that universal kriging is producing the most reliable prediction surface with RMSE of 14.065. A notable trend in variation of classified data values, especially in the southern part of the residential area. At Ravi road and Shahdara concentrations of TDS have been estimated below 292 mg/L. Whereas maximum concentration values have been found near Anarkali and Gulberg region, as can be identified through *Figure 3*. Total dissolve solids (TDS) is an important quality parameter which shows the measure of existing combinations of solid particles in accordance to lithology of examined groundwater samples. An emerging overriding trend in the current dataset of TDS shows a continuous spatial variation over the study area. It approves that Anarkali region and Gulberg have notable concentration levels of TDS. The prominent spatial affinity between Anarkali region and Baghbanpura also elevates a trend of contamination flow from north eastern side to south western edge. Gulberg has become the central commercial zone of residential area of Lahore due to the recent growth of modern infrastructure. Over extraction of groundwater has generated a depression zone in middle of city due to which degradation of water table is arising with the passage of time (Akhter and Tang, 2013; Kanwal et al., 2015; Mahmood et al., 2013). Usually, TDS rises in groundwater due to urban and rural runoff, desolation of natural sources, disposal of sewage and industrial waste into the rivers (Akhter et al., 2014).

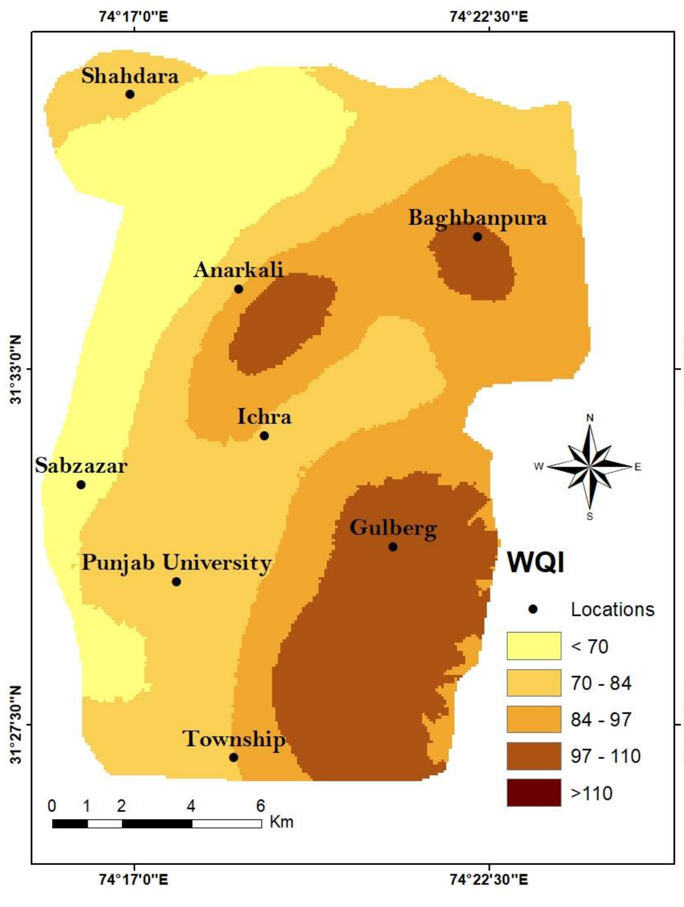

Figure 4. *Spatial distribution of water quality index, classified as per WHO standards*

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

Water quality index (WQI)

Thematic maps of selected physical (TDS, pH) and chemical (Mg, Ca, Cl, HCO_3) water quality parameters interpret spatial variability of their concentration levels at study area. Whereas, index map of groundwater quality shows current status of groundwater quality at any region, as given in *Figure 4*. Results of resampled interpolated rasters of current study have been classified in five equal number of classes. All the classes have been categorized as < 70 (good), 70–84 (moderately good), 84–97 (marginally good), 97–110 (moderate) and >110 as highly poor water quality. In present scenario, almost whole residential area lies with in the categories of marginally good, moderate and poor water quality. Spatial analysis of WQI map indicates that Anakali region is prominent and permanent source of highly poor groundwater quality. Since previous studies have also revealed the consistent growth of concentrated zone of contamination across this region. Low recharge and high abstraction rates are exerting high pressure on the aquifer, particularly near Anakali region. High concentration level of groundwater quality parameters can be caused by ground lithology that is composed of carbonates of magnesium and calcium. Upper northeastern region of study area at Baghbanpura shows an order of "moderate" water quality. A couple of studies show that the seepage of contaminants from Mehmood Booti landfill site are effectively deteriorating the groundwater quality of Lahore (Butt and Ghaffar, 2012; Mahmood et al., 2014). Leachate transfers through porous soil layer and flows towards local aquifer with the aid of groundwater. Mahmood et al. (2016) infers that lowering of water table tends water to take relatively longer path to travel. As longer will be the path, greater will be possibility of dissolution of minerals (soluble ions) in to water, which ultimately rise the alkaline characteristics in groundwater. Areas exits in the northern edge (Shahdara, Sabzazar and Punjab University) shows almost good and moderately good water quality due to the availability of high recharge zone, but comparatively low consumption rate. In south eastern edge, high proportions of contamination have been estimated which evaluate the expansion rate of urban area. Rapidly growing population and modern ways of civilization are degenerating the availability of permeable surfaces as given above.

Conclusion

This study illustrates current status of groundwater quality in the second largest metropolitan city of Pakistan, named Lahore. GIS based spatial analysis has been found very useful and effective in studying potential regions of groundwater contamination as well as their spatial distribution over study area. Along with having an insight analysis of the spatial distribution of considered water quality parameters, i.e. calcium, magnesium, chlorine, pH, bicarbonates and TDS, the WQI, as a sole representative of quality status, has been found very helpful in segregating water quality zones. Leaching through landfill sites (particularly Mahmood Booti) and disposal of industrial and municipal waste into river Ravi are major contributors of groundwater pollution in the area. WQI of 2016 along with previous similar studies of the region concludes that Anarkali region has become the permanent source of high contamination rate that is expanding towards the north eastern direction of study area. A directional impact of high concentrations from upper Baghbanpura region towards Anarkali region has been observed through TDS and WQI maps. High proportions of contaminants in groundwater under Anarkali could be due to dilution of calcium, magnesium and their

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DOI: http://dx.doi.org/10.15666/aeer/1702_39733988

carbonates that are abundant in underlying lithology. Deepest level of groundwater at Anarkali caused recharge water from outskirts of the area to flow towards it under gravity. As longer will be the travelling path for lateral flow of recharged water, more chances will be of soil-water interaction. Rapidly growing population and transforming of infrastructure in Lahore have lessened the availability of pervious land sites. Due to which south eastern region of study area explicitly shows high contamination ratios. Although, these factors generate temporary plumes in any concerned area but if unplanned infrastructure keep continues to develop, it will defiantly put long term effects on availability of safe groundwater. In general, groundwater quality of Lahore residential area is being deteriorating due to poor waste management system, involuntary developments in urban infrastructure, use of heavy pesticides in agricultural land surrounding the residential area and high-water extraction rates. Use of GIS based analysis in the study has highlighted potential regions of high concentrations of pollutants in the year of 2016, providing continuity of previous work done in this regard. Such a mapping of groundwater quality zones and their segregation can play a primary role in the decision-making process of local authorities for the sustainable solutions of drinking water in the region for which the only available source is the local groundwater. Therefore, this study concludes that an effective system for groundwater monitoring needs to be introduced by the local administrators and government authorities. Such issues are common in all over the developing world where lack of financial resources always hinder scientific knowledge-based solutions for sustainable development. Therefore, this study also concludes that the use of existing wells for sampling along with proper GIS can be an economical alternate in this regard.

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GERMINATION INDICES AND ANTIOXIDANT ACTIVITY ENZYME RESPONSES OF MADAGASCAR PERIWINKLE (*CATHARANTHUS ROSEUS* (L.) G. DON) UNDER PRE-TREATMENT BY SALICYLIC ACID

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(Received 11th Nov 2018; accepted 11th Jan 2019)

Abstract. The present factorial experiment was conducted based on completely randomized design (CRD) with three replications in order to determine the optimum time and concentration for *Catharanthus roseus* seed priming with salicylic acid. Treatments included five different levels of salicylic acid (0; 0.01; 0.1; 0.5 and 1 mM) as well as five different periods of time (0, 6, 12, 24, and 48 hours). Results indicated that the effects of concentration and time of priming were significant on all the studied traits. In addition, the effect of the interaction between concentration and priming time was significant on the mean time of germination, seedling length vigor index, chlorophyll content, and the activity of antioxidant enzymes. Further, the germination percentage increased by 24.1% and 22.3% in the treatment of 1 mM, and 48 hours, respectively, compared to the control. The highest germination speed was in 1 mM and 48-hour treatment, which had a 40% higher increase than the control. The interaction effect on the antioxidant activity indicated seed priming with 1 mM concentration in 24 hours, leading to 84% and 62.6% increment in the catalase (CAT) and peroxidase (POD) activities, respectively, compared to the control. Furthermore, 0.1 mM concentration in 24 hours led to 61.2% and 66.2% increment in chlorophyll a and total chlorophyll, respectively.
Keywords: *catalase and peroxidase enzymes; photosynthetic parameters; phytohormone; seed priming*

Introduction

Catharanthus is regarded as a medicinal ornamental plant. *Catharanthus roseus* is a perennial plant, semi-shrub or herbaceous and is related to the Apocynaceae family (Nejat et al., 2015). This plant is indigenous to Madagascar but is now widely distributed, and cultivated in China, India, Indonesia, Australia, North, and South America (Yang et al., 2011) (Verma et al., 2012). *Catharanthus* is known for its anti-cancer, anti-hypertensive, anti-diabetes, and antimicrobial properties. This plant contains about 130 bioactive Terpenoid Indole Alkaloids (TIAs), among which vinblastine and vincristine are regarded as the most important (Nejat et al., 2015). *Catharanthus* has a fairly long vegetative period due to its slow initial growth. It takes 180-200 days from cultivation to the maturity of the seed. (Omidbegi, 2006). The long growth period of the plant is considered as one of the limitations of its cultivation on a larger scale (Muthulakshmi and Pandiyarajan, 2013).

The process of germination of non-dormant seeds occurs in three phases including (I) imbibition, (II) lag phase, (III) the protrusion of the radicle through the testa (Bewley et al., 2013; Rosental et al., 2014). The duration of the third phase has great importance because the germination is completed and the embryo begins to develop concurrently during this period. Rapid seed germination and stand establishment are critical factors

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39894005

affecting crop production. After expanding our understanding of the processes involved in the germination methods used for changing the aforementioned factors, development of agriculturally applicable seeds began. The most frequently used method is known as "seed priming" (Bewley et al., 2013; Paparella et al., 2015). Seed priming is a controlled hydration technique which initiates the pre-germination metabolisms without actual germination (Hussain et al., 2015). In addition, it is considered as one of the most effective, pragmatic and short-term approaches to improve seed vigor (Batool et al., 2015), rate and percentage of seed germination (Santini and Martorell, 2013) synchronization of germination, crop establishment (Pouramir-Dashtmian et al., 2014; Paparella et al., 2015) and the seedling growth of many crops (Farooq et al., 2009) Further, it is associated with a wide range of metabolic and physiological improvements (Shehab et al., 2010) and cellular and molecular changes (Siri et al., 2013).

When the primed seeds are sown, the imbibition phase and lag phase of water absorption are shortened (Khan et al., 2009) to reduce growth time, in order to increase the productivity of agricultural land in such a way that the reported Indian farmers could harvest three products in one year (Harris et al., 2001).

The induced priming increase in the rate of seed germination was associated with the initiation of germination related processes, repair processes and increased levels of various free radical scavenging enzymes such as superoxide dismutase (SOD), CAT and POD (Vazirimehr et al., 2014). Furthermore, the positive effects of priming on seed germination in various species of plants are related to inducing biochemical mechanisms, repairing, and rebuilding cells (Di Girolamo and Barbanti, 2012) by activating the synthesis of many proteins and enzymes involved in cell metabolism (Varier et al., 2010). Salicylic acid (SA) is a phenolic compound, known as "phytohormone", which contributes to the regulation of the growth and development processes, especially seed germination, photosynthesis, respiration, flowering, and senescence (Rivas-San Vicente and Plasencia, 2011). Some studies indicated that priming strategies can enhance the activities of free radical scavenging enzymes such as CAT, POD, and SOD (Sharma et al., 2014). These enzymes reduce lipid peroxidation at the germination stage and increase the percentage and rate of germination (Baalbaki et al., 1999).

Recently, it has been reported that there are some encoding antioxidant enzymes such as CAT, SOD, and Ascorbate peroxidase (APX) preventing the overproduction of reactive oxygen species (ROS) among genes stimulated by SA (El-Esawi et al., 2017), Moreover, SA may act directly as an antioxidant, scavenging ROS (Popova et al., 2009). The utility of SA in inducing the plant defense mechanisms depends on its concentration and the method of its application (Huang et al., 2016). Hussein (2015) evaluated the effect of seed priming with SA (100 mg/L) for four hours at 25 °C on the germination of okra (*Abelmoschus esculentus* L.). The result indicated that seed priming with SA can induce the highest germination percentage and seed vigor index. Based on the results of previous studies, the effect of the interaction between time and different priming concentrations with gibberellic acid and SA was significant on germination rate (Azarniya et al., 2016).

There are many factors related to the effects of seed priming, which depend on the concentrations of priming solutions, the time and the temperature during priming (Khan, 1992). Thus, the optimization of the priming technique is important to achieve the best results (Maiti and Pramanik, 2013). Regarding the long growth period of this plant, the small size of the seed and poor establishment in the field with semi-heavy and heavy textured soils, the present study aimed to evaluate the effect of seed priming with SA in

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different concentrations and periods of time on improving *Catharanthus roseus* seed germination indexes.

Materials and methods

Preparation, Priming of seeds and germination process

The present study was conducted in order to assess the priming effects of time and various concentrations of SA on germination parameters, *Catharanthus* seed (*Photo 1*), with a factorial experiment based on completely randomized design (CRD) with three replications at the Laboratory of Seed Science and Technology of Shahed University in Tehran. Treatments included five different concentrations of SA (0, 0.01, 0.1, 0.5 and 1 mM) and five periods of time (0, 6, 12, 24 and 48 hours). The seeds were purchased from the company named Green Farm, and were surface-sterilized using 70% ethanol for 30 seconds, washed with distilled water three times, transferred to a solution of 50% Clorox (containing 5.25% NaOCl) for 15 min, and were finally rinsed three times with sterile distilled water (Taha et al., 2009). The seeds were placed in SA solutions with different concentrations, and for different times (6, 12, 24, and 48 hours) at 25 °C in darkness (Pandey, 2017). After removing the priming solution with distilled water, seeds were re-dried to the initial moisture content (Ratikanta, 2011) at 25 ºC for 24 hours in the laboratory. In every sterilized Petri dish (90 mm diameter), 50 seeds were sowed with two-layer Whatman filter papers moistened with 5 ml appropriate solutions. Thus, untreated seeds were used as control. In order to prevent water evaporation, the Petri dishes were closed with parafilm and placed in a germinator at 25 ± 1 °C (Prabhu and Rajeswari, 2017) with a photoperiod of 12/12 h day/night light (Senbagalakshmi et al., 2017). The germinated seeds with a root length longer than 2mm were counted (Liopa-Tsakalidi et al., 2012).

Photo 1. *Catharanthus seed germination in a germinator at the laboratory*

Each day germinated seeds that had a root length over 2 mm were counted (Kaya et al., 2006). At the end of the experiment (10 days) (Barkat et al., 2017) five samples were randomly selected from each petri dish (Huang and Redmann, 1995) and seedling length, plumule length, radicle length, seedling dry weight (Panwar and Bhardwaj, 2005) (Liopa-Tsakalidi et al., 2012), mean germination time (Ranal and Santana, 2006), mean daily germination (Hunter et al., 1984), seedling length vigor index and seedling weight vigor

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index (Hu et al., 2005) were calculated according to the following equations (*Eq.1-5*) (Hunter et al., 1984):

$$Germination\ percentage(GP) = (N \times 100) / M \quad \text{(Eq.1)}$$

$$Mean\ germination\ time(MGT) = \sum(Ni\ Di) / \sum N \quad \text{(Eq.2)}$$

$$Mean\ daily\ germination(MDG) = GP / T \quad \text{(Eq.3)}$$

$$Seedling\ length\ vigor\ index = Germination\ percentage \times Seedling\ length \quad \text{(Eq.4)}$$

$$Seedlingweightvigor\ index = Germination\ percentage \times Seedling\ dry\ weight \quad \text{(Eq.5)}$$

N: the sum of germinated seeds at the end of the experiment, M: the total number of planted seeds, Di: The time from the start of the experiment to the i^{th} observation, Ni: the number of germinated seeds at time Di, ∑N: Total germinated seeds at the end of experiment, T: Period of germination.

Antioxidant enzymes assay

An enzyme extract was prepared by the method of Mukherjee and Choudhuri (1983). A 0.5 gr leaf sample was homogenized in 10 ml of 100 mM phosphate buffer (KH_2PO_4/K_2HPO_4) pH 7.0, containing 1% (w/v) insoluble polyvinylpyrrolidone (PVP). The homogenate was centrifuged at x 15,000 *g* for 10 min at 4 °C, and the resulting supernatant was collected and stored at 4 °C for CAT, POD assays.

Peroxidase assay

The POD activity was assayed following the method of Gueta-Dahan et al., (1997). The reaction mixture contained, 50 mM potassium phosphate buffer (pH 7.0), 1% guaiacol, 1% H_2O_2, and 0.3 ml enzyme extract. The change of absorption due to guaiacol oxidation were recorded at 470 nm for 3 min per 15 s with spectrophotometer**.** In the next procedure, the enzyme activity was calculated using the extinction coefficient of tetra-guaiacol (26.60 mM^{-1} cm^{-1} extinction coefficient).

Catalase assay

Catalase activity was determined using the method of Chaoui et al., (1997). The reaction mixture consisted of 125 mM potassium phosphate buffer (pH 7.0) and 100 mM H_2O_2, and 25 µL of the enzyme extract in a total volume of 3 mL. The absorbance at 240 nm for 1 min at 25 °C was recorded spectrophotometrically**.** The enzyme activity was expressed in ΔOD g^{-1} FW min^{-1}.

Chlorophylls and carotenoid content

0.5 g of fresh leaf tissues were ground in 5 ml of 80% (v/v) acetone using mortar and pestle. The suspension was centrifuged at 6,000 g for 10 min at 4°C. Absorbance of the solution was then measured at 645 and 663 for chlorophylls and at 470 for carotenoids.

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The chlorophyll and carotenoid contents were determined using the formula given by Arnon (1967), and the following formulas (*Eq.6-9*):

$$Chlorophyll\ a\ (mg / gr\ fresh\ weight) = [19.3\ (A_{663}) - 0.86\ (A_{645})] v / 100w \quad (Eq.6)$$

$$Chlorophyll\ b\ (mg / gr\ fresh\ weight) = [19.3\ (A_{645}) - 3.6\ (A_{663})] v / 100w \quad (Eq.7)$$

$$Total\ chlorophyll\ (mg / gr\ fresh\ weight) = [20.8\ (A_{645}) + 8.02\ (A_{663})]\ v / 100w \quad (Eq.8)$$

$$Carotenoids = [(100\ (A_{470}) - 3.27\ (mgchl\ a) - 104\ (mgchl\ b)] / 227 \quad (Eq.9)$$

A= absorption of light at wavelengths of 663, 645 and 470 nm
V= the volume upper solution of centrifuges
W = weight of the sample in grams

Statistical analysis

After normalization test (Kolmogorov-Smirnov and ShapiroWilk test)**,** the data were analyzed with SAS 9.1 (SAS Institute, Cary, NC, USA). Means were compared with Duncan's multiple range tests in SAS at 5% statistical probability levels. The graphs were drawn with MS-Excel.

Results

Germination percentage

According to the analysis of variance (*Table 1*), the effect of duration and concentration of seed priming on germination percentage was significant at P≤0.01. However, the interaction between duration and concentration of seed priming failed to have a significant impact on germination percentage (*Table 1*). The comparison of the mean of results indicated that by increasing the duration of priming, germination percentage also becomes higher (*Table 3*). In addition, the highest germination percentage (83.2%) was shown in the 24 hour treatment, while the lowest germination percentage (64.6%) in control (*Table 3*). Further, the highest germination percentage was detected in 1 mM SA indicating a 24.1% increase, compared to the control, which allocated the lowest amount (control= nonprimed) (*Table 3*).

Mean germination time and mean of daily germination

Based on the results of ANOVA (*Table 1*), priming duration and concentration had a significant impact on mean germination time and mean of daily germination. Furthermore, the interaction effect between treatments has a significant influence on mean germination time at p≤0.05 (*Table 1*). The results of mean comparison showed that the highest mean germination time (4.5 days) was in the case of the control while treatment 0.01 mM SA+ 48 h hours with mean germination 2.7 days had the fastest germination (*Fig. 1*). The mean seed germination time is considered a very important trait in planting establishment and exploiting environmental conditions.

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Table 1. *Analysis of variance effect of timing and concentration priming with salicylic acid on Catharanthus roseus seed germination indexes*

		Mean square							
S.O.V	**df**	**Germination percentage**	**Mean germination time**	**Mean of daily germination**	**Seedling length**	**Plumule length**	**Radicle length**	**Seedling weight vigor index**	**Seedling length vigor index**
Concentration	4	1045.07**	2.64**	12.89**	1.26*	0.07 n.s	0.84 n.s	3422.03**	26028.51**
Time	4	904.13**	2.97**	11.15**	1.59**	0.16*	1.38*	1135.96**	13174.6**
Concentration × time	16	105.03 n.s	0.33*	1.3 n.s	0.37 n.s	0.03 n.s	0.39 n.s	269.28 n.s	3863.39*
Experimental error	50	110.50	0.14	1.36	0.35	0.04	0.49	225.58	1987.32
Coefficient of variation (%)	-	13.88	9.72	13.87	14.00	11.63	28.48	21.49	14.01

ns, * and **: non-significant, significant at 5 and 1%, respectively

Table 2. *Analysis of variance effect of timing and concentration priming with salicylic acid on Catharanthus roseus*

		Mean square					
S.O.V	**df**	**CAT**	**POD**	**Chl a**	**Chl b**	**Total Chl**	**Carotenoide**
Concentration	4	35.69**	43.55**	4.7**	0.30**	4.67**	0.25**
Time	4	77.62**	23.52**	2.43**	0.24**	3.26**	0.19**
Concentration × time	16	8.76**	6.2**	0.44**	0.02**	0.41**	0.02**
Experimental error	50	1.64	1.15	0.013	0.001	0.002	0.0007
Coefficient of variation (%)	-	29.63	14.80	2.99	7.12	1.12	4.7

ns, * and **: non-significant, significant at 5 and 1%, respectively
Chl: Chlorophyll; CAT: catalase activity; POD: peroxidase activity

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Table 3. *Mean comparison of the effects of concentration and priming time on germination characteristics*

Characteristics	Germination percentage (%)	Mean of daily germination (day)	Seedling length (cm)	Plumule length (cm)	Radicle length (cm)	Seedling weight vigor index
Concentration SA (mM)						
Control 0	64.66c	7.18c	4.55a	1.77ab	2.78a	56.18b
0.01	71.6bc	7.95bc	3.94b	1.73b	2.20a	55.71b
0.1	74.53b	8.28b	4.048b	1.75ab	2.31a	66.07b
0.5	82.66a	9.18a	4.57a	1.91a	2.65a	83.2a
1	85.2a	9.46a	4.18ab	1.78ab	2.44a	88.06a
Time (hour)						
0	64.66c	7.18c	4.55a	1.77b	2.78a	56.45b
6	71.33bc	7.92bc	4.55a	1.93a	2.64a	66.67ab
12	77.33ab	8.59ab	3.80c	1.84ab	2.00b	71.96a
24	83.2a	9.24a	4.32ab	1.72b	2.6a	78.44a
48	82.13a	9.12a	4.05bc	1.67b	2.38ab	75.77a

In each column, means sharing at least one letter, are not significantly different according to Duncan's multiple range test (p≤0.05)
*SA: Salicylic acid

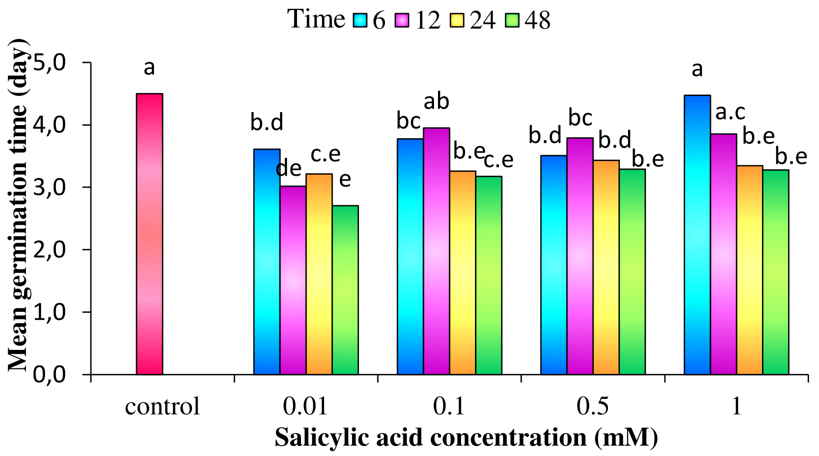

Figure 1. *Mean comparison of the effect of time and concentration on mean germination time*

The effect of different periods of time indicated that the highest and lowest required time for mean daily germination observed in the control and 24 hour long priming, were 9.2 and 7.1 days, respectively (*Table 3*). Furthermore, the speed of daily germination in the case of 24 hour treatment was 22.8% higher, compared to that of the control, although it was observed in the same statistical group with 12 and 48 hour treatments (*Table 3*). Results comparing the mean of the effect of different concentrations of SA showed, that increasing the concentration lead to a raise in mean daily germination and maximum mean daily germination achieved in 1 mM concentration SA which it did not has a meaningful difference with 0.5 mM concentration SA (*Table 3*).

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Seedling length

The results indicated that the main effects of priming time and concentration of SA played a significant effect on seedling length (*Table 1*). The highest and shortest seedling lengths were observed in 6 hour and 12 hour treatments, respectively (*Table 3*). The results showed that the maximum seedling length (4.57 cm) occurred in the 0.5 mM SA treatment and the lowest seedling length (3.9 cm) was found in the 0.01 mM SA treatment (*Table 3*).

Plumule length and radicle length

The results of ANOVA revealed that priming at different times could significantly influence the plumule and radicle length at P ≤0.05, but the concentrations of SA had no significant effect on these characteristics (*Table 1*). In addition, 6 hour seed priming had the highest plumule length and a significant increase occurred in the control treatment (*Table 3*). The shortest radicle length (2cm) occurred in the12 hour treatment priming, and had a significant difference compared to other treatments (*Table 3*).

Seedling weight and length vigor index

The effect of concentration and time on seedling weight and length vigor index was significant at the significance level of p≤0.01 (*Table 1*). In addition, the interaction between concentration × time on the seedling length vigor index was also significant (*Table 1*). The highest seedling length vigor index was observed during 1 mM SA + 24 hour priming (*Fig. 2*). Among the main effects of concentration and time priming, 24 hour treatment and 1 mM SA concentration revealed the highest seedling weight vigor index (*Table 3*).

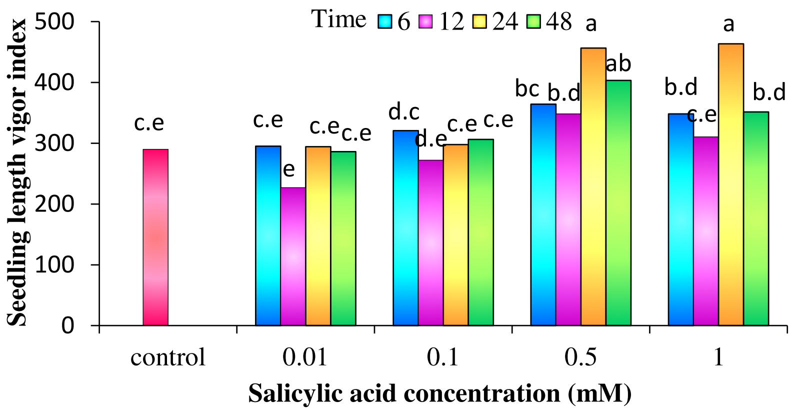

Figure 2. *Mean comparison of the effect of time and concentration on seedling length vigor index*

Chlorophyll content and carotenoids

According to the results, the effects of priming time and concentration, as well as interaction of concentration × time on chlorophyll a, b, total chlorophyll, and carotenoids were significant (p≤0.01) (*Table 2*). The highest chlorophyll a, b and total chlorophyll contents were related to the concentration of 0.1 mM SA, (*Fig. 3, 4, 5*) and higher carotenoid content was related to the treatment of 1 mM SA (*Fig. 6*). The results of this

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experiment showed that among the different levels of priming, 24 hours of treatment and control had the highest and lowest levels of chlorophyll a, total and carotenoids, respectively (*Figs. 3, 5, 6*).

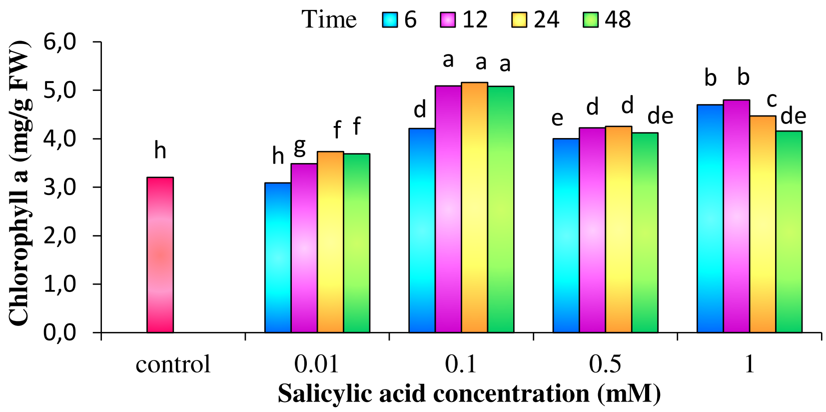

Figure 3. *Mean comparison of the effect of time and concentration on chlorophyll a*

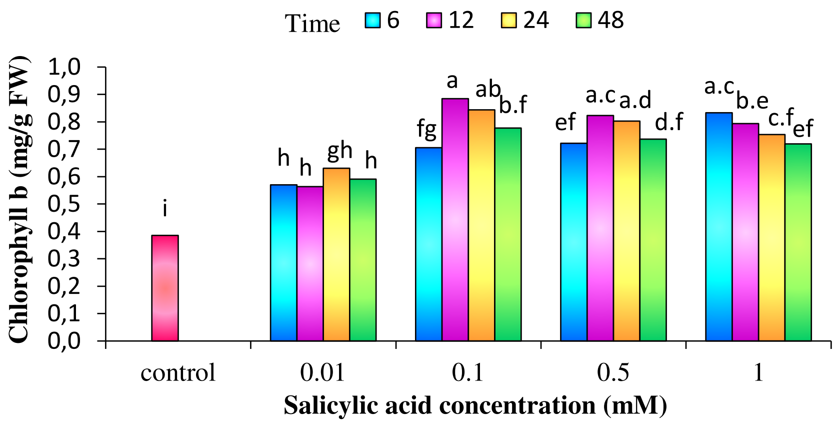

Figure 4. *Mean comparison of the effect of time and concentration on chlorophyll b*

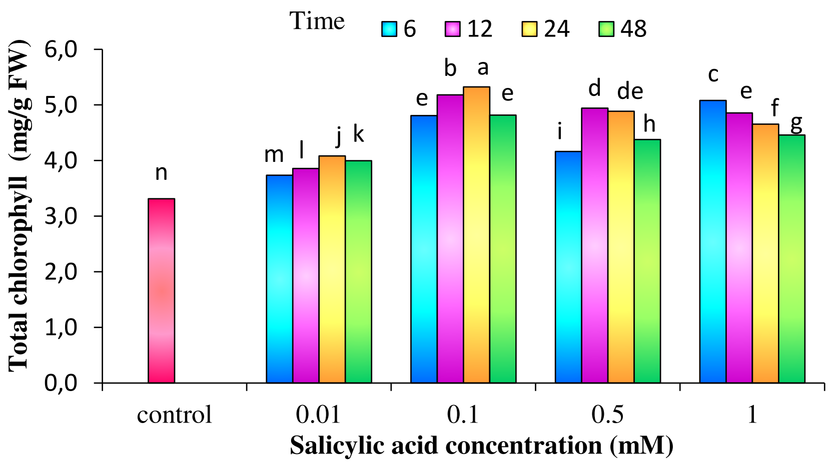

Figure 5. *Mean comparison of the effect of time and concentration on Total chlorophyll*

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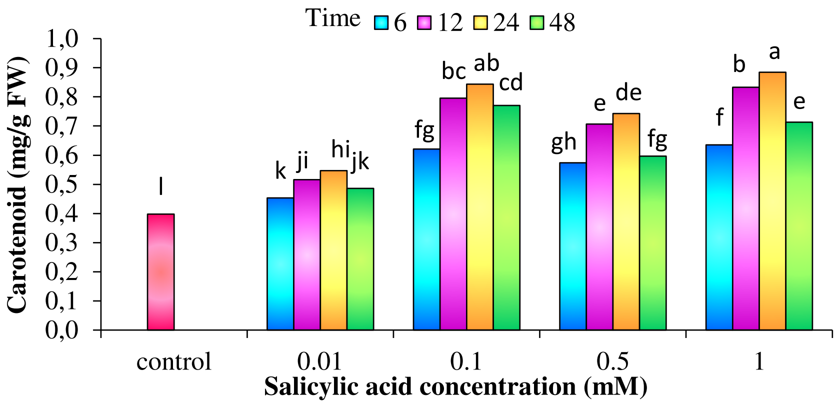

***Figure 6**. Mean comparison of the effect of time and concentration on carotenoid*

Furthermore, the highest and lowest levels of chlorophyll b were observed in the of 24 hour and control treatments (*Fig. 4*). In addition, interaction between concentration × time of priming indicated the highest concentration of chlorophyll a and total was at 0.1 mM SA + 24 hour seed priming (*Figs. 3, 5*) and the highest content of chlorophyll b and carotenoids was at 0.1 mM SA + 12 hour and 1 mM SA+ 48 hours of priming, respectively (*Figs. 4, 6*).

Antioxidant enzymes activities

The effects of time, concentration levels of priming, and the interaction between time × concentration on the activity of CAT and POD enzymes were significant at least at the significance level of 1% (*Table 2*). The highest activity of CAT enzyme was related to 1 mM SA treatment, which indicated a 84% higher activity than that of the control. Further, no significant difference was observed between the SA concentration of 0.1 and 1 mM (*Fig. 7*). Among the different priming times, 24-hour treatment and control had the highest and lowest activity of antioxidant enzymes, respectively. Furthermore, the interaction effects of 1 mM SA + 24 hours had the most significant effect on the activity of CAT enzyme (*Fig. 7*).

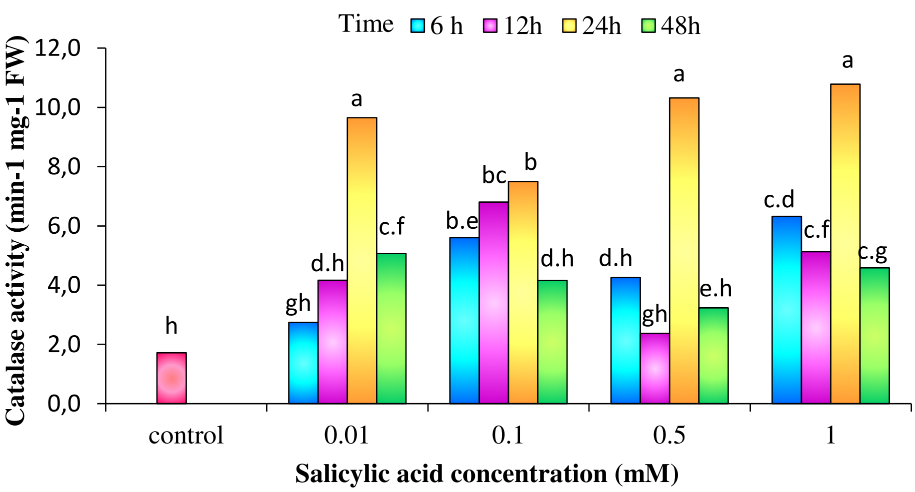

***Figure 7**. Mean comparison of the effect of time and concentration on catalase activity*

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Based on ANOVA results, the highest POD enzyme activity was related to 1 mM SA concentration and 12 hours of treatment (*Fig. 8*). In addition, the means of the comparison of the interaction between SA concentrations × time indicated higher POD enzyme activity when the seeds primed with 1 mM SA + 24 hours (*Fig. 8*).

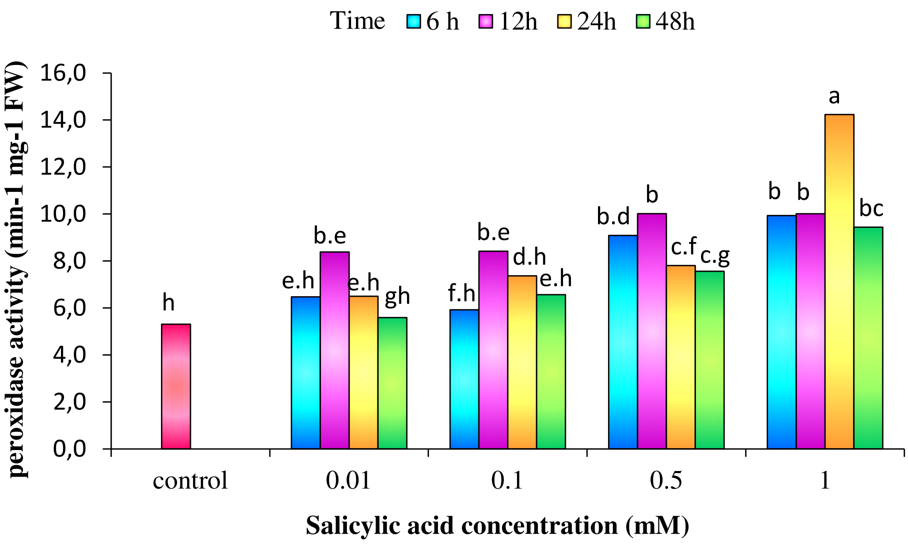

Figure 8. *Mean comparison of the effect of time and concentration on peroxidase activity*

Discussion

Several reports have been published demonstrating the role of SA in various physiological processes. Some of these reports demonstrated the protective or damaging effect of exogenous SA (Szalai et al., 2011). Priming pepper seeds in acetyl-SA improved the final germination percentage, and led to higher shoot and root dry weights (Korkmaz, 2005). However, it should also be noted that a dramatic inhibition of the germination process was reported above the concentration of 1 mM in maize (Guan and Scandalios, 1995).

Results indicated that by increasing the SA concentration germination percentage also becomes higher (*Table. 3*). Gorzi et al. (2017) reported that the seed priming with SA improved the germination parameters and seedling growth of stevia. Furthermore, SA may stimulate the seed germination via biosynthesis of gibberellic acid and induce thermogenesis (Shah, 2003). Previous studies reported that isocitrate lyase and malate synthase were found to be more abundant in seeds germinated in the presence of SA than in its absence. Isocitrate lyase and malate synthase are two key enzymes of the glyoxylate cycle, which play a crucial role in synthesizing carbohydrates from storage lipids during seed germination and seedling establishment (Eastmond and Graham, 2001).

Khoramdel et al. (2012) reported that an increase in SA concentration from 0.1 to 1 mM leads to an increase in germination percentage, and a reduction of the germination percentage occurred after increasing the concentration. It seems that different effects may be observed in different concentrations since SA is considered as a plant growth regulator. The time of contact with these substances is very important, along with the use of plant hormones. Thus, the amount or concentration of hormones should be lower in long time periods and it is better to consider the higher concentration of growth regulator

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substances in shorter periods of time. By increasing the duration of priming, hormones can better penetrate into seeds, leading to an increase in the activity of germination stimulating enzymes, as well as the percentage and speed of seed germination (Azarniya et al., 2016). Shahverdi et al. (2017) reported the highest germination percentage among the priming duration and different concentrations of boric acid were observed under 24 hours of priming. Application of 0.01 mM SA during a 48 hour priming showed the lowest mean seed germination time (*Fig 1*).

Reducing the mean germination time due to seed priming is related to an increase in the rate of cell division in primed seeds because the synthesis of RNA, DNA, and protein were completed in many physiological stages and the seed was placed at the threshold germination during seed priming (Foti et al., 2008; Brancalion et al., 2008). The results of the present study indicated that the 6 hour seed priming treatment had the highest seedling length and plumule (*Table. 3*). Compared to non-primed seeds, an important feature of primed seeds is that they quickly germinate and produce radicle and plumule in optimal conditions. Thus, the seedling length of primed seeds is considerably higher than that of non-primed seeds (Murungu et al., 2003). The maximum seedling length was assessed during the 0.5 mM SA treatment. The results of this study are in line with previous studies that indicated applying SA was more effective by soaking the seeds, and the best results were obtained using 0.5 mM SA solution (Baninasab, 2010). In addition, SA regulates elongation and cell division in collaboration with materials such as auxin. Furthermore, the exogenous application of SA causes cell division in the terminal meristem of the primary roots, which results in increasing longitudinal growth (Serraj and Sinclair, 2002).

Seedling length vigor index is derived from both multiplication germination percentage and seedling length. Therefore, every treatment increasing these two components leads to an increase in seed vigor. Thus, an increase in seed vigor using priming treatments can be related to increasing the activity of the enzymes involved in germination. Consequently, an increase in the consumption of seed reserves and length of seedling can be attributed to increased energy in primed seeds (Omid and Farzad, 2012). The results of our study indicated that 24 hours treatment and 1 mM, SA concentration resulted in the best seedling weight vigor index and seedling length vigor index (*Table. 3*).

Azarniya et al. (2016) reported that the effect of time and concentration of priming was significant on seed vigor and the most effective duration of priming was 12-hour priming. They also concluded that primed seeds have a more favorable germination vigor than non-primed seeds. Seed priming is considered as one of the strategies to improve the amount of chlorophyll a and b (Rehman and Ali, 2015). Furthermore, seed priming with SA has positively affected biochemical traits such as total chlorophyll (Singh et al., 2018).

Seed priming with the concentration of 0.1 mM SA indicated the highest chlorophyll a, b and total chlorophyll content (*Fig. 3.4.5*) and higher carotenoid content was related to higher a concentration of SA (1 mM) (*Fig. 6*). The results are consistent with the results of (Yanik et al., 2018), who reported that the total chlorophyll content increased under 10 μM SA. Higher concentration of SA resulted in decreasing total chlorophyll and increasing carotenoid contents. Moharekar et al. (2003) indicated that different concentrations of SA can activate the synthesis of carotenoids and xanthophylls while a total chlorophyll content reduction occurs in wheat. It seems that decreasing chlorophyll content due to the SA-induced excess leads to the accumulation of ROS and the inhibition of plant growth and development. (Ma et al., 2013). However, the results of other studies

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indicated that the SA pretreatment leads to a significant increase in the chlorophyll and carotenoid content (Saidi et al., 2013), which is beneficial for improving photosynthesis (Zhang et al., 2015). Catalase is one of the most important antioxidant enzymes, which can be increased in plants using a seed priming technique (Fallah et al., 2018). The results of the present study indicated that treating seeds with 1 mM SA for 24 hours had the most significant effect on the activity of CAT and POD enzymes. Yang *et al* (2016) investigated that treating seeds with SA could enhance the defense mechanisms of *Helianthus annuus*, leading to an increase in germination indices by increasing the activity of antioxidant enzymes (Omid and Farzad, 2012).

Conclusion

The results indicated that the priming technique can improve the seed germination components of *Catharanthus roseus.* Furthermore, a significant difference was reported between different priming times. Additionally, seed priming time is regarded as one of the important factors in seed treatment and determining the appropriate priming time may prevent the negative effect of pretreatment on germination characteristics and seedling growth of primed seeds. In order to have a positive effect, the best time for pretreatment on improved germination and seedling growth indices should be determined for every plant. In the present study, the results indicated that an increase in the pre-treatment time results in improving most of the components related to germination. In this experiment, the most beneficial length of time was 24 hours for pre-treatment of *Catharanthus roseus* seeds. In addition, the best results were related to 0.5 and 1 mM SA concentrations. Therefore, determining the best concentration and time for seed priming is considered as a key factor to implement seed priming effects in the most beneficial way.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_39894005

DETERMINATION OF ACRYLAMIDE AND HYDROXYMETHYLFURFURAL (HMF) VALUES AS AFFECTED BY FRYING DURATION AND TEMPERATURE LEVELS DURING THE PREPARATION OF TRAY KADAYIF DESSERT

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(Received 12th Nov 2018; accepted 14th Feb 2019)

Abstract. In this study, a wire kadayıf on a tray was fried at 175, 200 and 225 °C for 40, 50 and 60 min. The temperature levels affected hydroxymetylfurfural (HMF) content, color values (L, a, b), outward appearance, interior color, taste, texture, aftertaste and general acceptability values at significant levels ($p \leq 0.01$); as well as pH and aroma ($p \leq 0.05$). As the temperature increases the HMF content, color value a, outward appearance, interior color, taste, odor, aroma, texture, aftertaste and the general acceptability values have increased. Whereas the moisture, pH and L color values decreased. The frying time had an effect on moisture. The changes in the color brightness (L), red color (+a), and the yellow color (+b) values of the kadayıf were highly significant ($p \leq 0.01$); and the changeswere also significant ($p \leq 0.05$) on the HMF content, texture, aftertaste and general acceptability values. As the frying time increases HMF content, a color value, texture, aftertaste and the general acceptability values increased whereas the moisture, L and b color values decreased.
Keywords: *acrylamide, HMF, frying temperature, frying duration, tray kadayıf*

Introduction

Kadayıf is a delicious Turkish sweet pastry, consumed in different provinces of Turkey such as Mersin, Hatay, Erzurum, Diyarbakir, and Gaziantep. Tray kadayıf and creamy kadayıf can both be prepared with either whole hazelnut, walnut or pistachio (Anon, 2012). According to the “standard of tray kadayıf” (T.S-10344, 1992), it is a semi-processed product made by adding drinking water to sifted flour and baking it on a plate. However, tray kadayıf is offered in raw and fired varieties in markets. Flour for making Kadayıf should have the following properties: humidity 14.5% (max), protein 8-10% (F = 5.70), the essence of age 22% (max), water holding capacity 54% (max), stability 2 min (max), softening temperature 150 BU (max) and minimum value of the biscuits perchance show maximum level in kadayıf. In recent years, tray kadayıf has mostly been produced in small enterprises, but has also been manufactured in large quantities to a lower extent (Pekak, 2006).

Acrylamide was first prepared in 1893 by Moureu at 10 °C on a saturated solution of acrylic chloride in benzene, followed by adding dry ammonia and boiling for mean duration, then ammonium chloride was refluxed by filtrating, cooling, and precipitating acrylamide (Carpenter and Davis, 1957). Neurotoxic effects of acrylamide were detected in a laboratory with animal testing. In addition, genotoxic and carcinogenic effects of acrylamide have been identified in recent years, its presence in many processed foods has been a hazard to human health (Joint, 2005). Acrylamide as a chemical substance was synthesized in 1949, it is white, odorless, crystalline, solid and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

soluble in water (255 g/L of water) (Thomas and Thomas, 2012), its melting and boiling point is 87.5 °C and 125 °C, respectively (Gölükçü and Tokgöz, 2005). It has no burn inducing or irritating properties, low acidity, and weak basic characters. It has a monomeric structure of two different functional groups; double bond and weak amid group as the electron, and these groups are joined through chemical reactions (Can, 2007). In food items, acrylamide has a simple structure which occurs as a result of reactions taking place among carbohydrates, proteins, amino acids, lipids, or other food components (Gölükçü and Tokgöz, 2005). It is usually formed during frying, baking, and roasting (Svensson et al., 2003). The content of acrylamide in the product can be identified by some factors, such as preparation methods, compositions, ambient temperature, and pH. Asparagine appears to be the primary amino acid involved in the production of acrylamide via the Millard reaction (LoPachin and Canady, 2004).

According to Zyzak et al. (2003), there is a linear relationship between free asparagine contents and forming of acrylamide in the product. Gölükçü and Tokgöz (2005) indicated that asparagine content of the product is the most important factor in the formation of acrylamide in cereals. The duration of heating initiates the Millard reaction, in which sugars (simple monosaccharides capable of carrying out reduction reactions) present in starchy foods are reduced with amino acids to produce acrylamide. Applying high temperature (more than 120 °C) caused a raise in the acrylamide contents above 1 mg/kg in food items with high contents of carbohydrates. Therefore, there is a relationship between acrylamide in the food and temperature. Controlling the temperature range can reduce the amount of acrylamide formation. Food which is prepared through boiling has a low content of this compound, thus it should be preferred to frying, baking, roasting, and grilling; using methods such as frying or roasting at elevated temperatures for a long duration should also be avoided (Gölükçü and Tokgöz, 2005).

Beside acrylamide, hydroxylmethylfurfural (HMF) is another harmful compound which occurs as a result of the Millard reaction in biscuits. HMF is formed from an aromatic alcohol, aromatic aldehyde, and furan ring, and as an intermediate product in the hydrolysis of hexose in an acidic medium or in the Millard reaction (Seyyed Cheraghi, 2014). The concentration of HMF varies significantly and in some food items it can even reach 1 g/Kg (Rada-Mendoza et al., 2004; Akkan et al., 2001). The formation and amount of HMF are affected by temperature and pH value, also the quality deterioration of a wide range of edibles is determined by Hydroxymethylfurfural (HMF) (Gökmen and Şenyuva, 2006). Beside the harmful compounds which are the result of Maillard reactions, antioxidant compounds are also formed (Yıldırım, 2010). Some researchers have stated that HMF has possibly genotoxic and mutagenic effects on human cells, possibly inducing liver and colon cancer (Svendsen et al., 2007; Monien et al., 2012; Zhang and An, 2017); so the objective of this study was to determine the effect of temperature and duration on acrylamide and hydroxymethylfurfural formation during the preparation of tray kadayıf.

Material

As the ingredients of tray kadayıf, butter and hazelnut were acquired in the markets (Erzurum, Turkey). The ratio of grout which has been used was 2:1 sugar mixed with water. After boiling, approximately (2.0 ml) lemon juice was added to prevent

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

crystallization. The tray kadayıf had the following values: pH = 6.43, humidity = 20.53%, L (96.19), +a (0.53) and +b (16.89).

Method

Processing of tray kadayıf

200 g of butter was melted on a very low temperature and was then mixed with the kadayıf for a 4-5 min period; and about 500 g was laid with a homogeneous thickness. After seating on the tray, a special medium-density fiberboard (MDF) wood and an overall 10 kg heavy weight was placed on it. The tray kadayıf was fried at 175 °C, 200 °C and 225 °C for 40, 50, 60 min respectively. After that, the previously prepared syrup was poured onto it; then sliced and crushed nuts were placed on top, and was then presented to panelists. The analysis samples were prepared from the upper part of the fried tray kadayıf samples after grounding it into very small pieces.

The analyses performed on tray kadayıf samples

Measurement of the color intensity

In the above-mentioned samples, the color densities, which are brightness of kadayıf color (L; 0-100: dark-bright) red color (+a), yellow color (+b) were measured using three parallel Minolta Colorimeter device. Results were evaluated according to the International Commission on Illumination (CIE Lab, Commission Internationale de l'Eclairage; Anonymous, 2012).

Acrylamide analysis

The analysis of acrylamide was performed according to Robarge et al. (2003). During the preparation of the sample the following steps were taken.

The acrylamide standard solutions, used as the sample calibration solutions were 1000 ppb, 500 ppb, and 50 ppb 100 ppb, and were subjected to the same processes to obtain a calibration curve.

Extraction: very small particles of tray kadayıf samples (1 g) were put into 10 ml demineralized water in a 50 ml Erlenmeyer, then was magnetically stirred for 20 min, at 5500 rpm and centrifuged for about 10 min. After centrifuging the supernatant, it was filtrated through a 0.45 µm nylon filter, then 200-300 µL 0.1 normality (N) potassium bromate ($KBrO_3$) was slowly added to 3 ml of the filtrate, followed by stirring and 1 hour of rest in an ice bath. The ice bathing tubes received one drop of 1.0 N sodium thiosulfate ($Na_2O_3S_2$) and were mixed slowly with, followed by adding 2 ml of ethyl acetate ($C_4H_8O_2$). The tubes which had Ethyl acetate added were centrifuged at 5500 rpm for an additional 10 min, then the vials were transferred for supernatant analysis.

Sample extracts and the standard solution were measured using a Gas Chromatography-Mass Spectrometry (GC-MS, Agilent) analysis device. Positron effect was analyzed with imaging method of the GC-MS, DB-225 column (30 m × 0.25 mm × 0.25 µm) and helium gas. The oven temperature was increased from 40 to 200 °C and in this analysis set which increasing 30 °C in a minute split-less mode is used.

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Sensory analysis

The sensory analysis of tray kadayıf was carried out at the laboratory of the Faculty of Agriculture, Food Engineering Department, Ataturk University, and panelists were selected from among the graduate and postgraduate students of the Food Engineering Department faculty. Slides which were cut from each tray kadayıf were served on coded plastic plates with drinking water to eight panelists, who were asked to evaluate them. The tray kadayıf was left waiting for 24 h at room temperature. Tray kadayıf samples' taste, aroma, color, texture and overall acceptability were subjected to evaluation. There was a 9-point hedonic scale for the evaluation of these parameters (1 = very bad, 9 = very good) for the panelists to rate each tray kadayıf (Kramer and Twigg, 1973).

Determination of hydroxymethylfurfural (HMF)

Hydroxymethylfurfural analysis was performed with a High-Performance Liquid Chromatography (HPLC) on samples prepared according to the method given by Rada-Mendoza et al. (2002). According to the aforementioned method, a 2 g sample was transferred to a 50 ml Erlenmeyer, to which 4 ml each of carrez I [15 g potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6] \times 3H_2O$) mixed with 100 ml ultra-pure water] and carrez II [30 g zinc sulfate heptahydrate ($ZnSO_4$ x $7H_2O$) mixed with 100 ml ultra-pure water] were added and flask was filled to 50 mL with deionized water. After stirring for 30 min, the contents of the Erlenmeyer were passed through a 0.45 µm filter. The samples were analyzed using the HPLC, Agilent 1100 system. UV-VIS detector and Nucleosil 5C18 (250 × 4,6 mm) column (Hichrom, Reading Berkshire, England) were used in this system. The samples were then placed at room temperature, and 10:90 (v/v) methanol-water was used with a flow rate of 1 ml/min to keep it mobile. Hydroxymethylfurfural concentration curve was measured using external calibration (55690-5-HMF, Fluka Chemika) at 280 nm.

Determination of pH

The homogenized 3 g tray kadayıf sample was placed into pure water and its pH was assessed. The sample's pH was previously measured to be pH 4.00 and pH 7.00 using an INOLAB pH 720 brand of Microprocessor (Torley et al., 2008).

Determination of dry matter

Homogenous samples of about 8-10 g were taken from the total samples of both acrylamide and HMF and then dried in a drying oven at 110 °C until a constant weight was achieved (Kotancılar, 2013).

Experimental plan

Trials were conducted with factorial arrangement by three different frying temperatures (175, 200 and 275 °C) and three different frying durations (40, 50 and 60 min) in complete randomized design (CRD) with two replicates (3 × 3 × 2).

Statistical analysis

The raw values received from trials were subjected to variance analysis in SPSS (SPSS1999), and the mean of the sources of the main variation has been found

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DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

important, as compared with Duncan Multiple Comparison Test (Yıldız and Bircan, 2003).

Results and discussion

Acrylamide values in tray kadayıf

In the present study, variance analysis (*Table 1*) indicated that there were significant ($p \leq 0.01$) differences among different temperatures and different durations based on acrylamide values. As shown in *Table 2,* the temperature increase does not cause any significant changes on the acrylamide content, as the lowest amount of acrylamide was (130.5 μg/kg) at 200 °C, and the highest amount was (137.7 μg/kg) at 175 °C. Mottram et al. (2002) determinate that in the asparagine/glucose model systems the increase of temperature from 120 °C to 170 °C increases the amount of acrylamide and above 170 °C it is reduced. The acrylamide content increases with the frying duration. The lowest amount of acrylamide (132 μg/kg) was determined after 40 min and the highest amount (137 μg/kg) was obtained with 60 min of duration. Tuta (2009) stated that if the temperature rises above 100 °C and the duration of frying increases in parallel, then the acrylamide content decreases. The highest value was achieved with a high-temperature heat treatment of low duration.

Table 1. *Variance analysis of acrylamide, L, +a, +b color values at different temperatures and duration*

SOV	SD	F			
		Acrylamide	**L**	**+a**	**+b**
Frying temperature (°C)	2	2.54	410.00**	12810.00**	3184.00**
Frying duration (S)	2	1.06	120.00**	3253.00**	976.00**
C × S	4	1.29	20.00**	59.00**	1467.00**
Error	9				

*, **: Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 2. *Mean comparison of acrylamide, L, +a, +b color values at different temperatures and duration*

Frying temperature (°C)	n	Means			
		Acrylamide (μg/kg)	**L**	**+a**	**+b**
175	6	137.70 a*	66.67 a	2.72 c	25.10 b
200	6	130.50 a	60.16 b	7.81 b	26.77 a
225	6	135.10 a	45.05 c	13.52 a	15.81 a
Std. error		± 2.3	± 0.547	± 0.048	± 0.105
Frying duration (S)	**n**				
40	6	132.00 a	63.36 a	5.45 c	25.44 a
50	6	134.00 a	57.16 b	7.72 b	23.23 b
60	6	137.00 a	51.36 c	10.87 a	19.01 c
Std. error		± 2.3	± 0.547	± 0.048	± 0.105

*The average shown with the same letters are statistically indistinguishable from each other ($p < 0.05$)

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DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

Color parameter's values of the tray kadayıf

Variance analysis (*Table 1*) of the tray kadayıf L (Brightness), +a (Redness), and +b (Yellowness) color values influenced by the frying temperature, duration and C × S interactions was highly significant ($p \leq 0.01$). The important variables of the frying temperature and duration including L, +a, and +b color values can be found in *Table 2*. The lowest amount of L color value (45.05) was assessed at 225 °C and the maximum amount (66.67) was obtained at 175 °C temperature. By increasing the duration of frying, L color values decreased. The lowest L color value (51.36) was measured after the 60 min mark and the maximum amount (63.36) was obtained after 40 min of duration. The lowest amount of +a color value (2.72) was detected at 175 °C and the maximum amount (13.52) was obtained at 225 °C temperature. By increasing the frying duration, +a color value would increase. The lowest amount of +a color value (5.45) was assessed after 40 min and the highest amount (10.87) was obtained at the 60 min mark. The lowest amount of b color value (15. 81) was measured at 225 °C and the highest amount (26.77) was obtained at 200 °C temperature. By increasing the frying duration, b color values decreased. The lowest amount of b color value (19.01) was detected after 60 min and the maximum amount (25.44) was obtained during the 40 min duration. The interaction between C and S, and its effect on L, +a, and +b color values are given in *Table 1*.

The result showed that the reduction in the L color value as a result of the increased temperature caused the tray kadayıf to gain a dark color. In parallel, +a surface color value has increased and has become more of a reddish color, while in +b value there was an increase at first, followed by a serious decline. While o the dark and intense red color was obtained at 225 °C the brightest and least red surface color was obtained at 175 °C. Colored compounds formed as a result of Maillard reaction are high molecular weight macromolecules called Melanoidins and low molecular weight molecules which have two or three heterocyclic rings (Ames et al., 1998). Color formation, temperature, duration, increase with pH and moisture content in the medium ($aw = 0.3$ -0.7). As shown in *Table 2*, with the increase in temperature and duration caused a decrease in the L value of tray kadayıf, which means the surface color got darker. In parallel, the increase in a value was observed, which means that the intensity of red increased. The brightest and pinkish colors were obtained when the tray kadayıf was fried for 40 min and the darkest red was obtained by frying for 60 min. By increasing frying duration b color value decreased. A decrease was observed in yellow color as well. In a study by Özkaynak (2006) each of the three oil varieties developed darker colors depending on the frying duration as a result of variations in the formation of acrylamide, and L was found to decrease.

Sensory analysis values of tray kadayıf

Variance analysis values of appearance, interior color, taste, odor, flavor, texture, aftertaste and overall acceptability are shown in *Table 3*. The effect of frying temperature on appearance, interior color, taste, texture, aftertaste and the overall acceptability of tray kadayıf was statistically highly significant ($p \leq 0.01$) whereas that on the aroma and smell was significant at a different level ($p \leq 0.05$). The frying duration's effect on the overall acceptability was highly significant ($p \leq 0.01$); on the texture and aftertaste it was significant ($p \leq 0.05$) while on the appearance, interior color, test, smell and aroma it was not significant. The effect of the interaction between

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

frying temperature and frying duration on the outward appearance of tray kadayıf, taste, texture, aftertaste and the general acceptability was highly significant ($p \leq 0.01$), while that on smell value was significant ($p \leq 0.05$) and was not significant on the interior color and the aroma. Significance values of frying temperature can be seen in *Table 3*, while the Duncan multiple comparison test results are shown in *Table 4*.

Table 3. *Variance analysis of tray kadayıf features at different temperatures and durations*

S.O.V	SD	F							
		Appearance	Interior color	Taste	Smell	Aroma	Texture	Aftertaste	General acceptability
Frying temperature (°C)	2	82.08**	41.17**	8.08**	3.56*	4.77*	39.56**	15.13**	12.99**
Frying duration (S)	2	1.25	3.33	1.47	0.22	1.90	4.27*	4.72*	3.51**
C × S	4	13.40**	2.71	8.00**	3.93*	1.60	7.76**	7.99**	9.75**
Error	9								

*, **: Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 4. *Mean comparison of tray kadayıf features at different temperatures and durations*

Frying temperature (°C)	n	Means							
		Appearance	Interior color	Taste	Smell	Aroma	Texture	Aftertaste	General acceptability
175	6	2.83 c	3.79 c	4.87 b	5.21 b	4.67 b	4.33 c	4.38 b	4.50 b
200	6	5.96 b	6.08 b	5.87 a	5.96 ab	5.88 a	5.67 b	5.79 a	5.96 a
225	6	7.00 a	7.54 a	6.21 a	6.25 a	6.04 a	6.75 a	6.17 a	6.04 a
Std. error		± 0.24	± 0.29	± 0.24	± 0.34	± 0.34	± 0.19	± 0.24	± 0.24
Frying duration (min)	**n**								
40	6	5.00 a	5.63 a	5.30 a	5.58 a	5.00 a	5.16 b	4.87 b	5.00 b
50	6	5.58 a	5.68 a	5.70 a	5.89 a	5.67 a	5.62 ab	5.54 ab	5.62 ab
60	6	5.18 a	5.79 a	5.95 a	5.93 a	5.92 a	5.96 a	5.91 a	5.87- a
Std. error		± 0.24	± 0.29	± 0.24	± 0.34	± 0.34	± 0.19	± 0.24	± 0.24

*The average shown with the same letter are statistically indistinguishable from each other ($p < 0.05$)

As shown in *Table 4*, increase in the temperature of tray kadayıf influenced the appearance, interior color, taste, odor, flavor, texture, and aftertaste positively and was also more appreciated by panelists. While the highest values of appearance, interior color, taste, odor, flavor, texture, aftertaste and general accessibility were obtained at 225 °C, the least admired was the tray kadayıf fried in 175 °C.

HMF, pH, and surface moisture values of tray kadayıf

Variance analysis of HMF, pH and the surface moisture under different frying temperatures and durations of tray can be seen in *Table 5*. As shown in *Table 5* the effect of frying temperature on HMF and humidity values was highly significant ($p \leq 0.01$), while on pH value was significant ($p \leq 0.05$). The effect of frying duration on humidity values was highly significant ($p \leq 0.01$) while on HMF values was significant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

($p \leq 0.05$). As shown in *Table 6*, the increase in the temperature caused an increase in HMF values (the lowest value of HMF was achieved at 175 °C, while the highest value was observed at 225 °C), and a decrease in pH and the amount of humidity on the surface. Depending on the increase in the temperature the moisture content decreased accordingly. Normal moisture content in particular decreased at temperatures above 50 °C and pH 4-7, while HMF formation rate increased (Borrelli et al., 2002; Oral, 2011).

Table 5. *Variance analysis of HMF, PH and humidity values under different temperatures and durations*

S.O.V	SD	F		
		HMF	**pH**	**Humidity**
Frying temperature (C)	2	14.34**	5.59*	239.00**
Frying duration (S)	2	2.89*	2.22	108.00**
C × S	4	1.40	0.64	24.00**
Error	9			

*. **: Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Table 6. *Mean comparison of HMF, PH and humidity values under different temperatures and durations*

Frying temperature (°C)	n	Mean		
		HMF (µg/kg)	**pH**	**Humidity (%)**
175	6	8.70 b	5.70 a	2.29 a
200	6	54.30 b	5.60 b	0.78 b
225	6	342.90 a	5.50 b	0.56 c
Std. error		± 47.8	± 0.1	± 0.1
Frying duration (min)	**n**			
40	6	53.00 b	5.68 a	1.87 a
50	6	135.00 ab	5.61 a	1.16 b
60	6	216.00 a	5.52 a	0.61 c
Std. error		± 47.8	± 0.1	± 0.1

*The averages shown with the same letters are statistically indistinguishable from each other ($p < 0.05$)

The increasing in frying duration lead to a pH decrease. However, this change was not statistically significant. The HMF value proved to be dependent on frying duration. The lowest HMF value was measured during the 40 min frying period, while the highest value was detected after the 60 min cooking (*Table 6*). Cooking duration in the production of dry fruit pulp is extremely important. The determination of HMF and acrylamide contents significantly increased with the increase in cooking duration (Boz, 2012). As a result of the increase of frying duration, moisture content decreased. Elmore et al. (2005) investigated how much the moisture content depends on frying duration of baked cakes of rye and wheat flour and potatoes. The moisture contents of potatoes and cakes made from rye and wheat flour in the first minute were 59.73, 41.60, and 39.60, respectively. With the 10 min duration, the ratio under 180° C decreased to 24.25, 3.86,

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DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

and 10.33. After 50 min, it decreased to 0.75, 0.70, and 0 (Elmore et al., 2005). The reduction was observed in the moisture content when the temperature of tray kadayıf was increasing.

As displayed in *Table 6*, with the increase of temperature, at the end of all three periods there was an increase in moisture content of tray kadayıfs. At 175 °C, 200 °C and 225 °C and with 40 min of frying duration the highest moisture content, but after 60 min it reached the lowest value. These properties were observed after the 50 and 60 min periods. When the temperature was raised from 175 °C to 200 °C, there was a rapid decrease in the moisture content. At 220 °C and 40 min of frying, there were not many changes to moisture content.

Conclusions

None of the factors has a statistically significant effect on the value of acrylamide. Increase in temperature does not cause any change on acrylamide content. While raising frying duration caused an increase. Increase in temperature levels of tray kadayıf reduced its *L* color value and a darker color was obtained.

The increase in temperature levels, caused an increase in HMF values, a decrease in pH and moisture content on the surface. Depending on the temperature rise a reduction in moisture content was observed. Increasing the frying duration caused an increase in pH values. Depending on higher temperature levels and longer frying duration, HMF values raised. With the increase of frying duration, the moisture content was reduced.

In this research, *a* color value of the surface increased and the kadayıf appeared much redder. In *b* color value there was an increase at first and then it had a serious decrease. Raising frying duration caused a decrease in *L* color value of tray kadayıf, which means that the surface color got darker. In parallel, an increase in the *a* color value was observed, which meant a more intensive red color. The *b* color value decreased by increasing frying duration. An increase in temperature levels had positively influenced the appearance, interior color, taste and smell and caused it to be appreciated by panelists. Increasing the duration of frying had no effect on the aroma of tray kadayıf and had a positive effect on texture, aftertaste and general acceptability.

Acknowledgements. This research was supported by The Research Fund of Ataturk University, Erzurum, Turkey, contract no. BAP-2012.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40074017

EFFECTS OF DROUGHT STRESS ON THE QUALITY OF MAJOR OILSEED CROPS: IMPLICATIONS AND POSSIBLE MITIGATION STRATEGIES – A REVIEW

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(Received 15th Nov 2018; accepted 12th Feb 2019)

Abstract. Oilseed crops are considered as one of the most essential dietary components in human consumption as well as animal feed. While drought is one of the most important abiotic stress that adversely effect on the yield and nutritional quality of major oilseed crops across the globe. Therefore, maintenance of nutritional quality in such crops under drought stress may offer good opportunities to

provide sufficient human food as well as animal feed. The current review is to quantify the adverse effects of drought on the qualitative and quantitative traits of oilseed crops, suggest strategies for enhancing drought tolerance in crops. Although drought stress-induced effects on seed oil quality vary with larger environments as well as crop species and their interactions. When short and long term drought occurs at the seed/grain filling stage of crops, it might negatively influence the grain yield as well as the quality of crops. In the present review, we highlight that there are two ways to mitigate the adverse effect of drought *viz.,* development of crop cultivars tolerant to drought and to follow improved management approaches including the exogenous application of osmoprotectants, essential macro and micronutrient management and using organic amendments (compost, green manures, cow manure and incorporation of crop residues) in soils in order to increase the yield and grain quality of oilseed crops.
Keywords: *abiotic stress, crop phenology, oil quality, osmotic stress, plant adaptation, protein content, stress resistance*

Abbreviations: ABA, abscisic acid; AC, ash content; APX, ascorbate peroxidase; AsA, ascorbic acid; AY, ash yield; CAT, catalase; chl a, chlorophyll a; chl b, chlorophyll b; CI, conventional irrigation; DSI, drought stress index; DW, dry weight; Exo, exogenous (ly); Exo-GB, exogenous glycine betaine; Exo-Pro, exogenous proline; Exo-app, exogenous application; Fe, iron; FW, fresh weight; GB, Glycine betaine; GW, grain weight; GY, grain yield; LAA, L-ascorbate; MDA, malondialdehyde; Mg, magnesium; NO, nitric oxide; P, phosphorous; PAL, phenylalanine ammonia lyase; PC, protein content; POD, Peroxidase; Pro, proline; Pri, priming; PY, protein yield; OC, oil content; OSP, osmoprotectants; OY, oil yield; RDF, recommended dose of fertilizers; RLWC, relative leaf water content; ROS, reactive oxygen species; RWC, relative water content; SA, salicylic acid; SC, starch content; Se, selenium; SI, supplemental irrigation; SNP, sodium nitroprusside; SOD, superoxide dismutase; STI, stress tolerance index; SY, starch yield; UV, ultra-violet radiation; Tre, trehalose; TW, test weight; Zn, zinc

Introduction

The impacts of climate change are the foremost thought in many developing countries, due to greater vulnerabilities and limited ability to mitigate the adverse effect of climate change for sustainable crop production (Ali et al., 2017). The economy of most developing countries is based on agriculture, while agricultural sector is fully depend on nature (IPCC, 2012; Mendelsohn, 2014).

The major abiotic stresses such as drought, salinity, heat stress and water-logging are the main climatic factors for declining crop production in many developing countries (Hossain et al., 2013; Jahan et al., 2019; EL Sabagh et al., 2019b). Among them, drought stress is considered the most important one that adversely effect on the yield and quality of many field crops including oilseeds also (Alqudah et al., 2010), through altering the growth, physiology and metabolic activities of plants (Islam et al., 2011). Growing demand for food, feed and raw materials for industries has stressed upon the production of good quality of agricultural products. It is well established that drought has a directly affect the quality of agricultural crops, however, the severity of the drought on the crop production generally depends on the soil moisture status and nutrients availability (Gandah et al., 2003) as well as crop cultivars (Cooper et al., 1987).

Scientists have been trying to overcome the impacts of environmental stresses including drought by employing different strategies, such as improving stress tolerance in crops through foliar application of plant growth regulators, osmoprotectants (OSP), organic and inorganic nutrients which are efficient, economical and environmentally sound approaches (Ahanger et al., 2017; Yakhin et al., 2017). To counteract drought stress plants synthesize osmolytes and perform osmotic adjustment as a frontier in drought tolerance (Blum, 2016). Hence, the adverse effects of drought stress on the fatty acid composition in oilseed crops are not yet deeply elucidated. Therefore, the current

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40194043

review aims to understand the crop responses to drought stress in terms of important quality traits of oilseed crops, as well as to find the appropriate management approaches to enhance stress tolerance in these crops.

Effects of drought on quality of major oilseed crops

Drought stress leads to reduce the seed quality by producing small and medium-sized seeds; while deteriorating the seed vigour and rate of germination (Alqudah et al., 2010). Fahad et al. (2017) observed that under drought stress, the enzymatic activities of plants are diminished, that eventually lead to decrease the yield and quality of oilseed crops. The reduction in oil content has been observed under water deficient conditions of soybean seed also confirmed by Maclagan (1993). Serraj and Sinclair (2002) revealed that the increasing level of soluble carbohydrate and proline (Pro) content in many drought-induced plant species have been associated with the decrease in the protein content and the increase in proteolytic activation, resulting in an increase in the total soluble nitrogen.

Abiotic stresses such as drought might make changes in oil content and compositions in plants (Bagheri et al., 2012), due to water deficit condition often alters the composition and biosynthesis process of fatty acids leading to the reduction of oil yield and composition (Baldini et al., 2000). While, Amini et al. (2014) found a positive effect of water-deficit stress on seed quality (particularly protein) of oilseed crops. The greater seed quality was associated with the physiological process of the plants under water-deficit conditions. Similarly, Foroud et al. (1993) observed the significant increase of seed protein content in soybean under soil moisture stress. The increased protein and the reduced oil contents in plants were associated with the changes in the embryo, endosperm and testa of the seed (Henry and MacDonald, 1978).

Nevertheless, the underlying mechanisms and the extent of how drought stress affect fatty acids composition in different crop species are under research (Nazari et al., 2017). A details description of the effect of drought (soil moisture stress) on major oilseed crops is described below.

Soybean (Glycine max L.*)*

Soybean is considered as an essential oil seed legume crop in the globe, as it provides good quality oils, vegetable protein (Akande et al., 2009; El-Mohsen et al., 2013) and also extraordinary qualities suitable for humans and animals feeding, such as 40-42% good quality protein and 18-22% oil (Arshad et al., 2006). Drought is considered as the most important limiting factor reducing seed protein content of soybean (EL Sabagh et al., 2015) (*Fig.1*), through decreasing photosynthesis (Smiciklas et al., 1992). Drought also altered the composition of fatty acid in soybean, which affected oil content and oil composition (Bellaloui et al., 2013). The fatty acid composition differed when soybean plant was subjected to drought stress, along with a decrease in palmitic, linoleic and linolenic acids, with an increase of stearic and oleic acids were noticed by Gao et al. (2009). Further, drought affects the fatty acid composition, oil stability and oil processing (Gao et al., 2009). The increase of total oil and oleic acid concentrations and a decrease of linoleic and linolenic acid concentrations may be due to water deficit stress altering the rate of oil and fatty acids accumulation by possibly affecting fatty acid desaturations. The water deficit stress during seed filling stage is critical because of the inverse relationship between oil and

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protein with temperature (Carrera et al., 2009). Drought also alters fatty acid composition (Gao et al., 2009) and affects oil stability (Brumm et al., 1990). The effect of drought on seed composition gives an opportunity to breeder and biotechnology researchers to develop drought-tolerant soybeans with stable desirable fatty acid traits.

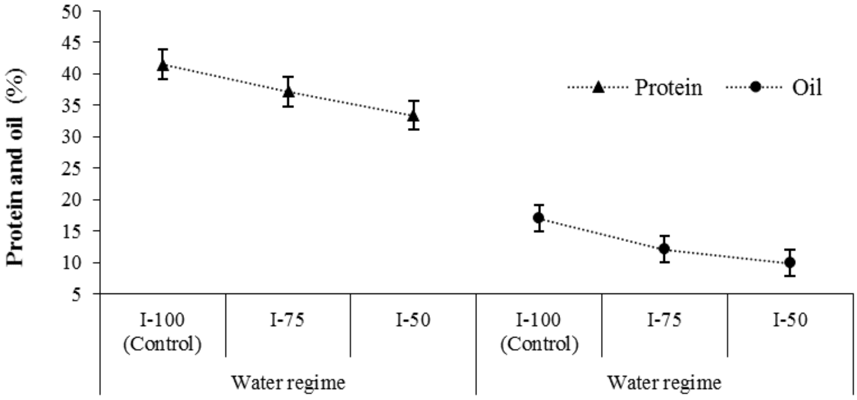

Figure 1. *Effects of proline, glycine betaine and compost application on yield traits and quality in soybean under water stress. Bars indicate standard errors. (Source: EL Sabagh et al., 2015)*

Protein content in seed depends on genetic and environmental factors, mainly drought through the grain filling stage (Tea et al., 2004). Drought stress during pod filling considerably reduced total protein and nitrogen content in seeds (Ghanbari et al., 2013). Drought stress occurs during critical periods of seed formation and seed filling stages reduced the oil content in seed. The reduction in the oil content under drought stress could be due to the oxidation of some of the polyunsaturated fatty acids (Singh and Sinha, 2005). Nonetheless, plants under drought stress increased protein contents by 2-23% as compared to unstressed plants (Rotundo and Westgate, 2010). The variations of the results among the investigations were due to the timing and intensity of the drought stress during the different stages (Carrera et al., 2009).

The decrease in oil content in soybean was also reported by Triboi-Blondel and Renard (1999). Whereas, the lowest oil percentage (20.8%) was obtained under drought stress at the grain filling stage (Smiciklas et al., 1992). Drought stress occurs during critical periods of seed formation and seed filling stages reduced the quantity and quality of seed oil (Maleki et al., 2013). A significant difference among soybean cultivars in terms of oil and protein percentage under drought stress was observed by Mohammadkhani and Heidari (2008). Franklin et al. (2010) also reported that the grain protein remarkably decreased with increasing water stress. The drought had little effect on the fatty acid composition of soybean seed (Dornbos and Mullen, 1992). The drought stress reduced the seed isoflavones in induced soybean plants (Al-Tawaha et al., 2007), while increased alfa-tocopherol concentration in seed (Britz and Kremer, 2002).

Under such situation, oil and protein percentages can be improved by external application of proline (EL Sabagh et al., 2016). Long-term interval of irrigation may build up water shortage in plants which generate organic acid contents in plants body. Boydak (2002) did not find any significant differences between 2-, 6- and 9-day irrigation intervals in the stearic acid content but an increase was observed with 12-day irrigation intervals in soybeans.

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Canola (Brassica campestris* L.*)

The most prominent constituents of cell membrane lipids are fatty acids which play a central role in cell permeability under the harsh and normal environment (Sculer et al., 1991). The composition of fatty acids in canola seeds fully depends on polyamines, by controlling the activity of some enzymes synthesis, which play a vital role in fatty acids metabolism (Talaat and EL-Din, 2005). Ullah et al. (2012) found that the basic level of erucic acid was higher in the canola seed and the erucic acid level was increased under drought stress, while another fatty acid such as oleic and linoleic acids and their composition ratio was not altered by drought stress. EL Sabagh et al. (2015, 2016) found that drought is one of the limiting factors that significantly influenced the quality parameters of canola. The water deficit condition significantly influenced the oil content and oil yield as well as protein yield of canola seed (EL Sabagh et al., 2017) (*Fig. 2*).

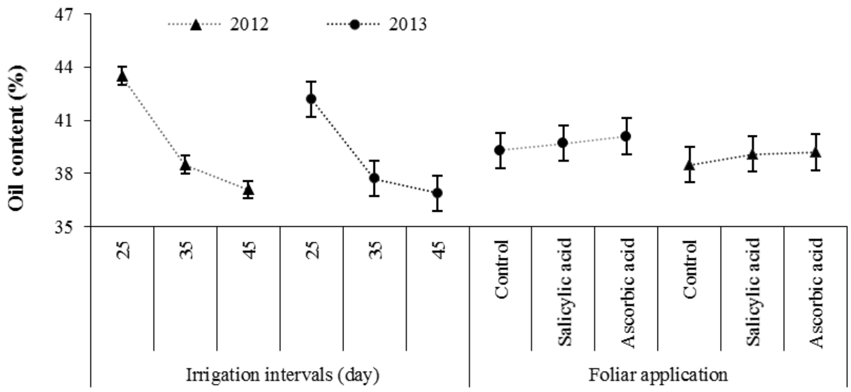

Figure 2. *Effect of irrigations intervals and antioxidants applications on the quality traits of canola during 2012 and 2013 seasons (Source: EL Sabagh et al., 2017)*

They also reported that irrigation at every 25 days intervals produced the highest values for these parameters followed by irrigation at every 35 days intervals. A similar type of reduction in oil content was also reported by Zhang et al. (2014) under-water deficient conditions, indicating that the reduction of oil contents is a common incident under drought stress. Zahedi et al. (2009) reported that drought stress caused a remarkable reduction in oil percentage, due to the reduction of the photosynthesis and the remobilization of assimilation.

Generally, the agronomic operations have an impact on the growth, yield and quality in different crops under different environmental conditions especially drought condition (Barutçular et al., 2016; Abdelaal et al., 2017; EL Sabagh et al., 2019a). Furthermore, optimum irrigation increases seed oil contents, whereas water deficit condition reduces the oil content of canola, due to the oxidation of some polyunsaturated fatty acids as a result of alteration in the physiological and biochemical activities of affected plants (Singh and Sinha, 2005). Similarly, a decrease in seed yield and deteriorating the seed oil quality of canola under drought conditions was also reported by Moghadam et al. (2011) and Rad and Zandi (2012). Reduction in linoleic acid and oleic content of canola under drought stress was also observed by Pritchard (2007).

Environmental stresses such as drought have significant effects on seed oil fatty acid composition of canola (Dawood and Sadak, 2014). Bouchereau et al. (1996) showed that water stress during the flowering stage affects the oil concentration as well as the

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fatty acid composition of canola seeds. Drought stress increased the percentage of palmitic acid and reduced unsaturated fatty acids (Aslam et al., 2009). In addition, severe drought stress reduced the oleic acid content of canola by 3.8% in a Mediterranean-type environment (Qaderi et al., 2006). A reduction of 2.0% in the linoleic content of canola has also been reported in a Mediterranean-type environment under severe soil water stress. This effect on oil production could be due to the oxidation of some fatty acids under drought stress conditions (Singh and Sinha, 2005). Moderate and severe drought stress increased the total saturated fatty acids and decreased the unsaturated fatty acids relative to control plants (Dawood and Sadak, 2014). Tohidi-Moghadam et al. (2011) mentioned that water deficit condition decreased the canola oil and linoleic acid contents, but increased stearic, α-linolenic, and gadoleic acids contents. Ullah et al. (2012) stated that drought stress reduced the oil quality of canola by decreasing oleic acid content and increasing the erucic acid content. However, the maximum erucic acid content was observed in the treatment where 60% irrigation was applied while, the minimum was found at 100% irrigation level (Ali et al., 2014). The accumulation of glucosinolates in rapeseed under drought stress during flowering stage has been reported by Bouchereau et al. (1996). Drought stress augmented the seed glucosinolates and oil erucic acid (C22:1) content of canola also reported by Ullah et al. (2012). The decrease in total phenolic contents of canola seeds under drought stress is also reported by Dawood and Sadak (2014). While, Gecgel et al. (2007) revealed that the effect of oilseeds genotype on fatty acids was greater than that of the environment stresses.

Sunflower (Helianthus annuus **L.***)*

Since sunflower has the capability to survive under stress condition than other oilseed crops to some extent, but it is highly sensitive to drought stress from flowering to grain filling stage due to inadequate availability of soil moisture and high ambient temperature in the arid and semi-arid region (Hussain et al., 2018). They also noticed that low soil moisture (water deficit) in combination with high air temperature from flowering to grain filling stages significantly reduced the seed yield and seed oil quality of sunflower. Oraki and Aghaalikhana (2012) noticed that low precipitation (drought stress) in the arid and semi-arid regions in combination with high ambient temperature significantly reduced the oil quality of sunflower through shortening the germination, anthesis and achene filling period, and also suppressing the stem elongation and leaf area (Soleimanzadeh et al., 2010; Babaeian et al., 2011; Fulda et al., 2011). The environmental factors during the seed-filling period can widely affect the oil percentage and fatty acid composition of oil (Anastasi et al., 2010). Drought leads to an increase in the oleic acid content of high oleic acid of hybrids sunflower, but a decrease in ordinary hybrids (reported by Baldini et al., 2000). The increased of linoleic and palmitic acid and decreased of oleic acid and stearic acid contents in sunflower seeds under water stress were also reported by Petcu et al. (2001). Jasso de Rodriguez et al. (2002) stated that the oil content of sunflower seeds showed a slight decrease when drought was occurred during grain filling to harvest time. They also found that water stress caused a significant reduction of oleic acid (about 8-14%) in a standard hybrid. While Baldini et al. (2002) and Flagella et al. (2000) found that water stress increased the oleic acid content but decreased linoleic acid content in both standard and high oleic content genotypes. The findings of the previous studies related to the adverse effect of drought on the seed and oil quality of sunflower also confirmed by Rondanini et al. (2003) and

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Lacombe et al. (2004), who noticed that under drought stress the composition of saturated fatty acids changed into unsaturated fatty acids. However, the linoleic acid (predominately unsaturated fatty acids) also changed through enzymatic processes governed by genes coding for oleoyl Δ-9 desaturase and oleoyl Δ-12 desaturase. Debaeke et al. (2017) and Hussain et al. (2018) confirmed that nitrogen (N) transport from root to shoot is restricted due to the weak absorbing capacity of the drought-affected crop which ultimately reduced the yield and oil quality of sunflower.

Flax (Linum usitatissimum **L.***)*

Flax is well known as linen flax or linseed around the World. It is grown in many countries for fibers as well as oilseeds. As linseeds' oil has been used as the basic component or additive of various paints and/or polymers industries (Shim et al., 2015). Likewise, seeds of flax contain about 36-40% oleic (omega-6), linoleic and linolenic acids (omega-3) (unsaturated fatty acids), which are very essential poly-unsaturated fatty acids for human beings and also for animals, because these acids cannot synthesize inside the body of both human and animals; thus it should ingest in human and animal diets (El-Beltagi et al., 2007).

However, under water deficit condition, flax plants produce less oil and fibre in comparison to irrigated condition (Bauer et al., 2015). Rashawn et al. (2016) observed a highly significant variation among the three tested flax cultivars of 'Sakha 1', 'Giza 9' and 'Giza 10' regarding protein and oil content under drought stress (*Fig. 3).* The highest oil yield was also reported at recommended (normal) irrigation, followed by limited irrigation at the stem elongation stage of flax also reported by Mirshekari et al. (2012). Considering on fiber-type flax, higher fiber contents along with the higher straw yield resulted in the high yielding fiber-type cultivars whose fiber yielding capacity is 60-70% higher than the seed-type cultivars (Bauer et al., 2015). The fiber quality (fineness and length), fiber percentage, overall yield and oil quality were adversely affected by drought (also reported by Abd El-Fatah, 2007; El-Refaey et al., 2010).

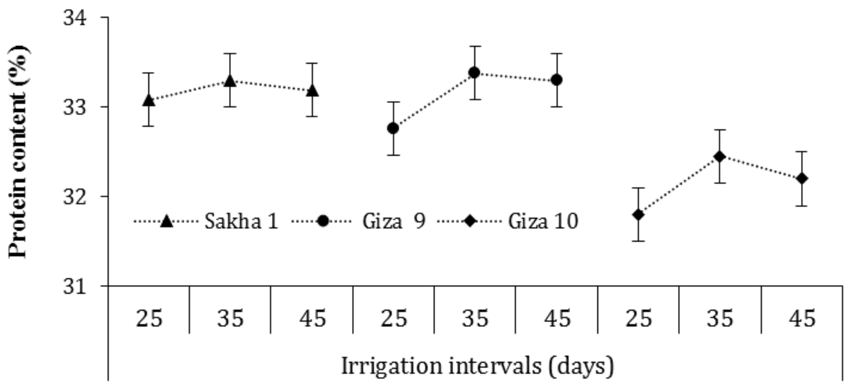

Figure 3. *Effects of irrigation intervals and flux cultivars on oil and protein content (Source: Rashawn et al., 2016)*

Safflower (Carthamus tinctorius **L.***)*

Safflower is an essential oilseed crop that can moderately tolerate against several environmental stresses including drought (Mohammadi et al., 2016). While, Amini et al. (2014) and Mohammadi et al. (2018) observed that the environmental conditions and

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DOI: http://dx.doi.org/10.15666/aeer/1702_40194043

management practices affected the oil content of safflower cultivars. Among the environmental stresses, water deficient condition increase protein and decrease oil contents of safflower (Nabipour et al., 2007; Amini et al., 2014). Drought stress during seed filling has been referred to as the major reason for reduced oil content in safflower (Mohammadi et al., 2018).

Ashrafi and Razmjoo (2010) found that safflower oil content was highly affected by different irrigation levels. They also reported that the oil contents of safflower cultivars were significantly reduced under limited irrigation (drought). Similarly, Bagheri and SamDaliri (2011) found a significant reduction of oil content in safflower seeds' as a result of different irrigation regimes, especially with the lack of water during the seed-filling stage.

Generally, seeds' oil of safflower contains a large amount of saturated (palmitic and stearic) and unsaturated (oleic, linoleic and linolenic) fatty acids and composition, which may be affected by abiotic stresses particularly due to drought stress (Fernández-Cuesta et al., 2014). While, Ensiye and Khorshid (2010) found that drought stress reduced the content of oil and oil composition in the seeds of safflower cultivars, due to a dramatic decrease in saturated fatty acid contents. The oil contents, palmitic, stearic, oleic and linoleic acid contents were reduced under drought during the process of flowering and seed development (also reported by Boydak et al., 2010). Further, drought reduces the palmitic, stearic, oleic and linoleic acid contents (Ashrafi and Razmjoo, 2010). Petcu et al. (2001) also found that the stearic acid content was reduced as drought levels increased. Although the palmitic acid content was decreased, but linoleic acid content was increased up to 14% under drought stress (Petcu et al., 2001). Higher oleic and linoleic acids and lower stearic and palmitic acids under drought stress were reported by Arslan and Kucuk (2005). Under water-stress, the early maturity of plants get a shorter period of grain filling duration, as well as shorter time for conversion of oleic to linoleic acid. This could be the main reason for linoleic acid ratio reduction under drought-stress condition (Nazari et al., 2017).

Peanut (Arachis hypogaea **L.***)*

Major factors influencing oil and other composition profile of peanut includes variety/cultivars/species and environmental conditions such as light, temperature, water stress and atmospheric constituents (Pattee, 2005). Chaiyadee et al. (2013) and Gulluoglu et al. (2016) found that genotypes, seasonal variation, location, air and soil temperature, planting date, soil nutrient, moisture availability, growing conditions and maturity affect the fatty acid content in peanut oil. However, under stress conditions, the changes even in the fatty acid composition and the decreasing order of the main fatty acids was in oleic acid, linoleic acid and palmitic acid, respectively (Amir et al., 2005). Other reports indicate that mid-seasonal drought had no effect, while late season drought significantly reduced the total oil and linoleic acid contents, and increased the stearic and oleic acid contents of peanut seeds (Dwivedi et al., 1993). Under water stress conditions, a large reduction in oil and oleic acid content in peanut was observed by Hashim et al. (1993) and also, reduction in linoleic acid, but an increase in oleic acid (Dwivedi et al., 1996).

A reduction in oil content of soybean and groundnut under drought stress were also reported by Dornbos and Mullen (1992) and Dwivedi et al. (1993); while a significant increase in the stearic acid content of peanut seeds under drought stress has been reported by Dwivedi et al. (1993). A reduction of 4-14% in the oleic content of

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sunflower but an increase in the oleic acid concentration of groundnut under drought stress have also been reported by Petcu et al. (2001). Reddy et al. (2003) observed the application of the different irrigation levels had different responses on the protein content of the seeds of groundnut; while the plants with adequate irrigation water not only gave more kernels but also produced the higher levels of total proteins and oil contents. Dwivedi et al. (1996) pointed out that the total oil and linoleic acid contents of peanuts decreased under water stress during the grain filling stage, but that total protein and stearic acid contents increased under this condition.

Management strategies of drought stress to improve the yield and quality of major oilseed crops

Plants induce stress tolerance mechanism by engineering/altering primary and secondary metabolites. Pant hormones and other compatible compounds such as carbohydrate, amino acid, polyamine, abscisic acid, phenolic compounds, glucosinolate, carotenoid and terpenoid derivatives , play a vital role to maintain the adaptation of plants through changing their membrane stabilization, osmoregulation, free radical scavenging, reduction of leaf area and leaf abscission, stimulation of root growth, electrolytic leakage etc. A details information related to crop-specific management and their survival mechanism is described under the following sub-headings.

Soybean (Glycine max **L.***)*

The most significant responses of plants to survive against drought stress are an accumulation of minerals and enhancement to OSP synthesis which are as a part of the metabolic activity of plants to enhance tolerance against drought stress (Samarah et al., 2004). The accumulation of OSP compounds such as Pro assists the stressed cells of plants through water preservation (Hare et al., 1998) and maintain the structure of cell membrane integrity (Conroy et al., 1988). Similarly, Ashraf and Iram (2005) reported that external application of OSP increased the osmotic adjustment in legumes (including soybean) for increasing the survival capacity against water stress. Metabolic changes in response to drought conditions play a significant role in the osmotic adjustment of metabolism and physiology of the soybean varieties to survive under drought stress (Silvente et al., 2012). One approach to increase oxidative stress tolerance in soybean plants on drought stress is the foliar application of exo-antioxidants through improving the total soluble protein, SOD, POD and H_2O_2 as compared to control (Hasanah et al., 2017). The increased seed oil and protein content were observed due to foliar application of Pro under water deficit conditions (EL Sabagh et al., 2016). Exogenous application of Pro in drought-affected plants ameliorated the adverse effect through maintaining the osmotic adjustment and altering the metabolic activity under water deficit conditions, thereby maintaining high photosynthetic efficiency and finally increased the quality of seeds (Ali et al., 2007). Ali and Ashraf (2011) stated that exo-GB improved the quality of oil by reducing the un-saponifiable matter and improving oil saponification and iodine values, the measure of oil unsaturation. In this concern, EL Sabagh et al. (2016) observed that foliar applied GB improved seed oil and protein content in soybean under water deficit conditions. Compatible solutes like GB or Pro improved the seed quality of soybean as a result of their protective effect on cellular structures during fatty oil biosynthesis and storage, which occurs in liposomes or oleosomes in seeds during the grain-filling stage of the plant (Ali et al., 2013;).

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Similarly, EL Sabagh et al. (2015) also found that foliar application of GB, stimulated the protein content in stress affected plants by improving the osmoprotective impact on the photosynthetic machinery and regulation of ion homeostasis (Hussain et al., 2008). Under drought stress the OSP help to transport essential hormones like cytokinins for biosynthesis and also have a role in the transport of photo-assimilates (Taiz and Zeiger, 2006; EL Sabagh et al., 2015).

However, application of compost is more effective on seed oil content than applying mineral nitrogen. This might be due to the fact that nitrogen composes protoplasmic protein, which required for increasing the growth and then increasing the seed oil content and oil yield. The influence of organic manure by improving the physical structure of the soil and increasing available nitrogen, which reflects a good growth and, consequently, more absorption of nitrogen and more crude protein synthesis (El-Bana, 2000; El-Sadek, 2005). Compost application was more effective mineral nitrogen on seed protein content might be due to the fact that nitrogen composes protoplasmic protein, which required for increasing the growth and then increasing the seed protein content (Connor et al., 2011). Similarly, the relative increase in protein yield by the application of vermicompost as compare to mineral nutrients. Jaleel et al. (2009) indicated that application of vermicompost with inorganic fertilizers improved yield and protein of *Phaseolus vulgaris* (common bean) seeds.

Canola (Brassica campestris L.*)*

Application of antioxidants significantly influenced the protein percentage of canola seed; while non-significant variation was found in terms of the oil content. It was observed that the application of salicylic acid (SA) and ascorbic acid (AsA) in combination with irrigation intervals achieved superior yield of canola, as well as significant increase in protein and oil content of canola (*Fig. 4*).

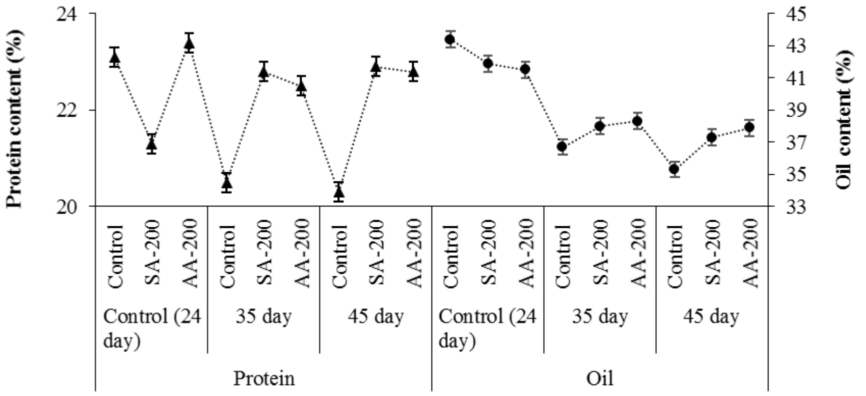

Figure 4. *Oil and protein content of canola as affected by the interaction of irrigation intervals and antioxidants applications during 2012 and 2013 seasons. SA, Salicylic acid; AA, Ascorbic acid (Source: EL Sabagh et al., 2017)*

Ahmadi et al. (2015) reported that spraying of 300 mg l^{-1} ascorbate peroxidase reduced the effect of drought-stressed on rape-seeds. Application of SA in an appreciative rate enhanced the antioxidant ability of cell and generated new protein synthesis in the photosynthetic apparatus (Tirani et al., 2013). According to EL-Tayeb (2005), SA application decreased the stress-induced loss in chlorophyll content and

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photosynthetic rate. AsA can also decrease the harmful result of oxidative stress and enhancing the plant growth under stress environment (Dolatabadian et al., 2009). Application of SA decreased the erucic acid rates in rape oil (Ullah et al., 2012). Application of GB leads to higher carbohydrate contents, oil contents, Pro contents, total soluble sugars and lower concentration of MDA and H_2O_2 under drought stressed in rape plants (Dawood and Sadak, 2014).Similarly, Ali et al. (2013) reported that compatible solutes such as GB or Pro improved the oil quantity and quality, due to their protective effect on cellular structures during fatty oil biosynthesis and storage, which occurs in liposomes or oleosomes in seeds during seed filling stage. Ali (2011) stated that exogenous GB improved the quality of oil by decreasing the un-saponifiable matter and increasing oil saponification and iodine values, the measure of oil unsaturation. Plant growth regulators are important compounds for the rapeseed yield and quality (Huberman et al., 2014). Putrescine, a new plant growth regulator, belonging to polyamines, is reported for its beneficial effects on plant growth under environmental stresses (Perez-Adamor et al., 2002).

Sunflower (Helianthus annuus **L.***)*

Sunflower plays a vital role in providing a ridiculous source of edible oil and protein for human consumption. However, drought stress severely reduced the growth, development, and yield of sunflower. Therefore, under changing climate particularly in drought stress in arid and semi-arid regions, it is important for the development of drought tolerant genotypes and also adaptive management approaches that are suitable for sustainable sunflower production. There are two ways to mitigate heat stress in crops, either by developing and practising improved stress management practices or by developing and using drought-tolerant cultivars (Farooq et al., 2011; Hossain et al., 2013; Hussain et al., 2018). Since, researchers around the globe have been trying to find-out the adaptive technologies which are appropriate to alleviate the adverse effects of drought (Bowsher et al., 2016; Sourour et al., 2017). Nonetheless, development of drought-tolerant cultivars through breeding programs is highly important for improvement of global sunflower production mainly in dry regions where a major portion of sunflower is cultivated under limited water (Rauf and Sadaqat, 2007; Hussain et al., 2018). The first step for improvement of drought resistance of crops is to assess the genetic variation that exists in wild types. Therefore, evaluation of these target resources in combination with genotyping and phenotyping traits under field conditions are directly related to the improvement in drought tolerance of sunflower (Rauf, 2008; Hussain et al., 2018). Considering phenotyping criteria, leaf area index might be preferred as first priority to identify genotypes under drought stress as it is highly correlated with net assimilation rate (Hemmati and Soleymani, 2014).

On the other hand, several stress tolerance indices (STIs), including stress tolerance, mean productivity, geometric mean productivity and stress susceptibility index, and can be used for identification high-yielding varieties with improved tolerance to stress (Ramirez and Kelly, 1998). These are used to determine stress tolerance in terms of minimizing the reduction in production caused by unfavourable versus favourable environments. STIs are measuring stress tolerance in genotypes by comparing GY under stressed environments (Rauf and Sadaqat, 2007; Hussain et al., 2018).

In case of management approaches, exo-application of phytohormones and OSP such as ABA, potassium chloride, SA and $CaCl_2$, sodium nitroprusside (SNP) a nitric oxide (NO) donor, triazole compounds, Pro and GB positively improved oil quality of

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DOI: http://dx.doi.org/10.15666/aeer/1702_40194043

sunflower through improving the physiological and biochemical processes of stress-induced plants (Babaeian et al., 2011). Zaidi et al. (2015) reported that foliar spray of SA significantly improved the Pro, sugars and proteins of sunflower under drought condition, indicating that exogenous SA plays an imperative role to survive plant under drought tolerance. Hussain et al. (2014) found that app of ABA at budding or flowering stages of sunflower helped to ameliorate the adverse effects of drought stress. Moreover, Narusaka et al. (2003) reported that ABA app regulates some stress reactive genes (*HaACCO*$_2$) and proteins in sunflower under drought stress. Similarly, Hussain et al. (2018) reported that exogenous ABA application induces the *HaACCO*$_2$ gene accumulated in leaves of sunflower, which is responsible for drought tolerant in sunflower. While, Rabert et al. (2013) found that triazole compounds like hexaconazole, tebuconazole and propiconazole when applied to sunflower under stress condition partially ameliorated the adverse effects of drought stress through improving the antioxidant enzymes i.e., SOD, APX and CAT activities. Filová (2014) reported that application of 28-homobrassinolide significantly improved the antioxidant enzymes and biochemical traits of sunflower to increase tolerance against drought stress. A sufficient nutrition of plants under stress conditions (including drought) improve tolerance against stress. Ashraf et al. (2015) observed that application of the major nutrients enhances the survival capacity against drought stress by improving protein synthesis, stomatal regulation, homeostasis and osmoregulation through reducing the ROS.

Application of micronutrients enhances sunflower performance against drought stress (Movahhedy-Dehnavy et al., 2009; Zafar et al., 2014) by improving the antioxidant defense, stay green, achenes weight and oil yield. Hussain et al. (2015) and Iqbal et al. (2015) found that exogenous application of $CaCl_2$ has a positive effect on relative leaf water content (RLWC), leaf pigments (such as chlorophyll a, chlorophyll b, carotenoids, anthocyanins, carotenoids), leaf minerals such as N, P, K and Ca, organic osmolytes (Pro and soluble sugars) and phenolic related enzymes such as phenylalanine ammonia lyase (PAL) and POD in sunflower against drought stress.

Similar to other management practices, organic manures have also played a positive role to improve the drought tolerance of sunflower when applied separately or in combination with chemical fertilizers or other OSP (Esmaeilian et al., 2012). Manures are the major source of many essential nutrients as well as enhance the nutrients availability by improving soil physicochemical properties (Hussain et al., 2018). Aowad and Mohamed (2009) and Esmaeilian et al. (2012) reported that when organic manure was applied either alone or in combination with synthetic fertilizers to drought-stressed sunflower, resulting in a significant improvement in drought tolerance.

Flax (Linum usitatissimum **L.***)*

Water deficit stress conditions (drought) is considered as one of the major harsh abiotic stresses that can inhibit the growth, development, yield and quality of flax. Some drought tolerant genotypes can abide stress by increasing the amount of secondary metabolites. It is reported that accumulation of secondary metabolites such as Pro, GB, sugars and some inorganic ions consider as a defense mechanism of plants to adapt the drought by altering their physiological and biochemical activities (El-Tayeb, 2006; Kiani et al., 2007). In addition, drought stress often leads to accumulating the excessive ROS that causes oxidative stress to the photosynthetic apparatus, which ultimately seriously damage the normal function of cells, finally reduced the total assimilation of plants (Niyogi, 1999; Fambrini et al., 1999). Bakry et al. (2012) found that application

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DOI: http://dx.doi.org/10.15666/aeer/1702_40194043

of SA @ 75 mg l^{-1} significantly increased the Pro and free amino acids content of two varieties of flax ('Olin' and 'Amon') under drought condition as compared to the corresponding control condition. Under drought stress, the significant increase in total carbohydrates in shoots of flax concurrently increased the growth rate of the flax that led to increase the photosynthetic efficiency in response to SA treatments and thus led to enhance biosynthesis of carbohydrates which are utilized in growth of flax plants under drought stress (El-Tayeb and Ahmed, 2010). Similarly, Kranner et al. (2002) demonstrated that SA is one of the antioxidant substances that concentrated in the chloroplast of the plant cell and protect the photosynthetic apparatus under drought stress, by reducing the excessively ROS species known as free radicals.

Accumulation Pro and free amino acids in plant tissues could be involved in the osmotic adjustment of plants under drought stress (Delavari et al., 2010) and could also be a protective agent of enzymes and cell membranes (El Tayeb and Ahmed, 2010). Harinasut et al. (2000) reported that when the plant is exposed to drought stress, they are maintaining their water content by the accumulation of compatible OSP, such as Pro, in their cytoplasm. Accumulation of Pro under drought stress produces hydroxyl radical scavenger for increasing stress tolerance in plant species under stress conditions (Hoque et al., 2007). Similarly, Arfan et al. (2007) found that SA is naturally produced in plants in very low amounts that acts as a potential non-enzymatic antioxidant as well as an endogenous plant growth regulator of phenolic nature. Exogenous application of SA helps in the activation of a range of plant defense genes for drought tolerance in plants (Sreenivasulu et al., 2007). Munns and Tester (2008) found that SA has a direct physiological effect through the alteration of antioxidant enzymatic activities by preventing oxidative damage in plants by detoxifying superoxide radicals, produced as a result of stress. The alleviation of oxidative damage and improve the tolerance to environmental stresses, at critical growth stages of the plant, are often correlated with an effectual antioxidative system, such systems may be induced or improved by SA application (He et al., 2005). This significant effect of SA could be attributed to improve in CO_2 assimilation and photosynthetic rate which improved mineral uptake by the plant (Noreen and Ashraf, 2008).

Safflower (Carthamus tinctorius **L.***)*

Plants adopt various strategic tactics to cope with water deficit conditions such as escape, avoidance and tolerance (Rasool et al., 2013). Among the above-mentioned cytosolutes, proline and glycine betaine constitute the most important organic solutes that have a multifunctional role in plants' defense for combating stresses (Hussain et al., 2015). Safflower has proved to be stress tolerant due to having more proline and GB application (Javadipour et al., 2013). Mona et al. (2012) is also in agreement with foliar application of SA during flowering in safflower that increased the production of oleic acid and linoleic acid.

The application of kinetin at 10-5 M as a foliar spray to safflower during flowering (140 days after sowing) was highly effective in increasing oil refractive index (Ullah and Bano, 2011), decreased oil acid value, free fatty acid content (% oleic acid) and specific gravity but increased oil pH. Kinetin was highly effective in increasing oleic acid (C18:1) but decreased the content of linoleic acid (C18:2) (Ullah and Bano, 2011). Foliar application in stem elongation stage obtained 6.02 percent more seed yield but in the flowering stage, fulvic acid spraying was achieved 35.5% more oil percent (Moradi et al., 2017). Boydak et al. (2002) reported that treating safflower plants by GA_3 had

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less oil content than the non-GA_3 treated plants. Spraying of Melagrow four times, at 60 or 40 ppm on safflower remarkably increased the oil yields, and percentages carthamin, carthamidin, oleic acid, and oleic/linoleic ratio (Hamza, 2015). Foliar application of zinc and manganese significantly improved the palmitic and oleic acids but these foliar applications reduced linoleic acid content. The palmitic acid content was increased and oleic acid content was decreased by drought stress (Dehnavi and Sanavy, 2008), while the foliar spray of potassium improved the oleic acid content of oil (Pandy et al., 2014).

Peanut (Arachis hypogaea L.*)*

Foliar application of LAA has a significant role in improving yield components, biochemical constituents and fatty acids composition in seeds of some groundnut cultivars (NC, Giza 6 and Gregory) grown in sandy soil (Mekki and Hussein, 2017). The addition of soaked poultry glaucoma as organic fertilizer gave the highest yield of peanut under drought stress, but the organic soaked pigeons' manure resulted in high percentage of oil (Abd El-Halim et al., 2016). The recommended dose of fertilizers (RDF) of P and K (100%) along with 50% of N basal and 50% of N as top dressing increased the yield attributes, yield and oil contents of groundnut as compared 100% RDF of N, P, and K to groundnut (Priya et al., 2009). Potassium was reported to play the main role on the oil content of groundnut (Charkaravarthy et al., 2002), and they also reported that potassium application during pod development stage increased the oil content in groundnut.

Phosphorus application in the form of single superphosphate has been shown to increase both oil and protein content of groundnut seed (Elsheikh and Mohamedzein, 1998). Rahman (2006) reported that oil and protein content of groundnut increased when calcium level was increased from 0-100 kg ha^{-1}. Recently, Vijayarengan et al. (2009) showed that cobalt at the rate of 50 mg kg^{-1} application in the soil had a beneficial effect on biochemical contents i.e., sugar, protein and amino acids of groundnut seeds compared with control plants. Applying P fertilizer obviously increased fat and protein content, applying N fertilizer mainly enhanced protein content, and applying K fertilizer mainly raised the content of soluble sugar. In addition, the application of NPK fertilizers also increased the contents of lysine and methionine which were inadequate in the protein fractions of peanut kernel, enhanced the contents of oleic acid and linoleic acid, raised the ratio of oleic acid to linoleic acid, improved nutritional quality of peanut, and pronged the shelf life of peanut products (Zhou et al., 2007).

Conclusion

Drought is the foremost abiotic stress that significantly influenced the qualitative and quantitative traits of major oilseed crops particularly in arid and semi-arid regions. Essential dietary components in human consumption, as well as animal feed, were significantly reduced as a consequence of drought stress through altering the physico-biochemical as well as enzymatic activities of crop plants. Hence, the negative effect of drought stress has been varied in almost all crops depending on crop species, genotypes, and their growth stages. The present review summarized that early growth stages followed by anthesis to grain filling stage of oilseeds crops are the most sensitive stages against drought. The review highlighted that there are different ways to mitigate the

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adverse effect of drought *viz.,* development of crops cultivar tolerant against drought and to follow improved management approaches including exogenous application of osmoprotectants, essential macro and micronutrients management and using organic amendments (compost, green manures, cow dung and incorporation of crop residues) in soils which lead to increase the yield and grain quality of oilseed crop. The findings of the current review will be helpful to plant researchers to make research programs for resolving the consequences of drought stress for sustainable quality food production in the era of climate change.

Disclaimer. We hereby declare that this review contains no material which has been accepted for the award of any degree or diploma in any university and that, to the best of our knowledge and belief, the review contains no copy of any material previously published or written by another person except where due reference is made in the text.

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A SYNOPSIS OF FIELD AND REMOTE SENSING BASED METHODS FOR STUDYING AFRICAN ELEPHANT (*LOXODONTA AFRICANA*) IMPACT ON WOODY VEGETATION IN AFRICA

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(Received 21st Nov 2018; accepted 28th Jan 2019)

Abstract. African elephants (*Loxodonta africana*) negatively influence woody vegetation, causing structural changes to ecosystems. Field-based survey methods used to monitor elephant impact, while valuable, are costly and time-consuming to execute. By applying distance-sampling techniques such as remote sensing technology, inaccessible areas can be surveyed. This overview provides insight into methods used by scientists to determine the impact of elephants on woody vegetation in sub-Saharan Africa. Data were sourced from a variety of research databases. Findings indicate that 87% (n = 92) of the reviewed studies used field-based methods and 13% (n = 14) used remote sensing-based methods. We explore the national affiliations of the lead and the last authors of the reviewed studies and the scientific journals that published them. Field-based is the dominant method used in the majority of published studies on elephant impact. The majority of these studies were published in European and American journals, instead of African journals, which are less represented. However, the majority of the lead and last authors' affiliations for both field-based and remote sensing based methods are affiliated with African institutions. We conclude that there is a need to improve the integration of remote sensing techniques into conservation and other ecological fields.
Keywords: *savanna, satellite imagery, national affiliation, accessibility, reviewed studies*

Introduction

African elephants (*Loxodonta africana*) are found in sub-Saharan Africa (Blanc et al., 2007), where they occupy savanna ecosystems, dense forests, Sahelian scrub and deserts (Nowak, 1999). Based on genetic information, taxonomists suggest that there are two sub-species of the African elephant namely, the forest elephant and the savanna elephant. Current literature indicates that there is no detailed knowledge available on the distribution of these sub-species or the presence of potential hybrid populations (Mondol et al., 2015). Savanna elephants are predominantly found in eastern and southern Africa, while the forest elephants are found mainly in the Congo Basin of Central Africa (Thouless et al., 2016). In this study, we considered African elephant as a single species found in sub-Saharan Africa (East, Central, West and Southern Africa).

Elephants, in general, utilize a wide range of landscape types when foraging. The distribution of resources across landscapes influences elephant home range use and size. Individual home ranges may include a variety of landscapes and ecosystems (Grainger et al., 2005). A home range is defined as an area traversed by an individual in its normal activities of food gathering, mating and caring for young (Burt, 1943). Elephant feeding

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40454066

behavior, densities, interactions with other herbivores, associated ecological and environmental factors such as fire damage, rainfall patterns, and plant growth rates, all influence elephant impact on vegetation (Dublin et al., 1990; Ben-Shahar, 1996b; Ferguson, 2014).

Adverse impacts associated with African elephants on ecosystems include reducing tree heights and densities, which leads to structural changes in the woody layer (Ben-Shahar, 1998; de Boer and Kohi, 2008). Changes in overall vegetation composition lead to a decline in biodiversity (Kelly, 2000). Guldemond (2006) found that elephant impacts in closed woodlands create canopy gaps, leading to reduced recruitment of young trees. Elephants require a daily average fresh food intake of approximately 173 kg across both the wet and dry seasons (Ruggiero, 1992). Since they feed mainly on woody plants, tree mortalities are common in areas they frequent. Woody plant species composition, tree species distribution, tree size, tree age, feeding preferences and habitat availability influences the consumption of woody vegetation by elephants (Gadd, 2002; Van Staden et al., 2016).

Protected areas are representative of natural vegetation that previously occurred in the broader vicinity around the protected area. This includes woodland spatial distribution, composition, and structure without anthropogenic influences (Guy, 1976; Gandiwa et al., 2011). The vegetation type, rainfall (Gandiwa and Kativu, 2009), management and conservation strategies and interventions (Hamandawana, 2012), differ for each protected area. Smit and Ferreira (2010) state that the availability and distribution of water sources remain different from one area to another, influencing elephant movement patterns. Variability between areas and their sizes, coupled with different elephant densities leads to fluctuating distribution patterns and intensities of elephant impact. The numbers of elephants have been steadily increasing in many protected areas in the sub-Saharan region, raising concerns of their impacts to biodiversity (Van Aarde et al., 2006; Van Aarde and Jackson, 2007). The focus on elephant management in many protected areas has been on their numbers in relation to the size of the area they occupy. According to Van Aarde et al. (2006), this approach gave rise to the establishment of many artificial water points, which modify the movements and home ranges of elephants.

Available methods for managing elephant populations include culling, relocation, and the use of contraceptives (Pimm and Van Aarde, 2001; Rubio-Martínez et al., 2014). Elephant culling remains a highly debated and sensitive subject in the scientific community (Mackey et al., 2006; Balfour et al., 2008; Lotter et al., 2008). Relocation of elephants is not a permanent option, but a temporary solution that can present adverse effects on the breeding behavior of female elephants (Raath, 1999). Relocated animals that breed can often not be accommodated by the parks or reserves they are moved to (Rubio-Martínez et al., 2014). Laparoscopic vasectomy is a costly contraceptive technique applicable to free-ranging male elephants. The long-term effects of this type of contraception are yet to be studied to determine how the elephant population demographics and dynamics are influenced (Rubio-Martínez et al., 2014). Additional contraceptive techniques available to wildlife managers for free-ranging elephant cows include pregnancy termination, the use of estrogen implants and immunocontraception (Poole, 1993; Whyte and Grobler, 1998). While these contraceptive techniques reduce population growth rates, they are often impractical and expensive to implement (Whyte et al., 1998).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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An increase in the number of studies that investigates the impact of elephants on vegetation structure and dynamics has been noticed over the three past decades (Ben-Shahar, 1996a, 1998; Shannon et al., 2011; Van Staden et al., 2016). Moreover, the impacts of elephants on vegetation has increasingly become one of the main concerns for environmental and protected area managers (Baxter and Getz, 2008; Wiseman, 2001). We identified two review papers on the impacts of elephants on vegetation in Africa (Guldemond and Van Aarde, 2008; Guldemond et al., 2017). Guldemond and Van Aarde (2008) reviewed 21 studies between 1961 and 2005 from 14 study sites in Africa. Although no opinion or broad generalizations were made on elephant management from the reviewed studies, there was no doubt that elephants have an effect on woody vegetation and that high elephant densities could result in adverse impacts. Guldemond and Van Aarde (2008) also highlighted the lack of published information on the effect of elephants on vegetation.

A current review paper by Guldemond et al. (2017) that looked at 51 peer-reviewed articles concluded that elephants have a significant influence on vegetation by changing tree structure and abundance. No overall adverse cascading effects for species that share space with elephants were reported. Both these reviews focus on the impact of elephants and not the methods applied. This makes it necessary to provide an overview of the studies and techniques that have been used to quantify the impacts of elephants on the environment. This would provide useful information for conservation managers, and would also identify opportunities for further research into practical and time-efficient methods for studying the impact of elephants on vegetation.

This paper aims to provide an insightful review of the types of methods used by different peer-reviewed studies to quantify and detect the impact of elephants on woody vegetation in sub-Saharan Africa from 1970 to 2017. For this paper, we grouped the studies into two different categories, a field-based (FB) approach and those that used a remote sensing-based (RS) approach. Field-based approaches involve the physical collection of detailed data using certain tools and techniques in the veld (Zimmerman, 2014). Remote sensing approaches provide a variety of imagery known for their different spectral, spatial, radioactive and temporal characteristics that are applicable for use in broad vegetation studies (Xie et al., 2008).

Our review provides information on the geographic location of each study (location of study area and spatial scale of research), the national affiliation of the lead and the last authors and whether the publication is local (within Africa) or international (outside the African continent). We further explore studies that use remotely sensed data in more detail and highlight the challenges and limitations of field-based and remote sensing-based methods.

Materials and methods

Database and literature search

For this review paper, we only included studies that related to the African elephant and made no distinction between forest and savanna elephants. We were interested in studies that explored the damage or impact of elephants on woody vegetation in conservation and protected areas. We have, therefore, excluded studies on immune-contraception, distribution, movement, population and densities of elephants. However, we sourced information on the densities of elephants in the sub-Saharan region from Thouless et al. (2016). Our review includes English; peer-reviewed articles containing

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original research from 1970 to 2017. This review excludes thesis documents and dissertations. We considered all studies from the four sub-Saharan regions (west, east, south and central Africa).

Relevant articles were identified using different keywords in different databases. Data were sourced from the ISI Web of Science during May 2018. “African elephant” was the first keyword used to retrieve studies published on elephants from the database. Two thousand two hundred and fifty-three (n = 2 253) studies dating back to 1972 were retrieved. We refined our search using keywords such as “elephant damage”, “elephant impact” and “woody vegetation” which resulted in 26 studies that matched these criteria. Since the Web of science database only lists articles between 1972 and 2018, we decided to search other scientific databases.

An additional list of 507 studies was sourced from Nexus, SA e-Publications, EBSCOHost, ScienceDirect, Taylor & Francis, ProQuest, BioOne, and SpringerLink using the keywords “elephant damage, “elephant impact,” “woody vegetation” and “Africa.” Studies from these electronic databases, repositories and search engines dated back to 1970, which is two years earlier than the data retrieved from the Web of Science website. Eighty-one (n = 81) studies from this search were relevant and were included in this review. Based on all the retrieved studies (n = 2 253 from ISI Web of science + 507 from above listed additional databases = 2 760), a sample of 106 (n = 26 + 80 = 106) studies were used for this review paper. These studies matched our search criteria and were grouped according to which part of the sub-Saharan region they belong to (eastern, central, western or southern Africa).

From the 106 studies, we extracted the year of publication, authors, methods used, country and location of the study area, and publishing journal and its location. We broadly recognized two types of methods from these studies, field-based (FB) and remote sensing-based (RS). Field-based methods have been extensively used due to their robustness in providing a widely reliable dataset (Buchanan et al., 2013). According to Liverman et al. (1998), field-based studies are generally not sufficient to quantify and analyze patterns of spatiotemporal change at an aggregated level. On the other hand, remote sensing techniques provide useful data sources for quantitatively measuring the dynamics of change processes at the landscape scale by analyzing change trajectories (Mertens and Lambin, 2000). Since this paper intends to provide insight into the types of methods used by scientists to study the impact of elephants on woody vegetation in Africa, we further discuss the limitations of each of the two approaches (field-based and remote sensing-based).

Content analysis

We determined the number of studies using either field-based or remote sensing-based methods per African region. Based on Thouless et al. (2016) report that estimated densities of African elephants in sub-Saharan Africa, we compared the relationship between the number of studies and the estimated number of elephants per region. Information on the national affiliation of the lead and the last author (Bhattacharya, 2010) was extracted for each of the studies. We included the lead author on the basis that the first author is generally the person who did most of the work, while the last author was included on the basis that he or she may have contributed to the work as a supervisor affiliated to an academic institution (Bhattacharya, 2010). We further identified the scientific journals that published all the studies included in this review and whether these were local (within African) or international (outside Africa).

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Results

For this review, we identified peer-reviewed studies on the impact of African elephants on vegetation in the sub-Sahara region, which is the natural distribution range for African elephants (Thouless et al., 2016) (*Fig. 1*). A total of (n = 106) papers was retrieved from different scientific databases (*Table 1*). Eighty-seven percent (87%) of the studies used field-based methods (n = 92) while 13% (n = 14) applied remote sensing-based methods for data collection. A list of all peer-reviewed studies, both field-based and remote sensing-based published between 1970 and 2017 is included as *Appendix A*. Field-based studies date back to 1970 and the oldest study that used remote sensing data to study elephant impact on vegetation was done in 1997, which is two decades after the field-based studies. A list of the estimated number of elephants (Thouless et al., 2016) for countries in Sub-Saharan Africa is included as *Appendix B*.

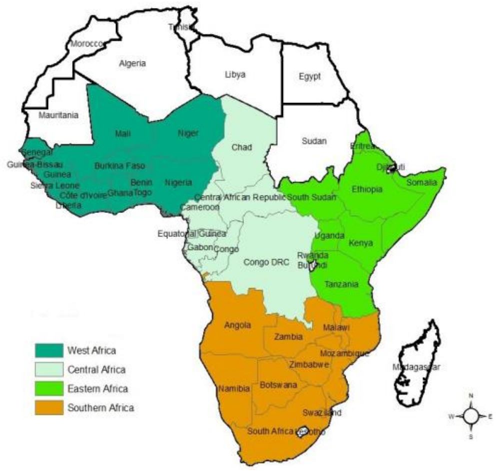

***Figure 1.** Distribution map of African elephants in the west, east, south and central countries of the sub-Saharan region in Africa (ESRI, 2014)*

***Table 1.** A number of peer-reviewed papers retrieved from different databases*

Database	Retrieved articles	Relevant articles in the sample
BioOne	14	7
EBSCOHost	9	5
ISI Web of Science	2253	26
Nexus (NRF Research)	12	12
ProQuest	49	13
SA e-Publications	55	6
ScienceDirect	203	16
SpringerLink	65	10
Taylor & Francis	100	11
TOTAL	**2760**	**106**

Field-based and remote sensing based methods

We determined countries with records of field-based and remote sensing-based studies and the number of elephants in each country (*Table 2*). Ninety-two (n = 92) of the field based studies are located in Africa, and one study was done in Gal Oya National Park, Sri Lanka (Ishwaran, 1983). This study is excluded in *Table 2* since it was not done in an African park or reserve. Another study area located in East Africa

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was not specific to a particular reserve or national park (Laws, 1970). Since we could not individually assign it to one specific study location, we have also excluded it from *Table 2*. Fourteen (n = 14) remote sensing-based studies were identified, and only one was located in Africa and not specific to a particular reserve or national park (Duffy and Pettorelli, 2012), and is therefore not included in the data represented in *Table 2*. There is a study that was done in Borgu game reserve, Guinea (Afolayan, 1976) on the impact of elephant activities on woody vegetation, and according to Thouless et al. (2016), there is no record of estimated elephant numbers for the country.

Table 2. *List of countries with a record of field-based and remote sensing-based studies and the number of elephants*

Region	Country	No. of FB*	% of FB*	No. of RS**	% of RS**	No. of elephants
West Africa	Burkina Faso	3	3	0	0	6 850
West Africa	Benin	1	1	0	0	2 984
West Africa	Ghana	1	1	0	0	994
West Africa	Guinea	1	1	0	0	0
Southern Africa	Botswana	10	12	5	38	131 626
Southern Africa	Zimbabwe	20	23	2	15	82 630
Southern Africa	Zambia	1	1	1	8	21 967
Southern Africa	South Africa	30	35	3	23	18 841
Southern Africa	Mozambique	1	1	0	0	10 884
Southern Africa	Malawi	1	1	0	0	1 307
Central Africa	Cameroon	1	1	0	0	6 830
Central Africa	Chad	1	1	0	0	794
Eastern Africa	Tanzania	10	12	0	0	50 433
Eastern Africa	Kenya	5	6	1	8	22 809
Eastern Africa	Uganda	3	3	1	8	4 923
Eastern Africa	Rwanda	1	1	0	0	88
		86	**100**	**13**	**100**	**354 126**

*Field-based studies, **Remote sensing-based studies

We compared the relationship between the top ten African countries with the highest number of elephants to the number of studies from each one of them (*Fig. 2*). We also summarised the type of data used in the remote sensing-based studies (*Table 3*).

Affiliation of the lead and the last author for FB and RS based methods

The majority of the articles' lead and last authors for both field-based and remote sensing-based methods are affiliated with institutions located in South Africa (26%). The United States of America follows South Africa with 17%, United Kingdom and Zimbabwe with 10%, and the rest of the other countries below 3% (*Fig. 3*). There is a relatively equal distribution of lead: last authors ratio affiliations in all the countries, except for Botswana, Netherlands and Uganda with higher numbers of last author affiliations: 3%: 2% for Botswana, 1%: 0% for the Netherlands and 3%: 1% for Uganda.

The highest percentage (6%) of lead and last authors affiliated with institutions in the United States of America were for studies that used remote sensing-based methods. This is followed by Botswana, South Africa and the United Kingdom with 2% each and the rest of the other countries at below one percent. Field-based methods were applied to 73% of the studies compared to 27% for the remote sensing-based methods (*Fig. 4*).

All studies, both field-based and remote sensing-based were published in 43 different journals. Eighty-four percent (n = 36) were in international journals, and 16% (n = 7) were published in local journals (*Table 4*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_40454066

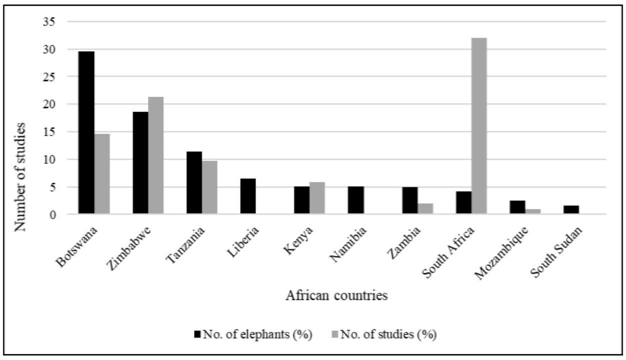

Figure 2. *The percentages of field-based and remote sensing-based studies in relation to the estimated percentage of elephants for the top ten African countries*

Table 3. *Summary of studies that applied the remote sensing-based method and the type of data used*

Satellite data	Number of studies	Percentage	Study Objective
Landsat TM	2	14	Impact of management strategies
Landsat MSS	1	7	Vegetation change over time
NDVI	2	14	Greeness versus elephant densities
MODIS	1	7	Piosphere effects
3-D	2	14	Vegetation structure
SPOT+NOAA+AVHRR	1	7	Ecological feature recognition
Aerial	2	14	Vegetation change over time
Aerial+SPOT	1	7	Vegetation change over time
Landsat+CORONA	1	7	Grazing pressures
Landsat+ETM+OLI	1	7	Impact of climate, fire and elephants
	14	**100**	

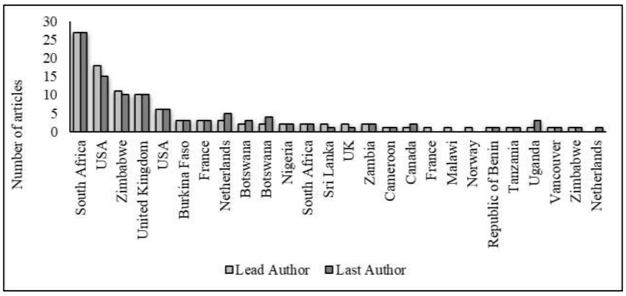

Figure 3. *National affiliation of lead and last authors from 106 reviewed papers*

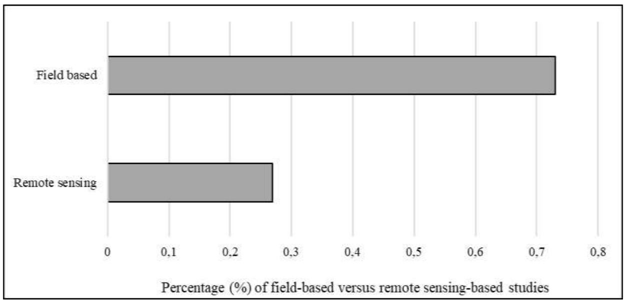

Figure 4. *The difference between studies that used field-based and remote sensing-based methods*

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Table 4. *Names of scientific journals where each of the reviewed studies were published*

	Name of Jnl.	Country	FBO**	RS***	All studies	Local	Intern.*
1	Jnl. of Tropical Ecology	United States	13	0	13	0	1
2	African Jnl. of Ecology	England	11	1	12	0	1
3	South African Jnl. of Wildlife Research	South Africa	7	0	7	1	0
4	Biological Conservation	England	5	1	6	0	1
5	Koedoe	South Africa	6	0	6	1	0
6	Ecological Applications	United States	4	0	4	0	1
7	Oikos	Denmark	4	0	4	0	1
8	Biotropica	United States	3	0	3	0	1
9	Ecography	Denmark	2	1	3	0	1
10	Ecology	United States	2	1	3	0	1
11	Int. Jnl. of Remote Sensing	England	0	3	3	0	1
12	South Africa Jnl. of Botany	South Africa	3	0	3	1	0
13	Int. Jnl. of Biodiversity and Conservation	?	2	0	2	0	1
14	Jnl. of Applied Ecology	England	1	1	2	0	1
15	Jnl. of Arid Environments	United States	2	0	2	0	1
16	Jnl. of Ecology	England	2	0	2	0	1
17	Jnl. of Vegetation Science	England	2	0	2	0	1
18	Pachyderm	Kenya	2	0	2	1	0
19	South African Jnl. of Science	South Africa	2	0	2	1	0
20	Tropical Ecology	India	2	0	2	0	1
21	Ambio	Sweden	1	0	1	0	1
22	Austral Ecology	Australia	1	0	1	0	1
23	Biodiversity and Conservation	Netherlands	1	0	1	0	1
24	Conservation Biology	United States	0	1	1	0	1
25	Ecological Research	Japan	1	0	1	0	1
26	Ecosystems	United States	1	0	1	0	1
27	Environmental Modeling and Assessment Jnl.	Australia	1	0	1	0	1
28	Forest Ecology and Management	Netherlands	1	0	1	0	1
29	International Forestry Review	England	1	0	1	0	1
30	Int. Jnl. of Applied Earth Observ. & Geoinfo	Netherlands	0	1	1	0	1
31	Int. Jnl. of Environmental Sciences	England	1	0	1	0	1
32	Jnl. of Animal Ecology	England	1	0	1	0	1
33	Jnl. of Ecology and the Natural Environment	?	1	0	1	0	1
34	Kirkia	Zimbabwe	1	0	1	1	0
35	Land	?	0	1	1	0	1
36	Plant Ecology	Netherlands	1	0	1	0	1
37	Proceedings of the National Academy of Sciences	USA	0	1	1	0	1
38	Regional Environmental Change	?	0	1	1	0	1
39	SpringerPlus	Germany	0	1	1	0	1
40	Systematics and Geography of Plants	Belgium	1	0	1	0	1
41	Transactions of the Royal Society of RSA	South Africa	1	0	1	1	0
42	Tropical Pest Management	England	1	0	1	0	1
43	Vegetatio / Plant Ecology	United States	1	0	1	0	1
			92	**14**	**106**	**7**	**36**

*International, **Field-based, ***Remote sensing, ?Unknown, Geoinfo-Geoinformation, Int.-International, Jnl.-Journal, RSA-Republic of South Africa, Observ.-Observation

Discussion

Southern Africa has the highest number of elephants on the African continent, with an estimated 71% (n = 293 447) of all the elephants on the continent. East Africa has 21%, Central Africa 6% and West Africa 3% (Thouless et al., 2016). The highest estimated elephant distribution range of 1 325 998 km² is in southern Africa, followed by 880 648 km² for East Africa, 783 085 km² for Central Africa and 142 500 km² for West Africa.

The top ten countries with the highest number of elephants include Botswana (131 626), Zimbabwe (82 630), Tanzania (50 433), Kenya (22 809), Namibia (22 754),

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Zambia (21 967), South Africa (18 841), Mozambique (10 884), South Sudan (7 103) and Gabon (7 058) (*Table 2* and *Appendix B*).

Our results indicate that South Africa produced the highest number of studies on the impact of elephants on woody vegetation and yet has a lower number of elephants compared to Botswana, Zimbabwe, and Tanzania. Botswana has the highest number of elephants but not as many research studies (15 studies) compared to South Africa (33) and Zimbabwe (22). According to Blanc et al. (2007), elephant numbers are increasing in Southern Africa, which may result in an irreversible impact on vegetation, especially in enclosed protected areas with high elephant densities.

South Africa's elephants are kept in fenced-off protected areas, which necessitates intensive management strategies. There are high levels of concern about how elephants influence vegetation and habitats. This explains the demand for increased research and resulting publications in South Africa. Another factor that could explain why South Africa has the highest number of studies could be that it is the most developed third world country on the continent, compared to for example Botswana. The presence and number of academic institutions, collaborative opportunities, and access to research funding, developed and maintained infrastructure, and a large number of protected areas and national parks all contribute towards the number of publications from the country.

Botswana has an estimated 131 626 number of elephants in an area covering approximately 228 073 km^2. South Africa is home to approximately 18 841 elephants in a 30 651 km^2 area (Thouless et al., 2016). The estimated density of elephants equates to 0.6 individual animals per square kilometer (0.6 /km^2) for both countries.

Field-based methods

Field-based methods have been used for many decades in ecological studies. Using field-based methods, information about the extent, spatial variation and resources species preferences by elephants can be studied in detail. Such information is essential for the development of effective management plans for conservation and protected areas. Although FB studies can be impractical and costly when applied in large areas and when large-scale datasets are required on a regular basis, they provide detailed ecological data at smaller scales and need to be simple enough to be widely applied in protected areas with limited capacity (Simms, 2009; Buchanan et al., 2013).

The highest number of studies on African elephants and their influence on vegetation using field-based methods emanated from southern Africa (*Table 2*). Almost half (48%) of the field-based studies are from South Africa, 32% from Zimbabwe and 16% from Botswana. Although Botswana and adjacent areas have the largest population of elephants in the world (Skarpe et al., 2004), only ten field-based studies on the impact of elephants on vegetation are from Botswana. South Africa and Zimbabwe are the most active countries regarding research on the interaction between elephants and plants, with 30 and 20 field-based studies, respectively (*Table 2*). The majority of reviewed studies located in South Africa were done in the Kruger National Park (43%, n = 13) and ten percent each (n = 3) were done for both Venetia-Limpopo Nature Reserve and Addo-Elephant National Park. All field-based studies in Botswana were conducted in the Northern-Botswana area along the Chobe River and Okavango Delta.

The lead and last authors for the field-based methods have affiliations with institutions in South Africa (26%), the US (17%) and the UK (10%). This shows that a significant proportion of field-based research on the impact of elephants is affiliated to Africa, while the US and UK researchers show active involvement.

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Remote sensing-based methods

Remote sensing techniques are cost-effective for application in large areas for collecting large-scale datasets (Duro et al., 2007). Limitations relate to limited funding to acquire satellite images, facilities, accessibility to data and the skills required to manage such datasets.

Botswana applies remotely sensed data more actively than all other countries in the sub-Saharan region (*Table 2*). In southern Africa, five out of 13 studies (38%) who used remotely sensed data were from Botswana, three (23%) were from South Africa, two (15%) from Zimbabwe and one (8%) from Zambia. Only one study per country in East Africa (Kenya and Uganda) used remotely sensed data, while there were no such studies from central and West African countries. Thouless et al. (2016) highlight factors that contribute to the accuracy of elephant densities in different countries, which may also influence the probabilities of executing remote-sensing research studies. Some countries may not have the financial means and expertise to conduct systematic elephant censuses on a regular basis, while countries that have political conflicts may not have the time or finances to do these surveys, let alone research.

Multispectral images such as Landsat and SPOT images, Aerial photographs, MODIS and Normalised Difference Vegetation Index (NDVI) were used in 13 remote sensing-based studies. Three of these studies were done in South Africa and were based in the Kruger National Park. One of them used panchromatic aerial photographs and digital SPOT imagery (Munyati and Sinthumule, 2016), while the other two used state of the art hyperspectral satellite imagery to capture the three-dimensional (3D) structure of vegetation (Asner et al., 2009; Levick et al., 2009). Both of the studies used LiDAR imagery to study the large-scale impact of herbivory on the structural diversity of vegetation. Levick et al. (2009) also highlight the value of 3D remote sensing in the assessment of conservation management outcomes.

The lead and last authors for remote sensing-based methods are mostly affiliated with institutions in the USA (6%), followed by Botswana, South Africa and the UK with 2% each. The low number of lead and last authors with affiliations in Africa may suggest that foreign institutions have financed the research conducted in Africa. Alternatively, this could relate to a slow transfer pace of technology and skills by developed countries to developing countries.

Publishing scientific journals

From the 43 scientific journals (*Table 4*) that published the reviewed studies in this paper, 84% (*n* = 36) were from outside the African continent, and only 15% (*n* = 7) were in journals from within Africa. The 14 studies that used remote-sensing data were published in journals outside the African continent, while 22 of the field-based studies were in African journals and 70 in journals outside Africa. The Journal of Tropical Ecology (United States of America – USA) published the highest number of studies (*n* = 13) followed by the African Journal of Ecology (England) publishing eleven. Within Africa, 19 of the field-based studies were published in five South African journals (Koedoe, SA Journal of Botany, SA Journal of Science, SA Journal of Wildlife Research and the Transactions of the Royal Society of South Africa), two studies in a Kenyan journal, Pachyderm and one in a Zimbabwean journal, Kirkia. No remote sensing studies were published in any of the African journals. The South African

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Journal of Wildlife Research published the majority of the South African studies (n = 7) followed by Koedoe (n = 6).

Elephants are major agents of change to woody vegetation as observed in some studies (Anderson and Walker, 1974; Caughley, 1976; Guy, 1976). Despite elephants modifying woody vegetation, Thomson (1992) alluded that no protected area in Africa is big enough to maintain elephants in a healthy state indefinitely without population management manipulation. Culling elephants, as a means of preventing woody vegetation loss is unlikely to produce the desired effects (Ben-Shahar, 1996a) since there is adequate production of browse available for elephants in drought years. The use of contraceptives is impractical for elephant cows due to the requirement for frequent follow-up treatments. Contraceptives are expensive to administer for both elephant cows and bulls. As our protected areas become overpopulated with increasing numbers of elephants, three things are bound to happen: (i) destruction of woodlands that will be replaced by mixed scrubland/grassland or degraded savanna; (ii) a catastrophic crash of elephant populations; and (iii) loss of plants and animals (Thomson, 1992).

Collecting ecological data on a regular basis from large, inaccessible and often dangerous areas (due to the presence of lion, leopard, rhinoceros, buffalo and elephant) using field-based methods can be challenging. Limitations relate to accessing rugged terrain, large areas to traverse, time constraints and financial implications. Using remote sensing techniques integrated with traditional field-based approaches, we can identify the biophysical characteristics of landscapes, predict species distributions, determine spatial variability in species richness, and monitor the impact of species on their environment (Kerr and Ostrovsky, 2003; Kohi et al., 2010) with less time spent in the field. Limitations of remote sensing techniques include the need to be integrated with field-based observations to validate results (Reinke et al., 2006). Collecting remotely sensed data could be very costly, with limited access in some instances. It is crucial to identify appropriate imaging characteristics (such as spatial and spectral resolution) suitable for processing and extracting relevant and vital information (Adam et al., 2010). Garrity et al. (2013) identified a shortcoming in detecting trees that are in various stages of morbidity due to the limited availability of archived satellite imagery. Regardless of the recognized limitations to using remotely sensed data, it is still a valuable tool that continues to prove its potential for use in a wide range of vegetation studies providing timely, up-to-date and accurate information for sustainable and effective management of vegetation (Adam et al., 2010).

We have noted the lack of available published studies on the use of remotely sensed data to map the small-scale impact of African elephant on woody vegetation. Only two reviewed studies used hyperspectral images to determine the large-scale responses of vegetation and ecosystem structure to the presence/absence of herbivory (Asner et al., 2009) and to gain insight into the influence of fire and herbivory to the structure and heterogeneity of savanna vegetation (Levick et al., 2009). Northern Botswana is known to have the highest density of elephants in the world (Skarpe et al., 2004) and the majority of studies from Botswana reported that elephant damage to vegetation is not significant. As noted by Ben-Shahar (1996b), the impact of elephants is generally distributed randomly. Some authors used field-based methods to study the effects of elephants on woodland modification and the implications this has on the diversity of bird species (Herremans, 1995) and the nesting sites of Southern Ground Hornbill (Henley and Henley, 2005). The placement of artificial water points influences tree biomass (Ben-Shahar, 1996a) and structure (Kalwij et al., 2010). Tall tree densities are

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affected by elephants in mopane woodlands (Ben-Shahar, 1998). From these studies, the impact of elephants was not significant, and in other instances, woody biomass and densities remained unchanged, except for new damage to vegetation during the dry season, which was recorded by Ben Shahar (1996b).

Conclusion

Field-based methods have been extensively used in ecology since the 1960s and are still effectively used. From reviewed studies on the impact of elephants on woody vegetation that applied field-based methods, we have noticed a steady increase from an average of two studies per year before the 20th century, to an average of four studies after the 20th century. These methods provide detailed and reliable datasets. This is an indication that field-based methods are still relevant today, even with advancements in technology in the form of remote sensing techniques.

Remote sensing and GPS technology have also been utilized to track elephant movements (Kahumbu, 2002; Bohrer et al., 2014; Xu et al., 2017). Field-based methods are utilized in combination with remote sensing techniques for field observations. Although remote sensing techniques offer cost-effective and repeatable ways for regular data collection and monitoring for trends, there is also a fieldwork element associated with it. However, using remote sensing techniques, time and labour required for field surveys, especially on an annual basis, are drastically reduced (Jensen, 2005). The results of our reviews indicate that there is a limited amount of studies that used remote sensing-based methods for studying the impact of elephants on woody vegetation. Even the available studies are sparsely distributed between 1997 and 2017, with three studies published in 2009, two of which were done in the Kruger National Park, South Africa and one in Hwange National Park, Zimbabwe.

Guldemond et al. (2017) call for the management of habitats used by elephants to maintain the heterogeneity of Savanna, with particular attention to important tree species of conservation value. These can only be achieved with improved and efficient methods of data collection. Remote sensing techniques can be useful and practical and should be used in conjunction with field-based methods for improved data collection, analyses and interpretation. However, there is a need for the transfer of technology and skills, capacity building and increased support to Africa. Access to quality remotely sensed data needs to be improved to promote the use of this technology and its integration into nature conservation disciplines to address conservation research questions about vegetation trends and responses to impact. Collaborative projects between skilled remote-sensing and field-based researchers are recommended to increase research capacity and the use of technology.

Based on the studies we reviewed our conclusions suggest that the use of remote sensing techniques in studying the impact of elephants on woody vegetation are currently limited. The limited number of studies that applied remote sensing-based methods are in Africa. We therefore encourage the integration of data collected from both remote sensing technology and field-based methods for monitoring the influence of elephants on woody vegetation. Remote sensing techniques are continuously improving and incorporating data collected with the latest technology will enhance habitat management strategies in the future. It is important to highlight that most African countries are in a developmental stage with limited resources and capacity. We also recommend that researchers should take advantage of available novel tools such as

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remote sensing technology to explore its practical and cost-effective applications to managing environments containing elephants, especially in a world that is becoming tech-savvy.

Acknowledgements. We would like to thank Mrs L Adriaanse from UNISA Library Services for providing and sourcing the list of articles from Nexus, SA e-Publications, EBSCOHost, ScienceDirect, Taylor & Francis, ProQuest, BioOne, and SpringerLink databases. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. We applied the "sequence-determines-credit approach" (SDC) for authors in this paper.

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APPENDIX A

A list of peer-reviewed studies published between 1970 and 2017

Studies that used remote sensing-based methods:

[1] Asner, G. P., Levick, S. R., Kennedy-Bowdoin, T., Knapp, D. E., Emerson, R., Jacobson, J., Colgan, M. S., Martin, R. E. (2009): Large-scale impacts of herbivores on the structural diversity of African savannas. – Proceedings of the National Academy of Sciences 106(12): 4947-4952.
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Studies that used field-based methods:

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APPENDIX B

	Region	Country	Number of elephants
1	Southern Africa	Botswana	131 626
2	Southern Africa	Zimbabwe	82 630
3	East Africa	Tanzania	50 433
4	East Africa	Kenya	22 809
5	Southern Africa	Namibia	22 754
6	Southern Africa	Zambia	21 967
7	Southern Africa	South Africa	18 841
8	Southern Africa	Mozambique	10 884
9	East Africa	South Sudan	7 103
10	Central Africa	Gabon	7 058
11	West Africa	Burkina Faso	6 850
12	Central Africa	Cameroon	6 830
13	Central Africa	Congo	6 057
14	East Africa	Uganda	4 923
15	Southern Africa	Angola	3 396
16	West Africa	Benin	2 984
17	Central Africa	DRC	1 794
18	Southern Africa	Malawi	1 307
19	East Africa	Etiophia	1 017
20	West Africa	Ghana	994
21	Central Africa	Equatorial Guinea	884
22	Central Africa	Chad	794
23	Central Africa	Central African Republic	702
24	West Africa	Mali	253
25	West Africa	Cote D'ivoire	189
26	West Africa	Liberia	124
27	West Africa	Nigeria	94
28	East Africa	Rwanda	88
29	Southern Africa	Swaziland	42
			415 427

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40454066

CHARACTERISTICS OF SOLAR ACTIVITY AND ITS POSSIBLE RELATIONSHIP WITH EAST ASIAN SUMMER MONSOON

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(Received 1st Dec 2018; accepted 20th Feb 2019)

Abstract. In order to study the variation of solar activity and reveal its relationship with the intensity change of East Asian summer monsoon (EASM), the sunspot time series and EASM intensity index are calculated by using sunspot relative number (SRN) data and sea level barometric data respectively. Then, Morlet wavelet analysis and correlation analysis are used to reveal the interdecadal characteristics of solar activity and EASM changes with their relationship. The results show that after the 1940s, the SRN and the fluctuation amplitude of the EASM index increased gradually, and reached the maximum from the 1950s to the mid-1960s. The influence of solar activity on the interdecadal variations of EASM is significant, with the same 40-year and 11-year cycles. The correlation between them is relatively good on the centennial scale, showing a "- + -" correlation with time change.
Keywords: *sunspot relative number (SRN), wavelet analysis, monsoon index, periodic signal, interdecadal variation*

Introduction

Solar activity is one of the main driving factors of global climate and environmental change. The relationship between solar activity and medium-term and long-term climate changes larger than or equal to 10 years has become a research hotspot in climatology in recent years (Zhao et al., 2012; Gray et al., 2016). Solar activity may affect regional climate in two ways. On the one hand, the abnormal changes of solar radiation affect the weather, climate and ocean thermal state. On the other hand, by stimulating the anomaly of the earth's magnetic field which fills the whole space of the earth, the motion state of the ocean and atmosphere changes, resulting in climate changes including weather scale, interannual scale, interdecadal scale and century scale (Haigh, 1996; Li et al., 2013; Yin et al., 2017). Study results have shown that solar activity is the basic factor affecting the temperature change over 10 years (Hood and Soukharev, 2012; Antico and Torres, 2015), and temperature change can lead to the change of zonal temperature gradient and Hadley circulation, and then affect the long-term change of monsoon (Li et al., 2018a, b). There is a strong correlation between solar activity and Asian monsoon activity. Neff et al. (2001) found that there is a strong correlation between the summer monsoon precipitation and the solar activity in the past 3000 years. The intensity of Indian monsoon in the past 1200 years corresponds well with the intensity of solar activity, and they have the same 200-year and 100-year cycles (Agnihtri et al., 2002). The results of Wang et al. (2005) showed that the rotation speed of the earth and the length of sunspot cycle are the main factors that restrict the interdecadal variations of Asian monsoon. The high-frequency variations of the East Asian summer monsoon (EASM) climate are driven by solar activity, and the 10-100-year monsoon climate

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40674080

variations are basically consistent with the solar activity cycle (Wu et al., 2006). However, the relationship between solar activity and EASM intensity, which has a significant impact on climate, remains to be studied.

China is a typical monsoon region in the world. The temperature and precipitation of interannual, interdecadal and even intercenturial climate changes in China are affected by the EASM (Hsu et al., 2011; Sun et al., 2015; Ding et al., 2018). Therefore, it is of great importance to study the interdecadal variations of EASM for understanding the causes of interdecadal climate variations of China. In order to further and quantitatively study the multi-time scale changes of monsoon and to have a consistent understanding of the characteristics of strong and weak monsoon, many scholars have established various indices reflecting the strength of EASM based on different data and methods (Guo, 1983; Wang et al., 2001; Sun et al., 2002; Zhang et al., 2003). For example, Guo (1983) defined the EASM index by using the East-West sea-level atmospheric pressure difference, which to a certain extent reflects the difference of sea land thermal power determining the EASM through the difference of land-sea pressure system, and the index has a relatively good effect in reflecting precipitation. These studies show that the period from 1951 to the mid-1960s has strong summer monsoon, after which the summer monsoon weakens, and after 1976, the intensity of summer monsoon weakens again.

It can be seen from the above that due to the lack of data before 1948, the time range of the study was concentrated in the later period, and the time series was short, so the long-term variation trend and interdecadal variation characteristics of EASM intensity were not known. Since only the UK's sea level pressure (SLP) data can be dated back to 1850, this paper uses the monthly SLP data after 1850 released by the UK's Hadley center to form a long-term EASM index series and study its interdecadal variation characteristics. At the same time, the relationship between solar activity and EASM is still unknown from the above analysis. Therefore, this paper attempts to study the characteristics of solar activity and monsoon intensity change on a centennial scale, to find the relationship between them, and to reveal the possible interaction and influence ways, so as to provide indicative information for the EASM forecast.

Data and methods

Sea level atmospheric pressure data (HadSLP2) released by the Hadley Center. Time: 1850-2006, monthly average; Space: 5 *5 degree grid global coverage. The research scope selected in this paper is "20-50°N, 110-160°E". This set of data is the second edition released by Hadley Center. It is the longest SLP data at present, and is conducive to calculating the East Asian summer monsoon index for more than 100 years.

The annual average sunspot relative number (SRN) data, published by the Solar Activity Data Analysis Center (SIDC), Institute of solar physics, the Royal Observatory of Belgium, time: 1700-2006.

The main methods used are Morlet wavelet analysis, correlation analysis and Butterworth bandpass filtering.

Morlet wavelet analysis has the characteristics of multi-resolution analysis, and is able to characterize the local characteristics of signals in both time and frequency domains. Wavelet transform decomposes the time series into the time-frequency domain, and then obtains the significant fluctuation patterns of the time series, i.e., the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40674080

periodic change dynamics and the temporal pattern of the periodic change dynamics. In essence, the signal is decomposed by a function's wavelet sequence, which is obtained by scaling and translation of a basic wavelet (wavelet base) or mother wavelet. The scaling and translation are carried out simultaneously in the mother wavelet function. Mother wavelet plays a key role in scale selection and wavelet transform. The applicability of the wavelet function should be fully considered in the selection of the wavelet functions, because the wavelet function used in the wavelet analysis is not unique, i.e., the wavelet functions are diversified. Analyzing the same problem with different wavelet bases will produce different results. At present, the results of signal processing and theoretical results of wavelet analysis are mainly used to evaluate the quality of the wavelet bases, and then the wavelet bases are selected. Morlet wavelet is a complex form of wavelet. It can separate the modulus and phase of the wavelet transform coefficients, where modulus represents the number of components of a certain scale, and phase can be used to study the singularity and instantaneous frequency of signals (Wang et al., 2005).

Correlation analysis method is to calculate the correlation coefficient of any two meteorological elements. It is used to measure the statistical quantity of the relationship between two meteorological elements. The correlation coefficient r_{xy} is the correlation between n time series x_i and another time series y_i. The calculation method is *Equation 1*.

$$r_{xy} = \frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)}{\sqrt{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2 \sum_{i=1}^{n}\left(y_i - \bar{y}\right)^2}} \qquad \text{(Eq.1)}$$

Variation characteristics of SRN

Sunspot is considered as the main body of solar activity, and its variation is very regular. It is often used as an important indicator of the intensity of solar activity. In this paper, the interdecadal variations of solar activity are studied by using the SRN data published by the SIDC of the Royal Observatory of Belgium. As can be clearly seen from *Figure 1,* the SRN has a marked upward trend on the whole, and the fluctuation amplitude of sunspot number in the past three hundred years increased gradually after the 1940s.

Results

Interdecadal variations of sunspot activity

In order to study the periodic variation characteristics of solar activity, Morlet wavelet analysis is performed on the SRN series. From the wavelet transform (*Fig. 2a*), it can be seen that there are obvious quasi-periodic oscillations at the levels of 80, 100, 40-50 and 11 years in the variation of the intensity of sunspot activity. At the 50-year level, the SRN has weakened since the 1960s, and the wavelet values and amplitudes have decreased significantly. The changes of 50-year cycles gradually weakened and disappeared, and turn into 40-year cycles. At the 11-year level: from the 1940s to the end of the 1980s, solar activity changes most dramatically. From 1950s to mid-1960s,

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the solar activity is the strongest in more than 300 years, which corresponded to the positive center of 180, 100, 40 and 11-year cycle changes. Secondly, from the 1970s to the 1990s, and from the 1930s to the 1950s, there are also two periods of strong solar activity. Since the solar cycle gradually changes from 50 years to 40 years around 1850, the wavelet variance series of 1700-1850 and 1851-2006 are analyzed respectively. From 1700 to 1850 (*Fig. 2b*), the most prominent period of solar activity is about 100 years, followed by 11 years and 50 years, and finally by 180 years. The 40-year cycle does not show up in this period. From 1851 to 2006 (*Fig. 2c*), the 11-year cycle becomes the most prominent cycle of solar activity, followed by the 100-year cycle, and then the 180-year cycle. The 50-year cycle no longer existed in this period, but a weaker cycle appeared in the 40-year scale. Han and Han (2001, 2002) pointed out that many periodic components of solar activity are not fixed, but have varying degrees of uncertainty and time-varying characteristics. That is, the period length, amplitude and phase change with time, which is consistent with the results of this paper.

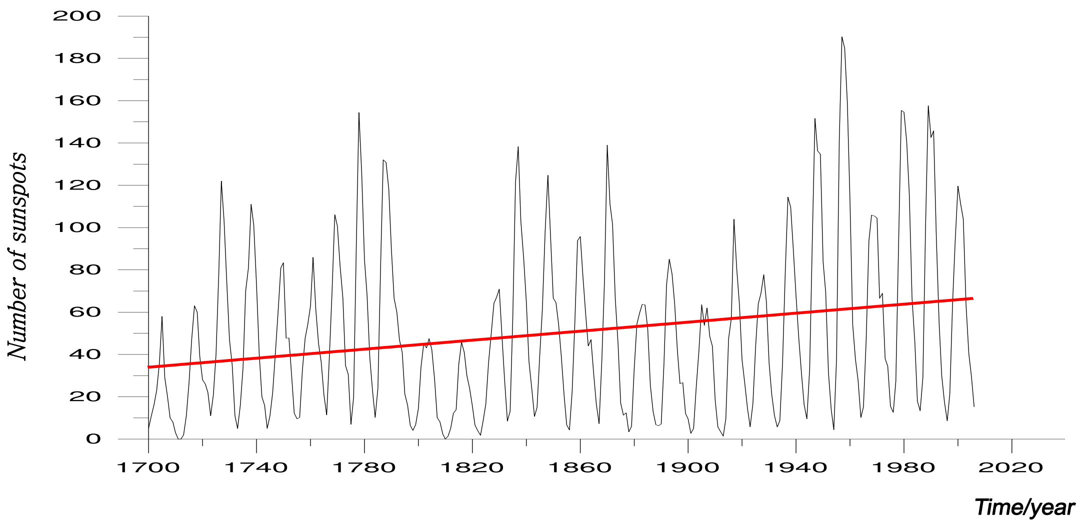
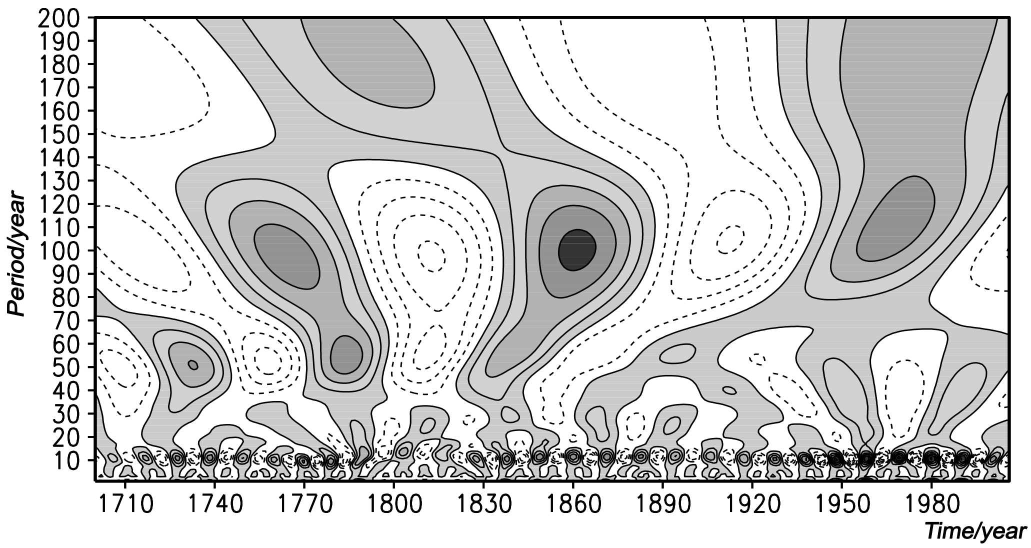


Figure 1. *Time series curve of SRN (Oblique line is trend line)*



a

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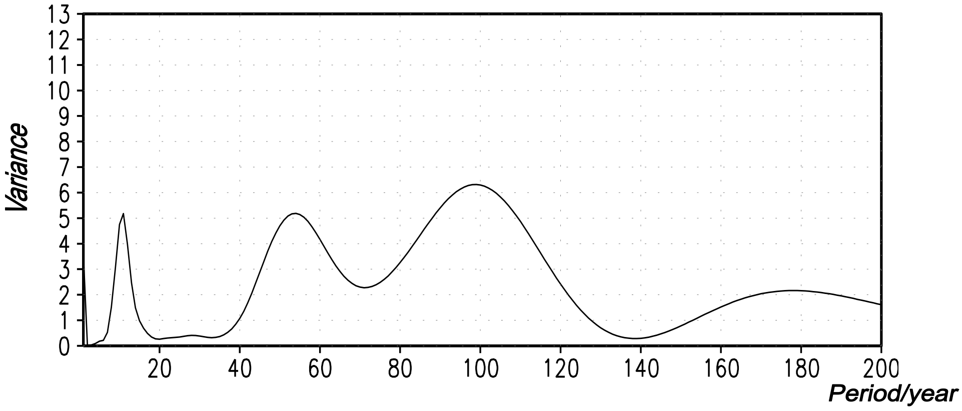

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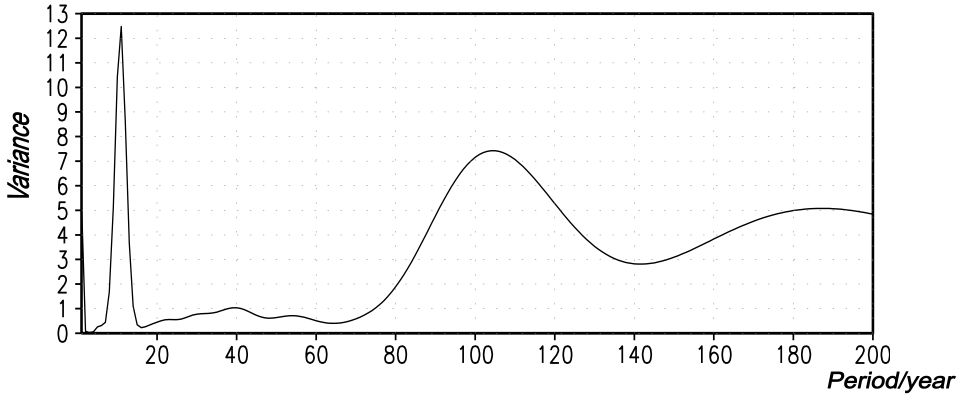

c

Figure 2. ***a*** *Wavelet transform map of SRN in 1700-2006.* ***b*** *Wavelet variance sequence of SRN in 1700-1850.* ***c*** *Wavelet variance sequence of SRN in 1850-2006*

Interdecadal variation characteristics of EASM

Intensity variation of EASM

In order to reveal the interdecadal variability of the EASM and its relationship with solar activity, the EASM index is selected and compared. Since the monsoon system is affected by the high and low latitudes systems at the same time, and there are interactions and constraints among various factors, each monsoon index can only describe one aspect of the characteristics of the monsoon better. At present, there is still no index that can describe the characteristics of monsoon variations in all aspects comprehensively. Considering the long-term nature of the time scale of the data, this paper chooses the method of calculating the EASM Index (EASMI) defined by Guo (1983), The strength of EASM is represented by the cumulative sum of △P ≤ -5 hPa at each 10 latitudes (∑△P) through the 110-160°E pressure difference (△P), between June and August 20-50°N. That is:

$$I_{\text{EASM}} = \sum \triangle P\left[SLP\left(20-50^{o}N,\ 110-160^{o}E\right)\right] \quad \text{(Eq.2)}$$

Figure 3 shows the East Asian summer monsoon index series calculated by *Equation 2* and normalized. As can be clearly seen from *Figure 3,* the EASM

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experienced four stages of strong-weak-strong-weak change in 1850-2006, which is non-linear and a long-time scale fluctuation. From the 1950s to the mid-1960s, the intensity of the EASM increased in general, and it reached its strongest stage in the 1950s and 1960s. This stage is also the strongest stage of solar activity analyzed in *Figure 2a,* which has been decreasing since the mid-1960s. The intensity of EASM changed abruptly in the mid-1960s and experienced a transition from the strongest to the weakest. Weak summer monsoon lasted until 2006, it reached its weakest stage after the mid-1970s. It is noteworthy that the fluctuation amplitude of the EASM increased gradually after the 1940s. Compared with the time series curve of SRN in *Figure 1,* it can be seen that the fluctuation amplitude of SRN has also increased gradually since 1940s. In conclusion, it can be concluded that the intensity of sunspot activity oscillation may be one of the reasons for the intensity oscillation of EASM.

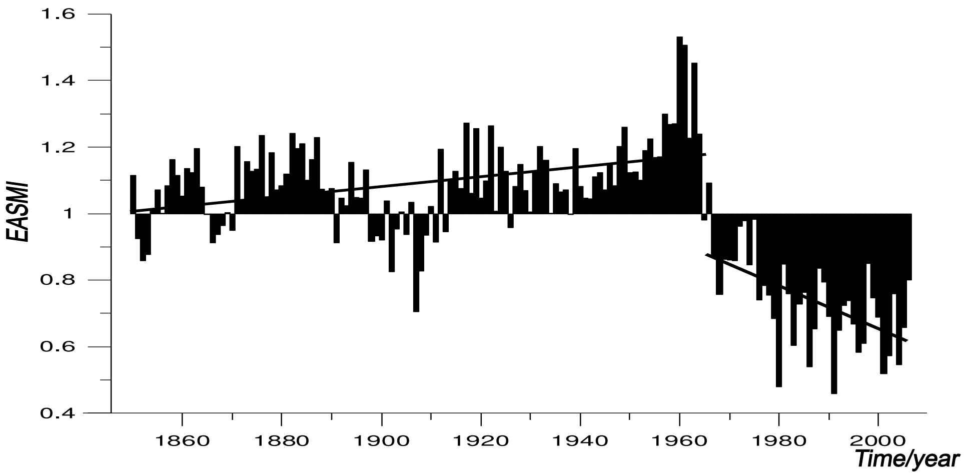

Figure 3. *EASM Index series (Dimensionless, oblique line is trend line)*

Interdecadal variation period analysis of EASM

From the previous analysis, we can see that the solar activity shows some interdecadal periodic changes. In order to study the possible relationship between solar activity and EASM, the wavelet transform and wavelet variance maps of the above-mentioned EASM indices are presented, and their periodic variation characteristics are analyzed.

As can be seen from *Figure 4a,* the EASM in 1850-2006 has obvious quasi-periodic oscillations in the levels of 80, 40, 20 and 10-13 years. The 40-year cycle has a decreasing trend since the 1950s; the 20-year cycle has increased since the 20th century, with an increasing trend of amplitude; and the 11-year cycle has also gradually increased since the 1950s. It can also be seen that the period from 1950s to mid-1960s was the strongest period of EASM. The density, numerical value and time level of the wavelet transform curve in this period was the largest in 157 years, and then followed by the weakest period of EASM, which is consistent with the previous analysis results. From the wavelet variance figure of *Figure 4b,* it can be seen that the main interdecadal period of the EASM is about 80 years, followed by about 40 years. In addition, there is a weak period of about 11 years. The period of about 20 years is not shown in the wavelet variance map because of the weak signal.

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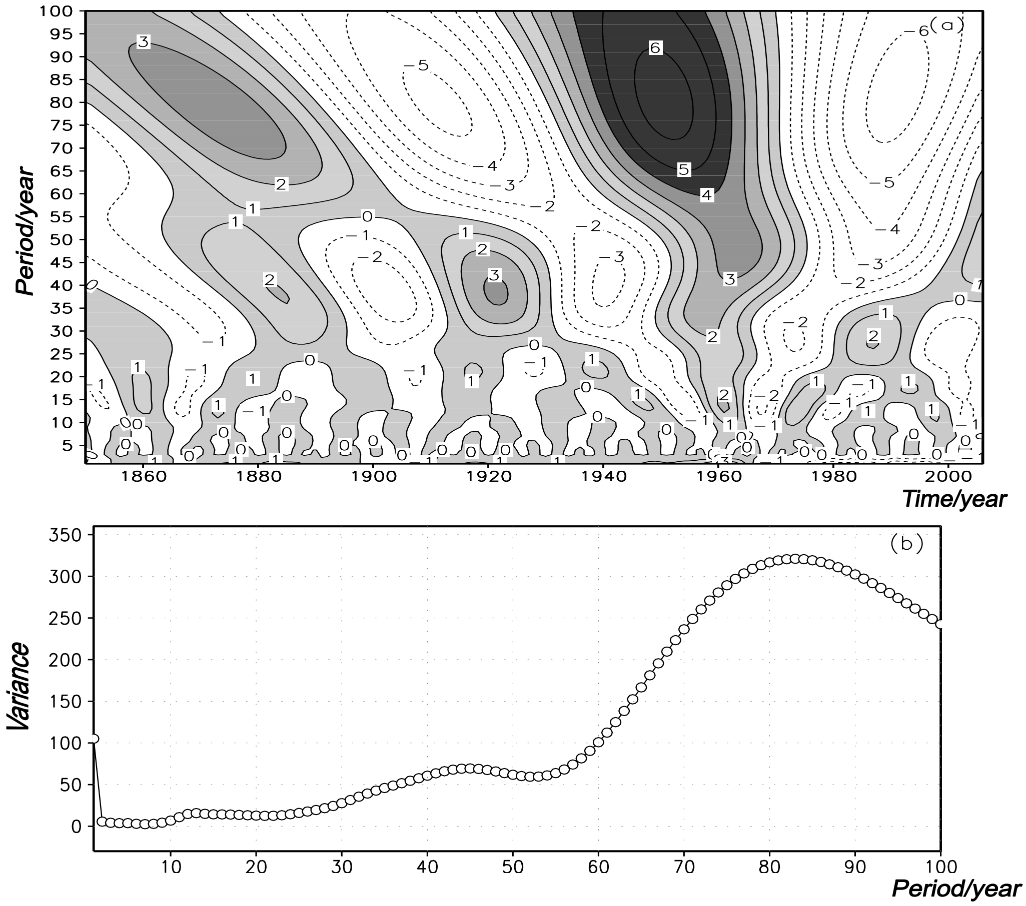

Figure 4. ***a*** *Wavelet transform map of the EASM index.* ***b*** *Wavelet variance map of EASM index. (In order to clearly show the period, the average wavelet variance in the figure is expanded by 10 times)*

Relationship between EASM and solar activity

The above analysis shows that the solar activity has the same 40-year and 11-year cycle as the EASM intensity change. EASM and solar activity reached the strongest in all years from 1950s to mid-1960s. Therefore, the influence of solar activity on the interdecadal variation of the EASM intensity does exist. Comparing the EASM index and sunspot number series from 1850 to 2006, it is found that before the mid-1960s, both of them had an increasing trend, and then they changed in the opposite direction. In order to discuss the interdecadal correlation between the two, the 157 EASM index is averaged by a nine-year moving window, and the interannual variation information is filtered out. Then the sunspot number is averaged by an 11-year moving window, eliminating its inherent 11-year cycle. In this way, the interdecadal relationship between EASM intensity and solar activity can be better reflected. The smoothed values are shown in *Figure 5*. The variations of the EASM and sunspot activity are not always consistent. The EASM and sunspot number curves show obvious negative correlation changes before the 20th century, then positive correlation changes from the beginning of 20th century to the mid-1960s, and then negative.

Kilcik et al. (2007) studied the influence of solar activity on climate from 1940 to 1984, and concluded that there was a positive correlation between solar activity

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parameters and terrestrial atmospheric temperature before 1966, but a negative correlation after 1966, which is consistent with the results of this study. The correlation coefficients between the moving averaged EASM index and the SRN are calculated. The results show that the correlation coefficients are -0.845 before 1910, 0.723 between 1911 and 1965, and -0.489 after 1966, all exceeding the reliability test of 0.001. Based on the wavelet analysis of sunspot activity, it is found that the correlation between EASM and sunspot activity is affected by the 100-year cycle of solar activity. When the sunspots number decreases from the peak in 1850 to the valley in 1910 on a centennial scale (*Fig. 2a*), there is a negative correlation between the sunspots number and EASM index, i.e., EASM is strong in the year with weak solar activity, while it is weak in the year with strong solar activity. When the sunspot number transits from the valley value in 1910 to the peak value in 1965, EASM index is positively correlated with solar activity, i.e., EASM is strong when solar activity is strong, and is weak when solar activity is weak. Similarly, since 1965, solar activity has shifted from peak to valley on a centennial scale, and the relationship between them has changed again. It can be concluded that solar activity not only has a strong influence on the intensity change of EASM at interdecadal scale, but also is an important factor that can not be ignored in influencing the intensity change of summer monsoon at centennial scale.

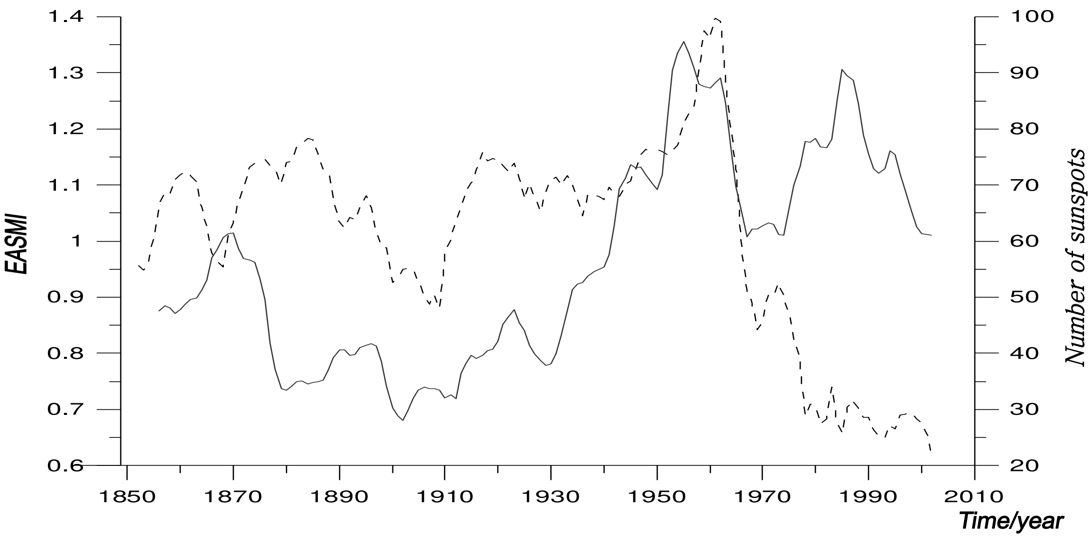

Figure 5. *Moving average curve of SRN (real line) and EASM index (dotted line)*

Analysis of solar activities affecting interdecadal variation of EASM intensity

The interdecadal variations of solar activity and EASM intensity are well correlated. Based on the previous analysis, the influence information of solar activity in the EASM index series is extracted and analyzed step by step in this section. When the information is separated from the concealed state, the relationship between them can be revealed, which provides a new basis for the interdecadal variation of the EASM.

The analysis of the EASM intensity index shows that in the mid-1960s, EASM changed dramatically. Before the mid-1960s, EASM generally strengthened. After reaching the strongest point in the mid-1960s, it suddenly changed and weakened, and then entered the period of weak summer monsoon. The trend line in *Figure 3* shows the trend changes before and after the sudden change of summer monsoon.

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When $i = 1, 2, 3......117$, $l_1(i)$ represents the trend state before the sudden change;
When $i = 118, 119......157$, $l_2(i)$ represents the trend state after the sudden change;
The general trend changes:

$$l(i) = l_1(i) + l_2(i) \qquad i = 1, 2, 3......157 \tag{Eq.3}$$

By subtracting the climatic trend $l(i)$ in *Equation* 3 from the original summer monsoon index series $I_{sm}(i)$, the summer monsoon index series without climatic trend, $Y_{sm}(i)$, can be obtained.

$$Y_{sm}(i) = I_{sm}(i) - l(i) \tag{Eq.4}$$

The mean square deviation of the original summer monsoon index series $I_{sm}(i)$ is obtained.

$$S = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(I_{sm}(i) - \overline{I_{sm}})^2} = 0.204 \tag{Eq.5}$$

where $\overline{I_{sm}}$ is the average EASM index for 157 years during 1850-2006. Similarly, the mean square deviation of EASM index series after the trend change is filtered out according to *Equation 5:*

$$S_1 = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(Y_{sm}(i) - \overline{Y_{sm}})^2} = 0.129 \tag{Eq.6}$$

where $\overline{Y_{sm}}$ is the average of EASM index series $Y_{sm}(i)$ defined in *Equation 4* after filtering out trend change during 1850-2006. Then the variance contribution of trend change to the summer monsoon index series is as follows:

$$\begin{aligned} SS &= [(S - S_1)/S] \times 100\% \\ &= [(0.204 - 0.129)/0.204] \times 100\% = 37\% \end{aligned} \tag{Eq.7}$$

In the formula, *S* and S_1 are calculated by *Equations 5* and *6* respectively. The variance contribution of the trend is 37%, i.e., the increase of EASM intensity before the mid-1960s and the subsequent abrupt decrease are the basic characteristics of the change in the whole 157 years.

The wavelet analysis of EASM index and SRN shows that there exists the same 40-year and 11-year cycle between EASM and sunspot activity. The 40-year periodic component series of EASM $l_{40}(i)$ is obtained by band-pass filtering. By subtracting 40-year cycle components from the original summer monsoon index series, the index series without 40-year cycle $Y_{sm}(i)$ can be obtained. The mean square deviation of $Y_{sm}(i)$

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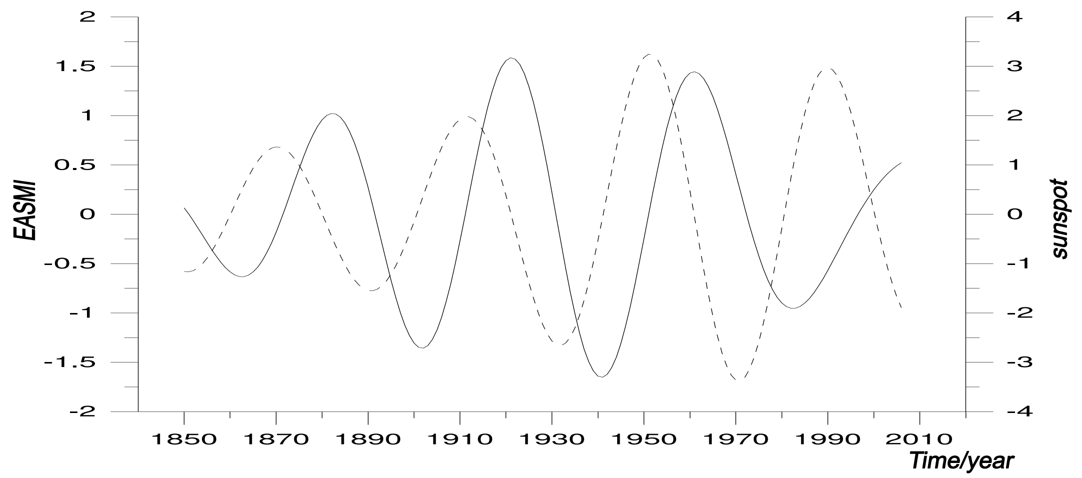
obtained from *Equation 5* is 0.159. According to *Equation 7,* the contribution of 40-year cycle change to the variance of summer monsoon variability is 22%.

It can be seen that the 40-year cycle is one of the main cycles of the EASM. Using the same method, the variance contribution rate of 11-year cycle is 14%, and that of 80-year cycle is 25%, which ranks only second to trend change.

The 11-year and 40-year cycles play an important role in interdecadal variations of the intensity of the East Asian summer monsoon. In order to further understand the influence of 11-year and 40-year cycles of solar activity on the variation of EASM, the wavelet component curves of the EASM index and SRN in the period of 40 and 11 years from 1850 to 2006 are plotted in *Figure 6*.



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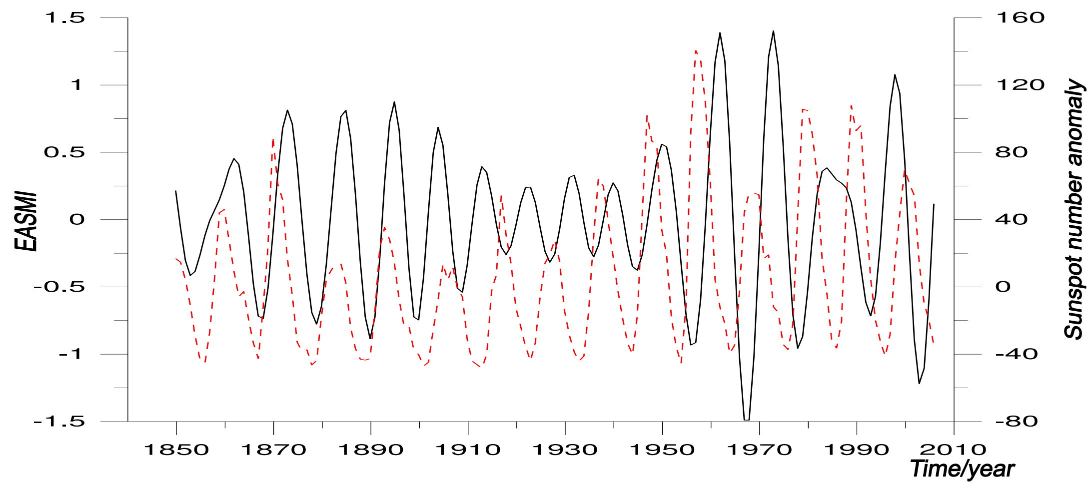

b

Figure 6. a *40-year wavelet periodic component curve of EASM index and SRN.* ***b*** *11-year wavelet periodic components of EASM index and anomaly values of SRN. (Real line and left coordinate are EASM index, while dotted line and right coordinate are SRN)*

As can be seen from the figure, there is a good positive correlation between the variation of EASM and the 40-year cycle of sunspot (*Fig. 6a*). The same is true for the magnitude. When the magnitude of sunspot variations increases, the magnitude of EASM variations also increases, and the sunspot number is about 10 years ahead of the

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summer monsoon. In the time scale of 11-year cycle (*Fig. 6b*), the variation curves of the two are generally similar. Before 1910, the variation of sunspot number is isotopic. The variation of sunspot number is 1-3 years ahead of that of EASM index. From 1910 to 1940, the two gradually changed into inverse phases, and the variation ranges were gentle. After 1940, the two phases gradually changed into the same phase. The magnitudes of the two increased obviously, but the magnitude of the EASM lagged behind the solar activity. The above analysis shows that the influence scale of sunspot activity on EASM variations is from 11 to 40 years, i.e., solar activity plays a decisive role in EASM variations on the “decadal” time scale.

Discussion

In this paper, the cycle analysis of the SRN and the EASM index variations shows that there is a close relationship between them: the fluctuation amplitude of the two shows a similar trend, with the same change period and strong correlation, and the variations of the EASM lag behind the changes of the SRN. The EASM seriously affects the weather and climate in China and East Asia. Therefore, it is of practical significance to analyze the solar activity to predict the intensity of the summer monsoon and then forecast the weather or climate conditions. At present, most studies focus on the characteristics of solar activity itself (Baldwin and Dunkerton, 2005; Frame and Gray, 2010) and the influence of solar activity on precipitation, temperature and other climatic factors (Maliniemi et al., 2014; Ge et al., 2016). Since some weather and climate systems, such as monsoons, directly influence precipitation. It is also beneficial to reveal the physical mechanisms of the sun affecting climate. Studies have yielded some useful results from this very point (Kerr, 2005; Verschuren et al., 2009), but there has been a lack of research on the East Asian monsoon. Because local climate systems vary from region to region, this paper is of certain value for the relevant research of EASM.

However, the effect of solar activity on interdecadal variations of EASM, and the law of solar activity affecting the variations of EASM are very complex, non-linear in different periods and time scales. Many problems still exist and need to be solved. Small changes in solar radiation over the 10-100-year scale can neither directly cause significant heating changes in the Qinghai-Tibet Plateau, nor affect the EASM. However, solar activity may affect the summer monsoon through atmospheric or oceanic circulation (Zhou and Chen, 2014). At present, the mechanism of solar activity changes affecting East Asian monsoon is not clear. More factors, such as sunspot magnetic field and total solar radiation (Qi et al., 2015), need to be considered to comprehensively study the ways and mechanisms of solar activities affecting EASM through atmospheric ocean circulation and ground radiation, etc.

Conclusion

In this paper, the main characteristics and main periodic variations of sunspot activity on a centennial scale are analyzed. The interdecadal variations of the EASM and their correlation with sunspot activity are discussed. Morlet wavelet technique is used to discuss them, revealing their common periods and analyzing them separately. The real change of climate factors is the combination of all effective signals and noise signals, i.e., the superposition of different periodic signals. Although the signal obtained by this

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method is only one of its “components”, the change of “original” information can be basically mastered by clarifying the change law of these basic periods. Therefore, it is significant to determine the interaction between these basic cycles for the analysis and prediction of interdecadal variations of EASM. The following conclusions can be drawn from this study:

From 1700 to 2006, SRN showed an obvious upward trend, and its fluctuation amplitude increased gradually after 1940s. On the centennial scale, before the 1960s, the sunspots number had significant 11-year, 50-year, 100-year and 180-year cycles, and then 50-year cycles changed to 40-year cycles.

There is a strong correlation between solar activity and EASM intensity variations on the centennial scale, showing a “- + -” correlation with time. The intensity of sunspot activity oscillation is one of the reasons for the intensity oscillation of EASM. Both of them reached their maximum from 1950s to mid-1960s, and they have the same 40-year and 11-year cycles.

The influence scale of sunspot activity on EASM variations ranges from 11 to 40 years. That is to say, solar activity plays a decisive role in EASM variations on the “decadal” time scale. The correlation between sunspot activity and EASM variations is regulated by solar activity on the centennial scale.

Acknowledgements. This work was supported in part by the China Civil Aviation Flight Technology and Flight Safety Research Base Open Fund Project (F2016KF02).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40674080

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40674080

HIERARCHIZATION OF ABIOTIC ENVIRONMENTAL PROPERTIES – METHODICAL STUDY FOR RESEARCH ON THE ECOLOGY OF PLANTS IN PEAT BOG HABITATS

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(Received 1st Dec 2018; accepted 18th Feb 2019)

Abstract. The objective of this study was to perform the hierarchization procedure of habitat physical-chemical properties of peat bog waters in a five-year research cycle. The identification of important abiotic habitat properties in the context of the analyzed boreal plant relics' occurrence determines the mechanisms of their active protection, and in the case of useful plants with high medicinal values – the possibility of their ecological cultivation. The procedure covers desk studies involving source data analysis, field reconnaissance with selection of study sites, botanical-phytosociological analyses, and laboratory analyses of the abiotic properties of the habitat. The complex statistical analysis of the obtained data including descriptive statistics, non-parametric Kruskal-Wallis test, and multidimensional methods permitted the designation of range values for properties favoring the occurrence of the studied plant species. The procedure finds practical applications for both for the active protection of endangered plant species and other useful plants.
Keywords: *boreal relic, herbs, peat bog, habitat, physical-chemical factors, research method*

Introduction

Relevant literature quite frequently discusses the issue of conducting habitat research in the context of protection of rare plant species or importance of ecological conditions for proper growth and development of useful plants (e.g. Aleric and Kirkman, 2005; Zhang et al., 2002; Kostrakiewicz, 2008; Reckinger et al., 2010). Depending on the species, specificity of their biology and ecology, and the study objective, authors consider different environmental factors, from light conditions, temperature, and humidity (Matsumoto et al., 2000), through biogenic compounds (Wassen et al., 2005), to a combination of many largely varied environmental properties, which combined determine the quality of the habitat (Kumar and Stohlgren, 2009).

An example of a broad application of results of habitat research in reference to the key processes of the biology of a species threatened with extinction is the paper by Hroneš et al. (2018). The determination of the specificity of habitat conditions at the mountain sites of occurrence of the population of a rare relic species *Salix lapponum* in the Czech Karkonosze Mountains involved the application of results of research on both abiotic and biocoenotic environmental properties. First, floristic inventories performed in 207 study plots were analysed in terms of habitat preferences of all plant species,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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based on the ecological index numbers of vascular plants (Ellenberg et al., 1991). The following factors were considered: temperature, light, continentality, moisture, soil reaction, nutrients. The co-occurrence of plant species in the studied communities was also analysed. The next stage was the analysis of 207 soil samples in terms of basic soil properties, i.e. pH, N ($g \cdot kg^{-1}$), P ($mg \cdot kg^{-1}$), Ca, and Mg ($mg \cdot kg^{-1}$). The study results were compared to data concerning blooming or lack of blooming of individuals of the studied species. The authors initially evidenced differences in conditions occurring in microhabitats in which individuals bloom more frequently.

Data concerning abiotic environmental factors are also used for the purpose of improvement of the state of preservation of endangered plant species. Adhikari et al. (2012) designated a potential area and habitat for the reintroduction of *Ilex khasiana* Purk by means of the algorithm of modelling maximum entropy distribution (MaxEnt). The model was developed with the application of 16 data on the location in native range and 16 environmental parameters.

The analysis of results of habitat research by Nicolè et al. (2011) among others permitted answering the question in what way the dynamics of the population of the endangered high mountain plant *Dracocephalum austriacum* depends on the local quality of the habitat and climatic variability.

The reconstruction of habitats does not always permit the reintroduction to the environment of rare plant species. According to Kleijn et al. (2008), it may partially result from the inability to reconstruct the specific biogeochemical conditions preferred by such species. Many variables concerning habitat conditions have been evidenced to affect the condition of plants – in this case rare species of heathers in Holland, but it remains unknown what their relative importance is and whether any biogeochemical variable acts as the key factor limiting their maintenance.

Habitat research is also used for the analysis of the possibility of future cultivation of useful plants. The objective of the study by Sugier et al. (2011) was the determination of the content of phenol glycosides in the bark of dark-leaved willow (*Salix myrsinifolia*). The bark of the species was determined to be a potential source of salicylates, hence the proposal of introducing the species to cultivation. The study involved among others the determination of habitat conditions of natural functioning of the species. Characteristics of plant communities with a contribution of the studied species, and selected abiotic properties of the habitat were determined through the analysis of soil samples in terms of content of organic matter, macro- and microelements, and reaction.

The objective of this study is to present a developed complex procedure of hierarchization of abiotic habitat properties related to groundwaters of peat bog ecosystems of the Łęczna-Włodawa Lake District (Polesie Podlaskie, East Poland). The procedure was developed in reference to research concerning the specificity of occurrence of protected relic species – *Salix lapponum*, *Salix myrtylloides*, and *Betula humilis* (Serafin et al., 2015a, b, 2018a); and species of plants with medicinal properties, namely *Menyanthes trifoliata* and *Oxycoccus palustris* (Serafin et al., 2017, 2018b) at sites representative of the region where the measurements were performed (subjective assessment).

The identification of abiotic properties of the habitat important for the occurrence of these groups of plants, related to groundwaters, can provide the basis for the selection of the right strategy of active protection of endangered plant species, e.g. in the case of their reintroduction. It also allows for the determination of the conditions of potential ecological cultivation of peat bog plants with high useful potential, e.g. medicinal, in

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this case for naturotherapy – obtaining a natural composition of biologically active substances in cultivated herbal material (feature of natural medicine).

The popularisation of the procedure of hierarchization of abiotic habitat properties therefore seems to be a pragmatic measure.

Methodological concept and practical application

The developed procedure covers multiple-stage research: desk studies including the analysis of source texts; field reconnaissance for the selection of the right locations for conducting research, field research for botanical-habitat analyses at selected study sites, and laboratory analyses for the determination of the value of physical-chemical water parameters.

The applied statistical methods permit the interpretation of results and identification of factors important for the proper functioning of the studied plant species.

Study sites and procedures

Ecological analyses of different plant species require a complex approach to the selection of the study area. An optimal solution seems to be the determination of sites of occurrence of the studied species representative of a given region, characterised by the occurrence of its numerous populations in specific biocoenotic conditions, particularly in phytosociological terms. This is of particular importance in the case of research on natural conditions of occurrence of a given species. In nature, in spite of subjectively similar habitat conditions, largely undetermined factors exist that may affect the size of population of a given species in different ways. Subjective perception of the quality of habitats (including their apparent similarities) frequently does not consider factors determining the dynamics of the population of the studied species to a greater extent.

The variable size of population of the studied species at sites differing in terms of abiotic and biocoenotic factors in a given region permits the determination of the potential range of ecological tolerance of the species to the analysed environmental factors.

In the scope of the adopted assumptions and based on the conducted desk and field studies on peat bogs of the Łęczna-Włodawa Lake District, seven research locations representative of the region were selected (*Fig. 1*). They were lake-peat bog complexes – Bikcze (B), Karaśne (K), Moszne (M), Długie (D), Lubowierz (L), and ranges: Blizionki (BZ) and Dekowina (DK), covered by areal forms of nature protection – particularly the Poleski National Park and its buffer zone (*Fig. 1*).

Research on habitat properties at the selected sites was applied in different configurations during the determination of the range of ecological valency of different plant species.

The verification of the accuracy of selection of study sites in reference to the preferences of a given plant species and naturalness of the occurring phytocoenoses was based on *in situ* botanical and phytosociological analyses.

In the scope of the adopted methodology, plant species inventories in selected locations of the Łęczna-Włodawa Lake District were performed still before the designation of particular study sites. The size of the eventually designated research plots was optimised, depending on specific conditions. The analysis of the naturalness of habitats was performed, e.g. based on the assignation of a specific plant species to

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historical-geographical and range groups, and determination of anthropogenic changes in the flora, articulated by the following ecological indices:

- total synanthropisation – ($\mathbf{W_{S\text{-}c}}$) = $\frac{Ap + A}{Sp + A} \times 100\%$; (Chmiel, 1993a)
- total anthropophytisation – ($\mathbf{W_{An\text{-}c}}$) = $\frac{A}{Sp + A} \times 100\%$; (Chmiel, 1993a)
- total apophytisation – ($\mathbf{W_{Ap\text{-}c}}$) = $\frac{Ap}{Sp + A} \times 100\%$; (Chmiel, 1993a)

where *Ap* - apophytes (taxa of synantropic plants of local origin, constituting part of the native flora, occurring in anthropogenic habitats developed as a result of human activity; *Sp* - spontaneophytes (taxa developed in a given area or imported, able to function with no human interference, existing of nature) + apophytes, *A* - anthropophytes (taxa developed in a given area as a result of human pressure, taxa imported to a given area by man, and species surviving at sites under constant human pressure) devised by Chmiel (1993a, b).

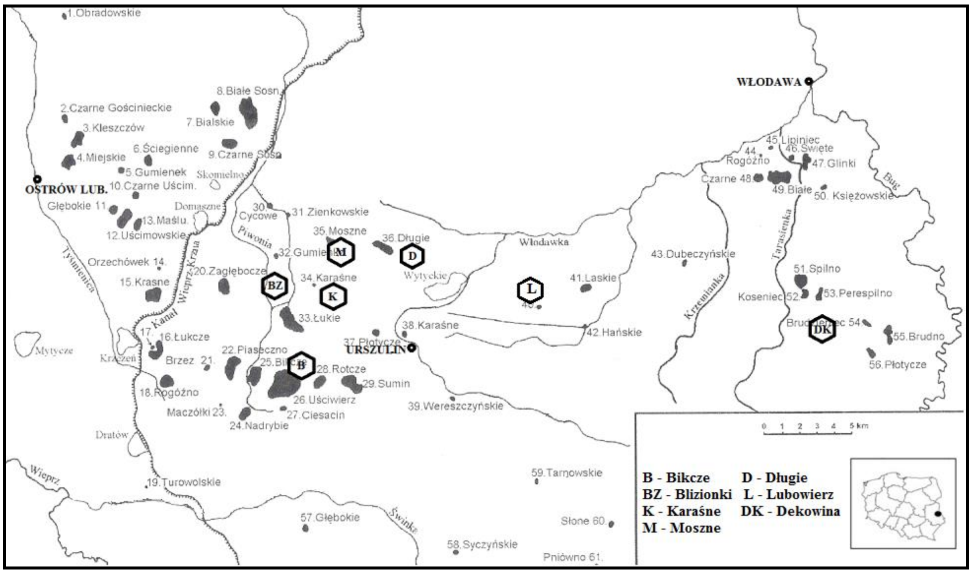

Figure 1. *Location of study sites related to the method of hierarchization of abiotic habitat factors in the Łęczna-Włodawa Lake District (Harasimiuk et al., 1998, changed)*

Low values of coefficients of anthropogenic changes in the flora suggest high biocoenotic naturalness of the selected locations, permitting the selection of specific study sites adjusted in terms of surface area to the local topographic and biocoenotic conditions, further described as subjectively representative (usually 100-625 m^2).

The selected study sites were characterised by a variable abundance of populations of the studied taxa, which allowed for grouping them in terms of contribution (%) of study objects in phytocoenoses.

Further analyses and presentation of results were performed for such designated groups. In the case of lack of considerable differences in the abundance of a given species in phytocoenoses of the selected sites, further analyses of all sites were performed for each site separately. Botanical research was supplemented with the

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following analyses: index of species similarity of phytocoenoses according to Jaccard (Piernik, 2008), and hierarchical classification, both for the sites, and for the species composition, and presented particularly in the form of tables (*Table 1*), dendrograms (*Fig. 2*), or their combinations with heatmaps (*Fig. 3*).

The aforementioned methods permitted a more thorough verification of the accuracy of selection of study sites in the context of species composition and phytosociological connections of peat bog habitats of selected locations. The final effect was the designation of groups of species of plants classified by frequency of occurrence at different study sites.

The determination of phytosociological affiliation of plants the most frequently accompanying the analysed objects permitted the final verification of the accuracy of selection of study sites in the scope of the adopted representative regional locations.

The next stage of research involved analyses of physical-chemical properties of peat bog waters. The selected factors covered the most important physical properties of waters: reaction (pH), electrolytic conductivity (EC); biogenic chemical factors: phosphorus fractions – total phosphorus (TP), phosphates ($P\text{-}PO_4$), and nitrogen fractions – total nitrogen (TN), ammonia nitrogen ($N\text{-}NH_4$), nitrates ($N\text{-}NO_3$), nitrites ($N\text{-}NO_2$); content of dissolved organic carbon (DOC); sulphates ($S\text{-}SO_4$), and selected ions: Na, K, Ca, Mg.

In order to perform such research, ground piezometers were dug in the central part of each site. They were perforated PVC pipes with a diameter of 0.1 m and length of 1 m, ending in a cup closed with an adjusted lid.

Depending on the research schedule, water samples for further laboratory analyses, performed by means of certified methods, were collected from the peat bog piezometers with specified frequency.

Ranges of values of the analysed properties in comparison with the variable contribution of the studied species in phytocoenoses of selected sites determined the range of its ecological tolerance in reference to the analysed properties and region of its occurrence (*Table 1; Fig. 4*).

The study results were based on selected statistical methods and presented in graphic forms depicting the found patterns.

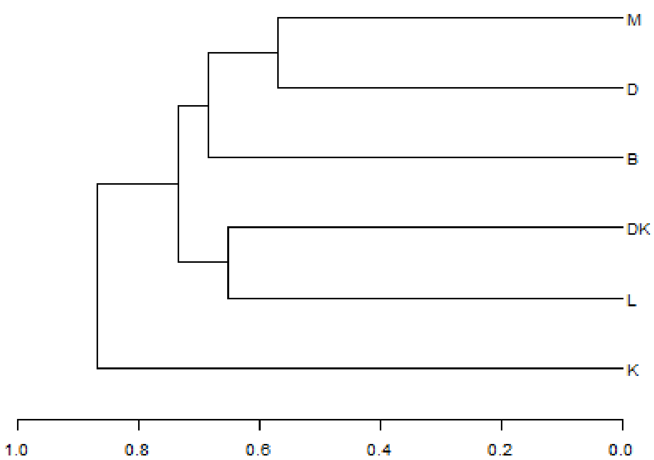

Figure 2. *Dendrogram plot for sites as part of research on the occurrence of Betula humilis on the Łęczna-Włodawa Lake Distict (Serafin et al., 2018a). M- Moszne, D - Długie, B - Bikcze, DK - Dekowina, L - Lubowierz, K – Karaśne*

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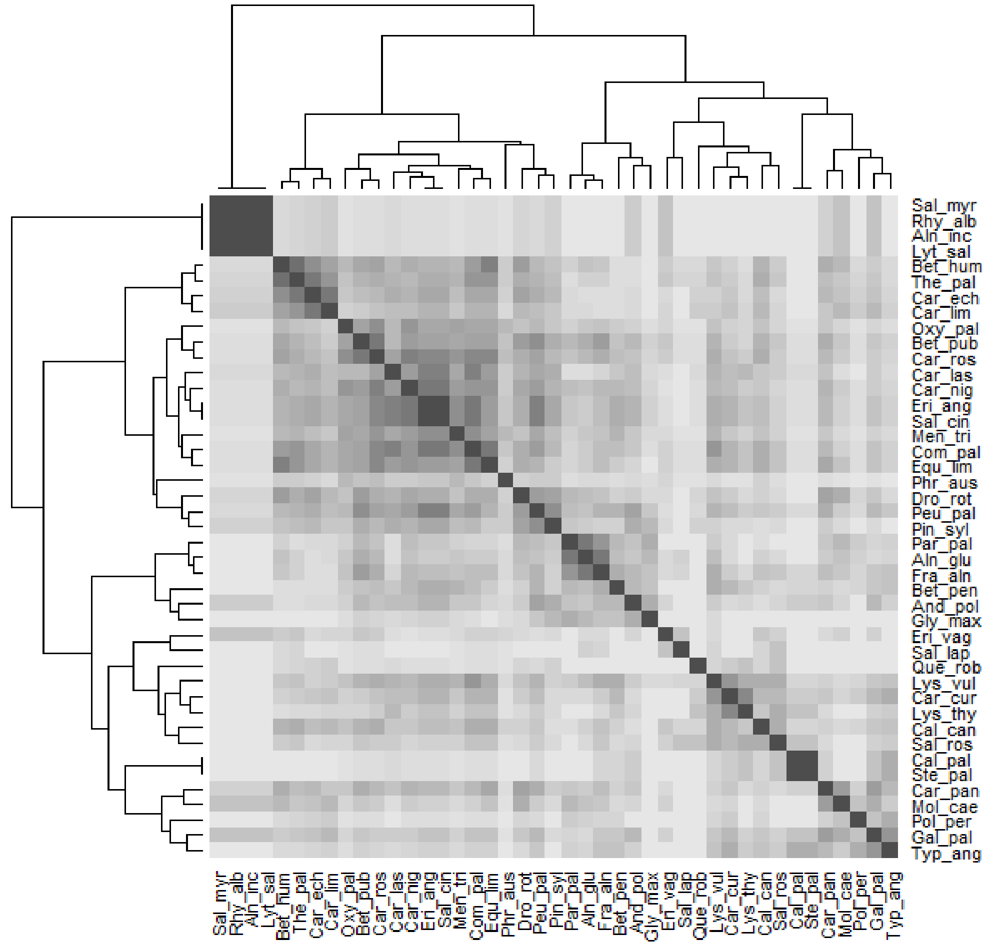

Figure 3. *Dendrogram and heatmap of hierarchical cluster analysis of 43 species based on the Jaccard coefficient and Ward method as part of research related to the occurrence of Oxycoccus palustris in the Polesie Podlaskie Region (Serafin et al., 2018b). Species composition of phytocoenoses: Alnus glutinosa, Alnus incana, Andromeda polifolia, Betula humilis, Betula pendula, Betula pubescens, Calamagrostis canescens, Calla palustris, Carex curta, Carex echinata, Carex lasiocarpa, Carex limosa, Carex nigra, Carex panacea, Carex rostrata, Comarum palustre, Drosera rotundifolia, Equisetum limosum, Eriophorum angustifolium, Eriophorum vaginatum, Frangula alnus, Galium palustre, Glyceria maxima, Lysimachia thyrsiflora, Lysimachia vulgaris, Lythrum salicaria, Menyanthes trifoliata, Molinia caerulea, Oxycoccus palustris, Quercus robur, Parnassia palustris, Peucedanum palustre, Phragmites australis, Pinus sylvestris, Polygonum persicaria, Rhynchospora alba, Salix cinerea, Salix lapponum, Salix myrtilloides, Salix rosmarinifolia, Stellaria palustris, Thelypteris palustris, Typha angustifolia*

Table 1. *Values of the Jaccard similarity index between study sites related to the occurrence of Menyanthes trifoliata in peat bogs of Polesie Podlaskie Region (Serafin et. al. 2017) B- Bikcze, M- Moszne, K- Karaśne, D- Długie, BZ- Blizionki, DK- Dekowina*

B-M	0.38	**M-D**	0.54	**D-K**	0.54	**K-BZ**	0.38	**BZ-DK**	0.33
B-D	0.51	**M-K**	0.52	**D-BZ**	0.41	**K-DK**	0.46		
B-K	0.56	**M-BZ**	0.31	**D-DK**	0.46				
B-BZ	0.34	**M-DK**	0.43						
B-DK	0.46								

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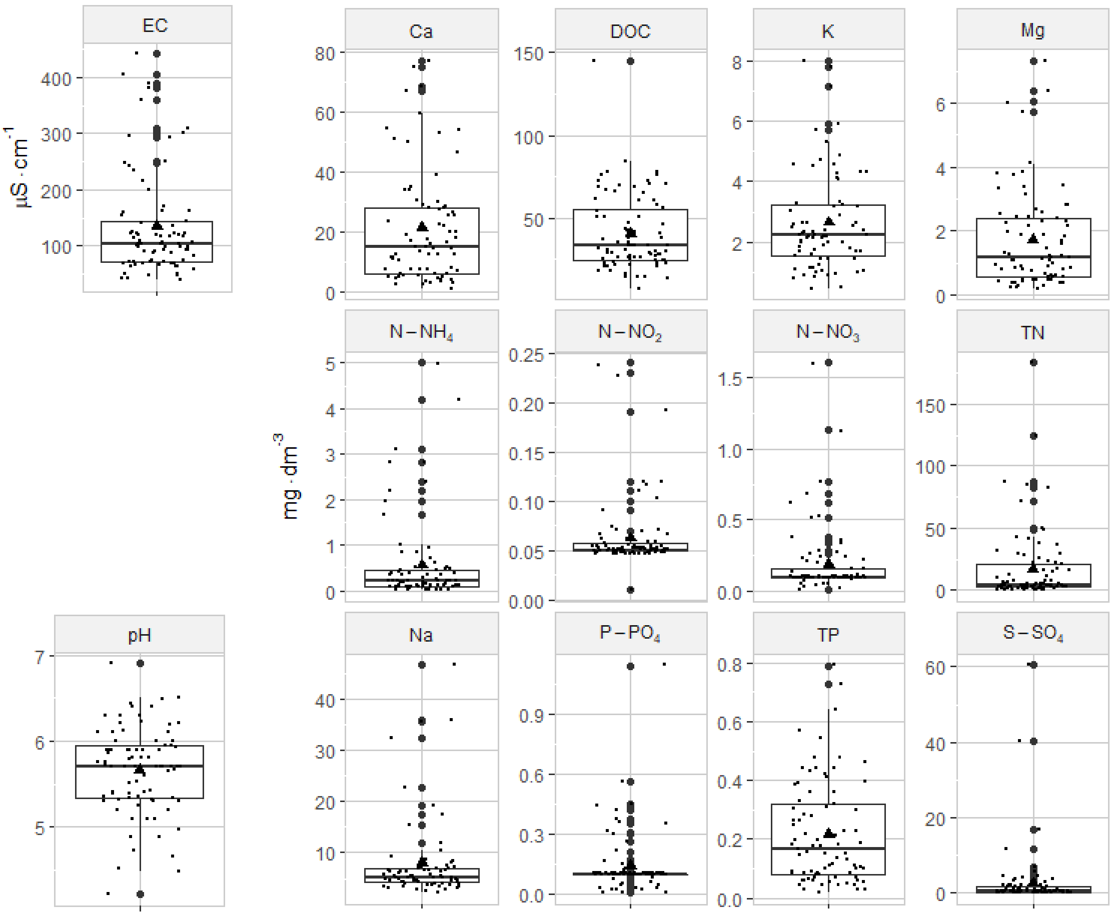

***Figure 4.** Distribution of values of the investigated physical-chemical properties of piezometric groundwater at the study sites in the period 2011-2014 for occurrence of Betula humilis on the Polesie Podlaskie peat bogs (Serafin et al., 2018a). (The box-and-whisker plots show the distribution of observations. In particular, the box represents the first and third quartiles. The horizontal line across the central region of the box represents the median. The mean value of the data is marked by a filled triangle. The whiskers are drawn to the most extreme observations that are located no more than 1.5 times the inter quartile range away from the box. Any observation not included between the whiskers is considered as an outlier and is plotted with an filled circle. When there are no outliers, the whiskers indicate the minimum and maximum values. The plot presents observed values of particular parameters, marked with small dots)*

Statistical methods

The statistical analysis of the species composition of the analysed phytocoenoses and values of physical-chemical properties of peat bog waters was divided into several stages.

The first stage concerning the analysis of the species composition of phytocoenoses at the study sites employed multidimensional methods, applied for distinguishing from the studied locations groups of objects uniform in terms of species composition (cluster analysis). Cluster analysis is a multidimensional technique permitting detecting similarities between analysed objects. Detection of similarities of sites in terms of species composition involved the application of the procedure of agglomerative hierarchical classification (Borcard et al., 2011). The practical and frequently applied in

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similar analyses Jaccard coefficient (Piernik, 2008) was adopted as the measure of similarity. The key element of the analysis, affecting the results of grouping, is the choice of the grouping procedure. In spite of a high number of publications concerning the issues (among others Sneath and Sokal, 1973; Jongman et al., 1995; Legendre and Legendre, 1998; Digby and Kempton, 2014), no universal algorithm of selection of the method of grouping of data exists. The final selection is usually determined by obtaining interpretable clusters. In the case of research employing the hierarchization procedure, the algorithm of minimum variance (Ward clustering) proved the most efficient for the species composition, as well as the algorithm of complete linkage for habitats (Serafin et al., 2015a, 2018a, b). The obtained results of the classification were presented graphically in the form of a dendrogram (diagram of another connection of objects – sites or species – in clusters together with distances for the connections) or heatmaps (intensity of colour representing among others differences in the frequency of joint occurrence) – compare *Figure 3*.

The next stage involved the statistical analysis of the structure of distribution of values of abiotic properties of a habitat for particular sites. Methods of descriptive statistics were applied for this purpose. Basic measures of distribution were determined for physical-chemical water properties. Additionally, the most important position statistics were presented in the form of boxplots – compare *Figure 4*. The analysis of the diagrams permitted the assessment of data distribution, identification of potential distribution skewness, and observations of untypical data or data suspected of untypical character. Then, the distribution of the analysed factors was performed for particular sites (or selected groups of sites with similarly higher and similarly lower % contribution of the analysed species). Due to the rejection of hypotheses on normality of distribution and homogeneity of variance, the comparison employed a non-parametric Kruskal-Wallis test.

A more thorough analysis of dependencies in the data set including the species composition of the analysed phytocoenoses (species composition) and physical-chemical properties of peat bog waters (environmental variables) at particular sites was possible with the application of the multidimensional direct ordination method (Jongman et al., 1995). The most popular methods of the type include redundancy analysis (RDA) and canonical correspondence analysis (CCA). The selection of the appropriate method was done based on the value of the length of the ecological gradient obtained by means of detrended correspondence analysis (DCA) (Gauch, 1982). DCA is indirect ordination method, and is performed based on data on species composition. In the discussed study, the length of the ecological gradient showed that the structure of the analysed data is of linear character (Jongman et al., 1995). Therefore, a method suitable for further research was the redundancy analysis (RDA). Before performing the analysis, for the purpose of meeting the assumption on normality, relevant transformation of data on environmental variables and their standardisation is required (Legendre and Legendre, 1998; Borcard et al., 2011). In the case of data on species, the necessity of their transformation was also considered (Legendre and Gallagher, 2001).

Prior to the analysis, redundant environmental variables (with linear correlation coefficient $|r| > 0.6$) were omitted to avoid collinear rite (Blanchet et al., 2008). The RDA output was presented graphically in the form of a triplot where sites and particular species are presented as points, and environmental variables as arrows. In the case of analyses performed with the application of the *vegan* package (Oksanen et al., 2018), in environment R (2017), depending on the used scaling method (scaling 1 or scaling 2), it

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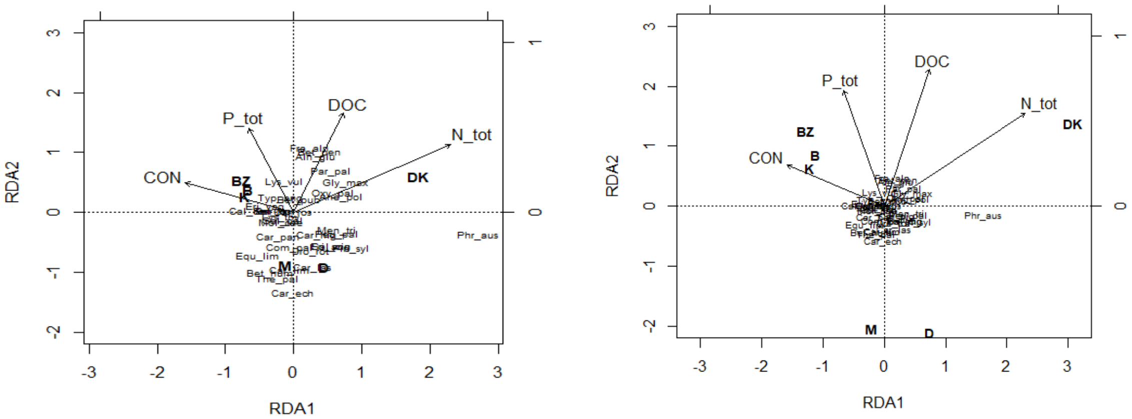
was possible to obtain two types of triplots (compare *Fig. 5*). The selection of the scaling method determined the interpretation of the obtained triplot. The triplot obtained for scaling 1, also called a distance triplot, permitted the analysis of dependencies in the context of Euclidean distances among objects. In the case of scaling 2, it is the so-called correlation triplot, where the primary goal was the assessment of the occurring correlations for environmental variables. A detailed interpretation of triplots for both scaling types was performed based on Legendre and Legendre (1998).



(a) scaling = 1 (b) scaling = 2

Figure 5. *Triplot of RDA with fitted site scores, species and environmental variables as arrows connected with occurrence of Oxycoccus palustris in the Polesie Podlaskie Region (Serafin et al., 2018b)*

In the case of analysis aimed at the detection of the structure and general patterns between the analysed physical-chemical water parameters at specific sites, indirect ordination methods should be applied. The performance of this type of analyses employed one of the oldest and most frequently applied methods, namely principal component analysis (PCA). The method was indicated by the length of the ecological gradient as suitable for the investigation of mutual relations of environmental data of the study area, irrespective if the species composition of the analysed biocoenoses, in the case of analysis of occurrence of relic species of willows (Serafin et al., 2015a, b). The PCA output was presented as a biplot where objects (sites) are presented a points, and environmental variables as arrows (*Fig. 6*). Similarly as in the case of RDA, the interpretation of biplots also depends on the selected scaling method (Legendre and Legendre, 1998).

Possibilities of interpreting results

Based on the performed procedure, competent study results were obtained permitting the following for particular plant species: 1) identification of representative study sites related to the preferences of species, analysis of association of the accompanying plants, and degree of naturalness of habitats; 2) estimation of the range of ecological tolerance in reference to the analysed habitat properties and region of occurrence of study objects, and 3) determination of statistically significant abiotic factors favouring the occurrence of their populations, and sometimes also ranges of values of the factors.

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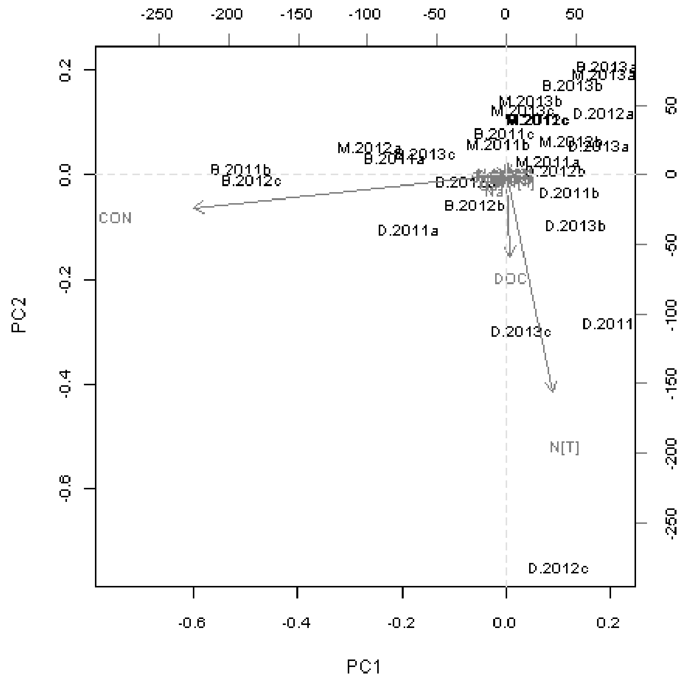

***Figure 6.** Projection of the original 14-dimensional scores onto the first two principal components for occurrence of Salix myrtilloides on the Polesie Podlaskie peat bogs (Serafin et al., 2015b). (The black labels indicate individual data items. Each variable (factor) is represented as a vector, while the direction and the length of the vector indicate how the corresponding variable contributes to a principal component)*

Study sites were designated for all plant species considered in the study in selected locations representative of the region. Their naturalness was evidenced by botanical-phytosociological analyses confirming the presence of plants from classes: *Scheuchzerio-Caricetea*, *Alnetea glutinosae*, and *Oxycocco-Sphagnetea* in the species composition of the analysed phytocoenoses strongly related to ombrogenic habitats of raised and transitional bogs, optimal in terms of occurrence of the analysed plant species (Serafin et al., 2017; Serafin et al., 2018a, b). Low values of anthropogenic coefficients of changes in the flora, high Jaccard coefficient indices, statistical hierarchical analysis of species similarity, as well as multidimensional analyses of the frequency of occurrence of characteristic species associations determined by preferences of the studied species additionally confirmed the accuracy of selection of natural study sites.

The specificity of occurrence of the studied species in reference to characteristic features of the peat bog environment in the region of Polesie Podlaskie (East Poland) permitted the determination that both boreal relics and medicinal plant species were characterised by a relatively broad amplitude of values of the majority of the studied physical-chemical properties of peat bog waters. This probably constitutes proof of the broad range of their ecological tolerance in reference to such factors and to such regional locations. The distribution of values of some of the analysed properties was favourable for the maintenance of the populations of the analysed species, whereas the distribution of the remaining ones should be considered as neutral or unfavourable. In the case of *Salix myrtilloides*, low level of values of nitrogen fractions, phosphorus fractions, studied cations, and DOC can be considered a group of conditions favouring

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the functioning of its population (Serafin et al., 2015b), whereas for the simultaneously studied *Salix lapponum*, such conditions were met by: low level of TN, phosphorus fractions and DOC, as well as a high level of Ca, pH, and EC (Serafin et al., 2015a).

For *Betula humilis*, values of statistically significant, favourable for its occurrence abiotic properties of groundwaters were provided in more detail also providing ranges of their values based on boxplots for which the upper and lower quartile referred to the Kruskal-Wallis test provide information on the range of values of physical-chemical water properties significant for the species. They included: TP: 0.08-0.32; P-PO_4: 0.1; TN: 2.2-21.2; N-NH_4: 0.1-0.46; DOC: 24.6-56.9 ($mg \cdot dm^{-3}$), as well as higher than average pH values, in a range of: 5.34-5.95; Ca: 5.67-28.1; Mg: 0.56-2.41 ($mg \cdot dm^{-3}$) m and EC in $\mu S \cdot cm^{-1}$: 72.1-142.3 (Serafin et al., 2018a).

For species of herbal plants with perspective possibility of ecological cultivation, additional research is necessary concerning the natural content of biologically active substances at natural sites of their occurrence (currently conducted). Nonetheless, also in their case, complexes of factors favouring their more abundant occurrence were determined.

For *Menyanthes trifoliata* they were - TN (N_{total}): from 4.16 to 27.4, TP (P_{total}) from 0.93 to 0.14 ($mg \cdot dm^{-3}$), EC (conductivity) from 70.4 to 112 $\mu S \cdot cm^{-1}$, and pH from 5.23 to 5.55 (compare *Fig. 7*).

Lower concentration values of important habitat parameters in the following ranges: TP = 0.17-0.36; P-PO_4 = 0.1, and DOC = 33.81-55.90 ($mg \cdot dm^{-3}$) can be considered as a state favouring proper functioning of *Oxycoccus palustris* individuals (Serafin et al., 2018b). Parameters important for the occurrence of a population of *Comarum palustre* in Polesie Podlaskie included: N-NH_4, Ca, Mg, DOC, EC, and pH (Serafin et al., in review).

The summary of the applied procedure of hierarchization of abiotic habitat properties in botanical research were PCA and RDA analyses supplementing the dependencies between groups of the studied elements: species composition of phytocoenoses, species similarity of study sites, and the values of physical-chemical properties of peat bog waters at the sites.

For example, in the case of *Salix lapponum*, the application of PCA analysis showed the highest correlations between chemical variables such as: P-PO_4-TP (0.9421), N-NH_4-TN (0.7607), and EC-pH (0.7533). It was also determined that EC, Ca, and pH were higher at site Karaśne (K) in comparison to sites Blizionki (BZ) and Lubowierz (L), and values of phosphorus fractions were higher for BZ in comparison to sites K and L, and that the separation of clusters K and L particularly results from the contribution of EC, Ca, pH, N-NH_4, TN, and DOC. Some data from sites B and L showed more uniform structure (Serafin et al., 2015a).

The analysis of data from research concerning *Oxycoccus palustris* based on the RDA method suggests that study sites located closer to each other (such as B, K, BZ, as well as D, M) have a similar species composition and similar values of habitat variables. Sites B, K, and BZ are characterised by high levels of EC and TP. Variable TN was related to the positive part of axis 1, and EC occupies the opposite part of the axis. The second axis is positively correlated with DOC and TP. The triplot suggests a positive correlation between TN and DOC, as well as a similar correlation of values between EC and TP, as well as between TP and DOC. In terms of species-habitat dependencies, the studied species was observed to be strongly positively correlated with TN (Serafin et al., 2018b).

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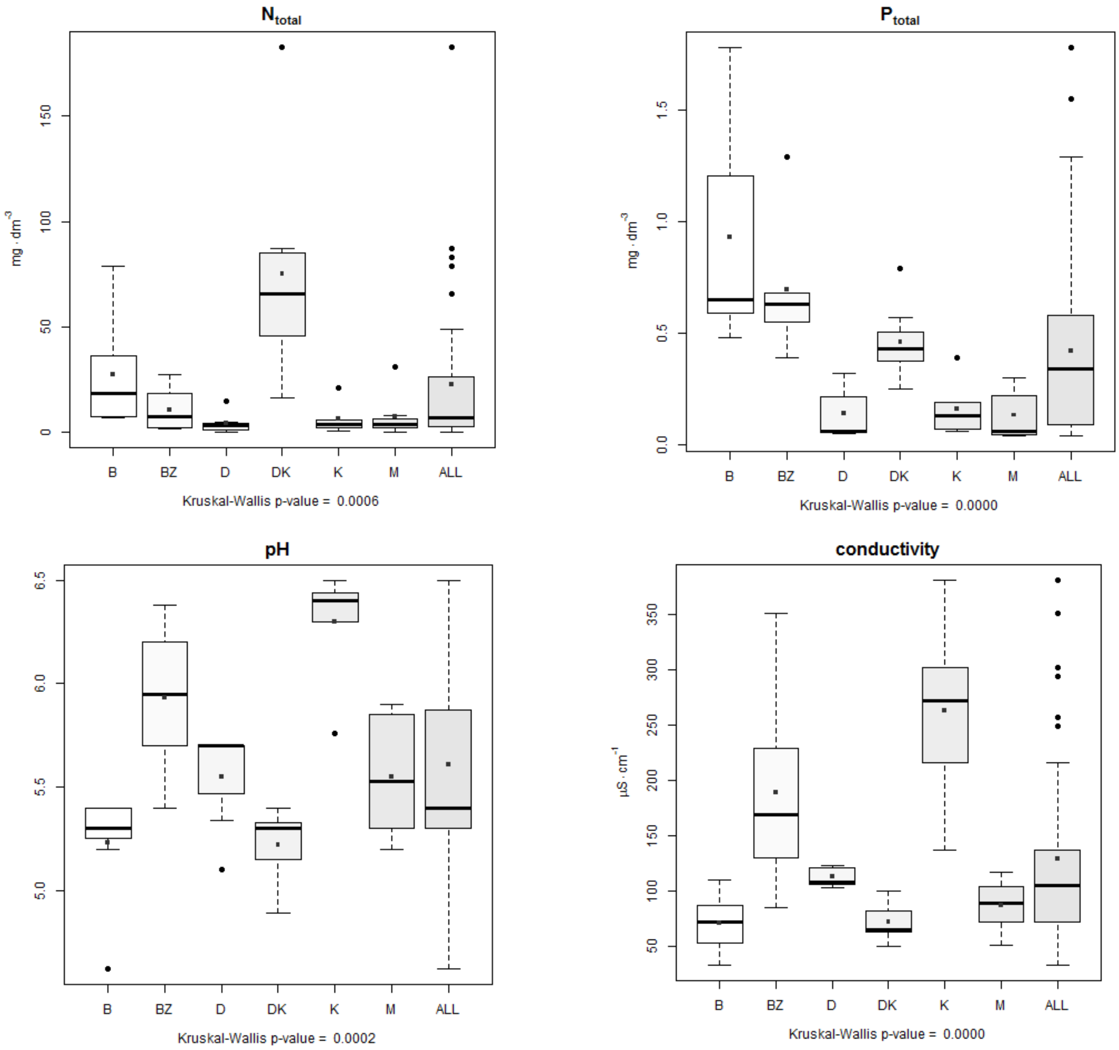

Figure 7. *Distribution of values of the investigated chemical factors of piezometric groundwater at the study sites in the period 2011-2013 (Serafin et al., 2017). (The box-and-whisker plots show the distribution of observations. The bottom and top of the box indicate the first and the third quartiles, respectively. The horizontal line across the central region of the box represents the median. The mean value of the data is marked by a filled square. The whiskers are drawn to the most extreme observations that are located no more than 1.5 times the inter quartile range away from the box. Any observation not included between the whiskers is plotted as an outlier with an circle. When there are no outliers, the whiskers indicate the minimum and maximum values. The abbreviations of the selected study sites are used to label tick marks on the x - horizontal axis. The x axis label provides the p-value derived from the Kruskal-Wallis test)*

Summary and conclusions

The modern dynamics of ecological patterns of different ecosystems require complex research methods accompanying the course of selected ecological phenomena and processes. Unlike in the case of comparative and reconstruction research, it is usually long-term, stationary research (in selected locations), also called process accompanying research. After a certain time, it provides material for many scientific fields, constituting the basis of essential source texts used in the planning and implementation of various research. In the case of the field of science based on botanical ecological analyses –

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dynamics of vegetation also called syndynamics – the basis for practical research methods was provided by among others Braun-Blanquet (1922); Collier et al. (1973, 1978); Faliński (1986); Burrows (1990); or Dierschke (1994). Irrespective of the character of the performed analyses, their success usually depends on three factors: consistency of interest, selection of the object, and continuity of financing (Faliński, 2001).

The procedure of hierarchization of abiotic habitat properties for botanical analyses in natural peat bog conditions meets all three conditions. It is based on constant interest in the study issue – particularly the occurrence of boreal relics in natural, although subject to increasing human pressure (global – climate change, and regional – impact of the Lublin Coal Basin, effect of the Melioration System if the Wieprz-Krzna Channel, as well as tourism and recreation in the Łęczna-Włodawa Lake District) habitats of Polesie Podlaskie (East Poland). Supplementary botanical research also focused on the conditions of occurrence of valuable species of herbal plants, and those with a perspective of ecological cultivation.

The selection of study objects resulted from conscious scientific interest of the authors particularly concerning boreal species of willows: *Salix lapponum* and *Salix myrtilloides*, subject to research already from the early 2000's (e.g. Pogorzelec, 2008, 2009). In the case of selected species of herbs: *Menyanthes trifoliata*, *Oxycoccus palustris*, and *Comarum palustre*, further research concerning the composition of biologically active substances in herbal material is currently conducted for different sites, and their occurrence in the aspect of performing an experiment of their ecological cultivation.

Eventually, financing of the said research was on the one hand related to resources available in the scope of project of NCN No. NN304385239: Population ecology and active protection of boreal relics from the *Salicaceae* (*Salix lapponum* and *Salix myrtylloides*) in Polesie Lubelskie Region, under the direction of D. Ph. Magdalena Pogorzelec, implemented in the years 2010-2014, and on the other hand to the statutory activity of the Department of General Ecology University of Life Science In Lublin (Poland), where Ph. D. Artur Serafin and Ph. D. Magdalena Pogorzelec were employed at the time.

The primary effect of the developed procedure are the possibilities of its practical application. In the scope of the restitution of the said species of willow in Polesie Podlaskie based on a project entitled: Active protection of particularly endangered species of relic plants from family Salicaceae in peat bog habitats (supervision Ph. D. Magdalena Pogorzelec), measures of their active protection have been implemented since 2018, involving their reintroduction. Places of introduction of new individuals of both species obtained from ex situ cultivation were optimised as habitats in terms of values of the analysed physical-chemical properties of peat bog waters in accordance with results of research based on the procedure described above.

In the case of the studied herbs, the perspective of their ecological cultivation depends on solving several issues. Firstly, in natural conditions of peat bogs, obtaining herbal material faces difficulties concerning the terrain conditions and safety of persons collecting plants. Secondly, part of herbal species are under species protection, and their collection is treated as an offence (*Menyanthes trifoliata*). Thirdly, the most natural habitats of occurrence of the plants are located in areas subject to different forms of areal protection (e.g. national park, landscape park, nature reserve, or ecological grounds), therefore sanctioning the possibility of collection of any plants in different

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DOI: http://dx.doi.org/10.15666/aeer/1702_40814096

ways. Finally, as a result of hydrotechnical economy, peat bog ecosystems are subject to shrinking in the territory of the majority of European countries (Ilnicki, 2002), resulting in a real threat of extinction of many species of plants related to this type of habitats. Ecological cultivation of medicinal species in optimal conditions of their natural occurrence provides a solution to the aforementioned problems. A very important issue in this aspect is the search for natural compositions of biologically active substances in herbal material for the purpose of development of a natural herbal medicine in accordance with the rules of the Good Manufacturing Practice (GMP) pursuant to the guidelines of the WHO (Borkowski, 1994; Drozd, 2012). It is particularly important in the times of search for different elements and measures limiting environmental impact and important for human health, especially in the context of naturotherapy, constituting a desirable alternative to synthetic pharmacology and dangerous polypharmacy (Oliveira et al., 2012; Senderski, 2015; Serafin et al., 2018b). Currently conducted research employing the procedure of hierarchization of abiotic properties of habitats for studied herb species preliminarily confirms higher values of biologically active substances from locations characterised by optimised natural values of the analysed physical-chemical water properties (Serafin, Dzida, unpublished data).

Due to the above, the developed procedure finds practical application both for active protection of endangered plant species and in the case of useful plants.

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DOI: http://dx.doi.org/10.15666/aeer/1702_40814096

GREEN SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES (AgNPs) FROM MAIZE (*ZEA MAYS* L.)

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(Received 4th Dec 2018; accepted 27th Feb 2019)

Abstract. In recent years, the biosynthesis (green synthesis) of metal nanoparticles such as silver nanoparticles (AgNPs) have become one of the safest, most cost-effective and environmentally friendly approaches. In this study, AgNPs were synthesized using maize (*Zea mays L.*) leaves. For the characterization of synthesized AgNPs different techniques were used, such as X-ray diffraction spectroscopy (XRD), Ultraviolet visible (UV-Vis) spectroscopy, transmission electron microscopy (TEM), Energy dispersive X-ray spectroscopy (EDX), Fourier-transformed infrared spectroscopy (FT-IR) and Thermal gravimetric and Differential thermal analysis (TGA-DTA). The XRD results showed that AgNPs had a mean diameter of 12.63 nm and a crystal-like appearance. In addition, antimicrobial activities of synthesized AgNPs were evaluated using 3 different antibiotics against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* bacteria and *Candida albicans* yeast. Antifungal activity of AgNPs with antibiotics has been observed to be better than the antibiotics against Gram-positive and Gram-negative bacteria. The minimum inhibitory concentrations were found to be 0.084, 0.337 and 0.021 mg mL^{-1} for *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, respectively. The results revealed that AgNPs synthesized from maize leaf extract have antibacterial activity against Gram-negative *Escherichia coli*, Gram-positive *Staphylococcus aureus* and antifungal activity against *Candida albicans* yeast, and that the produced AgNPs could be used in the production of biomedical products and in the pharmaceutical industry.
Keywords: *antimicrobial activity, green AgNPs, Escherichia coli, Staphylococcus aureus, Candida albicans*

Introduction

Scientific research and applications of nanotechnology have increased rapidly in recent years. The properties of nanoparticles such as durability, high diffusion, and versatile chemical and biological activities have gained importance in technological applications (Nabila and Kannabiran, 2018). Metal nanoparticle synthesis researches have been increasing in number due to the potential applications in nanotechnology (Isaac et al., 2013). Nanoparticles are used in many industrial fields, especially in electrical, biomedical, automotive and chemical sectors due to their superior properties (Gürmen et al., 2008). Generally, nanoparticles are defined as particles smaller than 100 nanometers (nm) (Kreyling et al., 2006).

The green technology used in the production of metal nanoparticles has become popular in recent years because of its being rapid, easy, cheap, sustainable and eco-friendly. Green synthesis, as an alternative to physical and chemical synthesis studies

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_40974105

for the production of AgNPs, is considered as an environmentally friendly and cost-effective method used by researchers from many different fields of science (Banerjee et al., 2014; Nartop, 2016; Majeed et al., 2018). In the biological synthesis of nanoparticles, organisms such as bacteria, fungi, seaweeds, plants, yeasts, and viruses are used (Khan et al., 2018). Among these organisms, plants are considered to be the most preferred organisms when they are evaluated in terms of cost and availability. In literature, antibacterial activity of metal or metal oxide such as zinc oxide nanoparticles (ZnO-NPs) has received significant interest worldwide (Gunalan et al., 2012). The most synthesized metal nanoparticles are copper (Cu), silver (Ag), gold (Au), zinc (Zn), nickel (Ni) and palladium (Pd). Edison and Sethuraman (2012) stated that the synthesized Ag and Au nanoparticles by *Magnolia kobus*, *Diospyros kaki*, *Ficus benghalensis* and *Citrus lemon* plant extracts are stable products. In studies with plants, the greatest phytochemicals, tannins, gallic acid, and gallate esters can be easily hydrolyzed (Swamy et al., 2015; Nabila and Kannabiran, 2018).

In this study, the plant extract obtained from maize plant leaves was used for the reduction of Ag^+ ions which are the basis of the synthesis of AgNPs to Ag^0 ions. Antimicrobial activity was evaluated by characterization of the obtained AgNPs. The green technology used in the synthesis of AgNPs in this study is cheaper, easier and faster than other applications.

Materials and Method

The plant extract used in the study was obtained from maize (*Zea mays* L.) plant. In the synthesis method, silver nitrate ($AgNO_3$, purity 99.8%) was used. The used antibiotics (Fluconazole, Vancomycin, and Colistin) were obtained commercially.

Preparation of plant extract

Collected maize leaves from Mardin region in Turkey were washed with deionized water, then dried under room conditions and afterward 50 grams of the ground samples were taken and boiled at 85°C with 500 mL of distilled water. After the formation of the extract, the sample was left to cool until reached the room temperature. Subsequently, filtration was performed using Whatman filter paper and the filtered extract was stored at 4°C.

Synthesis of nanoparticle

In general, the formation of AgNPs is determined by spectrophotometric measurements based on color change and time (Swamy et al., 2015; Jamdagni et al., 2016). A 1 mM $AgNO_3$ aqueous solution was used for the synthesis of AgNPs. In total, 125 mL of maize plant leaf extract and 500 mL of $AgNO_3$ solution were put into the reaction at room temperature in a 1000 mL flask. At the end of 40 minutes, the reduction of silver ions resulted in the change of light-colored Ag^+ ions to dark brown and the maximum absorbance value was determined at 461 nm wavelength by UV-Vis spectroscopy. The resulting dark solution was centrifuged at 6000 rpm for 20 min to remove the upper liquid phase and the remaining solid was washed seven times with distilled water. The obtained AgNPs were kept in a laboratory oven at 75°C for 24 hours and then left to dry and stored until the application of characterization tests.

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Characterization techniques

Ultraviolet spectra of synthesized AgNPs were determined in the UV-1601 220V Shimadzu spectrophotometer in the wavelength range of 350-800 nm. Size and morphology of AgNPs were determined using EVO 40 LEQ scanning electron microscopy. The crystal structures of AgNPs were analyzed with RadB-DMAX II computer-controlled X-ray diffractometer in the $3^o \leq 2\theta \geq 79^o$ range. SEM-energy dispersive X-ray (SEM-EDX) spectroscopy was used to verify the presence of AgNPs in the elemental composition. FT-IR analysis was performed in the 4000-400 cm^{-1} range with the Perkin Elmer spectrum one-brand device to determine which functional groups were involved in the reduction of plant extracts. Decomposition temperatures of AgNPs were determined by thermal gravimetric analysis at a constant heating rate (Shimadzu TGA-50) at 25°C min^{-1} (25°C-900°C) under the nitrogen atmosphere.

The evaluation of XRD phase and crystal structure analysis of green synthesized AgNPs is shown in *Figure 1*. The reflections (111), (200), (220) and (311) of the spherical structures of the AgNPs were calculated in the XRD analysis by 2θ (38.01°, 45.83°, 64.29° and 77.34°) values. It was determined that AgNPs have elemental (Ag^o) and spherical crystalline structure. The mean crystal size was calculated as 12.63 nm with the following Debye-Scherrer equation (Kanipandian and Thirumurugan, 2014):

$$D = K\lambda / (\beta \cos\theta) \quad \text{(Eq.1)}$$

In the equation, D is particle size (nm), K is constant (0.90), λ is wavelength X-ray (1.5406 °A), β is half of the highest peak value (FWHM) and θ is the refractive angle.

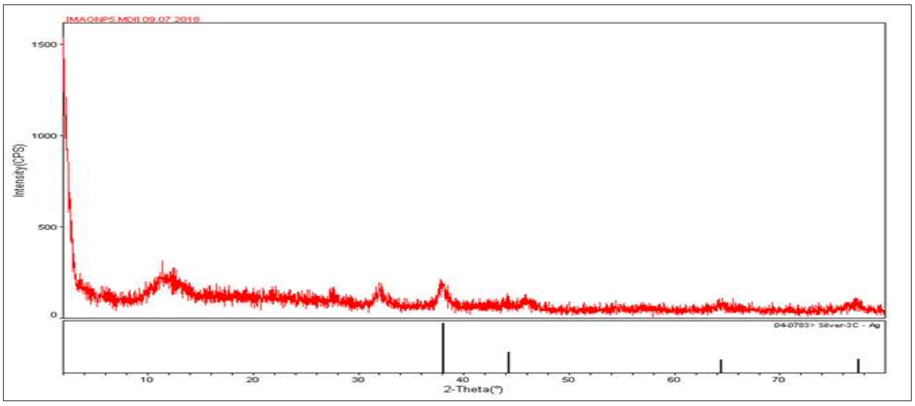

Figure 1. *XRD spectra of green method synthesized AgNPs*

Antimicrobial activity of silver nanoparticles

The antimicrobial assays were performed by the minimum inhibitory concentration (MIC) method (Wang et al., 2017). For this purpose, AgNPs were incubated overnight with Gram-positive (*S. aureus* ATCC 29213) and Gram-negative (*E. coli* ATCC 25922) bacterial and fungal (*C. albicans*) strains. Experiments were performed by the microdilution method and the predetermined medium was added to 96-well microplates. Following the preparation of a dilution series at adjusted concentrations, AgNP solutions were added on these dilutions.

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Then, the solutions including a certain amount of microorganism were prepared according to 0.5 McFarland standard, added onto the samples and the samples were incubated at 37°C overnight. After incubation, the lowest concentration without growth was determined as MIC (Nadaroglu et al., 2017; Abinaya et al., 2018). All experiments were repeated with four replicates per treatment.

Results and discussion

Analysis of ultraviolet-visible spectroscopy

The formation of AgNPs with samples taken at 5, 10, 15, 20, 25, 30, 35 and 40 minutes was observed with UV-Vis spectroscopy analysis. The synthesized AgNPs showed a sharp plasmon resonance at a maximum of 461.25 nm (*Fig. 2*). Similarly, AlQahtani et al. (2017) determined the surface plasmon resonance of AgNPs at 468.5 nm. Furthermore, the absorption spectra of AgNPs in the reaction medium were indicated to be in the maximum absorption in the range of 425 to 475 nm due to surface plasmon resonance of AgNPs (Banerjee et al., 2014).

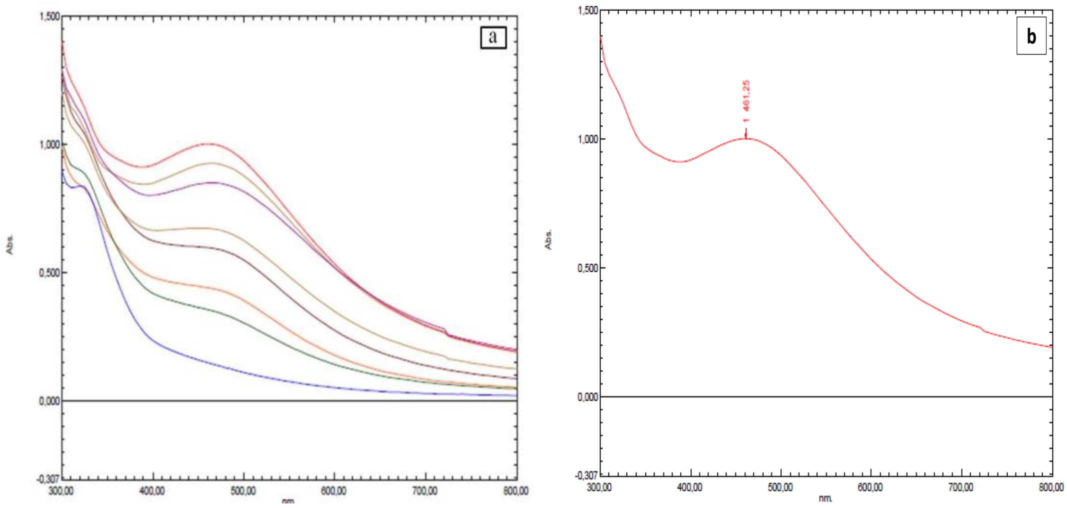

Figure 2. *a) Time-dependent formation of AgNPs in UV -Vis spectroscopy, b) Maximum absorbance value of synthesized AgNP in UV-Vis Spectrophotometer*

Fourier-transformed infrared spectroscopy analysis

The infrared spectrum in *Figure 3.* provides information about the functional groups of the synthesized AgNPs from maize leaf extract. The strong asymmetric flexural band of 3326.99-3321.54 cm^{-1} belongs to the -OH functional group on the leaf extract surface of the maize shifted due to the interaction between the extract and the silver metal. A frequency shifting of about 5 cm^{-1} occurred at the peaks around 3326.99 cm^{-1}. In the strong asymmetric stretch band at 2103-2106 cm^{-1}, a 3 cm^{-1} frequency shifting occurred between C≡C and C≡N groups. It was found that there was no considerable shifting in the 1636.29-1636.01 cm^{-1} band of the C-C groups belonging to the Amide I band and C-N functional groups and also the binding occurred on these three functional groups.

Shah et al. (2017) produced AgNPs by the interaction of $AgNO_3$ with an aqueous solution containing *Xanthorrhoea glauca* leaf extract and identified the peaks of carboxy

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and amide groups at 1642, 1556, 1536. They reported that the AgNPs demonstrated varying degrees of antibacterial activity against *Escherichia coli* and *Staphylococcus epidermis*, with *Staphylococcus epidermis* showing a larger zone of inhibition (11 mm).

The FT-IR spectrum measurements of AgNPs synthesized by Huang et al. (2007) showed the presence of five dense bands at 3422, 2922, 1629, 1381 and 651 cm^{-1}. It has also been stated that the maximum shifting was at 3422 cm^{-1}. In another study, it has been reported that the -OH stretch at 3430 cm^{-1} belongs to the functional groups of –C–N (peptide bone) at 2083 cm^{-1} and C-C at 1641 cm^{-1} and also the binding takes place on these two functional groups (Singh et al., 2017).

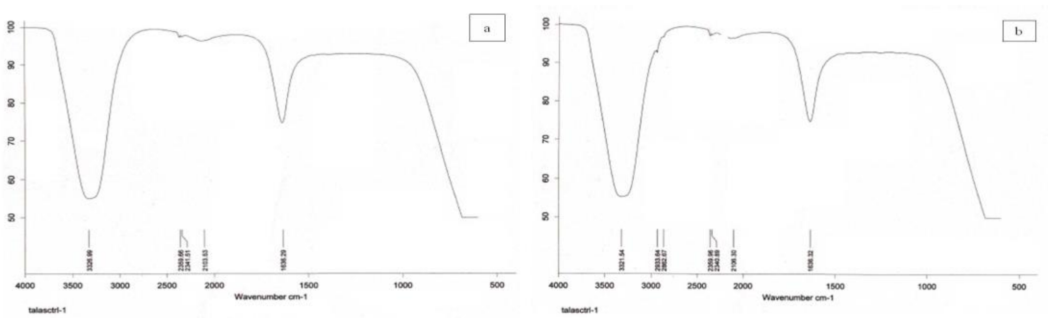

***Figure 3.** a) UV-Vis spectrum of plant extract, b) UV-Vis spectrum of synthesized AgNPs*

Scanning electron microscopy and energy dispersive X-ray spectroscopy analysis of silver nanoparticles

Morphological characteristics of AgNPs obtained from leaf extract of maize were investigated with scanning electron microscopy (SEM). The results clearly demonstrated the presence of spherically shaped AgNPs below 100 nm (*Fig. 4*).

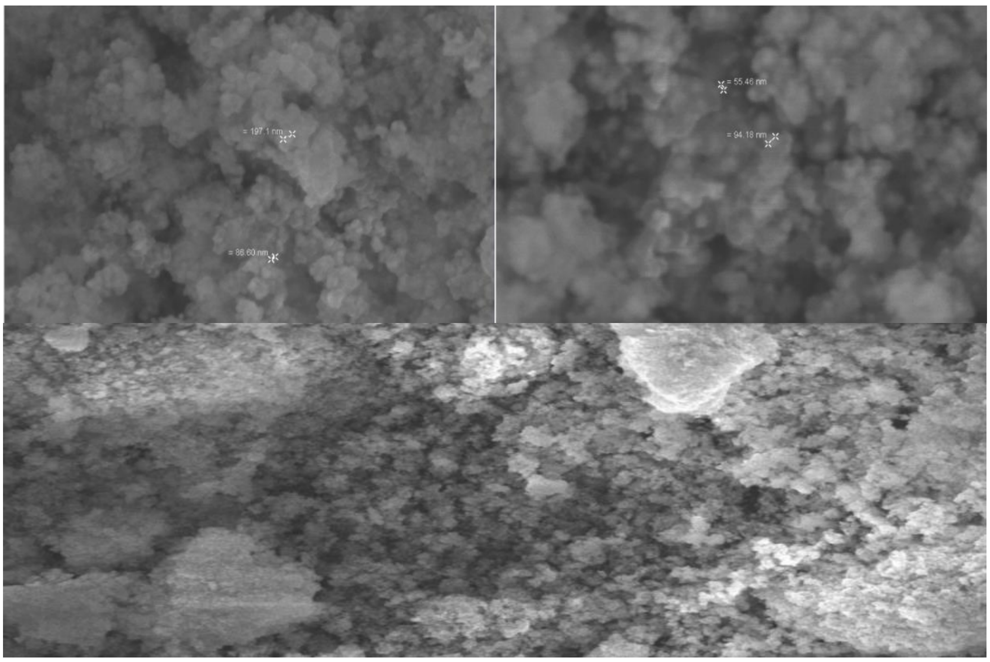

***Figure 4.** Images of SEM analysis of synthesized AgNPs*

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Nanoparticles produced with green synthesis by Phanjom et al. (2012) from *Myrica esculenta* resulted with different morphologies ranging from 45 nm to 80 nm and the Ag nanoparticles obtained from *Ziziphus nummularia* plant leaves by Khan et al. (2016) had spherical and homogenous shapes with diameters ranging between 30 and 85 nm. In similar SEM analysis studies, the size and morphological characteristics of AgNP are consistent with the results of this study (Swamy et al., 2015).

When the obtained results of this study were compared to the results of the study belonging to Kotakadi et al. (2013), it was seen that AgNPs have a spherical shape and the diameters of AgNPs varied between 27 and 50 nm. As can be seen in *Figure 5*, the energy-dispersed spectra of AgNPs obtained from the SEM-EDX analysis revealed pure silver particles.

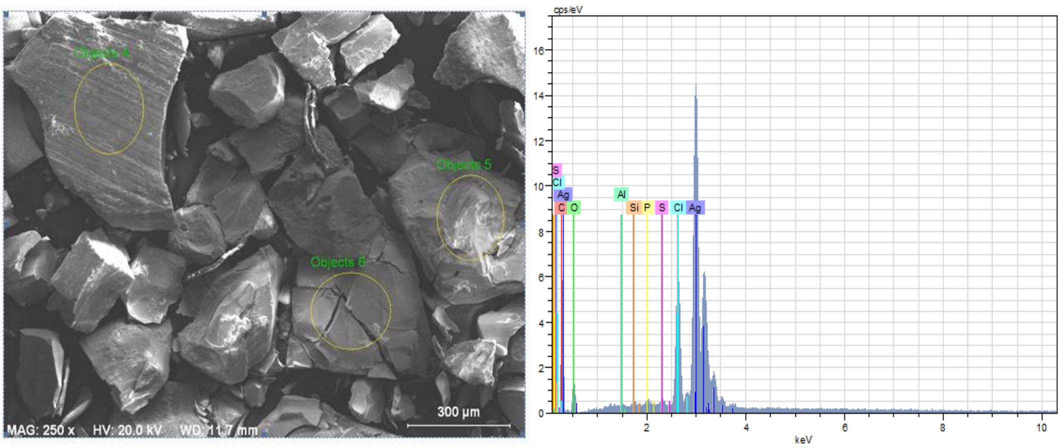

Figure 5. *SEM-EDX spectrum of AgNPs*

Thermal gravimetric and differential thermal analysis results

The TGA and DTA analyses of the nanoparticles prepared by green synthesis were evaluated between 25-900°C with a heating rate of 10°C min^{-1} and a flow rate of 20 mL min^{-1} in N_2 (g) atmosphere. The TGA curve indicates the mass loss due to thermal decomposition and the DTA curve shows the maximum decomposition temperature at each stage of the degradation (Baran, 2017).

As can be seen in *Figure 6*, the loss of mass at 37-100°C was caused by moisture, the loss of mass at 186-309°C was due to the cellulosic materials, and the loss of mass at 309-610°C was caused by phytochemicals from a plant extract. Finally, the substance was completely degraded at 610-900°C. The TGA data revealed that the mass loss of AgNPs synthesized in the study was between 50-800°C. In a similar study, when the TGA curve of AgNPs was examined, it was reported that there was a constant mass loss in the 50-800°C temperature range and this loss was due to the desorption of bioorganic compounds (Ali et al., 2015).

Antimicrobial assays of synthesized silver nanoparticles

In this study, the antimicrobial effect of AgNPs obtained from maize leaf was investigated by MIC method. The possible antibacterial effects of AgNPs on Gram-negative *E. coli* ATCC 25922 and Gram-positive *S. aureus* ATCC 29213 strains and antifungal activities on *C. albicans* were investigated. MIC values of AgNP and 1 mM

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DOI: http://dx.doi.org/10.15666/aeer/1702_40974105

silver nitrate were determined. The antimicrobial effect results of the produced AgNPs on *E. coli* ATCC 25922, *S. aureus* ATCC 29213 bacteria and *C. albicans* yeast were obtained as 0.084, 0.337 and 0.021 µg mL^{-1}, respectively (*Table 1*).

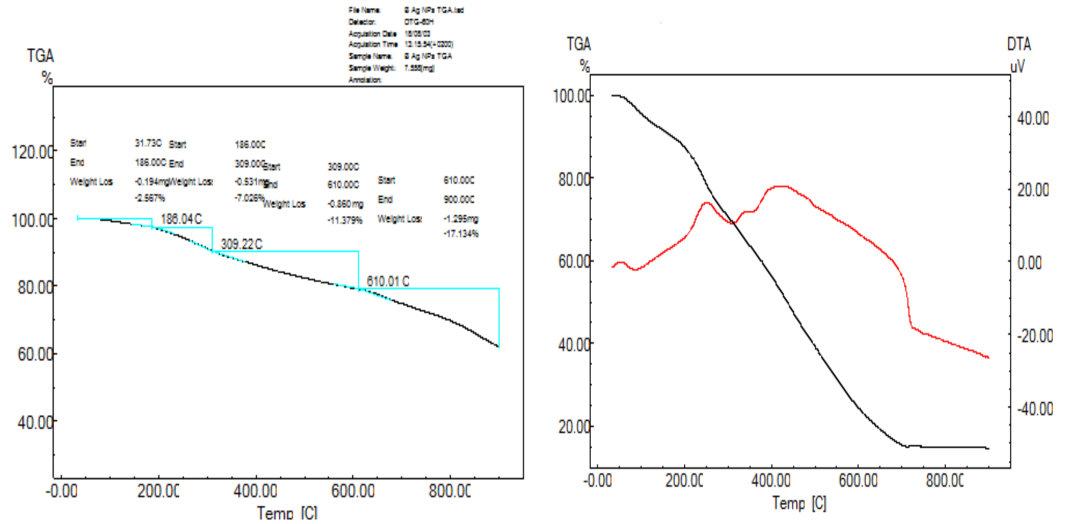

Figure 6. *TGA-DTA analysis result of the synthesized silver nanoparticle*

Table 1. *Minimum inhibitory concentration (MIC) values of AgNPs (µg mL^{-1})*

Organism	AgNP	Silver Nitrate	Antibiotic
***S. aureus* ATCC 29213**	0.337	0.500	0.500
C. albicans	0.021	0.500	0.500
***E. coli* ATCC25922**	0.084	1.000	0.125

Table 1 shows that AgNPs exhibit a more pronounced activity than silver nitrate. Wang et al. (2017) reported that AgNPs synthesized from green coffee grains showed excellent antibacterial activity against both Gram-negative and Gram-positive bacteria. Tippayawat et al. (2016) stated that the minimum inhibition concentration of AgNPs which they synthesized from *Aloe vera* plants was effective.

In another study, the nanoparticles synthesized with *S. potatorum* leaf extract were shown to be effective against on MDR human pathogenic bacteria namely, *S. aureus* and *K. pneumonia* (Kagithoju et al., 2015). Similar observations were reported on *Boswellia ovalifoliolata* and *Shorea tumbuggai* (Savithramma et al., 2011). Therefore, this study shows that the biosynthetic AgNPs have antimicrobial activity.

Conclusions and recommendations

Nowadays, different antimicrobial agents are being developed to be used on bacteria and yeasts which are resistant to antibiotics that still are in use in medicine and pharmacy. Therefore, many researchers have concentrated their studies on this field. In this study, the synthesis of AgNPs was performed by using maize plant extract. It has been determined that the synthesized AgNPs by maize leaf extract have a spherical structure. The synthesized AgNPs were characterized by UV, XRD, SEM-EDX, TGA-DTA and FT-IR methods. It is known that green synthesis is a cheap, simple and environmentally

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DOI: http://dx.doi.org/10.15666/aeer/1702_40974105

friendly method and the biosynthesis of AgNPs is an alternative method to the chemical synthesis method. Furthermore, AgNPs produced by biosynthesis demonstrated a good antimicrobial effect on different strains.

Green-synthesized AgNPs will open up a new field for the production of pharmaceutical products, the biomedical and industrial products in the pharmaceutical industry.

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DETERMINATION OF DROUGHT RESISTANCE OF SOME DURUM AND BREAD WHEAT CULTIVARS WITH AGRICULTURAL AND PHYSICOCHEMICAL PARAMETERS

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(Received 15th Nov 2018; accepted 14th Feb 2019)

Abstract. The aim of this study is to demonstrate the effects of water shortage, which may occur in different periods, on wheat grain yield and quality. One of the most important factors limiting wheat yield in the Southeastern Anatolia Region of Turkey is drought, which is caused by inadequate precipitation and irregular distribution over the year. Four different bread and durum wheat cultivars (Sarıçanak-98, Fırat-93, Pehlivan, Ceyhan-99), which are widely cultivated in the region, were used as the material of the study. The experiment was carried out with 3 replications, according to the random blocks design. The research was carried out under greenhouse conditions. The effects of four different drought conditions on the wheat's development, yield and mineral content were investigated under irrigation (K0), early drought (K1), late drought (K2) and full drought (K3). According to the results, a 9.12% loss in chlorophyll content was observed under irrigated conditions (K0). 21.68% leaf area, 28.98% plant green area, and 52.09% plant height declines were recorded. The amount of potassium (K) was found to change between 2.86 mg (K3) and 34.77 mg (K2), while the amount of calcium (Ca) changed between 0.67 mg (K3) and 33.23 mg (K3) and the amount of sodium (Na) changed between 0.20 mg (K1) and 27.05 mg (K0) in the root and stem of plants.
Keywords: *wheat, drought, drought applications, drought parameters, chlorophyll content, agricultural and biochemical characteristics*

Introduction

Most of the agricultural areas in the world are affected by different stress factors. Drought stress affects these areas the most at a rate of 26%, mineral substance stress ranks the second, affecting them at a rate of 20%, and cold and frost stress rank the third with 15%. Aside from these, 29% are affected by other stress factors and 10% are not under any stress (Kalefetoglu and Ekmekçi, 2005). In areas where there is drought risk, the decrease in the water amount available in the soil primarily leads to a reduction in the water potential of the plant. It is well-known that turgor pressure drops, stomas are closed, and leaf development and photosynthesis is decreased in the following stages (Monti, 1986).

It was reported that 55% of the wheat cultivation areas throughout the world are affected by drought stress periodically (Richards et al., 2010). The wheat yield in affected areas less than between 50% to 90% of yield potential of irrigable conditions (Reynolds et al., 1999). It would be useful to develop new and multidisciplinary methods together with classical cultivation methods used for the development of drought-tolerant wheat cultivars.

In Turkey, in the development studies for the drought-tolerant wheat cultivars, generally the "earliness" feature of the plants was used. The height of the plant and leaf

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

width are used as selection criteria. In later generations, drought-resistant cultivars are cultivated as a result of the yield level and "decisiveness". The severity of drought is a serious danger for the Southeastern Anatolian Region of Turkey. The reason of the danger varies depending on years, and it is not known how long the drought stress will last during the development of the wheat and how long its duration will be. In this case, the feature of "earliness" is not adequate for the yield in the conditions of the region, and genetic and some physiological properties are important in determining the resistance to drought. For this reason, it is desired that the cultivars that are developed have a yield potential which are capable of making use of the formation of optimum conditions meanwhile maintaining the yield at a certain rate compared to the sensitive plants when exposed to drought stress.

The yield of the cultivars that are not sensitive to drought stress stays the same even in the most suitable development conditions. The lack of photosynthesis area is shown as the reason for this. Due to this fact, in order to increase the yield, it is necessary to have high photosynthesis capacity as well as physiological durability. The efforts spent on developing a strong wheat variety require physiological tests which are inexpensive, simple, and repeatable and which may be considered as selection criteria with morphological parameters.

In Turkey, wheat plant is cultivated in conditions based on precipitation at a rate of 80%. The total rainfall is not adequate in dry agricultural areas where most of the total rain falls between November and April, and because of the irregular distribution, dry periods in different development stages cause the yield to be reduced.

The purpose of this research is to determine the endurance of bread and durum wheat cultivars produced in the Southeastern Anatolian Region of Turkey by using agricultural, quality and physicochemical parameters under different ecological conditions, which may be seen in different plant growth periods.

Materials and methods

This study was conducted in Dicle University, Agricultural Faculty, Field Plants Department, Medicinal and Aromatic Plants, Open Green House conditions in 2015-16 and 2016-17 cultivation periods. The greenhouse in which the study was performed was open in both sides and the top part was covered; and did not have any limiting factors that hindered air, light and wind. Two bread wheat (Pehlivan, Ceyhan-99) and 2 durum wheat cultivars (Sarıçanak-98, Fırat-93), which are cultivated widely in the region were used in the study as the study material. Fırat-93 and Pehlivan control cultivars have been produced in the region for many years, and have proven themselves to the drought and other negative conditions of the region. Ceyhan-99 and Sarıçanak-98 varieties are cultivated in large areas in the region. The cultivation of wheat in the province of Diyarbakır is made entirely in autumn. All cultivars used as materials were cultivated in autumn. Pehlivan is a kind of biological winter. The study was carried out according to the experimental design of random blocks with 3 replications.

In the Southeastern Anatolian Region of Turkey, precipitation is seldom in summer season. The relative humidity in the air is also quite low. The annual precipitation average of the region has been 485.7 mm and the relative humidity has been around 58% for many years. The trial soils in which the study was carried out is clayey and loamy; and the salt level is low. It is rich in potassium (0.42%), slightly alkaline (pH 7.77), normal limy (7.81% $CaCO_3$) and poor in organic matter (1.67%).

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DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

The pots which were used in the study were 0.085 square meters each (Diameter: 34 cm, height: 60 cm) and had 18 kg soil volume. The seeding process was done as 12 seeds per pot. After the seeding was done in mid-November as 450 seeds/m^2, the first irrigation for all of the pots was done for germination. In October, the pots were added 20 kg (DAP 18:46) and urea (46% N) as top-fertilizer.

Drought applications as follows: **Fully Irrigated Conditions (K0):** The plants were irrigated from the time of planting to maturity, when about 40% of the available water in the soil was consumed. **Early Drought (K1):** The plants were not irrigated and irrigation was avoided from the time when the second node appeared on the plant to the beginning of the *milking stage*. **Late Drought (K2):** The plants were grown in irrigated conditions until the beginning of the *milking stage*. Irrigation was avoided from the beginning of milking stage to the harvesting period and irrigation was not carried out. **Full Drought (K3):** Irrigation was avoided from the 2nd node on the plant base until the harvest time. No irrigation was made. Drought applications were determined based on "Feekes" development period scala values by using the gravimetric method (Zadoks et al., 1974; Cook and Veseth, 1991).

In this research, vegetative characters such as leaf area and index, plant green area, chlorophyll content, plant height, generative characters such as number of grain, grain weight and grain yield and some drought parameters were tried. Leaf area consists only of leaf (lamina) and leaf sheath (vagina), the plant green area consists of the sum of the fields of spike, leaf organs, node and internodes of stem.

In this study, the endurance to drought parameters like the yield, quality and mineral substance contents, Potassium, Calcium and Sodium Concentration in green parts and roots (mg/kg dry weight). Fresh leaf samples were washed with 0.1 % HCl and in pure water and then dried at 70°C for 48 hours. 200 milligrams of these samples were incinerated for 5 hours at 550°C in an ash furnace. The ash obtained was dissolved in 3.3% HCl and filtered using blue tape filter paper (Bremner, 1965). In the obtained filtrate, K, Ca, Na concentrations were determined by atomic absorption device (Atiunicam 929) in solution.

The chlorophyll content was measured in the 3rd and 4th leaves of plants by the SPAD meter device. the readings were made with the Minolta brand SPAD meter and, the chlorophyll content units were given as SPAD value.

With the data that were obtained as a result of the study, and by using the JUMP 7.0 Statistics Program, variance analysis was made, and the differences between the average scores were evaluated with the LSD Test.

Results and discussion

In this study, in terms of wheat cultivars, it was found that the highest leaf area average was in Ceyhan-99 variety with 28.70 cm^2; and the lowest leaf area average score was in Sarıçanak-98 variety with 25.18 cm^2. The highest leaf area in irrigated conditions (K0) was 31.36 cm^2, followed respectively by late drought application (K2) with a decrease of 13.23%; full drought application (K3) with a decrease of 16.77%; and early drought application with a decrease of 21.68% (*Table 1*).

In terms of the cultivars, the highest plant green area was determined in Pehlivan with 128.16 cm^2 and the lowest green area average was in Sarıçanak-98 with 110.85 cm^2. In terms of drought applications, the highest green area value was determined in irrigated conditions (K0: 138.7 cm^2), followed respectively by late

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

drought application (K2) with a decrease of 5.54%; early drought application (K1) with a decrease of 16.46%; full drought applications (K3) with a decrease of 28.98% (*Table 1*).

Table 1. *The average values of Leaf Area (cm^2), plant green area (cm^2) and the formed groups according to the LSD (least significant difference) test*

	Leaf Area (cm²)					**Plant Green Area (cm²)**				
Cultivars	**K0**	**K1**	**K2**	**K3**	**Average**	**K0**	**K1**	**K2**	**K3**	**Average**
Ceyhan-99	30.65ab	27.01cde	31.16ab	25.99def	28.70	125.6b-e	107.9e-g	157.6a	96.9g	122.0A
Pehlivan	34.23a	25.26def	23.80ef	25.54def	27.21	139.6a-c	142.5ab	134.5bc	96.0g	128.2A
Fırat-93	30.55bc	26.07def	30.63abc	25.33def	28.15	133.4bc	109.0d-g	122.5c-f	127.8b-d	123.2A
Sarıçanak-98	30.01bc	19.88g	23.26fg	27.56bcd	25.18	156.3a	104.2fg	109.6d-g	73.38h	110.8B
Average	31.36A	24.56C	27.21B	26.10BC		138.7A	115.9B	131.0A	98.5C	
LSD (C)	ÖD					7.14**				
LSD (D)	1.82**					9.45**				
LSD (C)x(D)	3.63**					18.89**				

It was determined that the highest leaf area index average was in Pehlivan with 2.06 and the lowest leaf area index average was in Sarıçanak-98 with 1.57. In terms of drought applications, it was determined the highest leaf area index value was 3.05 in irrigated conditions, followed respectively by late drought application (2.15), early drought application (1.32) and full drought applications (0.88) (*Table 2*).

Table 2. *The average values of Leaf Area Index, Chlorophyll Content (SPAD) and the formed groups according to the LSD (least significant difference) test*

	Leaf Area index					**Chlorophyll Content (SPAD)**				
Cultivars	**K0**	**K1**	**K2**	**K3**	**Average**	**K0**	**K1**	**K2**	**K3**	**Average**
Ceyhan-99	3.06	1.65	2.51	0.90	2.03A	62.40	52.69	59.60	52.27	56.74C
Pehlivan	3.73	1.76	2.04	0.71	2.06A	60.80	56.49	57.41	63.91	59.65B
Fırat-93	2.67	0.94	2.43	0.94	1.75AB	61.25	53.10	62.79	67.08	61.05A
Sarıçanak-98	2.75	0.94	1.61	0.98	1.57B	59.94	59.84	60.61	60.27	60.16B
Average	3.05A	1.32C	2.15B	0.88D		61.10A	60.88AB	60.10B	55.53C	
LSD (C)	0.03					0.67**				
LSD (D)	0.03					0.85**				
LSD (C)x(D)	ÖD					1.7				

In terms of average values of the cultivars, it was determined that the highest chlorophyll content was in Fırat-93 with 61.05 SPAD and the lowest chlorophyll content was in Ceyhan-99 with 56.74 SPAD. In terms of drought applications, it was determined that the highest chlorophyll content value was 61.10 in irrigated conditions (K0), followed respectively by early drought application (K1: 60.88), late drought application (K2: 60.10) and full drought applications (K3: 55.53 SPAD) (*Table 2*).

In the present study, it was determined that leaf area, plant green area and chlorophyll content were affected by drought applications. The highest values obtained from the irrigated conditions showed that especially the early drought applications were

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

affected more at a rate of approximately 50%. In fact, plants reduce the transpiration area to decrease water loss to a minimum level with their decreasing leaf surface area in stress conditions. Chlorophyll content is affected by drought stress and is decreased at a significant level when compared with normal irrigated conditions. Chlorophyll decomposition is increased towards the end of the development period due to drought stress (Aghanejad et al., 2015).

It was determined that the highest plant height average was 56.08 cm in Sarıçanak-98 and the lowest plant height average was 50.61 cm in Pehlivan. In terms of drought applications, it was determined that the highest plant height value in irrigated conditions (K0) was 69.93 cm, followed respectively by early drought application (K1) with a 3.07% decrease; late drought application (K2) with a 39.11% decrease; and full drought applications (K3) with a 52.09% decrease (*Table 3*).

The highest number of spike was determined in Pehlivan as 714 pieces/m^2 and the lowest number of ears value was determined in Fırat-93 variety as 611 pieces/m^2. In terms of drought applications, the highest number of ears in square meter in irrigated conditions was 967 pieces, followed respectively by late drought application (K2: 813), early drought application (K1: 506) and full drought applications (K3: 343) (*Table 3*).

Table 3. *The average values of plant height (cm), the number of spike and the formed groups according to the LSD (least significant difference) test*

	Plant Height (cm)					**The Number of Spike (pieces / m^2)**				
Cultivars	**K0**	**K1**	**K2**	**K3**	**Average**	**K0**	**K1**	**K2**	**K3**	**Average**
Ceyhan-99	70.93	50.53	62.80	32.33	54.15B	984	596	839	333	688B
Pehlivan	66.93	35.66	66.06	33.80	50.61D	1050	658	847	301	714A
Fırat-93	71.20	33.20	73.60	33.80	52.95C	914	368	792	372	611C
Sarıçanak-98	70.66	50.93	68.66	34.06	56.08A	921	404	776	364	616C
Average	69.93A	67.78B	42.58C	33.50D		967A	506C	813B	343D	
LSD (C)	0.79**					ÖD				
LSD (D)	1.85**					9.26				
LSD (C)x(D)	3.69					ÖD				

The highest fertile stem ratio was determined in Ceyhan-99 with 68.89 % and the lowest fertile stem ratio average was determined in Fırat-93 with 45.16%. In terms of drought applications, the highest fertile stem ratio in irrigated conditions was found as 93.72%, followed respectively by late drought application (K2) and early drought application (K1). In full drought applications (K3), the fertile stem ratio was determined to be 0% (*Table 4*).

In the present study, when the average values of the cultivars were examined it was determined that the highest thousand grain weight was determined in Pehlivan with 26.72 g and the lowest thousand grain weight was determined in Fırat-93 with 19.21 g; and in terms of drought applications, it was determined that the average value varied between 19.49 g and 42.71 g. The highest thousand grain weight value in irrigated conditions (K0) was determined as 42.71 g followed respectively by late drought application (K2) with a decrease of 21.33%; and by early drought application (K1) with a decrease of 54.37%. No grains were obtained in the Full Drought Application (K3) (*Table 4*).

DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

Table 4. *The average values of fertile stem ratio (%), the thousand grain weight and the formed groups according to the LSD (least significant difference) test*

	Fertile Stem Ratio (%)					The Thousand Grain Weight (g)				
Cultivars	**K0**	**K1**	**K2**	**K3**	**Average**	**K0**	**K1**	**K2**	**K3**	**Average**
Ceyhan-99	97.57	91.44	88.54	-	68.89A	43.97	28.47	31.04	-	25.87AB
Pehlivan	99.44	92.75	64.15	-	64.09A	43.69	27.84	35.33	-	26.72A
Fırat-93	89.99	-	90.64	-	45.16B	44.81	-	32.01	-	19.21C
Sarıçanak-98	89.86	84.67	91.06	-	66.40A	38.38	21.64	36.00	-	24.01B
Average	93.72A	67.22C	83.60B	-		42.71A	19.49B	33.60C	-	
LSD (C)	1.01**					0.06**				
LSD (D)	1.15**					0.05**				
LSD (C)x(D)	2.29					0.1				

It was determined that the highest number of grains in the spike was in Ceyhan-99 with 24.86 and the lowest number of grains in the spike in average was in Fırat-93 variety with 14.01. In terms of drought applications, the highest number of grains in the spike in irrigated conditions (K0) was 35.01, followed respectively by early drought application (K1: 31.52) with a decrease of 9.97%; and late drought application (K2: 12.67) with a decrease of 63.81%. No grains were obtained in the Full Drought Application (K3) (*Table 5*).

Table 5. *The average values of the number of grain per spike, grain yield (kg/ha) and the formed groups according to the LSD (least significant difference) test*

	The Number of Grain per Spike					Grain Yield (kg/ha)				
Cultivars	**K0**	**K1**	**K2**	**K3**	**Average**	**K0**	**K1**	**K2**	**K3**	**Average**
Ceyhan-99	43.02	19.35	36.40	-	24.86A	5964.7	1498.8	4765.9	-	3057.4A
Pehlivan	35.63	18.32	35.26	-	22.48AB	5895.3	1314.1	4657.6	-	2966.8AB
Fırat-93	26.92	-	27.73	-	14.02C	5238.8	-	4274.1	-	2378.2C
Sarıçanak-98	34.49	12.32	26.68	-	18.55	5324.7	983.5	4752.9	-	2765.3B
Average	35.01A	31.52A	12.67B	-		5605.9A	949.1C	4612.6B	-	
LSD (C)	4.10**					0.47**				
LSD (D)	3.73*					0.49**				
LSD (C)x(D)	7.45					0.99				

The highest average grain yield was determined in Ceyhan-99 variety with 3057.4 kg/ha and the lowest grain yield average was determined in Fırat-93 with 2378.2 kg/ha; and in terms of drought applications, the average values varied between 949.1 kg/ha and 5605.9 kg/ha. The highest grain yield value in irrigated conditions (K0) was determined as 5605.9 kg/ha followed respectively by early drought application (K1) with a decrease of 17.72%; and late drought application (K2) with a decrease of 83.07%. No grains were obtained in the Full Drought Application (K3) (*Table 5*). Early drought applications were found to be effective on plant height, especially grain number per spike and indirectly on grain yield. Grain yield was taken but remained quite low.

In terms of drought applications, it was determined that the highest potassium amount in the plant root tissues was in early and late drought applications (K1: 18.71

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

and K2: 18.20 mg), and the lowest potassium amount was 13.09 mg in full irrigated conditions. In the evaluation made in terms of variety-drought applications, the highest Potassium amount was determined in Sarıçanak-98 in the late drought application (K2: 18.23 mg), and the lowest Potassium amount value was found in Ceyhan-99 variety in late drought application (K2: 5.12 mg) (*Table 6*). In terms of the potassium (K) amount in the stem tissues of the wheat plants, when the average values of the cultivars were examined, it was determined that the highest Potassium amount was in Ceyhan-99 variety (24.47 mg) and the lowest Potassium amount was in Pehlivan variety (14.94 mg). In terms of drought levels, it was determined that the highest Potassium amount average was found in early drought application (K1: 18.71 mg), and the lowest Potassium average was found in irrigated conditions (K0: 13.09 mg) (*Table 6*).

Table 6. *The average values of potassium amounts in the plant roots and stems*

Potassium (K)										
	K0		K1		K2		K3		Average	
Cultivars	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**
Ceyhan-99	17.34	6.53	18.46	7.34	34.77	5.12	20.17	10.02	24.47	7.25
Pehlivan	15.81	5.86	19.74	13.54	22.22	6.62	2.86	12.53	14.94	9.64
Fırat-93	12.92	13.07	18.38	7.92	5.26	5.29	21.55	12.49	15.06	9.69
Sarıçanak-98	6.307	14.19	18.26	7.82	10.56	18.23	26.07	6.21	18.29	11.61
Average	13.09	9.91	18.71	9.16	18.20	8.82	17.66	10.31		

In the examinations on plant roots, it was determined that Fırat-93 variety had a value of 16.63 mg in terms of Calcium amount and Ceyhan-99 variety had an average of 6.15 mg. In terms of drought levels, the highest Calcium amount was determined in the late drought application (K2: 17.20 mg), the lowest Calcium average was determined in the full drought application (K3: 7.15 mg). In terms of variety-drought interaction, it was determined that the highest Calcium amount value was in Pehlivan variety in late drought application (K2: 29.40 mg), and the lowest Calcium amount value was in Fırat-93 variety in full drought application (K3: 0.670 mg) (*Table 7*).

Table 7. *The average values of Calcium amount in the plant roots and stems*

Calcium (Ca)										
	K0		K1		K2		K3		Average	
Cultivars	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**
Ceyhan-99	7.00	11.76	8.02	12.10	3.45	12.25	6.15	33.23	17.33	6.15
Pehlivan	6.66	13.84	14.29	15.66	29.40	9.52	7.92	10.20	12.30	14.57
Fırat-93	14.07	14.91	10.53	11.85	25.30	9.62	0.67	4.61	10.24	16.63
Sarıçanak-98	9.26	9.25	10.70	10.80	10.74	11.15	7.383	5.86	9.271	9.52
Average	9.25	12.44	10.88	12.61	17.20	10.63	7.15	13.47		

In terms of the Calcium (Ca) amount found in the stem tissues of the wheat plants, when the average values were examined in terms of the average values of the cultivars, it was determined that the highest Calcium amount was in Ceyhan-99 variety (17.33 mg) and the lowest Calcium amount was in Sarıçanak-98 variety (9.27 mg). In terms of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

drought levels, it was determined that the highest Calcium amount average was in full drought application (K3: 13.47 mg), and the lowest Calcium average was in late drought application (K2: 10.63 mg) (*Table 7*).

As a result of the analyses made on the plant root tissues it was found that the highest Sodium amount was in Ceyhan-99 variety (18.33 mg) and the lowest Sodium amount was in Pehlivan variety (2.22 mg). In terms of drought applications, the highest Sodium amount average was determined in irrigated conditions (K0: 14.51 mg), and the lowest Sodium average was found in late drought application (K2: 9.10 mg). In terms of variety-drought interaction, the highest Sodium amount was determined in Ceyhan-99 variety in early drought application (K1: 23.34 mg), and the lowest Sodium amount value was determined in Pehlivan variety in early drought application (K1: 0.20 mg). In terms of the Sodium (Na) in the stem tissues of the wheat plants, when the average values were examined, it was determined that the highest Sodium amount was in Ceyhan-99 variety (15.41 mg) and the lowest Sodium amount was in Pehlivan variety (0.69 mg). In terms of drought levels, the highest Sodium amount average was determined in full drought application (K3: 18.90 mg), and the lowest Sodium average was determined in early drought application (K1: 3.35 mg). In terms of variety-drought interaction, the highest Sodium amount was determined in Ceyhan-99 variety in irrigated conditions application (K0: 27.05 mg), and the lowest Sodium amount value was determined in Pehlivan variety in full drought application (K3: 0.21 mg) (*Table 8*).

Table 8. *The average values of Sodium amounts in the plant roots and stems*

Sodium (Na)										
	K0		**K1**		**K2**		**K3**		**Average**	
Cultivars	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**	**Root**	**Stem**
Ceyhan-99	27.05	18.61	4.90	23.34	2.89	0.14	26.81	13.05	15.41	18.33
Pehlivan	0.81	0.71	0.92	0.20	0.83	2.22	0.21	0.42	0.69	2.22
Fırat-93	18.73	20.42	4.04	2.59	8.49	9.10	17.54	16.54	12.20	12.16
Sarıçanak-98	4.17	4.51	1.11	1.39	17.88	0.83	12.41	1.01	8.89	2.30
Average	16.65	14.51	3.35	9.11	9.76	9.10	18.90	10.20		

Conclusion

The effects of drought on the development and yield of the wheat depends on the developmental period in which drought occurs, on the severity, and on the time of the drought. The negative effect of drought on the leaf area after the heading and blooming is considered as the sole reason of the decrease in the yield. The drought occurring at the spike formation stage causes reductions in the number of grains in spike, and the one occurring after the blooming affects negatively the increase in the mass weight of the grains after a certain point.

The increase of Potassium concentration in plant tissues increases the durability of the plant to stress factors (Hsiao and Lauchli, 1986). It is known that the genotypes which have more K and Ca ions in green parts and roots have increased endurance to stress conditions, and in addition, this also causes increases in the enzyme activities in the genotypes during drought stress (Kusvuran, 2010). One of the basic elements that is necessary for growth and development is K and the other one is Ca ion. Abiotic stress affects the K and Ca intake negatively. In the present study, it was observed that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41074115

drought stress increased the K and Ca levels in the plant however, it was also observed that this level changed according to different drought applications. It is possible to claim that especially in the late drought application, the substance coverage of both minerals increased. Again, it was also determined that the cultivars showed different K and Ca levels in different drought applications. It is known that the Sodium ion causes necrotic stains on the shoots and leaves starting from the old leaves in drought conditions (Aktas, 2002; Dasgan et al., 2006). As seen in the K/Na and Ca/Na rates in the green parts and roots, it was determined that the genotypes with higher Na and lower K and Ca contents were more susceptible to damage.

As a result, Ceyhan-99 cultivar which show the best average of 7 agricultural and biochemical characters, were superior to all other wheat cultivars in Southeast Anatolia conditions of Turkey. However, these drought studies should be continued for longer years and changing climate conditions, the drought resistance of the varieties, produced in the region should be determined.

Acknowledgements. This research was supported by a grant from Dicle University Research Funding (DUBAP; Project No. ZIRAAT.17.001).

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TRANSFERRING THE RESPONSIBILITY OF FOREST PROTECTION TO VILLAGE ADMINISTRATION: A CASE STUDY OF IKIZDERE, RIZE, TURKEY

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(Received 5th Dec 2018; accepted 28th Feb 2019)

Abstract. Effective public participation is considered essential to making sound decisions for the protection and sustainable management of natural resources. This paper aims to analyze the preferences of forest villagers to protect state forests within the boundaries of villages and generate income for their livelyhoods. As a case study area in Turkey, forest villages of Rize-Ikizdere town were selected to carry out the research. In the study area, intensive forest protection activities are carried out under the administration of Trabzon Regional Directorate of Forestry. A questionnaire form was developed and applied to the villages of the town on a face-to-face basis. According to the survey results, while most of the participants preferred to hand over the forest protection responsibility from state to legal village administration, they indicated some concerns about taking over the responsibility of forest protection that would bring some risks under the proposed policy. In conclusion, most of the participants envisioned that the new policy was likely to develop mutual understanding of participation, thoughtfulness, worthiness and stewardship about forests rather than foreseeing it as a source of income generation, and help lessen the forest degradation to a certain extent.

Keywords: *public participation, natural resources, forest villagers, survey, conservation*

Introduction

One of the basic objectives of all national forest policies include conservation with a focus on protection, maintenance, sustainable utilization, restoration and enhancement of the forested lands (Alemagi et al., 2013); however, meeting the objectives set by these policies has rarely been fully achieved (Makarabhirom, 1999). One of the impediments has been the lack of public participation in processes and mechanisms which enable stakeholders to be part of decision-making in all aspects of forest resource management, including policy formulation processes. Effective public participation is thus considered essential to making sound and better quality decisions about sustainable forest management (Beierle and Cayford, 2002; Buchy and Hoverman, 2000; Diaw et al., 2009; Kozak et al., 2008; Mendoza and Prabhu, 2000; MPWG, 1999; UNCSD, 1992; WCFSD, 1999) through which the socio-economical, ecological, and cultural values of the public can fully be taken into consideration regarding forest use and management (Buchy and Hoverman, 2000; Duinker, 1998; Pretty and Smith, 2004; Shindler and Neburka, 1997). This can be achieved by ecologically acceptable and socio-economically feasible approaches involving processes and mechanisms that enable stakeholders in forest resources to be part of decision-making in all aspects of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

forest management, including policy formulation (Cantiani, 2012; FAO, 2012 Kaskoyo et al., 2017).

Over the last several decades, public participation has become an important topic for environmental agencies throughout the world, and has been increasingly incorporated into forest management decision-making, with, in many cases, legal provisions for public input (Ahmed and Jana, 2017; Alemagi, 2007; Buchy and Hoverman, 2000; Cantiani, 2012; Coskun and Elvan, 2003; Duinker, 1998; Sinclair and Doelle, 2003). All these developments have been driven by pressures brought about by certain realities of ecology and economics and our increased demands for multiple use; expectations from and changing attitudes towards forests; and the need for the development of new policies and more effective management systems. The first examples of public participation in the management of forests were seen both in the developed and developing countries; however, objectives set and methodologies employed were different, due to the complex nature of the problems associated with the ownership of the lands and land use objectives.

In Canada and the US, for example, the onset of the participation process in as early as 1960 resulted in an increase in public awarenes of environmental issues among wider sections of the population (Cantiani, 2012; Germain et al., 2001; Grumbine, 1994; Tabbush, 2004; Vining and Tyler, 1999). Among other countries in Europe, Finland has been an important example in applying the effective and structured participation in forestry through establishing a number of working groups, represented by a wider range of people and the associated political parties to develop forest policies and prepare forestry related projects (Anonymus, 1995).

In some developing countries, public participation has been exercised in a more cohesive way and with more focus on the empowerment of local populations emphasising principles of equity and social justice (Cantiani, 2012). This approach assumes partial or full responsibility of local people in the management of resources within their jurisdictions. In this regard, a new concept of “Forest management by the village” in Tanzania (Warner, 1997), “Joint Forest management (JFM)” concept of India (Ahmed and Jana, 2017; SPMU, 2015), “Social Forestry Programme” of Nepal (Gimmour and Fisher, 1991) can be given as important examples.

Turkey also represents a similar and a unique example. Public participation is a major concern in the management of forest resources. With about seven million people living in about 20 thousand villages in and around forested lands, most forest villagers depend on forests for their livelihoods. Almost all forested lands (99.9%) in the country belong to the state and are managed and protected by the General Directorate of Forestry (GDF). State policies for the protection of forestlands and natural resources have been developed with little emphasis on the needs, demands and expectations of society and forest communities. Although many different protection measures have been taken and implemented, including “forest soldiers” (Bingöl, 1990) and “centralized protection centers” (Ayanoglu, 1981), success has been limited. In the search for a better alternative, a method/policy that empowers the legal village administration to protect the forests within the boundaries of villages with a certain amount of budget and a bilateral protocol has been explored for some two decades now. However, there have been no comprehensive research endeavors, aside from some theoretical studies, documenting the pros and cons of handing over forest protection activities to the village administration compared to the current practices. It is quite uncertain how much benefit the alternative methods may provide to the forest villagers and to the sustainable

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

management of the forest resources. Besides, the expectations, demands and preferences of the forest villagers would be unknown when the new protection policy would be in effect.

The objective of this research is to analyze the attitudes and preferences of forest villagers regarding the transfer of forest protection activities, currently conducted by the forest protection unit of the state forest organizations, to the legal entity of villages. The study also focuses on revealing the probability of success of the newly proposed approach in the eyes of the forest villagers. The forest villages of Rize-Ikizdere town were selected as a case study area. Here, the forests are intermingled with both residential areas and agriculture lands creating complex and intense forest protection activities.

Material and methods

The case study area is located between 40°57'12"- 40°50'46" N latitudes and 40°24'15" - 40°55'07" E longitudes in the northeastern part of Turkey (*Fig. 1*). The total surface area of Ikizdere town within the province of Rize is about 89,800 ha, of which 29,698 ha (33%) is forested. The population of Ikizdere, which is the highest of all the towns in Rize, was nearly 5,600 in 2017. 68% of the population (3,814) lives in the villages and the outskirts of Ikizdere town (URL-1, 2018). According to article 31 of Turkish Forest Law 6831, 24 villages of Ikizdere Town are in the status of “forest villages” that have certain privileges and status over other ordinary villages.

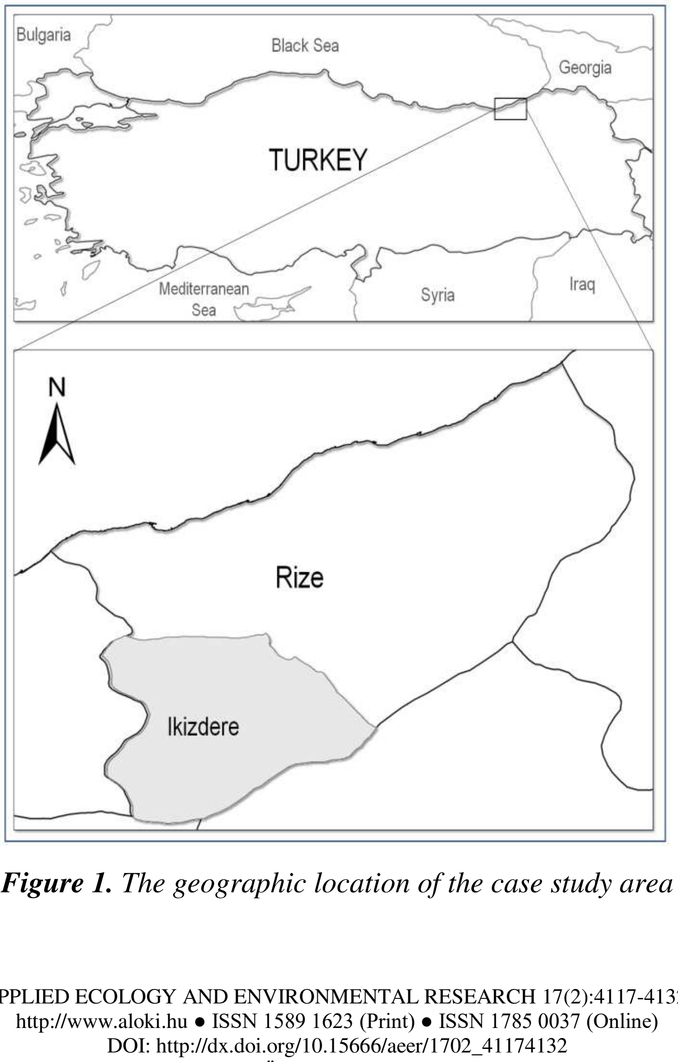

Figure 1. *The geographic location of the case study area*

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DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

The research method employed in this study entailed the use of structured questionnaires administered in the forest villages located within the study area. The questionnaire form consisting of 12 structured questions was tested with an initial pilot application in the study area prior to the implementation of the study. As a result, the number of questions were reduced to eight and revised accordingly in the process. The individuals interviewed were selected with a simple random sampling method. The survey was conducted by one researcher in the field on a face-to-face interview basis. A total of 104 households from 24 potential forest villages were contacted for the interview. Only one person per household (the household representative) was interviewed to avoid repetition and achieve some level of consistency in answers from respondents of the same household (Asante et al., 2017). Four to five household representatives from each village were included in the study. The number of respondents required (sample size) in this study was calculated to be 96 using the formula (*Eq.1*):

$$n = \frac{F \times t^2 \times P \times Q}{\left(F \times m^2 \right) + \left(t^2 \times P \times Q \right)} \quad \text{(Eq.1)}$$

where,
n: sample size
F: Population size
t: Confidence level (95%)
m: Margin of error (10%)
P and Q: Percentage value (0,5 × 0,5).

The questionnaire form was designed to acquire both personal information of forest villagers such as the number of individuals in the household, the age, education level of the respondents and the annual income of households, and other information in line with the objective of the study, such as the possibilities, advantages and disadvantages of transferring the rights of state forest protection activities to the forest villagers, as well as gathering the opinions of the villagers about the current status of forest protection. The questions of the survey were designed in the form of a series of closed-ended, single-choice questions. The respondents were asked to select one alternative among all alternatives provided within the survey form. In addition to the close-ended questions, the form also included an additional alternative under the heading of the “other” to allow the respondents to suggest different alternatives freely. The results of the survey were statistically analyzed using the Statistical Package for Social Scientists (SPSS version 16). The socio-economic and demographic characteristics of respondents were summarized and presented using simple descriptive statistics. Moreover, to see if there is a relationship between socio-economic and demographic characteristics of respondents and their choises, Chi-squared test was employed. In addition to the analysis of the questionnaire results, the qualitative data from the interviews was also evaluated to get the ideas and preferences of the respondents regarding the protection of the state forest resources.

Results

In this survey, two out of eight questions asked to the respondents were about the socio-economic and demographic structure of the villages. The rest of the questions were about the opinions and preferences of the respondents who were asked in order to select the method that people would see as appropriate.

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Results about the households surveyed

The results related to the number of individuals in the households, the age, educational level, annual income of households are listed in *Table 1.*

Table 1. *The demographic information about the respondents interviewed and households surveyed*

Individuals in a household		
Person	**Number**	**Percent**
1-2	19	18
3-4	22	21
5-6	34	33
>7	29	28
Total	104	100
Age (year)		
Year	**Number**	**Percent**
0-30	6	6
31-50	42	40
>50	56	54
Total	104	100
Level of Education		
Level	**Person**	**Percent**
Illiterate	5	5
Literate	1	1
Elementary school	76	73
High school	17	16
University	5	5
Total	104	100

Table 2. *Employment and the annual income level of the household per individual*

Occupation		
Occupation[1]	**Number**	**Percent**
Worker	26	25
Officer	8	8
Private sector	61	58
Other	9	9
Total	104	100
Annual income per person		
Amount ($)	**Number of households**	**Percent**
0-1000	56	54
1001-2000	2	2
2001-4000	3	3
>4001	43	41
Total	104	100

[1]Worker: person working for a state organization as a worker; Officer: person employed by a state organization as a civil servant; Private sector: any person not employed by a state organization; Other: person retired or having no job.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

According to *Table 1*, there are primarily 6-7 individuals in a household and the average age of the household representatives is over 50 years. The literacy rate of the household representatives is about 95%. This is almost the same as the average literacy rate of the country (96%) (URL-2, 2018).

The survey included questions regarding the employment and the income level of the households. The type of employment and the salaries of the respondents were directly asked in the household survey. Additionally, where agriculture is not the main source of income, it was thought that income from agricultural activities would also be valuable to determine the total income of the households more realistically. Thus, the amount of agricultural products produced were included in the questions asked, and the results were processed with the current unit prices in order to determine the income level of the households. *Table 2* shows the employment and the annual income level of the households. The US dollar currency of the date when the survey was conducted was used in the conversion.

About 54% of the households surveyed in this study has an annual income level under 1000$ per person, indicating a monthly income of less than 100$. The agricultural income is quite low due to the scarcity of agricultural land and the poor productivity of the land used for agricultural purpose. However, the presence of officers and workers in households helps increase the annual income level in the case study area. Some of the "private workers" are working only in agricultural activities and animal husbandry; the others are operators or small businesspersons in the town facilities or tourism facilities. The "other" category of occupation in *Table 2* refers to the persons who are either housewife or jobless.

Opinions of the household representatives about the forest and forestry

In this category of the survey, five closed-ended questions were asked to the representatives of the household and the results were analyzed in the following sub-headings.

Who should protect the state forests?

The appropriate choice among the possible alternatives for the protection of the state forests within their village boundaries was asked to the forest villagers. For that purpose, four alternative methods of forest protection were determined and placed into the survey. The first of the four alternatives in the questionnaire form refers to the current alternative of "continuation of the current method of forest protection (by the state forest sector)", the second alternative is about the "protection of forests by the civil authorities", the third one refers to the "forest protection by the squads of forest soldiers experienced in a certain period in the history of Turkish forestry" and the last one is the "protection of state forests by the villagers". The answers to these important questions are given in *Table 3*.

Table 3. *Alternative forest protection methods presented in the survey*

Alternative methods for better protection of forests	Number	Percent
Forests should be protected by the state forest organizations	47	45
Forests should be protected by the legal village entities	57	55
Total	104	100

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According to *Table 3*, the majority of the respondents (55%) preferred the choice of forest protection by the forest villages. The rest of the respondents (45%), however, favored the alternative of forest protection by the state forest organizations as currently practiced in Turkey. In fact, there was no significant difference between the two alternatives. Surprisingly, the other two alternatives (forest protection by the civilian authorities and forest protection by the squads of forest soldiers) developed in the survey form were not selected by the respondents at all. Aside from the four alternatives, no additional alternative was suggested by the respondents either.

Evaluating the pros and cons of forest protection by the villages

Table 4 presents the responses about the priority or degree of the possible consequences of transferring the state forest protection activities to the legal village entities.

Table 4. *Benefits of transferring forest protection to the forest villages; first option: "Development of desire to safeguard the forest and increased feeling of affection for them"; second: "A decrease in forest related crimes, due to resulting self-control"; third: "Less illegal forest utilisation"; fourth: "Generation of additional income for the villagers*

Benefits of forest protection by forest villages	Rank	Number	Percent
Develops the spirit for safeguarding and feeling of affection for the forests.	1	82	80
Decreases forest related crimes as it brings self-control	2	37	36
Lessens illegal forest utilization.	3	49	47
Helps generate additional income for the villagers.	4	75	72

Based on the interpretation of *Table 4*, the majority of the respondents (80%) believes that the principle benefit of forest protection by the forest villages would be the choice "develops the spirit for safeguarding and feeling of affection for the forests", that shows the level of forest stewardship. About 36% of the respondents indicated that forest protection by the forest villages will cause decreases in forest related crimes as it brings self-control. The third priority benefit of forest protection by the forest villages is indicated by 47% of the respondents to be the "lessens illegal forest utilization". At the bottom of the priority list, 72% of the respondents indicated that forest protection by the forest villages will help generate additional income for the villagers.

On the other hand, potential disadvantageous of forest protection by the forest villages were also questioned in the study. The opinions and the concerns of the villagers were gathered in the survey as well. The results are presented in *Table 5*.

Table 5. *Ranking the disadvantages of forest protection by the forest villages*

Disadvantages of forest protection by forest villages	Rank	Number	Percent
Inefficiency in forest protection by the villagers may arise due to the lack of knowledge about forest ecosystems in general.	1	35	34
Disputes with neighboring villages or villagers may be inevitable and this may fuel hostility, should village administrations take biased or prejudiced decisions.	2	64	62
Severe confrontations may be indispensable in the election of chiefs for the villages.	3	27	26
Illegal forest utilization may increase.	4	41	39

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According to *Table 5*, nearly 34% of the respondents indicated the most important concern about forest protection by the forest villages was “inefficiency in forest protection by the villagers may arise due to the lack of knowledge about forest ecosystems in general”. The second important concern indicated by the 62% of the respondents is the possible “disputes with neighboring villages or villagers may be inevitable and this may fuel hostility, should village administrations take biased or prejudiced decisions”. Of course, the chief or managers of forest village administration will become responsible and powerful with the initiation of forest protection by the villages. The candidates for the chief position may be confronted by serious challenges to get the rights and power of the village administration. Such situation may cause hostilities among the villagers. This concern is ranked third among the disadvantages of forest protection by the villages. Finally, 39% of the respondents believed that “illegal forest utilization may increase” as a forth concern when forest protection activities are transferred to the forest villages.

Forest villagers´ Expectations from the agreement between the state and the village administration upon the transfer of forest protection to forest villages

The opinions of the villagers were gathered in the survey in order to determine the conceivable expectations of forest villagers from the transfer of forest protection activities to forest villages. The results are summarized in *Table 6*.

Table 6. *Expectations of forest villagers from the agreement upon the transfer of forest protection to forest villages*

Expectations	Rank	Number	Percent
Social security coverage for the villagers working in forest protection activities.	1	95	91
Higher payments	2	62	60
Training of forest villagers who will work in forest protection activities about the rights and the responsibilities.	3	60	57
Provision of clothes, guns and other equipment to the forest villagers in charge of forest protection	4	57	55

Table 6 shows that the most important expectation is the “covering of the villagers working in forest protection activities under social security programme.” reflected by the 91% of the respondents in the survey. The second level of expectation is the high level of payment for the villagers who will work in forest protection activities. Training of forest villagers and provision of clothes, guns and other equipment to the forest villagers in charge of forest protection are among the latter expectations.

Evaluation of legal rights and responsibilities upon the transfer of forest protection to the forest villages

The villagers empowered with the rights to protect the forest resources need to know their legal rights and the responsibilities to carry out the activities effectively. The opinions and the concerns related to the legal rights and the responsibilities for the villagers are summarized in *Table 7*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

Table 7. *Results related to the legal rights and responsibilities of forest villages entitled to protect forest resources*

Evaluations by the forest villagers	Number	Percent
Forest villages should be empowered with the same rights and responsibilities of the current forest rangers.	38	37
The duty of protection should exist, yet administrative and judicial penalty provisions for forest related crime and lawbreakers still need to be exercised by public officers.	31	30
Forest villages and the forest rangers need to cooperate in enforcing the penalties incurred for the incurred by lawbreakers.	42	40
No legal rights regarding the about the administrative and judicial penalty provisions should be granted to the forest villages	6	6

According to *Table 7*, forest villagers seem to abstain from undertaking legal rights and responsibilities for forest protection activities. Only 37% of the local people indicate their desire to empower the forest villages with the rights and responsibilities of the current forest rangers. Others either would like to cooperate with the public officers and do not want to get involved, as an intervener, in prescribing and enforcing the associated penalties for the lawbreakers.

Opinions about causes of committing forest crimes or misconducts

The fundamental causes of committing forest crimes in state forests in the eyes of forest villages are evaluated here. While four different questions were designed and asked to the forest villagers in the survey, only two of them were responded appropriately.

Table 8. *The results and the rates of the causes of committing forest crimes*

The causes of committing forest crimes	Number	Percent
Inadequacy of the recruited forest rangers	5	5
Poverty of forest villagers	99	95
Total	104	100

Table 8 clearly shows that the fundamental cause of forest related crimes or misconducts is apparently the poverty of the forest villagers (95%). The inadequacy of the recruited forest rangers is only represented by 5% of the respondents. However, the alternatives of “attitude to gain improper personal benefit” and “desire to proclaim forest land for agricultural activities” were not selected by the respondents at all. While there was the “other” alternative for the respondents to provide further causes of forest crime, it was left blank.

Discussion

This paper highlights the preferences of forest villagers to protect state forests within the boundaries of villages in the northeastern part of Turkey. It also outlines the perceptions of the local people in public participation and identifies challenges in forest protection in Turkey. The study is limited in terms of coherence and extent, but

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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provides new perceptions in forest protection in state-owned forests. Almost all forests are owned by the state in Turkey, and General Directorate of Forestry (GDF is the primary state organization in the country responsible for both managing the forest resources including all forest related actions and patrolling the forest lands and protecting its properties against any illegal activities in all forests across the country. The forest protection activities have long been carried out by the forest rangers established by the state forest sector for over 60 years. While the forest crimes in the country have decreased for the last three decades, more than 120,000 forest misconducts have been registered over the last decade (2008-2017). The number of forest misconducts in 2016 alone constituted nearly 10,000 cases according to the official reports (GDF, 2016). Moreover, it is suspected that majority of forest misconducts have not been reported and registered in the official records, indicating an inadequacy in overall protection activities.

The questionnaire administered in the selected case study areas identified both demographic status of the respondents and the opinions and expectations of forest villagers about the transfer of the authority in forest protection to the villages as per an agreement between the state and the village administration. According to the survey results, the literacy level of the people and the size of the household in the case study area were found to be close to the country averages (URL-2, 2018). When the income is evaluated, nearly 55% of the people has an annual income of less than 1,000$ per individual. However, the annual income per individual is considerably higher in households having public officers and private job owners such as salesmen or small entrepreneurs.

People living in the forest villages consider that the primary reason for committing a forest crime in state forests around villages is the poverty in rural areas. Only the 5% of the respondents indicated that forest destruction is realized due to inadequacies or vicious attitudes of the forest rangers authorized in forest protection activities. These results are in agreement with poverty related forest crimes in the developing countries (ATIF). Forest management and protection programmes through participation have mostly been developed in response to both high number of forest crimes and the reality of the forest villagers being among the poorest people of the country (UNCSD, 1992; Duinker, 1998; WCFSD, 1999; MPWG, 1999; Buchy and Hoverman, 2000; Coskun and Elvan, 2003; Pretty and Smith 2004; Alemagi, 2007; Kozak et al., 2008; Cantiani, 2012). In this regard, public participation in forest management may be an important step towards the sustainable use and protection of forest resources (Alemagi, 2007; Kozak et al., 2008; Diaw et al., 2009; Cantiani, 2012; Ahmed and Jana, 2017). One of the practical alternatives seems to be the transfer of the forest protection activities to the forest villagers under certain provisions as suggested in this study. Such that, forests would be protected more effectively than previously and there would be a relative increase in the income of forest villagers.

Almost 45% of forest villagers involved in the survey favored the maintenance of current operation or method in forest protection. However, the majority preferred the transfer of forest protection authority to the villages. The contract of forest protection can be considered to be a source of income particularly for the unemployed villagers or the households involved in agriculture and animal husbandry. As a matter of fact, almost 70% of the respondents expects that the annual income of the forest villagers will considerably increase due to the contract of the forest protection taken by the villages. Several programmes implemented in many countries provided evidences to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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support this conclusion (Kozak et al., 2008; Vergara-Asenjo and Potvin 2014; Kaskoyo et al., 2017; Ahmed and Jana, 2017). The results also indicate that additional sources of income may be generated for the common expenses of the villages such as roads, bridges, mosques and common village mansions. In addition to additional source of income for individuals and fund raising for common village expenses, it was understood with 80% of acceptance level that the protection of forest lands by forest villages would enhance the relationships between people and their forest, internalize the proposed forest management concept and help increase an interest in forests. Additionally, *Table 6* indicated that forest villagers authorized in forest protection activities would like to have payment and social security covered by the new alternative as they work in state forest management endeavors. These findings are in close agreements with other reports. In Panama, for example, it was reported that public participation and the provision of additional sources of income (Vergara-Asenjo and Potvin, 2014) and increased use of state forests by the villagers would increase the success in forest protection (Thang et al., 2010). Moreover, these provisions may foster and lead to the acceptance of new rights for the poor forest villagers (Scheba and Mustalahti, 2015).

Surprisingly, the assumption that the number of unaccounted and of reported forest crimes may be lowered as a result of transferring the forest protection activities to the forest villages due to self-control was not favored by the majority of the respondent forest villagers. Even, as seen in *Table 5*, almost 39% of the villagers showed a deep concern about the possible increase in illegal forest utilization. Besides, clothes, guns and other equipment was also requested for the forest villagers in charge of forest protection.

Forest villagers taking part in the survey are doubtful about fulfilling appropriately or enforcing the duty of forest protection given to them. Almost 57% of the forest villagers would like to be trained by the state forest organizations on the expected rights and the responsibilities. Trainings were reported to improve the succes of such programmes (Kaskoyo et al., 2017). In contrast to that, only 37% of the forest villagers thinks that they would be able to enforce the duty of forest protection if the related legal basis is established. The majority would like to cooperate with the public officers to conduct forest protection activities and indicated that administrative and judicial penalty provisions for the forest related crime and the lawbreakers still need to be exercised by the public officers.

The forest villagers show serious concerns about possible confrontations with neighboring villages as well as with their own during the prevention of forest crimes and struggle with lawbreakers. Furthermore, people worry about the fact that the challenge may spark off undesired confrontations among households during the elections for the village administration.

As for the relationship between socio-economic and demographic status of the respondents and protection alternatives, Chi-squared statistic did not reveal any significant relationships between the responses of villagers and the socio-economic and demographic variables. However, although not statistically significant, unlike expectations, results indicated that older people were more of the opinion that protection activities should be transferred to villages. This can be ascribed to the fact that young people have a tendency to move to metropolitan areas to find better job opportunities. The results also indicated a parallelism in the tendency of forest protection by the villagers as the number of households increase.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

Conclusions

General conclusions reached can be summarised as:

- Villagers, in general, seem to be ambitious and prepared to use the rights and shoulder the responsibilities for the protection of forests within the boundaries of their villages with a certain amount of negotiated budget and a bilaterally agreed protocol.
- Most of the participants in the survey envisioned the new policy proposal to provide some opportunities to develop mutual impressions about participation, thoughtfulness, worthiness and stewardship in forest protection and, to a lesser extent, generate a source of income.
- Training is necessary for the successful implementation of the proposed program. In this regard, villagers who will take part in forest protection activities need to be trained systematically about the due rights and responsibilities. And,
- An effective administrative controlling process seems to be inevitable. State forest administration should continue to cooperate with the forest villagers in struggling with the forest lawbreakers as well as preventing any conceivable prejudiced decisions of the villagers and likely corruptions. It is also crucial and necessary that the state forest organizations enforce the duty of both administrative and judicial penalty provisions for the forest related crimes.
- Transferring the forest protection activities to the forest villages is considered timely and necessary in Turkish forestry provided that the required precautions are taken and some cases are designed and implemented in pilot areas to foresee any possible complications and problems, before the widespread implementation of the approach across the country.
- Given the area studied and issues contained in the study, this study can be considered extremely limited. Community involvement in any planning requires that local conditiones be also included in the overall planning process. Therefore, new studies are required for other regions before a wisespread implementation of the proposed program. The new studies should involve socio-economical, physical, ecological aspects of community involvement in future forest management plannings.

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Questionnaire

1. The interviewee:

Age: …………………………………………………
Educational status:………………………………….
Occupation: …………………………………………
Number of population in the household: ……..…….

2. Total annual income of the household:

I. Salary: (for employees)……………...............……... $/year
ii. Agricultural income: ………………………………… $/year
iii. Livestock income:…………………………………… $/year
iv. Other incomes (self-employment):…………………... $/year

3. Alternative forest protection methods presented in the survey

a. Forests should be protected by the state forest organizations
b. Forests should be protected by local governors
c. Forests should be protected by troops
d. Forests should be protected by the legal village entities
e. other (please specify ....)

4. Benefits of forest protection by forest villages

a. Develops the spirit for safeguarding and feeling of affection for the forests
b. Decreases forest related crimes as it brings self-control
c. Lessens illegal forest utilization.
d. Helps generate additional income for the villagers.
e. other (please specify ....)

5. Disadvantages of forest protection by forest villages?

a. Inefficiency in forest protection by the villagers may arise due to the lack of knowledge about forest ecosystems in general.
b. Disputes with neighboring villages or villagers may be inevitable and this may fuel hostility, should village administrations take biased or prejudiced decisions.
c. Severe confrontations may be indispensable in the election of chiefs for the villages.
d. Illegal forest utilization may increase.
e. other (please specify ....)

6. Expectations of forest villagers from the agreement upon the transfer of forest protection to forest villages

a. Social security Coverage for the villagers working in forest protection activities.
b. Higher payments
c. Training of forest villagers who will work in forest protection activities about the rights and the responsibilities.
d. Provision of clothes, guns and other equipment to the forest villagers in charge of forest protection
e. other (please specify ....)

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7. Results related to the legal rights and responsibilities of forest villages entitled to protect forest resources.

a. Forest villages should be empowered with the same rights and responsibilities of the current forest rangers.
b. The duty of protection should exist, yet administrative and judicial penalty provisions for forest related crime and lawbreakers still need to be exercised by public officers.
c. Forest villages and the forest rangers need to cooperate in enforcing the penalties incurred for the incurred by lawbreakers.
d. No legal rights regarding the about the administrative and judicial penalty provisions should be granted to the forest villages
e. other (please specify ....)

8. What are the main reasons for forest crimes?

a. Easy and unfair gain
b. Inadequacy of the recruited forest rangers
c. Efforts to provide new agricultural areas
d. Poverty of forest villagers
e. Other (Please specify…………………………………….)

APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):4117-4132.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41174132

SPATIOTEMPORAL VARIABILITY OF INDOOR ATMOSPHERIC EMISSIONS IN THE MEGA-CITY OF PAKISTAN

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(Received 7th Dec 2018; accepted 28th Jan 2019)

Abstract. Many sites of the world are currently faced with the prospects of air pollution. It is well established that photocopying machines generally contribute to the indoor emissions of Volatile Organic Compounds (VOCs) and Ozone that strongly influence the health of individuals. The current study aimed to discern the spatiotemporal variations of indoor Total Volatile Organic Compounds (TVOCs) and Ozone using a real-time monitoring approach in photocopying centers over mega-city of Pakistan. The real-time monitoring transcribed the diurnal variability of TVOCs and Ozone in photocopying centers (n = 200) during working hours (8:00 AM to 8:00 PM). Spatially, a high degree fluctuation was observed in the indoor level concentrations of TVOCs and Ozone. A typical diurnal cycle with a minimum value in the morning and a maximum in the noon with a gradual decrease until night was observed for TVOCs and Ozone suggesting that the photocopying emissions were a major source of generation. A significantly strong positive correlation was found between TVOCs and Ozone ($r = 0.627$, $p < 0.001$). The findings were compared with the available standards indicating the potential of health hazards to workers.
Keywords: *air pollution, ozone, photocopying machines, indoor environment, total volatile organic compounds*

Introduction

In the current era of mechanization, photocopying machines are among the imperative accessories in the office setup. Globally, photocopying industry is a source of earning for millions of workers. A prominent modification with regards to the technical aspects, basic material, convenience, yield and skillfulness in the photocopying process has also encountered (Elango et al., 2013; Kasi et al., 2018).

In spite of commercial ease and viability, photocopiers are considerable initiators of air pollution. During functional mode, photocopiers release particles of toner, volatile and semi-volatile organic compounds, particulate matter, particles of paper, nano-particles, ammonia, ozone, oxides of nitrogen, metals including arsenic, iron, chromium, titanium, zinc, nickel, cadmium, elemental sulphur and silicon (Durga Ch and Gokhale, 2015; Lee et al., 2006; Bar-Sela and Shoenfeld, 2008; Barthel et al., 2011; Bello et al., 2013; Wensing et al., 2008; Morawska et al., 2009; Adetunji et al., 2009; Pirela et al., 2013; Kleinsorge et al., 2011).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41334146

The tracking of Volatile Organic Compounds is required to evaluate the air quality. Spatiotemporal fluctuation of VOCs with respect to source is crucial in alleviating photochemical pollution (Huang et al., 2014). The chemical deterioration of VOCs gives rise to secondary air pollutants including peroxyacetyl nitrate (PAN), secondary organic aerosols and aldehydes. Sunlight as an auxiliary component in the presence of nitrogen oxides and volatile organic compounds promotes the generation of ground level ozone. The profile of VOCs and ozone reveals that the generation of ozone is VOC restricted (Srivastava et al., 2005; Lee et al., 2002; Toro et al., 2006). The association between the atmospheric emissions in terms of Nitrogen oxides and VOCs couples with the generation of ozone (Xu et al., 2017).

Ozone is a predominant component of the atmosphere that is of crucial importance to global climatic variation and tropospheric chemistry (Reddy et al., 2012). The precursors and elevated ozone levels are of great concern owing to the deleterious effects on the human health, forests, plants, natural and anthropogenic materials (Mavroidis and Ilia, 2012; Fann and Risley, 2013; Szyszkowicz et al., 2012; Kheirbek et al., 2013; Kumar et al., 2014a, b; Singh et al., 2014).

The traditional methods for the detection of organic compounds can require weeks to systematize, execute, delineate and interpret results. Direct reading methods (DRMs) have been initiated in workplaces as a substitutive approach with the benefits of swift measurement of the concentration (Brown et al., 2016). Direct reading methods using a portable real-time monitor for the detection of VOCs is a convenient tool (Coy et al., 2000; Peng et al., 2007). For the measurement of Volatile Organic Compounds at workplace, commercially available PID has been used for several decades (Driscoll, 2013).

Various kinds of compact portable VOC monitors are available nowadays. A monitor employing photoionization detector (PID) is able to detect Total Volatile Organic Compounds (TVOCs) with the intervening time of few seconds. The sensitivity of the measurement was outlined to range from ppb to ppm (RAE Systems, 2005). The PID-supported VOC monitor is anticipated to be a beneficial tool for the accurate measurement of TVOC exposure (Mizukoshi et al., 2010). As a segment of an effort in comprehending the role of photocopying process in the generation of TVOC and Ozone, the current study aims to perform real-time monitoring of TVOC and Ozone in photocopying centers and to evaluate the temporal and spatial variation in different areas of Lahore, Pakistan.

Materials and methods

Sampling area

Lahore is a megacity of Pakistan with the inhabitants of about 10+ million people (Razi et al., 2017). It is situated between 30.25° N to 31.5° N and 73° E to 74.5° E and a provincial capital of Punjab covering an area of 1, 172 km^2 (ul-Haq et al., 2016). Lahore is characterized by the semi-arid climatic conditions with very hot and wet summer and ordinarily cool and dry winter. In the month of June, the recorded highest maximum temperature was 48 °C; whereas, the lowest temperature was recorded to be -2 °C in January. Lahore is documented with an annual rainfall of 489 mm (Ali et al., 2014). The photocopying centers (n = 200) in the vicinity of Lahore were selected randomly. For the convenience, the sampling areas were divided into two different zones. The

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sampling areas of the current study are outlined in *Figure 1*. For the collection of baseline data of photocopying centers, a self-structured questionnaire was designed.

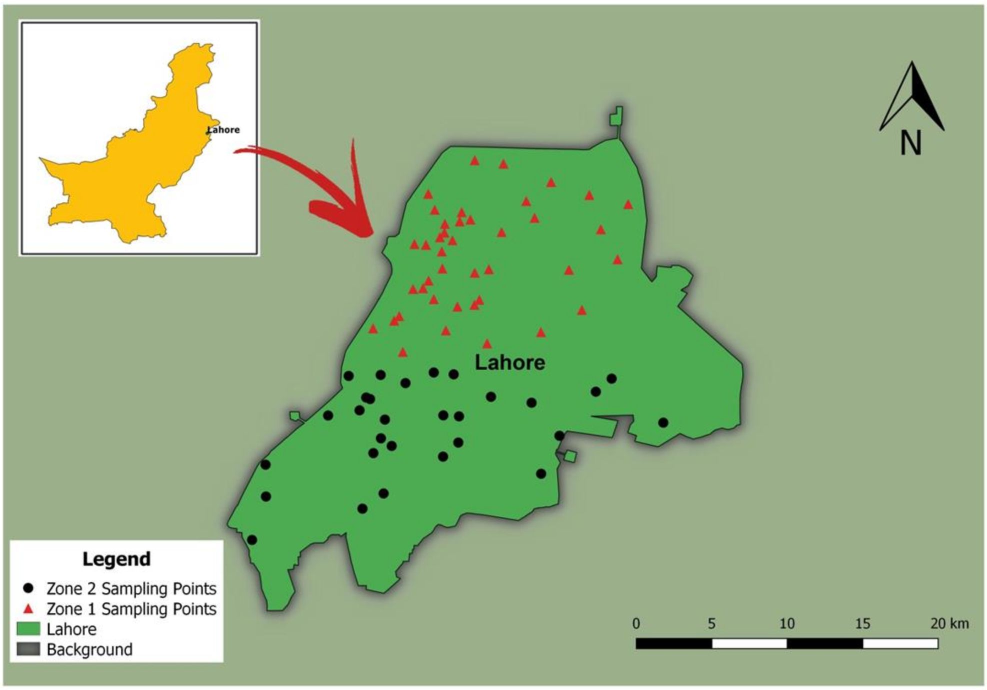

Figure 1. *Sampling areas of Lahore, Pakistan*

Monitoring instruments and technique

Monitoring was performed for the Total Volatile Organic Compounds (TVOCs) and Ozone (O_3) during the working hours in photocopying centers. A hand-held digital real-time VOC monitor with PID detector [ToxiRAE Pro PID (RAE Systems by Honeywell, 4.6” H X 2.4” W X 1.2” D, 235 g)] was employed as shown in *Figure 2a*. The monitor was characterized by the photoionization sensor with 10.6 eV lamp. The response time of the VOC monitor was <15 s. The monitor was turned on and the self-testing was performed by the instrument. Following the normal start-up process, the main measurement screen was displayed. The instrument specified the readings in parts per million (ppm). The documented values were converted to isobutylene equivalent concentrations in mg/m^3. Since Isobutylene is conventionally used for the calibration of PID detectors (RAE Systems, 2005).

For the monitoring of Ozone, portable single gas detector [BW Technologies GAXT-G-DL Gas Alert Ozone (1.1” X 2.0” X 3.75”, 82 g, by BW Technologies)] was employed with the concentration range of 0–1.00 ppm as shown in *Figure 2b*. The response time of Ozone monitor was 5 s. The self-testing was executed by the detector after being turned on. The normal start-up screen was displayed. The readings of Ozone were converted to µg/m^3. The TVOC and Ozone monitors can measure the concentrations every second. For the average concentration of TVOC and Ozone in an hour, the average concentration of 10-min interval derived from original 1-min interval reading was utilized. The TVOC and Ozone measuring instruments were placed inside the photocopying centers at the height of approximately 1.1–1.5 m above the ground

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(Meciarova et al., 2017). Meanwhile, it was ensured that the instruments are away from the direct pollutant sources and ventilation openings (Moreno-Rangel et al., 2018).

a b

Figure 2. *Monitoring instrument for* ***a*** *TVOC and* ***b*** *Ozone*

Data analysis

The mean TVOC and Ozone concentrations at a particular sampling point were shown in the maps. In order to analyze the spatial variability of TVOC and Ozone, the local interpolation method i.e., Inverse Distance Weighting (IDW) was employed using the QGIS Desktop 2.18.12.

Statistical analyses were executed by using SPSS [IBM SPSS Statistics Version 20 for Windows, SPSS Inc., Chicago, IL, USA]. The normal distribution of the data was determined by using Shapiro-Wilk Test. For categorical variables, Chi-square test was executed. For the data with no normal distribution, Mann Whitney U test was employed. Spearman's Rank correlation test was performed to quantify the correlation between testing variables. The Friedman Test was applied for assessing the difference in the concentrations of TVOC and Ozone with time of the day. The p-value < 0.05 was considered to be significant.

Results and discussion

The baseline attributes from photocopying centers were documented using a self-structured questionnaire as presented in *Table 1*. No significant difference was observed in terms of average working hours, number of machines and nature of area in photocopying centers located in both zones.

Spatial variability of total volatile organic compounds and ozone

The spatial variations of Total Volatile Organic Compounds (TVOCs) in mg/m^3 and Ozone in $\mu g/m^3$ are presented in *Figures 3a, b* and *4a, b.* A high degree fluctuation was observed in the concentrations of both variables.

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***Table 1.** Baseline attributes of photocopying centers*

	Total (n = 200)	**Zone 1 (n = 92)**	**Zone 2 (n = 108)**	**p-value**
Average working hours	11.97 ± 0.47	12.02 ± 0.55	11.93 ± 0.38	0.194 *
No. of workers	2.30 ± 1.80	2.21 ± 2.25	2.38 ± 1.31	0.027 *
No. of machines in the working centers	2.90 ± 1.52	2.72 ± 1.61	3.05 ± 1.44	0.063 *
No. of black and white machines	2.63 ± 1.24	2.47 ± 1.29	2.76 ± 1.183	0.052 *
No. of colored machines	0.29 ± 0.58	0.25 ± 0.59	0.31 ± 0.57	0.206 *
No. of machines in working condition	2.73 ± 1.57	2.59 ± 1.62	2.84 ± 1.52	0.144 *
No. of machines not in working condition	0.19 ± 0.50	0.13 ± 0.40	0.25 ± 0.57	0.093 *
Presence of computer	Yes = 189 (94.5%) No = 11 (5.5%)	Yes = 82 (89.1%) No = 10 (10.9%)	Yes = 107 (99.1%) No = 1 (0.9%)	0.002 **
Nature of area	Well ventilated = 30 (15%) Ventilated = 55 (27.5%) Congested = 68 (34%) Dark and congested = 34 (17%) Dark and very congested = 13 (6.5%)	Well ventilated = 14 (15.2%) Ventilated = 22 (23.9%) Congested = 30 (32.6%) Dark and congested = 21 (22.8%) Dark and very congested = 5 (5.4%)	Well ventilated = 16 (14.8%) Ventilated = 33 (30.6%) Congested = 38 (35.2%) Dark and congested = 13 (12%) Dark and very congested = 8 (7.4%)	0.331 **
Air conditioner	Yes = 12 (6%) No = 188 (94%)	Yes = 9 (9.8%) No = 83 (90.2%)	Yes = 3 (2.8%) No = 105 (97.2%)	0.038 **
Average No. of copies per day	2860.63 ± 5601.65	2280.71 ± 6650.59	3354.63 ± 4495.12	0.000*

*Mann Whitney U test, **Chi square test

Diurnal variability of total volatile organic compounds

The diurnal variation of Total Volatile Organic Compounds (TVOC) in mg/m^3 is presented in *Figure 5*. The average TVOC (mg/m^3) during working hours in photocopying center of Zone 1 and Zone 2 was found to be 0.674 ± 0.699 and 0.691 ± 0.786, respectively. The average TVOC concentration in photocopying centers located in Zone 1 was marginally lower than the centers in Zone 2. However, regarding the concentration of TVOCs, no significant difference was observed in the photocopying centers located in two different zones. In Zone 1, the diurnal variation revealed a similar pattern throughout the day with the lowest value observed at 8:00 am in the morning when the photocopying centers were opened. In case of Zone 2, the highest value of TVOC was observed at 12 Noon. Thereafter, it decreases gradually with the lower value during closing time of the photocopying centers. Several studies reported the generation of Volatile Organic Compounds in the photocopying centers (Stefaniak et al., 2000; Lu et al., 2015; Kowalska et al., 2015). A minimum TVOC concentration was observed during morning that gradually started increasing till noon. A progressive decline was found with the passage of time with the lower value at 8:00 pm.

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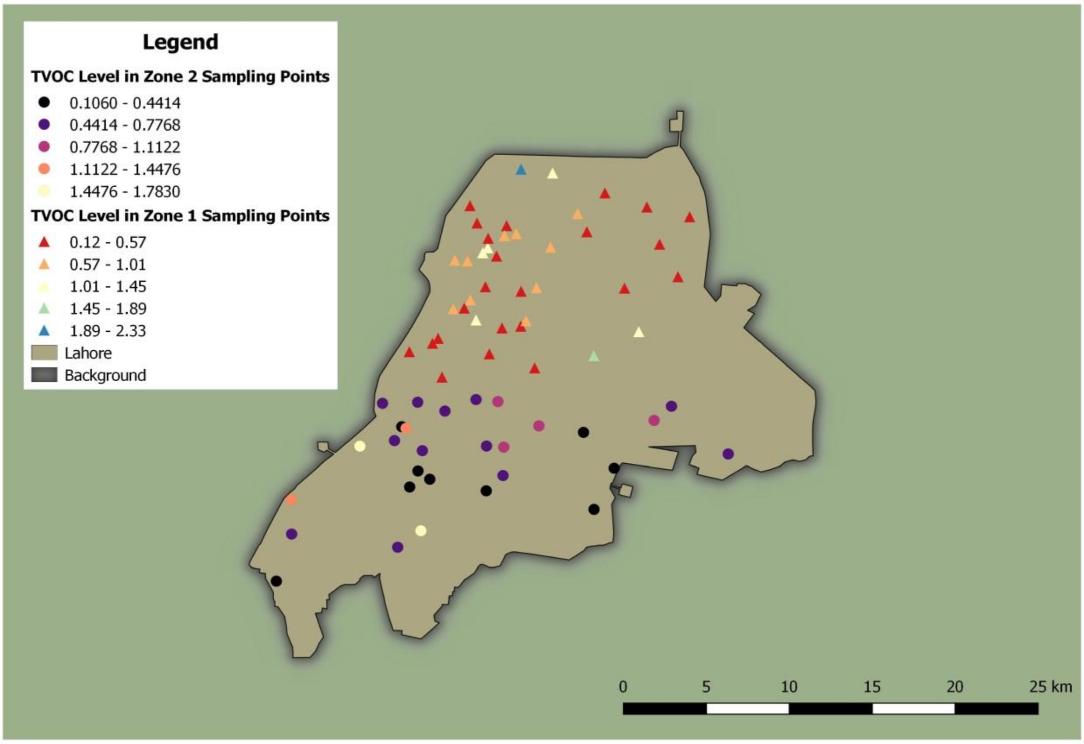
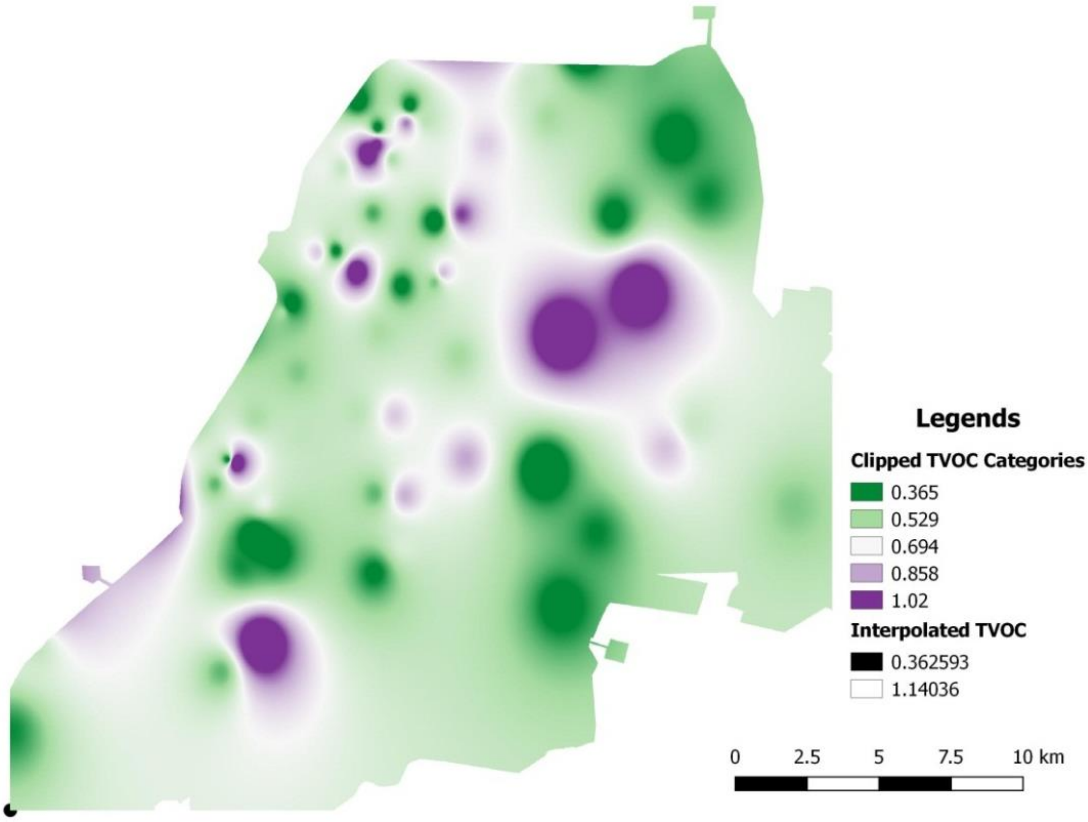
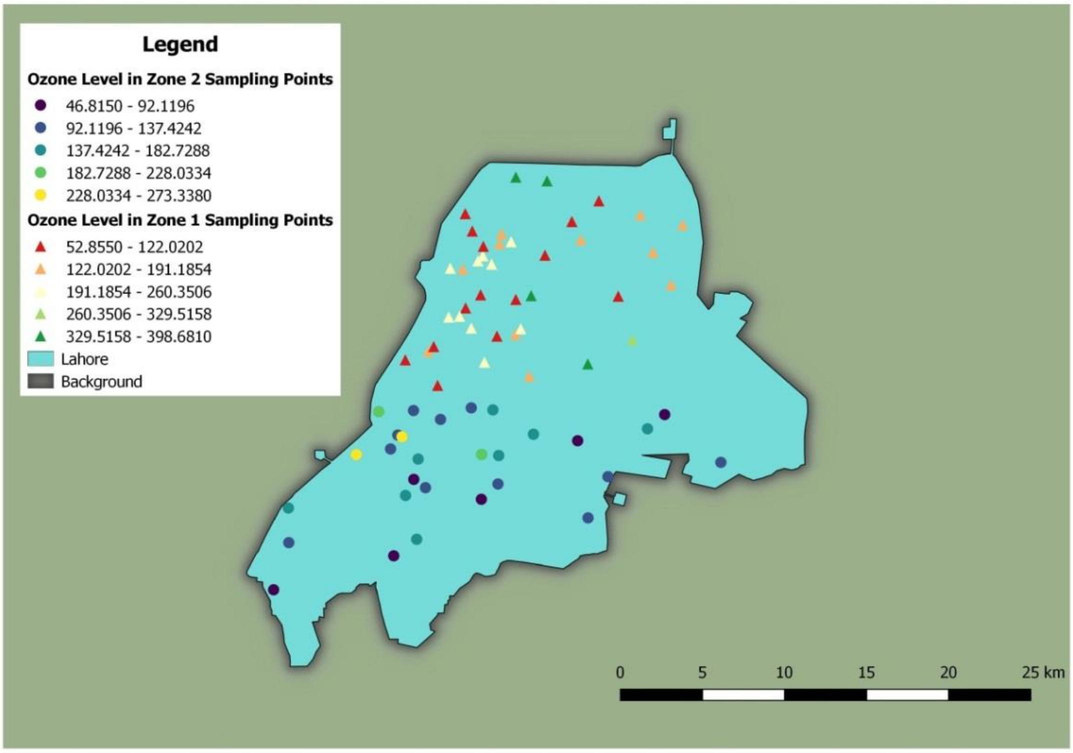


a

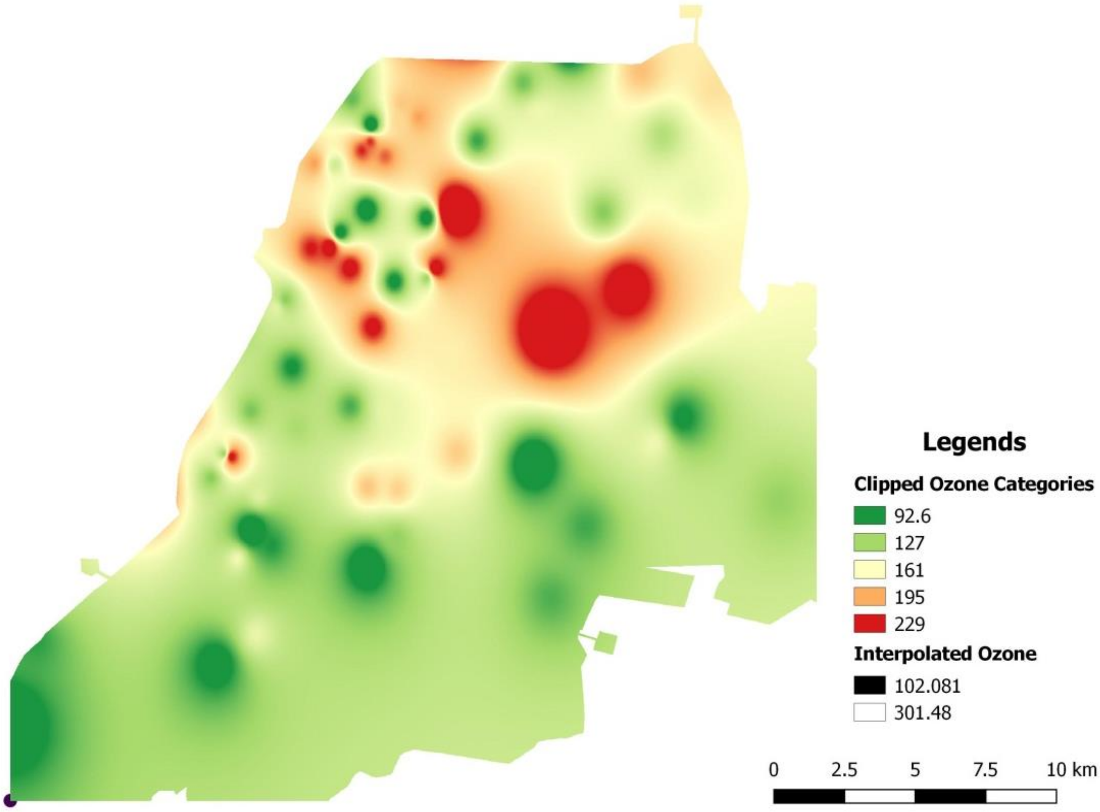


b

***Figure 3. a** Spatial distribution of mean TVOC concentrations in photocopying centers of Lahore. **b** Spatial distribution of TVOC concentrations by IDW method*



a



b

***Figure 4. a** Spatial distribution of mean Ozone concentrations in photocopying centers of Lahore. **b** Spatial distribution of Ozone concentrations by IDW method*

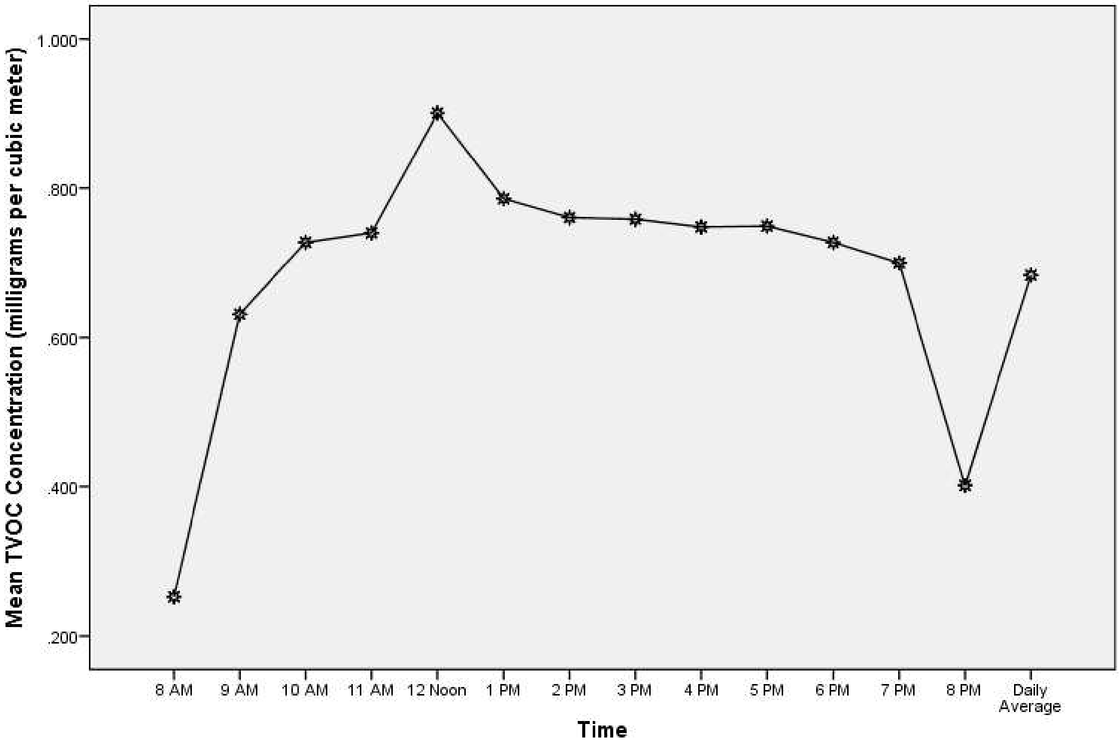


Figure 5. *Diurnal pattern of TVOC (mg/m^3) in photocopying centers*

Diurnal variability of Ozone

The observed diurnal variability of Ozone at different photocopying centers is shown in *Figure 6*. The average concentration of Ozone (µg/m^3) in Zone 1 and Zone 2 was found to be 187.50 $\pm$ 119.28 and 139.08 $\pm$ 83.56 respectively with the significant difference ($p < 0.05$) between both the zones. A relatively steep slope was recorded for the concentration of ozone. The diurnal variation of Ozone revealed that the minimum concentration was observed at 8:00 in the morning. It indicates that the maximum concentration was observed at 12 noon. Thereafter, it gradually declines and reaches the minimum value during the night. The similar diurnal variability was documented in other studies (Xue et al., 2017; Adame Carnero et al., 2010; Kumar et al., 2014b).

The Spearman's correlation was determined to evaluate the association between the TVOC and Ozone concentration (*Fig. 7*). A positive and highly significant correlation exists between the concentrations ($r = 0.627$, $p = 0.000$). The ozone precursors including oxides of nitrogen (NOx) and Volatile Organic Compounds (VOCs) vigorously affect the ozone profile (Filella and Penuelas, 2006).

In order to verify whether the time of the day makes any significant impact on the concentrations of TVOC and Ozone in photocopying centers, the Friedman test was employed. The significant results for both the concentrations indicated that the time of the day did have a significant impact on the concentrations of TVOC ($\chi^2 = 599.311$, Df = 13, Asym. Sig 0.000) and Ozone ($\chi^2 = 1357.799$, Df = 13, Asym. Sig 0.000).

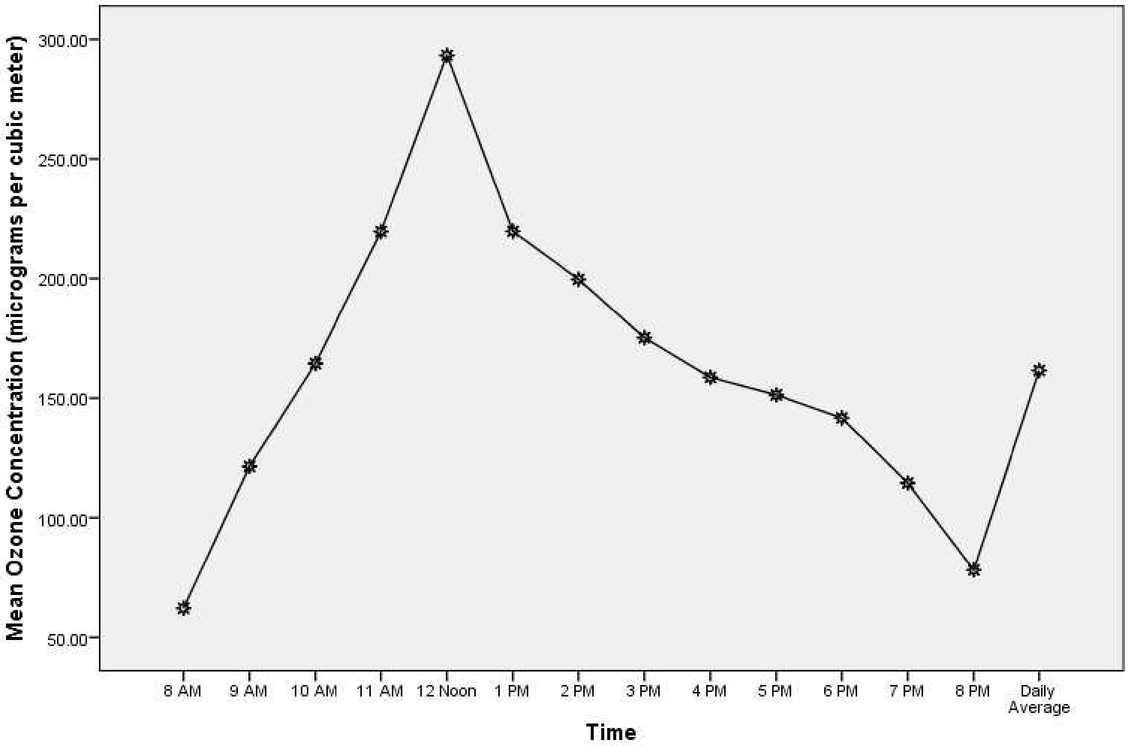


Figure 6. *Diurnal pattern of Ozone (µg/m^3) in photocopying centers*

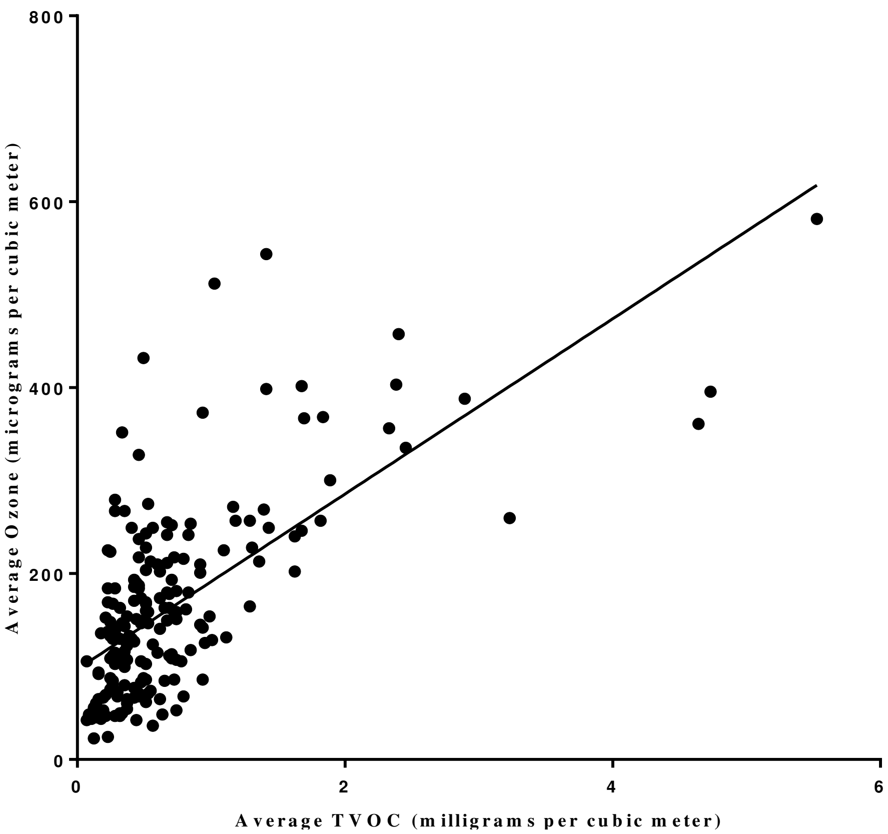


Figure 7. *Correlation between average TVOC (mg/m^3) and Ozone (µg/m^3)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_41334146

The average number of copies made in each photocopying center per day was found to be positively correlated with the concentrations of TVOC and Ozone. A highly significant and positive association was found between the average number of copies per day and the concentration of TVOC ($r = 0.556$, $p = 0.000$). Likewise, the average number of copies per day was computed to be positively and significantly correlated with the Ozone concentrations ($r = 0.333$, $p = 0.000$).

Concentration of TVOC and Ozone with respect to nature of area

In order to compute the association between the concentration of the testing variable and the nature of the area, Kruskal-Wallis test was employed. Based on the observation, the nature of the area was divided into 5 main categories i.e., Well Ventilated, Ventilated, Congested, Dark and Congested, Dark and very Congested. A highly significant association ($p < 0.01$) was observed between the nature of area and the concentrations of TVOC and Ozone (*Figs. 8* and *9*).

The observations of the current study revealed the variation in TVOC concentration at photocopying centers with respect to the nature of area. The maximum concentrations of TVOCs and ozone were observed in dark and very congested photocopying centers representing the significant role of ventilation in the declined TVOC concentration. Hori et al. (2013) reported the reduced concentration of TVOCs with the elevated ventilation.

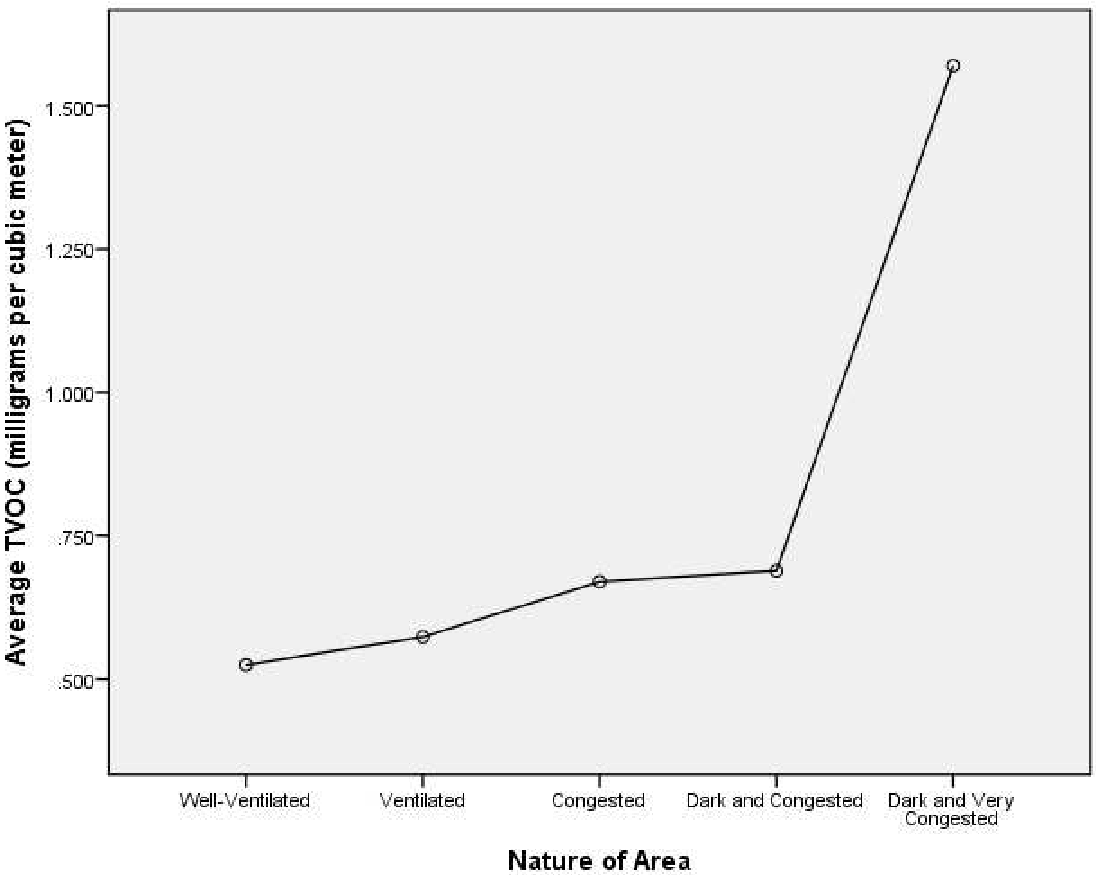

Figure 8. *Association between the nature of area and average TVOC (mg/m^3)*

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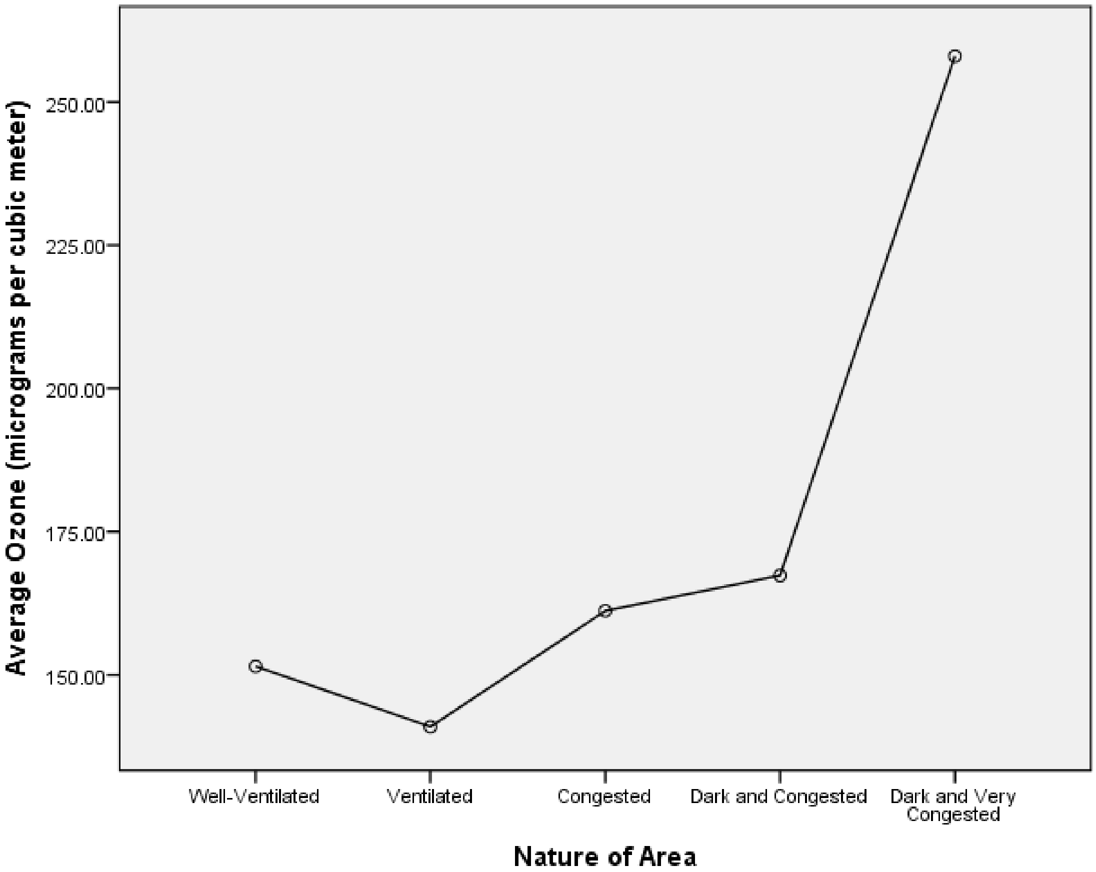

***Figure 9.** Association between the nature of area and average Ozone (µg/m^3)*

The concentration of ozone and its precursors may have a significant effect on the health of human beings (Kumar et al., 2014b). Keeping this in view, the results were compared with the available standards. In non-industrial indoor environment, discomfort and irritability ranges are defined by Molhave (1991). The concentration of VOCs below about 0.2 mg/m^3 is considered to be the comfort range. Between 0.2–3.0 mg/m^3, Irritation and discomfort is possible if other exposures are encountered. The results of the current study with reference to the concentration of TVOCs indicate that it lies within the range of multifactorial exposures i.e. 0.2–3.0 mg/m^3. As specified by the National Environmental Quality Standards for Ambient Air by Pak- EPA, the one hour average allowable concentration is 130 µg/m^3. However, in the current study, the average concentration exceeded this allowable limit that may result in ill-health of the workers.

Conclusion

In this research, the spatial and temporal variations of the total volatile organic compounds and ozone concentrations measured at photocopying centers in Lahore are presented. The variations in the concentrations of TVOC and Ozone between sampling sites indicated the role of photocopying emissions in generating indoor air pollution. The daily cycle presents variations with respect to the time of the day. The maximum

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concentrations of TVOC and Ozone was observed at 12:00 Noon. The daily minimum concentration was monitored at 8:00 am and 8:00 pm with fluctuating values throughout the day. The findings from this research highlight the importance of monitoring strategy to ultimately develop effective control measures. The present research can be used to find the association with the health impacts in the workers. Furthermore, it is recommended to perform comprehensive research using an appropriate risk assessment strategy.

Acknowledgements. The authors are thankful to the Chairperson, Department of Environmental Science for administrative support during the study and staff of lab of the department. Meanwhile, the authors are thankful to Pak-Green Enviro-Engineering for the provision of instruments to conduct this research.

Conflict of interests. There is no conflict of interests among the authors about the research work.

Research funding. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41334146

THE EFFECTIVENESS OF RECOMBINANT CHITINASE OBTAINED FROM BARLEY (*Hordeum vulgare* L.) AGAINST POTATO PATHOGENS

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(Received 8th Dec 2018; accepted 8th Feb 2019)

Abstract. Globally, fungi and fungi-like organisms, with over 8,000 species, cause more plant diseases than any other group of plant pests. Chitinases possess an anti-fungal role in disease resistance. They represent the pathogen-related protein group that cleaves β-1,4-glycoside bond of chitin present in the cell wall of filamentous fungi by catalysing the hydrolytic cleavage. In this study, we have amplified 672 base pair (bp) coding regions of the plant chitinase gene of *Hordeum vulgare*, over-expressed in *Eschercia coli* (*E. coli*) host BL21 DE3 strain by 0.5mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. In western blotting, the recombinant chitinase protein transferred on nitrocellulose membrane was detected at 26 kDa with histidine-tagged antibody reactions. By applying the nickel charged sepharose column affinity chromatography method, a single purified band of recombinant chitinase protein was found at 26 kDa in SDS-PAGE. The purified recombinant chitinase protein during the agar well diffusion method inhibited the growth of three phytopathogenic fungi in a significant way; *Alternaria alternate*, *Rhizoctonia solani* and *Fusarium oxysporum* in qualitative in-vitro antifungal assays. The purified recombinant chitinase protein showed up to 82.6% reduction in *A. alternate* hyphal growth; 77% reduction of hyphal growth in *Rhizoctonia solani* and 78% reduction of Fusarium's hyphal growth in in-vitro quantitative antifungal assays, thus depicting a strong antifungal potential. Conclusively, plant derived chitinase genes are superior in protecting the crops from phytopathogenic fungi if cloned in a suitable plant vector and expressed.
Keywords: *antifungal response, early blight, Fusarium oxysporum, Rhizoctonia solani*

Introduction

Fungal diseases have a deleterious impact on crops and consequently on the economy in direct, measurable terms. The extent of yield losses for major crops such as rice, wheat, maize, potatoes and soybean as the result of an epidemic outbreak vary, but a major threat to food security persists globally (Ramirez-Cabral et al., 2018). Oerke (2006) found that 26-30% of global yield losses of crops including sugar beet, cotton and wheat are due to fungal pathogens, while this number is 40% for potatoes and 37% for rice. Currently, fungal infections destroy approximately 125 million tons of crops including rice, wheat, maize, potatoes, and soybeans each year. Some of the world's greatest famines and the resulting human suffering can be blamed on plant pathogenic fungi and fungi-like organisms (FLOs). Fungicides are primarily used on fruit and vegetable crops and approximately 97% of potato crops are treated with fungicides

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41474157

(Kaonga et al., 2018). The fungicides including Maneb, Mancozeb, cymoxanil, dimethomorph hexaconazole, iprodione, isoprothiolane and thia bendazole are sprayed on vegetables (Bentley et al., 2006; Stadlinger et al., 2018).

The most devastating among them include *Alternaria alternate*, *Rhizoctonia solani* and *Fusarium oxysporum*. *Alternaria alternate* causes early blight in tomato and potato, has a worldwide distribution and has been considered one of the most important foliage diseases in areas with warm wet conditions in the early crop season. The symptoms include tuber blight and leaf spot on potato; fruit rot and stem lesions on tomato. *Rhizoctonia solani* is a soil-borne fungus that causes economically important diseases in many crop species, such as sugar beet, potatoes, tomatoes, rice, rape, maize, soybean and others (van Lenteren et al., 2018). The disease caused by *Rhizoctonia solani* include stem canker and black scurf in potatoes, fruit rot in tomatoes, sheath blight in rice, Rhizoctonia crown and brace root rot in maize, root and stem rot in soybean crop and root rot in canola (Melzer et al., 2016). *Fusarium oxysporum* is a soil borne phytopathogen cause economical loss around the globe. It not only causes yield reduction but also accounts for dry rot on potato, sudden death syndrome in soybean, crown rot and head blight on the wheat and bakanae disease on rice (Bentley et al., 2006; Munkvold, 2017).

Various chemical fungicides and breeding methods for selection of natural resistant varieties are in practice to meet the fungal problem. But the selection of resistant variety is not up to mark to control fungi. The excessive use of chemical spray with about15-20 application per growing season, not only have a harmful effect on targeted pest but also on beneficial organisms in addition to hazardous environmental effects. The cell wall of the fungus is a complex structure, composed of chitin fiber and glucan held together by protein. Chitin is considered to be the second most abundant polysaccharide in the universe, composed of N-acetylglucosamine(Nakkeeran et al., 2016). The concentration of the chitin differs among fungi, as yeast cell wall composed of 1-2% chitin while Aspergillus contains 10-20% of chitin by dry weight. *F. oxysporum* contain 25-35% chitin in its cell wall (Becker et al., 2016). The primary role of chitin is to provide structural integrity but it also functions as linkage between cell wall and the capsule. Disruption of the fungal cell wall has severe effect on the morphology resulting in osmatic instability of the cell wall which leads to cell death (Cushion et al., 2018). It is, therefore, hypothesized that chitinase can have potential as an antifungal agent.

Chitinase an enzyme which cleaves β-1,4-glycoside bond of chitin by catalyzing its hydrolytic cleavage (Jitonnom et al., 2011). Chitinases belong to pathogen-related protein (PR) group in plants. Initially, when discovered, PR protein was considered as defense protein only as it was induced only in response to an infection but later it was revealed that PR protein has a potential role in abiotic stress also (Lincoln et al., 2018). The fact that plant chitinases act as a shield against plant fungi is well established (Fesel and Zuccaro, 2016). Plant chitinases are part of various groups of enzymes which differ with respect to their structure, enzymatic properties, and cellular localization. They are endo-chitinases and are present in stem, seed, tuber and flowers. Plant chitinases are categorized in 5-6 classes based on amino acid structure.

The aim of the present study was to isolate and over express barely chitinase II gene and evaluate its antifungal activity against major plant fungi including *Alternaria alternate*, *Rhizoctonia solani* and *Fusarium oxysporum* that cause yield loss to important crops.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Materials and methods

Isolation of chitinase gene from barley

Seeds of Barley (*Hordeum vulgare* L.) variety Haider-93 were kindly obtained from Ayub agriculture research center, Faisalabad, Pakistan and were grown in CEMB field, Lahore, Pakistan. Total RNA was isolated from the young, immature leaves using TriZol reagent (Invitrogen). cDNA was prepared by cDNA synthesis kit (Thermoscientific) according to the manufacturer instructions. Chitinase II gene specific forward (BLP-F) and reverse (BLP-R) primers were used to amplify 889 bp gene fragment (*Table 1*). For amplification, reaction mixture comprised of 1× PCR reaction buffer, 0.1 mM of dNTPs, 1 pmole each of forward and reverse primer, 1 unit of Taq DNA polymerase (Thermoscientific) and 50 ng of template cDNA. PCR reaction was carried out in a thermal cycler (GeneAmp PCR system 9700, ABI) with the following thermal cycling parameters: initial denaturation at 95 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final extension of 7 min at 72 °C. Amplified products were resolved on 1% agarose gel and visualized under UV transilluminator (Biotop, Pakistan). Amplified gene fragment was cloned into pCR2.1 TA cloning vector and transformed into *E. coli* DH5α competent cells. The cloning was confirmed by restriction digestion and sequencing. Further, the deduced gene sequence was submitted to GenBank database for accession number.

Cloning and over-expression of chitinase gene

For over-expression of chitinase II gene in *E. coli*, pET 30a vector (Novagen) was used. For directional cloning, *BglII* and *NcoI* restriction sites were added at 5’ in BLPetF and BLPetR primers (*Table 1*) to amplify 762 bp gene fragment. Cycling conditions were the same as above. Further, the amplified 762 bp chitinase gene fragment was codon optimized from IDT (Integrated DNA Technologies) and cloned in fusion with His tag in the pET30a vector. Further, the ligated product was transformed in chemically competent *E. coli* BL21 (D3) strain for expression studies.

Recombinant protein expression

Approximately, 22 different transformed colonies of BL21 (D3) were screened for over-expression of recombinant protein. Out of these, only three colonies were subjected to enhanced protein expression. Fast induction method opted for suboptimal yields of the recombinant protein by using a different concentration of IPTG (0.1 mM to 1.5 mM) and induction time.

Purification of His tag recombinant protein

The induced culture was pellet down and sonicated at 50% duty cycle with short pulses of 35 s each for 15 min. The tube was centrifuged and the pellet was resuspended in 20 mM Tris-HCl (pH-8) supplemented with 2 M Urea, 2%triton, 1 mM EDTA and 500 mM NaCl. After centrifugation, the pellet was washed with 1XPBS Buffer inclusion bodies were resuspended in 1 XPBS buffer containing 0.5% Triton X-100 and 2 M urea). This was kept at room temperature for 35 min. The detergent was removed by five times washing with sterilizing distilled water. Inclusion bodies were finally solubilized in buffer containing 6 M guanidine HCl, 20 mM Tris–HCl, pH 8.0 and 0.5 M NaCl stirred subsequently for 1 h at room temperature. Crude protein was

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quantified by (Kruger, 2002). Finally, the protein was purified using Chelating Sepharose Fast Flow (Amersham) through Nickel. The column was equilibrated with 6 M guanidine HCl and protein was loaded on equilibrated column at the rate of 1ml/min. then washing was done with 1X PBS containing 0.5 M NaCl and 20 mM imidazole. The protein was eluted in 1X PBS containing 0.5 M NaCl and 200 mM imidazole. The eluted protein was Dialysed against 50 mM phosphate buffer pH 6.5 for 24 h at 4 °C and diluted in refolding buffer (100 mM Tris-HCl pH 8, 0.5 M L-arginine, 0.2 mM EDTA) with constant stirring.

Table 1. *Sequence of primers used to amplify the chitinase gene fragment from H. vulgare*

Name	Sequence (5'-3')
BLP-F	TAGTTAGCATGGCGCGGTA
BLP-R	TCATACGACACGATCAACTGG
BLPetF	GGCAGATCTGTCATCACGCAATCGGTGTA
BLPetR	GGCCCATGGGAAGTTTCGCTGGGTGTAGC

Western blot

After purification of the recombinant protein SDS-PAGE was run. The protein was transferred on nitrocellulose membrane using semidry Trans Blot (Bio Rad) transfer. The transfer was carried at 12 V for 40-50 min. The membrane was blocked in 5% skimmed milk overnight at 4 °C. Following day, membrane was removed from blocking solution and membrane was washed three times with 1X PBS buffer for 10 min each. The membrane was incubated with His tag anti rabbit primary antibody (1:500; Santa Cruz) in 1X PBS for 1 h with gentle shaking at room temperature. After washing the membrane with 1XPBST the Alkaline Phosphatase conjugated secondary antibody was added at 1:15000 and incubated at room temperature for 1 h with gentle shaking. After washing the membrane with 1XPBST the purple color was developed by NBT/BCIP tablet (Sigma). The reaction was stopped with autoclaved distilled water.

In-vitro antifungal assay

Qualitative assay

The phytopathogenic fungi *Rhizoctonia solani*, *Alternaria alternate* and *Fusarium oxysporum* were kindly obtained from USDA. For the antifungal assay, cylinder plate method of (Kirubakaran and Sakthivel, 2007; Toufiq et al., 2018) was adopted with some modifications. The 2 mm patch of the fungi to be tested was inoculated on potato dextrose agar (Oxoid) plate in the centre and kept at 28 °C for 72 h for growth. Three cylinders were made around the plate edges which refer to control, 70 µg and 100 µg of purified chitinase protein. In the control cylinder, sterilized water was poured. Resulting fungal growth retardation/inhibition of fungi was observed after 48 h of protein inoculation. The results were repeated in triplicate.

Quantitative assay

The inhibitory effect of recombinant chitinase protein on the hyphal growth of *A. alternata*, *R. solani* and *F. oxyporum* was evaluated as previously described by (Khan et al., 2017; Plascencia-Jatomea et al., 2003). Wells were made on potato dextrose agar

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(Oxoid) plate and filled with 40 µl of respective fungal spores suspension (10^4 spores per ml) followed by incubation at 28 °C for overnight. Further, in each well inoculated with respective fungi, 30 µg, 50 µg, 70 µg, 100 µg of the purified chitinase protein was added and incubation was continued at 25 °C for 5 days. The control well was contained only the fungal inoculum and instead of recombinant protein, protein extraction buffer was added. Subsequently, the radial growth of respective fungi was recorded every 24 h for 5 days. The distance between the control hyphal edge to the centre – the distance between the treated hyphal edge to the centre) / the distance between the hyphal edge to the centre in the control] × 100% (Plascencia-Jatomea et al., 2003). The results were replicated in triplicate.

Results

Over-expression of recombinant chitinase gene

Full-length Class II chitinase gene of 889 bp was successfully amplified from barley. Deduced sequences from recombinant TA clone were analyzed for the potential coding region through the CDD (Conserved Domain Database), and the sequence was submitted to NCBI Genbank and accession # KC899774 was obtained. *Figure 1* depicts the isolation of chitinase gene and restriction digestion of chitinase gene in pET30a vector.

Further, the amplified 762 bp gene fragment was directionally cloned in pET30a, under the control of T7 promoter by using *BglII* and *NcoI* restriction enzymes. Successful cloning was depicted by restriction digestion of the recombinant plasmid pET:ChiII. The appearance of the vector at ~ 5.4 kb and gene fragment at ~ 762 bp verified the construction of pET:ChiII (*Fig. 1*).

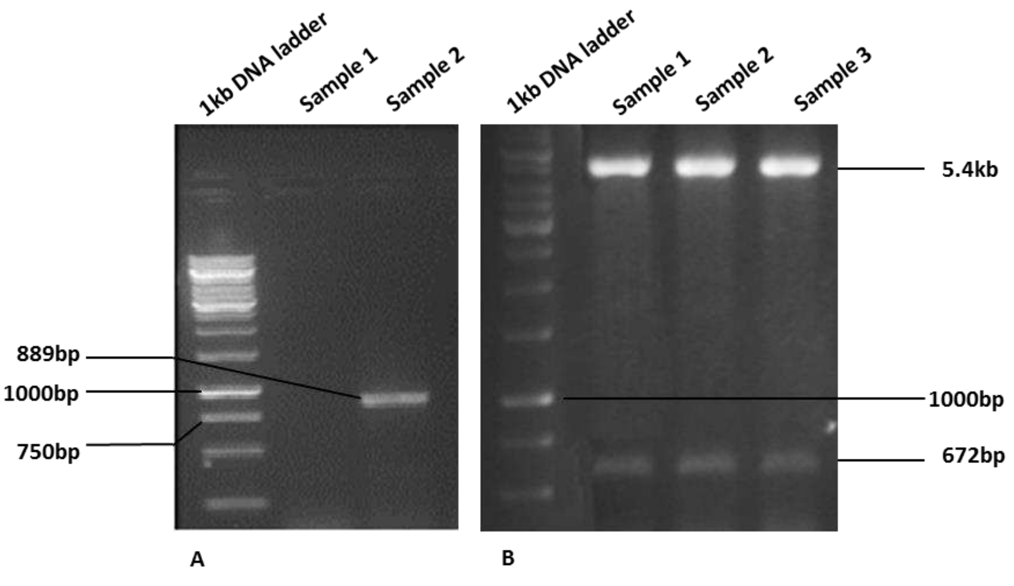

Figure 1. *Amplification and restriction digestion of chitinase gene. A) amplification of chitinase gene from barley, lane1 = 1 Kb ladder, lane2 = negative control, lane3 = amplification of 889 bp gene from barley. B) restriction digestion of chitinase gene into pET vector30a, lane1 = 1 Kb ladder, lane 2-4 = Restriction digestion of potential pET clones to verify the construct*

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Several transformed colonies of BL21 strain were selected for recombinant protein expression onto SDS-PAGE. Out of total 22 colonies selected, only three were selected for further expression enhancement. Recombinant proteins over-expressed in bacteria often form insoluble proteins called inclusion bodies. It was found that recombinant chitinase protein was present inside the inclusion bodies while expressing in the *E. coli* host. Maximum expression of the recombinant chitinase protein was achieved when samples were induced with 0.5 mM IPTG while optimized induction time was 4 h. *Figure 3* depicts the expression patterns of all three selected colonies. A Clear difference in the expression pattern of induced versus uninduced sample can be seen (*Fig. 2*). For each treated colony, the recombinant protein expressed more as compared to the control, un-induced sample. However, maximum expression was achieved in colony # 2 where unwanted proteins did not express (*Fig. 2*). Subsequently, colony # 2 was selected for purification through column chromatography.

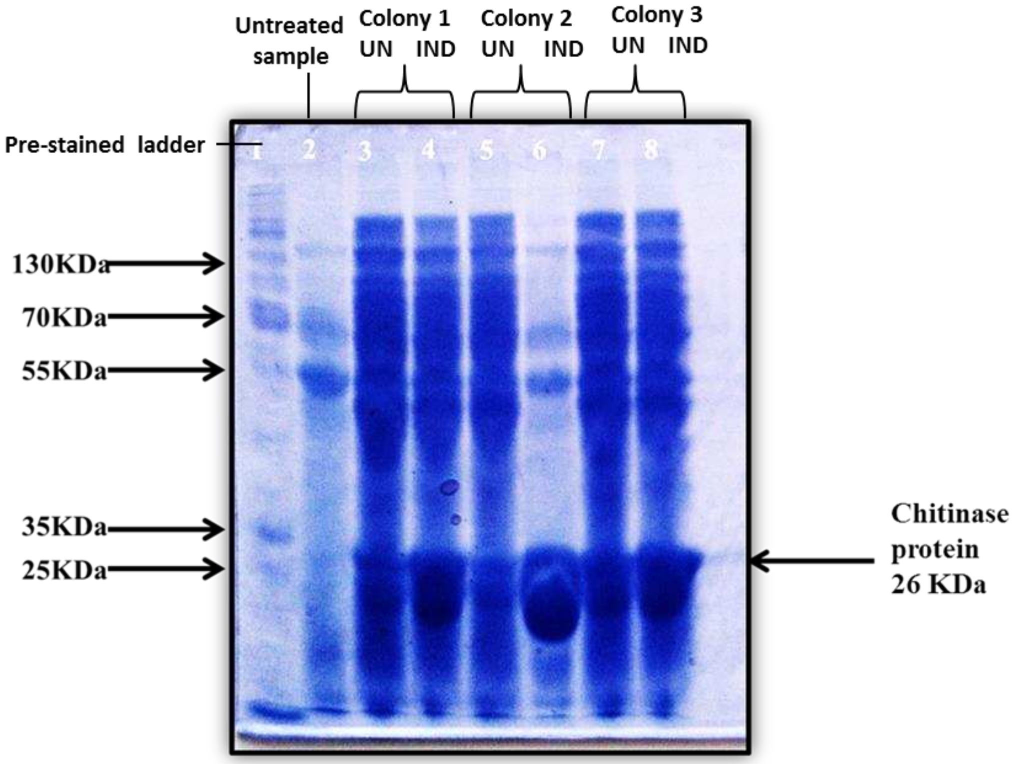

Figure 2. *SDS-PAGE of crude protein samples showing recombinant protein expression in different induced/uninduced colonies of BL21 strain. Un-induced cultures were also run in parallel. In each well, approximately 10 µg protein was loaded while pre-stained protein marker was used to define protein size. In the figure, UN refers to uninduced colony culture and IND refers to induced culture of the same colon while in total, three colonies were screened for expression of the recombinant protein*

As the recombinant protein was present in inclusion bodies, purification of the protein was convenient. The inclusion bodies were purified to avoid co-purification of contaminants. Solubilization was done by 6 M guanidine HCl and further subjected to refolding after elution using Ni-NTA IMAC purification system. The purified protein was resolved on 12% SDS-PAGE followed by western blotting using anti- His antibodies as shown in *Fig. 3A* and *B*.

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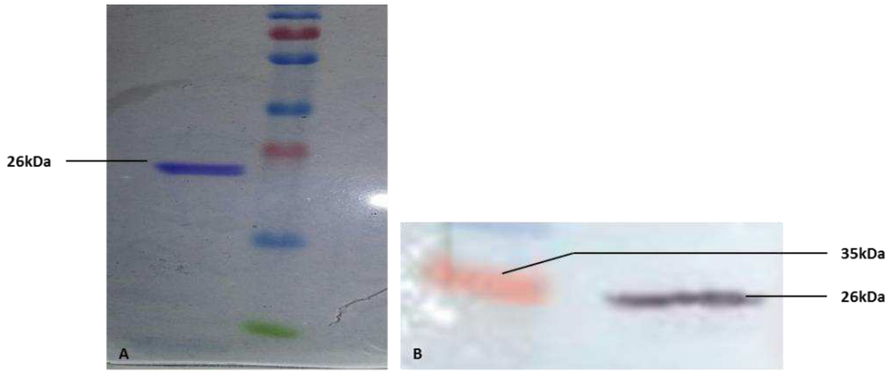

Figure 3. *A) Purification of the recombinant chitinase protein purified through IMAC fast flow affinity chromatography. A single purified protein fraction at 26 kDa was obtained. B) Western blot of chitinase protein over-expressed in BL 21 strains. The blot was developed through antigen antibody reaction where Histag anti-rabbit primary antibody and secondary goat anti-rabbit AP conjugated antibody were used*

Recombinant chitinase protein inhibited the hyphal growth to a significant extent

In *in vitro* qualitative assay, cylinder plate antifungal assay was performed where three phytopathogenic fungi; *Rhizoctonia solani*, *Alternaria alternate* and *Fusarium oxysporum* were tested against purified protein. Pathogen inhibition assay revealed that recombinant chitinase protein was effective in controlling the inoculated fungi. Growth retardation of *Rhizoctonia solani* was inhibited to a significant extent when treated with purified protein at 100 µg concentrations while fungus inhibition was more as compared to the 70 µg concentration of the protein where relatively less inhibition was observed. While in control sample, no inhibition was observed at all. Similar findings were met when *Alternaria alternate* and *Fusarium oxysporum* were inoculated and treated with purified recombinant protein. Maximum inhibition of the fungi growth was achieved at 100 µg protein concentration; less was in 70 µg concentration as compared to the control sample where no inhibition was observed (*Fig. 4A, B* and *C*). Conclusively, the fungus inhibition was found to be directly linked with increasing concentration of the recombinant protein, as, at maximum protein dose (100 µg), fungus inhibition was maximum.

In the *in-vitro* quantitative antifungal assay, percentage reduction of hyphal growth in *A. alternate* was 40% at a 30 µg concentration of the purified recombinant chitinase protein; 52% at 50 µg; 78% at 70 µg; 82.6% at 100 µg protein concentration (Fig. 5A). Similarly, purified recombinant chitinase protein inhibited the hyphal growth of *R. solani* maximally at a100 µg concentration where inhibition was 77% while the percentage inhibition was 42, 62 and 76 at 30 µg, 50 µg and 70 µg protein concentration (Fig. 5B). In parallel, chitinase protein reduced the hyphal growth of *Fusarium* to 78% at 70 µg and 100 µg concentrations while comparatively less reduction was observed at 30 µg and 50 µg protein concentration where inhibition was 52% and 66.6% (*Fig. 5C*). The findings of *in vitro* quantitative assay showed that there was a significant difference among control and different concentrations in standard value. ($P < 0.05$; $n = 3$). In summary, at 70 µg and above a concentration of the protein, fungal growth was inhibited maximally and the fungi were unable to grow across the well while at lower concentrations, the reduction was observed at a lesser extent. The control, untreated sample did not exhibit any inhibitory effect and the respective fungus grows maximally.

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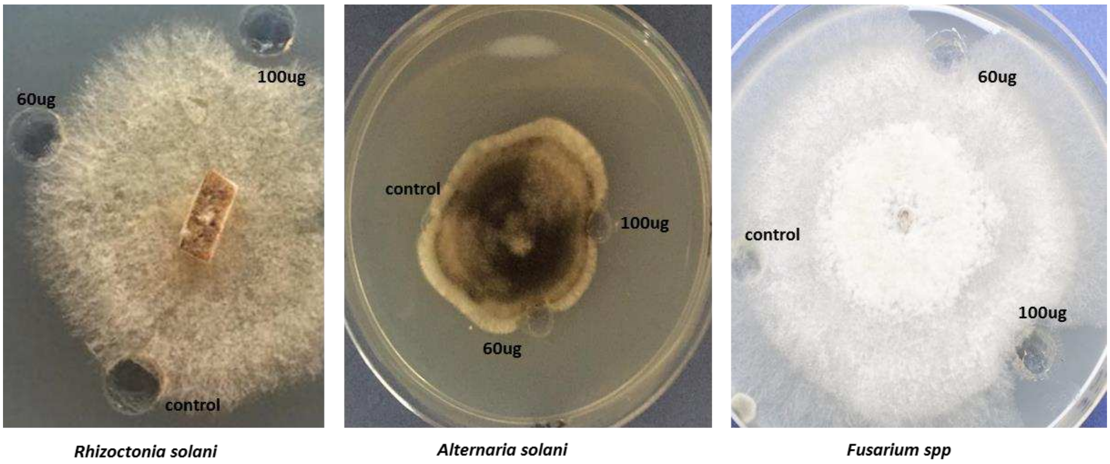

Figure 4. *In-vitro fungal inhibition assay. Alternaria alternata, Rhizoctonia solani and Fusarium oxysporum were tested in qualitative assay against purified recombinant chitinase protein. Zone of inhibition are clearly visible*

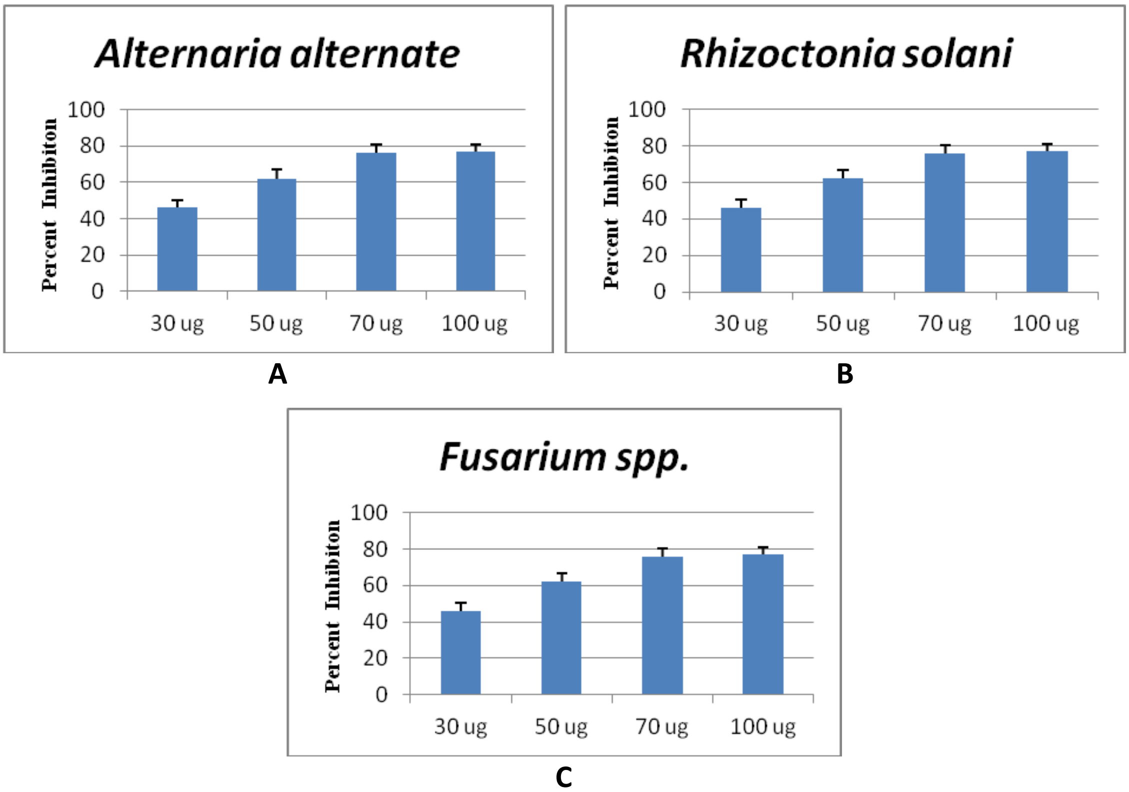

Figure 5. *A) Percentage reduction in the hyphal growth of Alternaria alternate exhibited by the purified recombinant chitinase protein. B) Percentage reduction in the hyphal growth of Rhizoctonia solani exhibited by the purified recombinant chitinase protein. C) Percentage reduction in the hyphal growth of Fusarium oxysporum exhibited by the purified recombinant chitinase protein. The data were obtained in in-vitro quantitative antifungal assays. Control sample did not exhibit any reduction. The y-axis represents percentage inhibition percentage of fungi while the x-axis represents different concentration of purified chitinase. The bars represent the standard deviation. Statistically, the results showed that there was significant difference among control and different concentrations in standard value. ($P < 0.05$; $n = 3$)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_41474157

Discussion

The aim of the present study was to isolate a plant based chitinase gene, characterize it and express it into the *E. coli* BL21 strain. The full-length gene was successfully isolated from total RNA of barley. Roberts and Selitrennikoff (1988) proposed that bacterial chitinases are difficult to interact with fungal cell wall so they have less resistance against fungi, whereas plant chitinases show high antifungal activity. The plant chitinases are usually endochitinases and contain lysozyme activity (Islam and Datta, 2015). In contrast, the bacterial enzymes are exochitinases and hydrolysed the chromogenic trisaccharide analogue as a substrate (Horn et al., 2006; Roberts and Selitrennikoff, 1988; Toufiq et al., 2018). So plant chitinases are usually preferred for increasing antifungal activity in the host. For this purpose, a plant chitinase was selected in this study.

Subsequently, we over-expressed chitinase gene in *E. coli* host and after purification of recombinant protein, the inhibitory effect against the three most devastating pathogenic fungi was evaluated in-vitro. The purified recombinant protein was used for inhibition of *Rhizoctonia solani*, *Alternaria alternate* and *Fusarium oxysporum* Different studies showed the inhibitory effect of chitinases in-vitro against fungi containing chitin. Kirubakaran and Sakthivel (2007) used class 1 chitinase from barley to inhibit pathogenic fungi of broad range in-vitro. In another study, Eissa et al. (2017) showed protection of transgenic wheat by using class II chitinase from barley seeds, against powdery mildew. Similarly, Liu et al. (2013) expressed two chitinase genes, LbCHI31 and LbCHI32 from *Limonium bicolor* in *E. coli* and reported their antifungal activity against *Alternaria alternate*. While Shenoy et al. (2006) have reported a 29 kDa chitinase with antifungal activity in the bulbs of plant *Urginea indica*. Additionally, Garg and Gupta (2010) purified chitinase from moth bean having an inhibitory effect on *Macrophomina phaseolina* strain 2165. Tariq et al. (2018) transformed chitinase II gene to sugarcane and obtained significant resistance against *C. falcatum* compared to control. While Khan et al. (2018) expressed plant chitinase II gene and developed potato variety resistant to *A. solani*.

Conclusion

Conclusively, the potential inhibitory effect of barley derived chitinase II gene was exploited against phyto-pathogenic fungi which, in future, can be used to develop transgenic plants tolerant towards these devastating fungal pathogens.

Acknowledgements. This research work was funded by Higher Education Commission (HEC), Pakistan under "Indigenous Ph.D. Fellowship' scheme".

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41474157

KLEBSIELLA PNEUMONIAE DISINFECTION WITH ULTRASOUND AND HYDROGEN PEROXIDES

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(Received 16th Dec 2018; accepted 22nd Feb 2019)

Abstract. Water-borne epidemics caused by pathogenic microorganisms endanger public health especially in countries where access to clean water sources is difficult. Also, the conventional disinfection methods have various disadvantages on environmental health and they can be ineffective against some pathogenic microorganisms. For these reasons, the disinfection process applied in water has gained great importance and new alternative treatment technologies have to be developed to replace traditional methods used to protect public health. In this study, the effects of ultrasound (US) and hydrogen peroxide (H_2O_2), which have been used for water disinfection in recent years, have been examined whether or not they can remove *Klebsiella pneumoniae* from water. This study has concluded that the combination of US and H_2O_2 can effectively remove *Klebsiella pneumoniae* bacteria from water. When the application of ultrasound was performed with a H_2O_2 concentration of 5 mg l^{-1}, the disinfection efficiency of *Klebsiella pneumoniae* was 4.22 log after 60 min.
Keywords: *water disinfection, ultrasound, hydrogen peroxide, coliform*

Introduction

Water is significantly contaminated by human or other environmental factors. Water, which is required for the survival of living organisms, are polluted by anthropogenic sources, such as, chemical, physical and microbial contaminants. Especially, water storage tanks can be contaminated significant pollutants, such as leaks, mixing of sewage sludge from soil cracks, agricultural runoffs, unsuitable industrial applications, mining applications, underground injection of waste chemicals, and corrosive waters (WHO, 2004, 2011). The legal regulations to ensure clean and reliable drinking and potable water must be very strict in application and control, and so clear and easy to update in terms of compliance with scientific developments (WHO, 2004, 2011).

Pathogens that cause waterborne diseases include; bacteria, viruses, protozoa and algae (Ashbolt, 2004; Moe, 2007). Water-borne epidemics diseases have significantly reduced by chlorinating drinking water since 1900 (Akin et al., 1982) but can be ineffective against to some pathogenic microorganisms. *Klebsiella pneumoniae* is stationary, non-spore, short, rounded, 1-2 µm in length and 0.5-0.8 µm in with basil. *Klebsiella pneumoniae* is best grown at 12-43°C and at pH 7 using bloody agar, endo agar and EMB agar media in their analysis, which can show capsular, aerobic and facultative anaerobic character in Gram negative polysaccharide structure. Because it is a bacterium found in the upper respiratory tract and stool flora in humans, pathogenicity is released as an opportunistic pathogen in the face of inappropriate conditions (Dutka, 1973). The sources of pathogenic microorganisms in drinking water are surface waters and groundwater contaminated with fecal matter (Shiddamallayya and Pratima, 2008; Gray, 2014). Drinking water supplies are contaminated with fecal *Klebsiella pneumoniae* via domestic wastewater, meat treatment processes wastewater and the surface flow from farms (Percival, 2014). In addition, *Klebsiella pneumoniae* are responsible for hospital

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

infections (Percival, 2014). *Klebsiella pneumoniae* is listed as total coliform in the international guidelines (Arbuckle et al., 1976). As for standard of WHO, the number of total coliform in 100 ml of untreated ground water is limited to less than 10 cells (WHO, 2004), and it is indicated in the guideline that there should be no fecal coliform in water samples (LeChevallier and Au, 2013).

Because of the various disadvantages of conventional disinfection methods on the environment and human health, the use of advanced treatment methods for disinfection have gained importance with effective inactivation of pathogenic microorganism to prevent disinfection by-product formation in conventional disinfection methods especially (Sirivedhin and Gray, 2005). At the beginning of advanced treatment methods are ultrasound, Fenton oxidation, UV, ozone, hydrogen peroxide applications. UV oxidation technologies; (titanium dioxide) in a homogeneous environment by the addition of a suitable oxidizing agent (hydrogen peroxide, ozone) (Stasinakis, 2008; Koparal, 2018). Homogeneous processes (UV / H_2O_2, UV / O_3) and heterogeneous processes (photolysis of semiconductor particles) as an advanced treatments methods also used for water disinfection but these alternative methods has some limits (Venkatadri and Peters, 1993). For examples, UV disinfection equipped with lamps producing 254 nm wave length UV rays is used to inactivate the microorganisms in the water, but it is difficult to obtain effective inactivation ratio in water with high turbidity (Qualls et al., 1983; Christensen and Linden, 2003; WHO, 2004). Also ozone application needs addition of chlorine after effective disinfection in order to supply residue protection (Langlais et al., 1991).

Hydrogen peroxide (H_2O_2) is a powerful chemical disinfection agent (Falagas et al., 2011). Disinfection efficacy of H_2O_2 depends on its concentration, contact time and microorganism (Alasri et al., 1992). H_2O_2 has wide-ranging activity against viruses, bacteria, yeast, bacteria spores. However, the effect of hydrogen peroxide is more effective against gram-positive, rather than gram-negative bacteria. Hydrogen peroxide is a powerful oxidizing agent because it produces free hydroxyl radicals. High-level disinfection can be obtained with hydrogen peroxide in the critical patient care units (Arrage et al., 1993).

Ultrasound or, in other words sonication, is one of the alternative advanced treatment technologies for water disinfection (Stasinakis, 2008). The inactivation of microorganisms depends on the time and power of the ultrasonic application. The ultrasound effect on bacteria is based on the destruction of the cytoplasmic membrane (Mason et al., 2003). When the ultrasonic wave passes through a liquid, very small bubbles are formed and this is called cavitation. The collapse of these cavitation bubbles creates high temperatures and pressures locally. These sudden changes in temperature and pressure cause the cell wall structure to deteriorate. When the US is combined with conventional chemical treatment, it increases the potency of the oxidizing chemical in the microbial cell membrane (Jatzwauk et al., 2001). Factors affecting ultrasound removal efficiency are temperature, wave frequency, wave amplitude, applied power, medium viscosity, contaminant type, surface tension of solution and solution vapor pressure (Mason and Peters, 2002).

In this study, the synergistic effects of ultrasound (US) and hydrogen peroxide (H_2O_2) have been examined on the removal of *Klebsiella pneumoniae* from water in order to eliminate the limited applications of these two methods. It has been shown that the combination of US and H_2O_2 can effectively remove *Klebsiella pneumoniae* bacteria from water.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

Materials and Methods

This research was conducted in Department of Environmental Engineering, Faculty of Engineering, Eskisehir Technical University, and Eskisehir, Turkey. In this study, the disinfection efficiencies of ultrasound (US), hydrogen peroxide (H_2O_2) and hybrid application of these methods.

Microbiological studies

Klebsiella pneumoniae working solution of 100 ml and a concentration of 1×10^5 CFU ml^{-1} was disinfected at three ultrasonic frequencies and three H_2O_2 concentration. The disinfection studies were performed in a sterile cabinet (Heraeus KSP-18 Class II) at room temperature. The bacterial concentration was determined with a simple plate count method during all disinfection studies using serial dilution of the samples. All the samples were inoculated on plate count agar (PCA, Merck-Germany) solid media, and the plates were incubated at 37°C for 18-24 hours (Karel, 2016). The bacterial inactivation ratio was calculated as the difference between the initial bacterial concentration and final bacterial concentration for each disinfection study. The disinfection efficiencies of systems were determined with the average bacterial concentration of samples taking into account *Eq. 1* accounting to logarithmic term.

$$E\ (log)\ =\ log\ (Co - Ct) \qquad \text{(Eq.1)}$$

where:
E (log) = Disinfection efficiency in logarithmic term,
C_0 = Initial average bacterial concentration (CFU ml^{-1}),
C_t = Average bacterial concentration at t time (CFU ml^{-1}).

The results of microbiological studies were expressed as average of three independent experiments with error bar using standard deviation (SD). Each experiment was performed with three parallels. Statistical significance of the difference of the counted bacteria in in plate count agar among each trials was determined by analysis of variance (ANOVA) using two-way test. In order to show reliability of measurement, p value obtained from two-way ANOVA test was given for each disinfection study.

Ultrasonic disinfection studies

Disinfection studies were carried out in a W-113 Ultrasonic multi-cleaner (Honda Electronics Co., Ltd., Aichi, Japan) given in *Fig. 1* using batch flow condition. This batch type of ultrasonic reactor was operated at ultrasonic frequencies of 28, 45 and 100 kHz and at a power of 100 W. Ultrasonic disinfection studies were performed for a contact time of 60 minutes.

Disinfection with hydrogen peroxide

Hydrogen peroxide is a strong oxidizing agent and has a bacterial inactivation effect. The bacterial inactivation effect of hydrogen peroxide on *Klebsiella pneumoniae* was examined for concentrations of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} in the working solution. Hydrogen peroxide stock solution of 500 mg l^{-1}, 1000 mg l^{-1} and 2000 mg l^{-1} were freshly prepared before the hydrogen peroxide disinfection studies. These working concentrations of hydrogen peroxide (5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1}) were set in the

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DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

working solution using 1 ml of suitable stock solution of each concentration. The disinfection studies were performed with three different concentrations of hydrogen peroxide, and the effect of bacterial inactivation was determined in the same working solution at similar flow conditions and contact time.

Figure 1. *Batch type of ultrasonic reactor was operated at ultrasonic frequencies of 28 kHz, 45 kHz and 100 kHz*

Disinfection with hydrogen peroxide and ultrasound

The hybrid disinfection studies of ultrasound and hydrogen peroxide were conducted in an ultrasonic reactor with H_2O_2 concentrations of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} and applying a 28 kHz ultrasonic frequency. To determine the synergetic effect of H_2O_2, the US disinfection studies were repeated with similar conditions as the previous disinfection process.

Results and discussion

The effect of ultrasonic frequency on *Klebsiella pneumoniae* disinfection process at an average of 1×10^5 CFU ml^{-1} is shown in *Figure 2*.

When a 28 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae,* a 3.52-log disinfection efficiency was attained with a 60-min disinfection period. When a 45 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae,* a 1.52-log disinfection efficiency was attained with a 60 min disinfection period. When a 100 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae,* a 1.1-log disinfection efficiency was attained with a 60 min disinfection period. The results of ultrasonic disinfection studies showed that the maximum bacterial inactivation rate at an ultrasonic frequency of 28 kHz.

In the present study, the highest inactivation efficiency of *Klebsiella pneumoniae* was determined at an ultrasonic frequency application of 28 kHz after a 60-min disinfection

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DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

period as the bacterial inactivation ratio is dependent on the mechanical disruption effect on the cell membrane from ultrasonic frequency. Gao et al. (2014b) studied *Enterobacter aerogenes*, *Bacillus subtilis, Staphylococcus epidermidis, S. epidermidis* and *Staphylococcus pseudintermedius* microorganisms at ultrasonic frequency 20 kHz and 100 kHz applying 13 W power. It was observed that the highest bacterial inactivation rate was determined to be 4.5-log after 20 min continuous sonication at 20 kHz ultrasonic frequency differing microorganisms(Gao et al., 2014a).

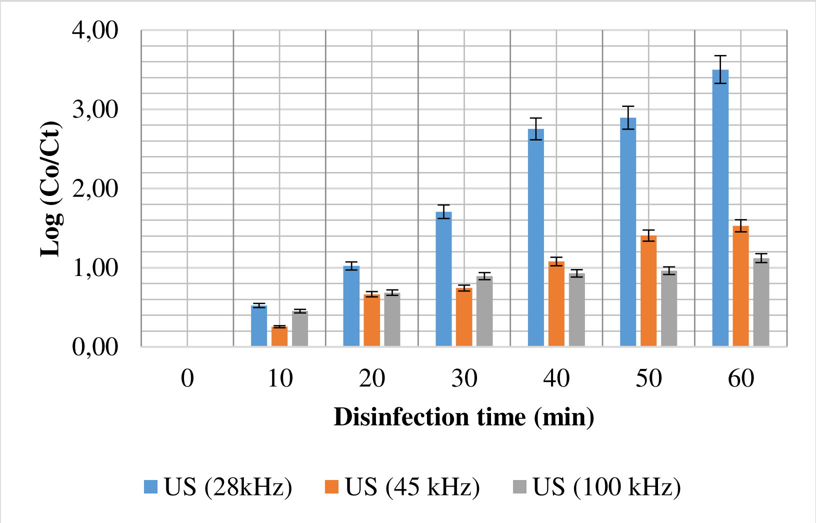

Figure 2. *Effect of ultrasonic frequency on the Klebsiella pneumoniae disinfection process at a concentration of 1 × 10^5 CFU ml^{-1} (p value was calculated as 0,334 using two-way ANOVA test. This value showed that there is no significant difference of each measurements among each three disinfection experiment because p>0.01)*

This ultrasonic frequency effect was disgusted in previous research(Gemici et al., 2014; Karel, 2016) by high-power ultrasound frequencies, which are described as using low ultrasonic frequencies between 16 kHz and 100 kHz because high-power ultrasound is more effective than high-frequency (500 kHz-1 MHz) ultrasound when performing bacterial inactivation(Joyce et al., 2003). The chemical effects of high-frequency ultrasound used in this study leading to thermal decomposition of water molecules into OH^- and H^+ radicals was probably insufficient for the inactivation of *Klebsiella pneumoniae,* although the physical effect of low-frequency ultrasound enhanced the efficiencies of bacterial inactivation(Mason et al., 2003).

The result of hydrogen peroxide disinfection studies is shown in *Fig. 3*. When a 5 mg l^{-1} $H_2O_2^+$ concentration was used to inactivate *Klebsiella pneumoniae*, a 0.04-log disinfection efficiency was attained with a 60-min disinfection period. When a 10 mg l^{-1} H_2O_2 concentration was used to inactivate *Klebsiella pneumoniae*, a 0.04-log disinfection efficiency was attained with a 60 min disinfection period. When a 20 mg l^{-1} $H_2O_2^+$ concentration was used to inactivate *Klebsiella pneumoniae*, a 0.08-log disinfection efficiency was attained with a 50 min disinfection period. These results showed that increasing the hydrogen peroxide concentration raises the bacterial inactivation ratio as expected (Loraine et al., 2012), however, the they indicated that obtained inactivation ratio of *Klebsiella pneumoniae* was not enough for effective disinfection.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

The disinfection study performed in an ultrasonic reactor with addition of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} of H_2O_2 during a 60 min disinfection period under 28 kHz ultrasonic frequency is shown in *Fig. 4* and Anova: two-way test results of final disinfection study were summarized in *Table 1*.

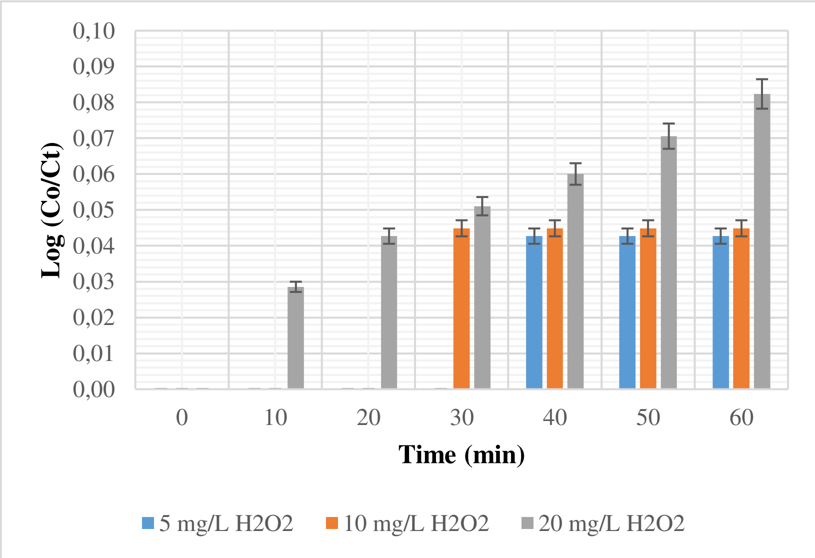

***Figure 3.** Effect of hydrogen peroxide concentration on the Klebsiella pneumoniae disinfection process (5, 10 and 20 mg l^{-1} H_2O_2; 1 × 10^5 CFU ml^{-1} of Klebsiella pneumoniae and 60 min disinfection period under batch conditions) (p value was calculated as 0,000000009 using two-way ANOVA test. This value showed that there is a significant difference between and within of hydrogen peroxide disinfection experiment because $p<0.01$)*

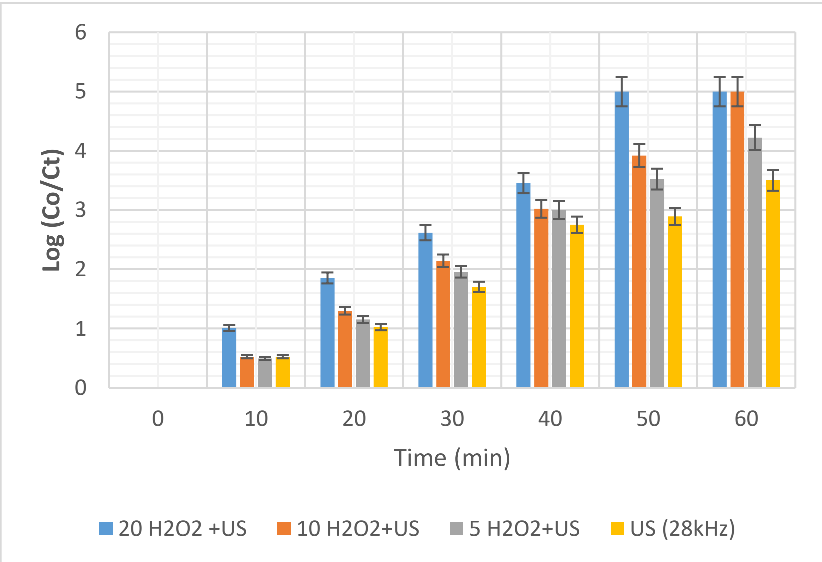

***Figure 4.** Effect of hydrogen peroxide concentration on the ultrasonic disinfection process (an ultrasonic frequency of 28 kHz and 100 W power at 5, 10 and 20 mg l^{-1} H_2O_2; 1 × 10^5 CFU ml^{-1} Klebsiella pneumoniae and 60 min disinfection period under batch conditions) (p value was calculated as 0,071 using two-way ANOVA test. This value showed that there is no significant difference between and within of hydrogen peroxide disinfection experiment because $p>0.01$)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

Table 1. *Anova table and descriptive statistical values of the effect of hydrogen peroxide concentration on the ultrasonic disinfection process*

ANOVA: TWO-WAY				
Summary	*Number*	*Total*	*Average*	*Standard deviation*
***Trial* 1**	5	100407	20081.4	2E+09
***Trial* 2**	5	100538.3	20107.65	1.99E+09
***Trial* 3**	5	120645.9	24129.18	2.87E+09
Initial bacteria concentration	3	320000	106666.7	1.33E+08
20 mg l^{-1} H_2O_2 + US (28kHz)	3	119.7	39.9	32.83
10 mg l^{-1} H_2O_2+ US (28kHz)	3	335.35	111.7833	330.6158
5 mg l^{-1} H_2O_2+ US (28kHz)	3	375	125	625
US (28kHz)	3	761.1	253.7	5117.07

ANOVA- ***descriptive statistical values***						
Standard deviation Resources	*SS*	*df*	*MS*	*F*	*P-value*	*F -criteria*
In groups	54263193	2	27131596	1.02183	0.402524	4.45897
Between groups	2.72E+10	4	6.81E+09	256.4678	1.78E-08	3.837853
Error	2.12E+08	8	26551961			
Total	2.75E+10	14				

When the application of ultrasound was performed with a H_2O_2 concentration of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1}, the disinfection efficiency of *Klebsiella pneumoniae* was 4.22 log after 60 min, 5 log after 60 min and 5 log after 50 min, respectively. This indicates that *Klebsiella pneumoniae* disinfection with hydrogen peroxide and ultrasound decreased the disinfection period and increased inactivation ratio according to a single hydrogen peroxide application. The physical effects of low-frequency ultrasound improved the bacterial inactivation ratio by enhancing the disinfection effect of hydrogen peroxide through synergistic effects of ultrasound. The results of microbiological studies were expressed as average of three independent experiments with error bar using standard deviation (SD) using three parallels studies of all disinfection process. The results of analysis of variance (ANOVA) using two-way test, p value was calculated 0,402524 within groups and p value was calculated 0,0000000178 between groups These value showed that there is no significant difference within groups of each three disinfection experiment because p value was greater than 0.01. To concluded, two-way ANOVA test indicated that US+ H_2O_2 hybrid applications gave reliable and repeatable results.

Image of *Klebsiella pneumoniae* on petri dish from disinfection with hydrogen peroxide and ultrasound studies was given in *Fig. 5* as initial bacteria concentration in *Fig. 5A*, 5 mg l^{-1} H_2O_2+ US 28 kHz in *Fig. 5B*, 10 mg l^{-1} H_2O_2+ US 28 kHz in *Fig. 5C* and, 20 mg l^{-1} H_2O_2+ US 28kHz in *Fig. 5D*. These results indicate that ultrasound and hydrogen peroxide reduced the hydrogen peroxide concentration to obtain an eligible bacterial inactivation efficiency in water because ultrasound accelerates the contact of OH^- and H^+ radicals, which are the decomposition products of H_2O_2, with bacteria.

Ultrasound can also facilitate breakage of microorganism agglomeration, thereby increasing the efficiency of other chemical disinfectants (Karel, 2018). This effect was clearly shown in literature. Gao et al. (2014a) was studied disinfection of *E. aerogenes*, *Bacillus subtilis*, *S. epidermidis* and *A. Pullulans* with hydrogen peroxide (30%) and t-butanol in 85 kHz ultrasonic reactor. As a result of their study, it was observed that the addition of H_2O_2 provided microorganism inactivation much shorter than ultrasound disinfection (Gao et al., 2014b). Lakeh et al. (2013), was disinfected *Anguillicola crassus*,

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Paramecium sp. and *Artemia sp*. with low-frequency US and UV-C methods. They found that the mean size of suspended solids in the water culture with low frequency US decreased and therefore the UV-C inactivation rate increased by 0.6 log. Comparison of UV-C radiation with low-frequency US did not cause a decrease in the number of colonies in fresh water, but the antiseptic effect of UV-C in turbid water increased by 0.6 log. When colony count values were compared only with UV-C and low-frequency US with UV-C, pretreatment with low frequency US showed significant effect. The US sonication of 0.2-18.8 kJ/L low frequency dose range varies from 7% to 95% depending on the inactivation rate of paramecium (Lakeh et al., 2013). Ayyıldız et al. (2011) have demonstrated the effects on *E. coli* and total coliform inactivation when used in combination with chlorine dioxide (ClO_2, 2 mg /L) and US (150-300 W/L) in wastewater disinfection. Raw waste water and synthetic water are used. US (vibra cell 505) 500 W power and 20 kHz frequency were used. The log removal was 1.4-1.9 in single treatments and the combined use of consecutive US (150 or 300 W/L) and ClO_2 (2 mg/L) resulted in 3.2 - A 3.5-month fix was provided. Using the US and ClO_2 disinfection methods together, high concentrations of particles in the raw wastewater and their breakdown under shock noise waves have been achieved (Ayyildiz et al., 2011).

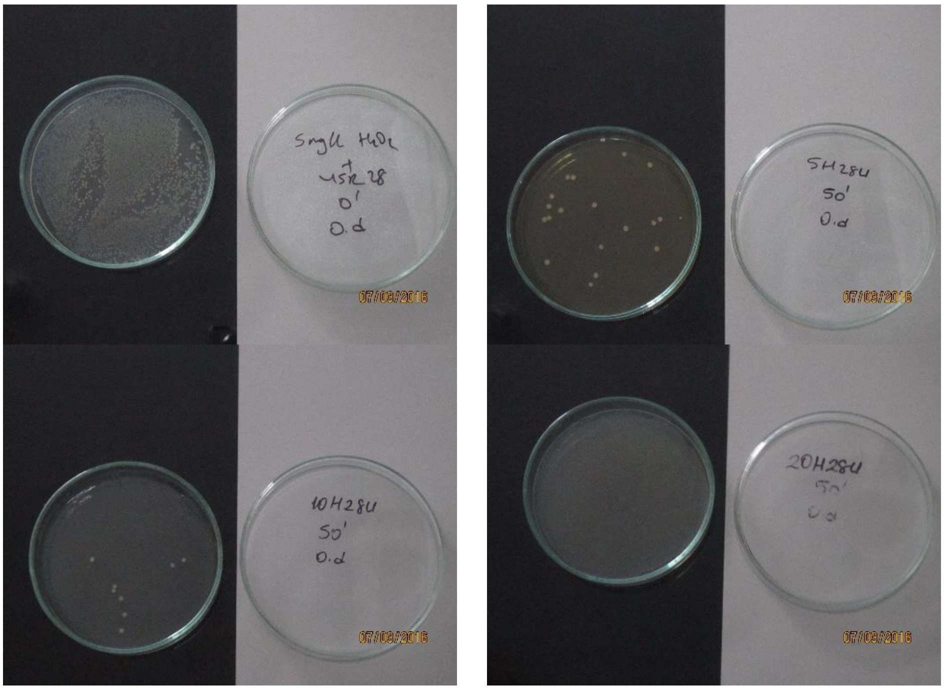

Figure 5. *Image of Klebsiella pneumoniae on petri dish from disinfection with hydrogen peroxide and ultrasound studies (A: initial bacteria concentration, B: 5 mg* l^{-1} H_2O_2*+ US 28 kHz, C: 10 mg* l^{-1} H_2O_2*+ US 28 kHz, D: 20 mg* l^{-1} H_2O_2*+ US 28kHz)*

Hydrogen peroxide, which has a significant effect on the disinfection performance of the ultrasound, can be generated simultaneously during the treatment of water and wastewater depending on the conditions under which the treatment takes place and the ultrasonic system parameters (frequency, power, amplitude, power density, etc.). This is most likely to be the production of OH-H radicals at ultrasonic frequencies in the range of 100 kHz to 1 mHz and the sonochemical process called H_2O_2 oxidation(Suslick, 1988), but it is seen in the literature that high power and retention times should be applied for

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the production of these radicals (Rae et al., 2005). In this study, H_2O_2 could not be produced by using effective sono-chemically at the 28 kHz ultrasonic frequency, US-H_2O_2 disinfection studies were also showed the effect of H_2O_2 addition to the ultrasonic system.

This study was showed that ultrasound and its hybrid application with hydrogen peroxide. It has been seen that, when US combined with H_2O_2 with the accelerator effect of ultrasound on mass transfer as seen in other oxidation methods, bacterial inactivation ratio increased with less chemical usage. Also, the disadvantages of ultrasound eliminated with H_2O_2 hybrid studies because it requires high energy density for high inactivation ratio by the use of ultrasound alone. Due to the catalysis effect of the ultrasound, a significant reduction in the amount of H_2O_2 required for the disinfection resulted in H_2O_2 disinfection for high bacterial removal efficiency. As a result of the study, it has been determined that the *Klebsiella pneumoniae* in water and other ecosystem effects on human health can be successfully treated with hybrid US- H_2O_2 process.

Conclusion

The effects of different frequencies and added H_2O_2 on microorganism removal performance in ultrasonic system were investigated in this paper. It is clearly stated that this study will be an important development in drinking and potable water disinfection to reduce limitation of these alternative methods. It was concluded that water could be microbiologically treated by ultrasound providing less energy consumption and less chemical usage. Microorganism inactivation can be recommended in higher yields by employing the ultrasonic system as a hybrid. The studies have been carried out in a small scale in the laboratory environment and this study can be given as a result of this study in pilot scale and larger systems. It can also be tested in the ultrasound system with separate studies for other microorganisms that can be found in the targeted water environment.

Acknowledgements. This study was supported by the Anadolu University Scientific Research Projects Commission under [Grant number 1309F321].

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41594169

EFFECTS OF LEAD STRESS ON THE CHLOROPHYLL CONTENT AND PHOTOSYNTHETIC FLUORESCENCE CHARACTERISTICS OF *VALLISNERIA NATANS*

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(Received 9th Dec 2018; accepted 4th Mar 2019)

Abstract. In order to reveal the effects of lead stress on the chlorophyll content and fluorescence characteristics of *Vallisneria natans*, six Pb concentration levels were applied to a culture in clean river sand and water. The results showed that Pb could promote the synthesis of chlorophyll at a low concentration (Pb $\leq$ 10 mg·L^{-1}) in one day. *V. natans* had certain tolerance to the stress of low Pb concentration. With the prolongation of stress time and increasing Pb concentration, the leaves of *V. natans* gradually lost their green color. Chlorophyll a decreased more than chlorophyll b. It had the least effect on carotenoids. Fv/Fm, Fv/Fo, qP, Y(II) and ETR decreased significantly (Pb > 10mg·L^{-1}) in seven days. However, *qN*, *Y(NO)* and *Y(NPQ)* showed an upward trend. The efficiency of the leaves using light energy decreased noticeably, and the electron transport of PSII was blocked severely. It can be speculated that *V. natans* may be used in the phytoremediation of waters contaminated by a low concentration of lead.
Keywords: *Vallisneria natans, lead stress, photosynthetic pigment, fluorescence characteristics, rapid light curve*

Introduction

In recent years, heavy metal pollution has become prominent with mining, smelting, electroplating, and different types of wastewater, with solid wastes being discharged into water bodies (Ji et al., 2018). Due to the fact that these cannot degrade or be decomposed, heavy metals are accumulated in living organisms, impair the health of animals and humans with biological amplification through the food chain (Xu et al., 2003). As one of the “five poisonous” heavy metal elements, lead (Pb) is a non-essential element utilized during plant growth and metabolism, and otherwise has toxic effects (Sharma and Dubey, 2005; Bisht et al., 2013; Ansari et al., 2017; Shahid et al., 2016), inhibiting the progress of photosynthesis and reducing the activity of chlorophyllase.

Submerged macrophyte, as important primary producers in aquatic ecosystems, not only provide food, habitat and breeding sites for aquatic animals, but also can get rid of N, P and other nutrients (Song et al., 2011). It also has ability to adsorb and accumulate heavy metals (Pan et al., 2011; Chen et al., 2017), used to remove Pb from water (Li et al., 2011). The nutrient absorption, secondary metabolism and antioxidant response of *Vallisneria natans* (*V. natans*) under Pb stress were studied (Wang et al., 2011, 2012) Under Pb stress, malondialdehyde content increased and total chlorophyll and carotenoids decreased. *V. natans* had a certain resistance to Pb stress, and the key enzymes of nitrogen and phosphorus metabolism were more sensitive to the response of Pb stress (Yu et al., 2016). At present, the research focus on mainly the enrichment of

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DOI: http://dx.doi.org/10.15666/aeer/1702_41714181

heavy metals (Liang et al., 2016; Xue et al., 2010), physiological and biochemical effects (Yu et al., 2016; Xu et al., 2006; Min et al., 2012), ultrastructure (Xu et al., 2004; Shi et al., 2000), etc. While the mechanisms of submerged macrophyte enduring heavy metals from photosynthetic and chlorophyll fluorescence are rarely reported.

V. natans is a perennial herbaceous plant perennial in China with high economic value (Wang et al., 2006), strong regeneration ability, used in water ecological restoration project widely (Gu et al., 2017; Wang et al., 2009). In 1980, professor Schreiber invented the pulse amplitude modulated. Due to the rapid, simple, sensitive, reliable and non-interference characteristics of the modulated fluorescence technology, it can reflect the "intrinsic" characteristics of the photosynthetic system (Hu et al., 2017), regarded as the effective probe in studying the relationship between plant photosynthesis and the degree of environmental stress (Janssen et al., 1992). The aims of this study were to investigate the intrinsic mechanism of photosynthetic system response under Pb stress, and provide some basic data and theoretical basis for the ecological restoration of Pb contaminated waters.

Materials and methods

Materials cultivation and treatment

Whole plants of *V. natans* were collecting from Nanjishan nature reserve of Poyang Lake, China, on June 5th in 2017. Plants (20 cm length) were planted in plastic buckets, adding 1/10 Hoagland nutrient solution, and acclimated for two weeks. Then, *V. natans*, healthy and consistent growth, were transplanted into eighteen transparent glass jars (50 cm × 40 cm × 40 cm) with 10 cm thick river sand paved at the bottom (*Fig. 1*). 50 L distilled water and Pb solution with different concentration levels were injected into the jars. Pb was added in the form of $Pb(NO_3)_2$ with a concentration level of 0, 1, 10, 20, 50 and 80 $mg \cdot L^{-1}$, 0 $mg \cdot L^{-1}$ was the control group (CK), three repetitions for each level.

***Figure 1**. Diagram of experimental device*

Determination of chlorophyll content

The determination of photosynthetic pigment was used by 95% ethanol extraction (Li, 2000). The value of absorbance was measured at 665, 649 and 470 nm, respectively. The concentrations of chlorophyll a (Ca), chlorophyll b (Cb), carotenoid (Cc) and total chlorophyll (Ct) in the extract were calculated respectively by *Equations 1-4*. The content of Ca, Cb, Cc and Ct ($mg \cdot g^{-1}$) was calculated by *Equation 5*.

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$$C_a(mg \bullet L^{-1}) = 13.95A_{665} - 6.88A_{649} \quad \text{(Eq.1)}$$

$$C_b(mg \bullet L^{-1}) = 24.96A_{649} - 7.32A_{665} \quad \text{(Eq.2)}$$

$$C_c(mg \bullet L^{-1}) = \frac{1000A_{470} - 2.05C_a - 114.8C_b}{245} \quad \text{(Eq.3)}$$

$$C_t(mg \bullet L^{-1}) = C_a + C_b \quad \text{(Eq.4)}$$

$$Chlorophyl\ content(mg \bullet g^{-1}FW) = \frac{C_t(mg \bullet L^{-1}) \times total\ amount\ of\ extract(ml)}{sample\ fresh\ weight(g)} \quad \text{(Eq.5)}$$

Determination of fluorescence parameters

The chlorophyll fluorescence characteristics of *V. natans* leaves were determined by the underwater modulation fluorescence instrument (DIVING-PAM), produced by WALZ company in German. Before the measurement, the dark clips were clamped on the leaves of *V. natans* in situ. After dark adaptation for 20 min, the clips and detection light were opened. First, the induction curve was determined, the minimum fluorescence (Fo) and maximum fluorescence (Fm) were obtained. The biggest actinic light efficiency of PSII (Fv/Fm), effective quantum yield (Y(II)), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN) and quantum yield of regulatory energy dissipation (Y(NPQ)) and quantum yield of non-regulatory energy dissipation (Y(NO)) were calculated automatically by the selected system mode. Fv/Fo = (Fm - Fo) / Fo. Then, the rapid light curve was measured. The gradients of photosynthetic active radiation (PAR) were 0, 100, 200, 300, 400, 600, 800, 1000 and 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ respectively.

Statistical analysis

Excel 2017 was used to process the experimental data and draw graphics. SPSS19.0 was used for one-way analysis of variance. The Duncan method was used for multiple comparisons. $P < 0.05$ means significant difference.

Results

Effects on photosynthetic pigment under Pb stress

As shown in *Figure 2A,* Ct, Ca and Cb after one day under Pb stress of 1 $mg \cdot L^{-1}$ were increased significantly compared with the control group (CK). The increase of Ct, Ca and Cb under Pb stress of 10 $mg \cdot L^{-1}$ was not significant compared with the CK ($P > 0.05$). Ct, Ca, Cb, Cc and Ca/Cb were reduced significantly when Pb was over 20 $mg \cdot L^{-1}$.

As shown in *Figure 2B,* Ct, Ca and Cb were lower slightly after seven days under 1 $mg \cdot L^{-1}$ Pb. Above 10 $mg \cdot L^{-1}$ Pb stress, Ct, Ca, Cb and Cc decreased significantly ($P < 0.05$). It was respectively 45.89%, 39.20%, 58.01% of the CK under Pb stress of 80 $mg \cdot L^{-1}$. Ca/Cb appeared in overall downward trend. Ca was more sensitive than Cb under Pb stress, and decreased sharply. The damage of Pb stress on chlorophyll was

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larger than carotenoids. The leaves of *V. natans* lost green color gradually with the increase of Pb concentration.

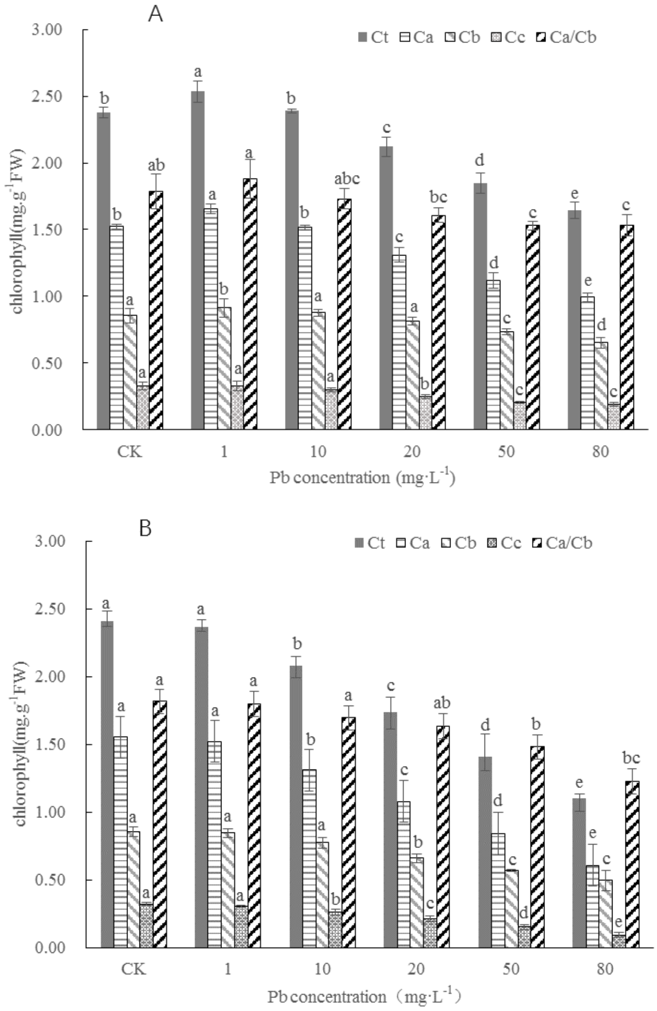

***Figure 2**. Varieties of Ct, Ca, Cb, Cc and Ca/Cb on V. natans under Pb stress. (Different lowercase letters represent the significant differences (P < 0.05) in different treatment groups, the same below. A represents one day stress, B represents seven days stress)*

Effect on Fo, Fm, Fv/Fm and Fv/Fo under Pb stress

As shown in *Figure 3,* Fo decreased with the increase of Pb compared with the CK after one day, but the difference was not significant. Fm was less than the CK. Fm had little difference at Pb stress of 20-80 mg·L^{-1}. Fv/Fm was lower than that of the CK except at Pb stress of 1 mg·L^{-1}. Fv/Fm had significant difference ($P < 0.05$) under Pb stress of 10-80 mg·L^{-1}. The change trend of Fv/Fo was similar with Fv/Fm.

After seven days, the increase of Fo was significant under the high Pb stress. Under Pb stress of 80 mg·L^{-1}, the increase of Fo was 28.32% compared with the CK. Fm was smaller than the CK, also smaller than that of the same Pb stress after one day. Fm was 39.58%, 37.23%, 31.47% of the CK at Pb stress of 20-80 mg·L^{-1}. Fv/Fm was lower than the CK. Conversion efficiency of PS II primary light energy decreased with the increase of Pb. Fv/Fo was also lower significantly than the CK ($P < 0.05$).

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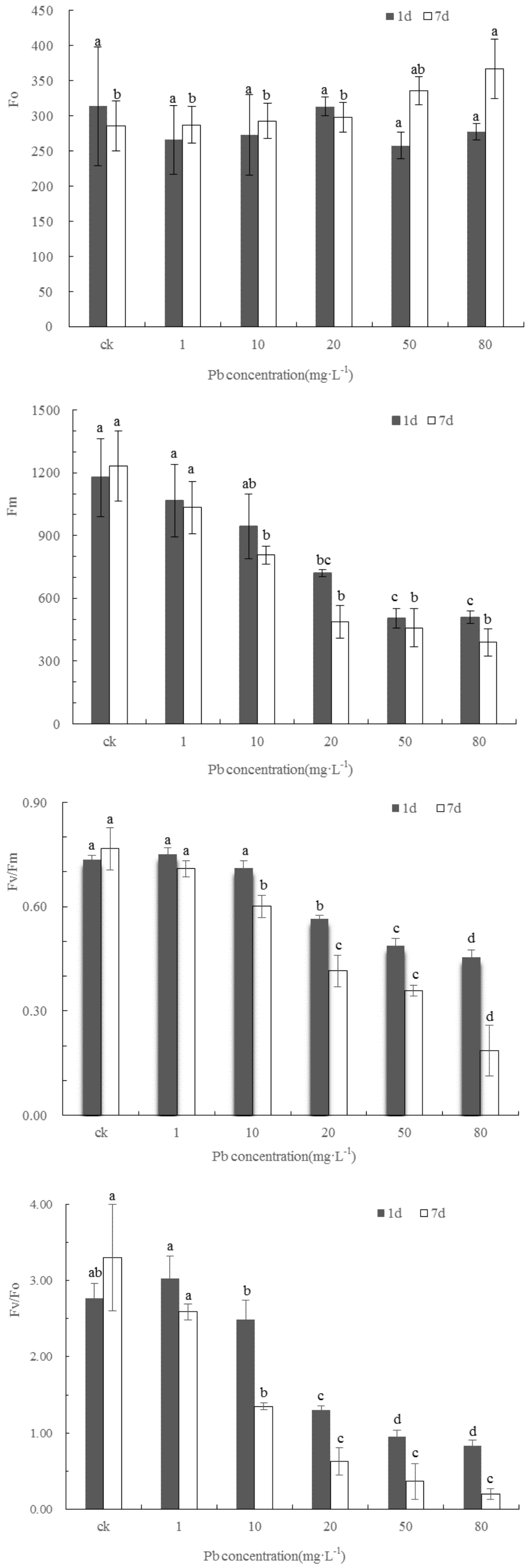

Figure 3. *Effects on Fo, Fm, Fv/Fm and Fv/Fo of V. natans*

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Effects on qP, qN on leaves of V. natans under Pb stress

The value of qP was in a downward trend with the increase of stress intensity (*Fig. 4*). It had no significant difference under Pb stress of 0-10 mg·L^{-1} (P > 0.05) after one day. It decreased to 82.67% of the CK under Pb stress of 80 mg·L^{-1} after one day, and 37.36% after seven days. The trend of qN was opposite to qP. qN increased slowly with the increase of stress concentration. It peaked under Pb stress of 80 mg·L^{-1}, 141.3% higher than the CK.

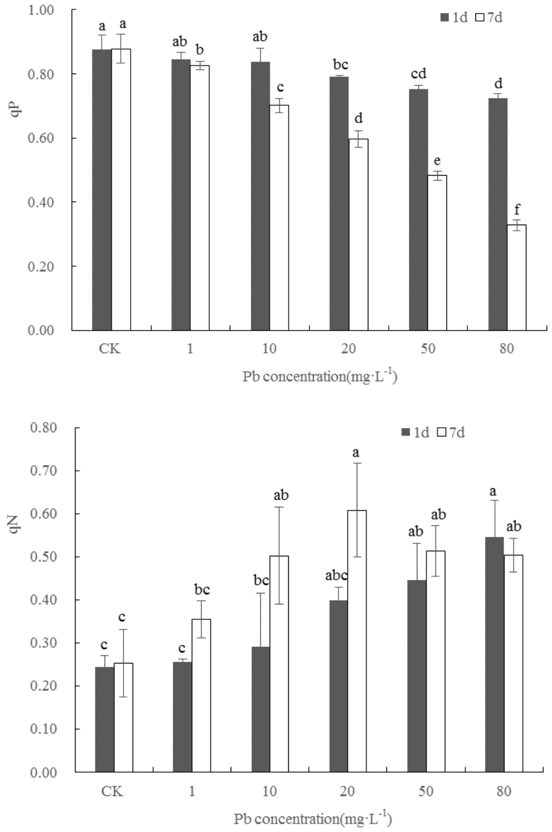

Figure 4. *Effects on qP and qN on V. natans under Pb stress*

Effect on Y(II), Y(NO), Y(NPQ) under Pb stress

Y(NO) increased slowly with the increase of stress concentration. The increase of Y(NO) was significant over 20 mg·L^{-1} Pb after one day. Under the same stress concentration, Y(NO) after seven days was higher than that after one day, which was 1.01, 1.01, 1.05, 1.08, 1.14 and 1.3 times respectively. Y(NPQ) showed an overall upward trend. Under Pb stress of 80 mg·L^{-1}, it peaked 4.05 times of the CK, then decreased significantly (P < 0.05) (*Fig. 5*).

Response of rapid light curves under Pb stress

The relative electron transfer rate (ETR) increased rapidly, then decreased and flattened. It behaved as light suppression. ETR increased compared with the CK under Pb stress of 1 mg·L^{-1} and 10 mg·L^{-1} after one day (*Fig. 6A*). It indicated low Pb could stimulate transmission of the electron. *V. natans* had certain resistance to low

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concentration. ETR was smaller than the CK under Pb stress of 20-80 $mg \cdot L^{-1}$. The maximum of ETR appeared when PAR was 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. ETR was 91.42%, 72.78%, 50.3% of the CK.

ETR in each treatment group was lower than the CK after seven days (*Fig. 6B*). ETR decreased significantly ($P < 0.05$) with the prolongation of stress duration. The change trend was similar to that of qP. The proportion of real electron transfer in light reaction center and absorbing light used in photochemical process reduced. Electron transfer in the leaves was significantly inhibited so photosynthetic efficiency weakened (Gan et al., 2017).

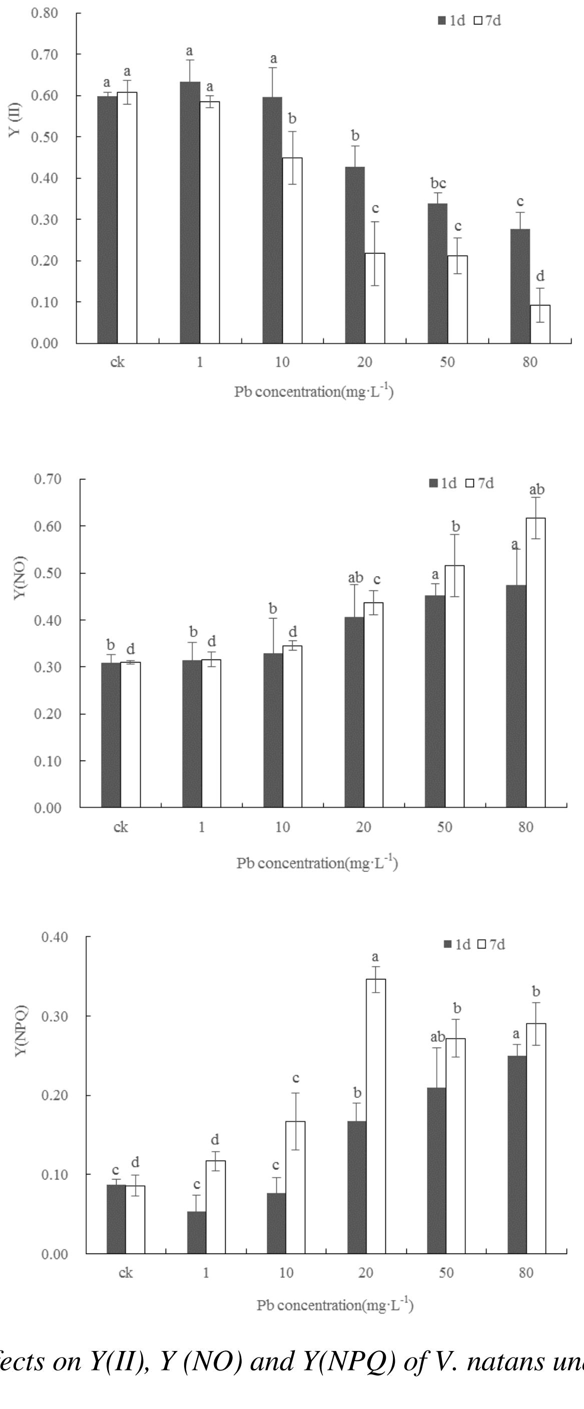

***Figure 5.** Effects on Y(II), Y (NO) and Y(NPQ) of V. natans under Pb stress*

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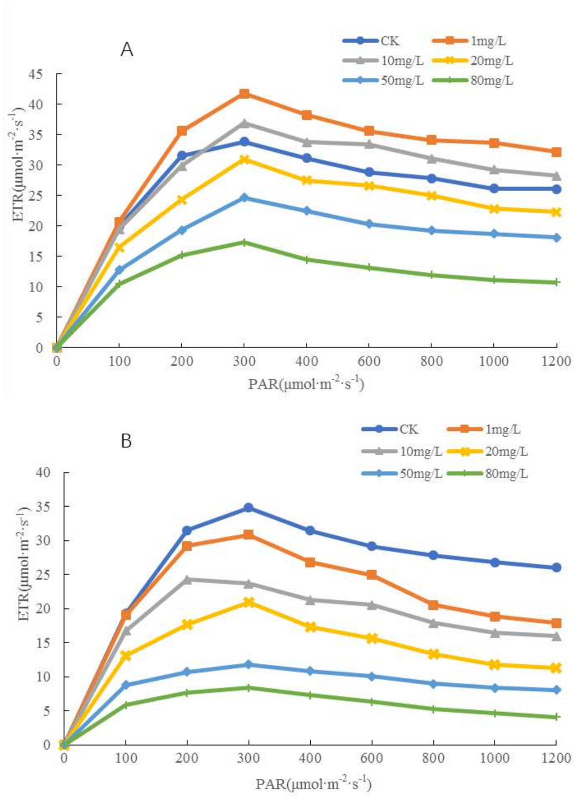

Figure 6. *Effects on ETR of V. natans under Pb stress. (A represents one day stress, B represents seven days stress)*

Discussion

Chlorophyll content is an important index to measure leaf senescence. The degree of reduction can also reflect the situation of Pb poisoning in plants. After one day under 1 mg·L^{-1} Pb, the synthesis of chlorophyll was enhanced because activated microorganisms in the water improved the supply condition of water nutrients (Yu et al., 2016), and Ca/Cb increased which may be a kind of resistance caused by Pb accumulation in *V. natans* to slow down the aging speed of the leaves.

After seven days, Ca decreased faster than Cb. Photosynthetic pigment decreased with the increase of Pb concentration. The main reason may be that Pb binds to the sulfhydryl of related enzyme in the chloroplast (Assche and Clijsters, 1990), destroyed the chloroplast structure. At the same time, with the accumulation of Pb, the combination of Pb and some enzyme (original chlorophyll reductase and porphobilinogen deaminase) that synthesize chlorophyll in cells (Asgharipour., 2011), make the enzyme activity blocked and chlorophyll synthesis inhibited.

The parameters of chlorophyll fluorescence kinetic have been widely recognized as one of the good indicators of plant resistance. The fluorescence parameters of plant growing under normal conditions were stable (Roháček, 2002). Fo was related with the activity of PS II light reaction center. In this research, Fo increased obviously when Pb concentration was over 20 mg·L^{-1}, which may be caused by the damage of the PS II reaction center of *V. natans* leaves (Gan et al., 2017). Fv/Fm indicated the light conversion efficiency of PS II reaction center. Fv/Fo indicated the potential activity of PS II. The change trend of Fv/Fo change trend is similar with and Fv/Fm. Fv/Fo is more

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sensitive to change of the photosynthetic efficiency. Conversion efficiency of PS II primary light energy decreased with the increase of Pb. The low Pb concentration had a weak effect on the potential maximum photosynthetic capacity of the leaves, and *V. natans* had a definite resistance to adversity. High Pb concentration hampered the light energy conversion efficiency of PS II reaction center, was unfavorable for the leaves to capture light energy into chemical energy.

After seven days, Fv/Fm, qP, Y(II) and ETR was 54.1%, 67.99%, 35.69% and 67.99% of the CK respectively under Pb stress of 20 $mg \cdot L^{-1}$, 24.25%, 37.36%, 15.3% and 24.14% of the CK respectively under Pb stress of 80 $mg \cdot L^{-1}$. This showed that with the prolongation of stress duration, the photosynthetic activity of PS II reaction center in *V. natans* leaves had been irreversibly damaged (Liu et al., 2017). It had basically lost photosynthetic capacity.

The decrease of qP indicated that the electron transportation from the oxidation side of PS II to the reaction center was blocked. The electrons used for photosynthesis decreased and the light energy dissipated in heat or other forms increased, which was consistent with the decrease of Y(II) (Zhang., 2016). qN reflects the plant's ability to dissipate excess light energy as heat and reflects the light protection ability of plants. The value of qN gradually increased with the increase of Pb level. *V. natans* would start self-protection mechanism, dissipate excess light energy absorbed by the antenna pigment as heat energy to reduce the damage of chloroplasts and other photosynthetic organs when it was threatened by the heavy metal (Qian et al., 2011; Wu et al., 2016; Janssen et al., 1992). With the prolongation of stress duration, the damage of leaves was severe under high Pb stress (>20 $mg \cdot L^{-1}$). Dead leaves appeared. It was beyond the scope of self-protection.

Light quantum of adsorbed by PS II reaction center transferred and dissipated in three ways (Kramer et al., 2004). The sum of all light quantum yield closed to 1, that is, Y(II)+Y(NO) +Y(NPQ)=1. The proportion of Y(II) was the smallest with the increase of Pb concentration. Excessive Pb directly inhibited the transportation of photosynthetic electron, thus reduce the percentage of energy conversion in photochemical way (Qian et al., 2011). Y(NO) is an important indicator of light damage. Its higher level indicates that the photochemical energy conversion and protective regulatory mechanisms (such as heat dissipation) are not sufficient to completely consume light energy absorbed by the plant. With the prolongation of stress time, it increased, indicating that *V. natans* had been damaged. Y(NPQ) is an important indicator of light protection. The higher of Y(NPQ) indicated that the light intensity accepted by the leaves was excess, the plant can adjust (such as the excess light energy dissipated into heat) in order to protect themselves. This research showed that Y(NPQ) after seven days was significantly higher than that after one day. It received excess light. The utilization of light energy by the plant was weakened under Pb stress. The plant consumed excessive heat to protect itself and adapt to the environment by increasing the quantum yield of regulatory energy dissipation (Li et al., 2005).

Conclusions

Vallisneria natans can carry out relatively normal physiological activities ($Pb \leq 10 mg \cdot L^{-1}$). It can be speculated that *V. natans* are used as repairing species in low concentration Pb contaminated waters. This study was conducted in the period of vigorous growth, the period of flowering and decay of *V. natans* should be studied in future.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41714181

Acknowledgements. This study was supported by Jiangxi Provincial Education Department (GJJ170978), National Natural Science Foundation of China (51369024, 51769016), Jiangxi Provincial Technology Department (20161BAB216109).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41714181

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SIMULATION OF SOIL WATER MOVEMENT UNDER BIOCHAR APPLICATION BASED ON THE HYDRUS-1D IN THE BLACK SOIL REGION OF CHINA

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(Received 9th Dec 2018; accepted 4th Mar 2019)

Abstract.
The soil water movement under different biochar treatments of the black soil region in Northeast China was simulated by the Hydrus-1d software in this paper. The simulation errors were between -5.12% and 9.15%, within the ±10% range. with the increasing amount of biochar applied, the residual soil moisture content and the shape parameter were decreasing, while the saturated soil moisture content and the reciprocal of air inlet value were increasing. The difference between the saturated and residual water content was getting bigger with the increasing amount of biochar applied, which improved the available soil moisture content, a beneficial factor of crop growth. With increasing the amount of biochar applied, the moisture rate in the soil profile clearly increased, soil water redistribution curve right shifted significantly, which indicated that biochar can greatly improve the water retention ability of the soil. Furthermore, the soil moisture content increased when the soil depth was more than 50 cm. The effects of biochar on soil moisture diminished rapidly.
Keywords: *farmland, Northeast China, soil water distribution, simulation model application, biochar application*

Introduction

Hydrus-1d is a software for the simulation of water, energy and solute movements in saturated and unsaturated porous media, developed by US Salinity laboratory. It has been widely used in the analysis of the processes of water movement and solute migration in unsaturated and porous media. Simunek et al. (1999) and Simunek et al. (1996) used it to calculate the parameters of soil water movement and solute migration. Tang et al. (2011) simulated the soil water infiltration process of the snow melting in the desert region based on the Hydrus-1d, and the simulation deviation was small. Chi et al. (2014) used the modified Richards equation to simulate soil water infiltration and solute migration processes. Ma et al. (2011) studied field water cycle regularities in Huabei Plain by means of Hydrus-1d, and the simulation precision of the model was high. Liu et al. (2018) simulated the soil water infiltration process for different soils, and revealed the relationship between the soil water distribution, soil types, and amount and rate of irrigation water after 24 h of irrigation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

Biochar is a substance in the state of solid particles, which is produced from some wastes such as crop straw, animal manure, etc. under high-temperature and hypoxia (Wei et al., 2018b). Many studies showed that biochar application could both increase and decrease the bulk density and the total porosity of the soil separately, and these changes could affect its water capacity (Wei et al., 2018a). Furthermore, due to the higher moisture absorption ability of biochar, soil water capacity could be increased (Xiao et al., 2015; Qi et al., 2014), along with the improvement of plant growth (Oguntunde et al., 2008). In addition, it also could affect soil organic matter, aggregate, pH value, etc. to some extent. It would also be possible to influence soil water diffusivity, dispersion coefficient and unsaturated hydraulic conductivity significantly, which in turn would affect soil water storage and movement (Mukherjee et al., 2013; Mankasingh et al., 2011; Novak et al., 2012; Wei et al., 2016; George et al., 2012; Li et al., 2014). As a result of previous studies some researchers are turning their attention towards the effects of biochar on soil water movement. When biochar is applied to clay, the soil water contents, soil water infiltration rates and accumulated infiltration water amount could all be increased (Cen et al., 2016). By changing the amount of biochar soil water infiltration rate and the amount of accumulated infiltration water were influenced at varying rates under different salinization degrees (Liu et al., 2017). When the soil moisture content was lower than 0.4 cm^3/cm^3, biochar could lower the soil water diffusivity, and when it was higher than 0.4 cm^3/cm^3, the results were the opposite (Liu, 2017). Few researches have studied and simulated soil water movement in the black soil region of China, and this research could prove helpful for soil water management in the region.

The soil water movement simulation and analysis in this paper would provide a basis for the efficient utilization of soil and water resources, and the protection of the black soil region in northeastern part of China.

Materials and methods

The experimental area

The experiment area was located in a sloping farmland with a 3 degree slope gradient in the Hongxing state farm of Heilongjiang province, China (*Fig. 1*).

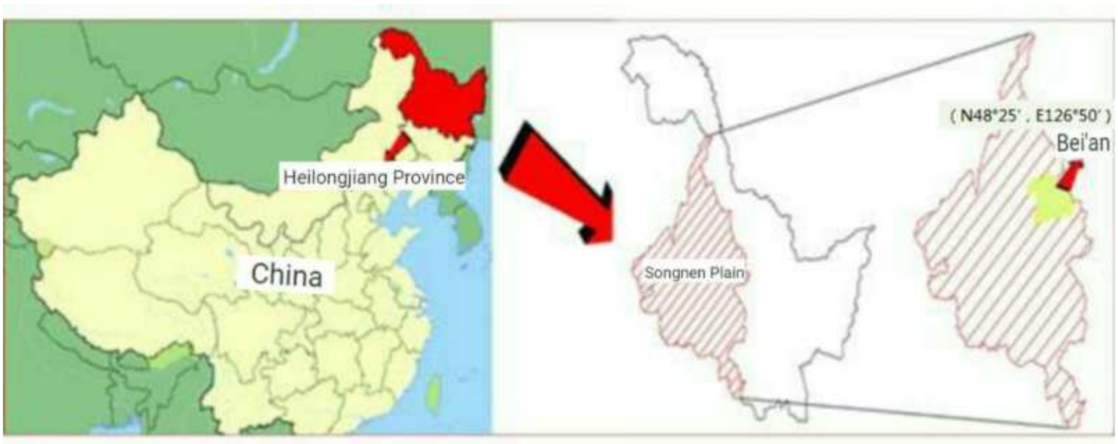

Figure 1. *Location of the experimental site*

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The soil type was meadow black soil with 21.93% of sand (>0.02 mm), 44.21% of silt (0.02~0.002 mm) and 33.86% of clay (<0.002 mm) in mass fractions, 86.3 g/kg organic matter content, 18.5 mg/kg, 18.5 mg/kg of ammonium nitrogen, 58.5 mg/kg of available phosphorus, 151 mg/kg of available potassium, and the pH value was 6.3 (Wei et al., 2016). The raw material of the biochar was corn straw and was purchased from the Jinhefu agricultural development company of Liaoning province near the experimental site. It was formulated under 450 °C temperature and anaerobic conditions. The particle size of the biochar was 1.5~2.0 mm, the contents of total C, total N, total P and total K were 70.21%, 1.58%, 0.73% and 1.66% respectively, and the pH value was 9.36 (Wei et al., 2016).

Experimental design

Five different amounts of biochar were applied (0, 25, 50, 75 and 100 t/hm^2) in different field plots with 5 m of width and 20 m of length, named CK, C1, C2, C3 and C4 treatments respectively, among which CK was the control treatment.

Before seeding, the top 0-25 cm of the soil layer was loosened, followed by the artificial mixing of biochar into the soil, after which a waiting period took place until the seeding of the soybeans. The soybean variety used was Heihe number 3.

The fertilizer supply was as follows; NPK compound fertilizer with 13% of N, 25% of P_2O_5 and 10% of K_2O was supplied at the seeding stage, with a supply amount of 450 Kg/hm^2.

The items and methods of the observations

Soil matric potential was measured using tensionmeters installed in the soil layers at 10, 20, 40, 60, 80, 100, 120 and 140 cm below soil surface.

Meteorological data was measured using automatic weather station located in the experimental site.

Soil water content was measured using the TRIME-T3 Pipe Soil Moisture Measurement System made in Germany.

Simulation calculation

Brief introduction of Hydrus-1d

Hydrus-1d is a model annunciated by the international center of ground water simulation (Liu et al., 1998) with 5 modules used for different calculations, including water movement, solution migration, heat conduction, plant-root water uptake and plant-root growth. The water movement module can be specifically used for unsaturated soil water movement simulation, and not only does it take plant-root water uptake into consideration, but also modifies the lagging of soil water holding capacity. It's widely used in soil water movement simulation (Wang et al., 2005, 2012; Lai et al., 2015) and the results are more precise. The boundary conditions of the model are comprehensive, in which the upper boundary conditions include constant water head, constant water flow, atmospheric boundary, variable water head, variable water flow and atmospheric boundary condition surface with run-off; while the lower boundary conditions include constant water head, constant water flow, variable water head, variable water flow, freedom drainage, deep layer drainage, leached surface and horizontal drainage. Some boundary conditions are variable-boundaries and correspondent variable conditions should be imputed.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

For the calculation of water movement the classical equation of Richards was used, taking the soil surface as the abscissa while the direction of the *Z* axis is downwards, then the basic equation of unsaturated soil water movement is as follows.

$$\frac{\partial \theta}{\partial t} = \frac{\partial}{\partial z}\left(D(\theta) \frac{\partial h}{\partial z} \right) + \frac{\partial k(\theta)}{\partial z} + S \qquad \text{(Eq.1)}$$

Where θ is soil water content; h is suction head; k is unsaturated soil water conductivity; S is plant-root water uptake, which would be 0 when no plants are present in the soil surface; and $D(\theta)$ is soil water diffusivity.

The model can be divided into a single pore model and a double pore model, and the Van Genuchten-Mualem model which was developed from the single models of Van Genuchten (1980) and Mualem (1975), its basic equation is as follows:

$$\theta(h) = \begin{cases} \theta_r + \dfrac{\theta_s - \theta_r}{\left(1 + |\alpha h|^n\right)^m}, h < 0 \\ \theta_s, h \geq 0 \end{cases} \qquad \text{(Eq.2)}$$

Where, θ_r is the residual soil moisture content; θ_s is saturated soil moisture content; h is pressure water head.

Considering the effect of suction head on plant-root water uptake, Feddes function was used to calculate the actual transpiration rate according to the potential transpiration rate. Its basic equation is as follows:

$$\alpha(h) = \begin{cases} 0, h \geq h_4, h \leq h_1; \\ \dfrac{h_1 - h}{h_1 - h_2}, h_2 \leq h \leq h_1; \\ 1, h_3 \leq h \leq h_2; \\ \dfrac{h - h_4}{h_3 - h_4}, h_4 \leq h \leq h_3; \end{cases} \qquad \text{(Eq.3)}$$

Where, h is soil water potential, *cm*; h_1 is the negative pressure value when the soil pores are full of water; h_2 is a negative value when the soil water reaches the water holding capacity; h_3 is a negative value when the capillary bond is disrupted due to plant consumption and evaporation from the soil surface; and h_4 is a negative pressure value when permanent wilting occurs.

Definite conditions

1) Initial conditions

The initial condition is the negative pressure value of every soil layer in the beginning.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

$$h(z,0) = h_0(z), z \geq 0 \tag{Eq.4}$$

2) Boundary condition

The experimental plots were in a sloping farm land with a 3 degree gradient, thus the soil surface boundary condition was:

$$-K\frac{\partial h}{\partial z}\bigg|_{\Gamma 1} = q(z,t) \tag{Eq.5}$$

There should be no surface water when the rain intensity is lower than the infiltration intensity, which is:

$$-k(\theta)\left(\frac{\partial \theta}{\partial z} + 1\right) = R(t), z > 0 \tag{Eq.6}$$

And surface water will accumulate when the rain intensity is higher than the infiltration intensity, which is:

$$h(0,t) = H(t), t > 0, z = 0 \tag{Eq.7}$$

Where, $q(z,t)$ is the water flux density in the soil surface boundary; $R(t)$ is the rain intensity; and $H(t)$ is the depth of surface water.

The parameters to be measured are residual soil moisture content θ_r; saturated soil moisture content θ_s; reciprocal of air-entry value α and shape factor n; soil water content θ; pressure water head h; saturated water conductivity K_S; soil texture and soil layer thickness d; inclined degree of soil layer; times at which the simulation is started and finished, etc..

The above soil hydraulic parameters were obtained from soil water characteristic curves which were determined using a Hitachi CR-21G3 centrifuge manufactured in Japan. 8 levels of speed were set (500, 1000, 1500, 2000, 3000, 4000, 5000 and 6000 r/min), and the centrifugal time for each speed level was 100 min. Ks was measured with the double-rings method in the field plots, and the K_S value was calculated according to Daxi law, K_S=0.75 cm/h.

Unsaturated soil water conductivity was determined with the following equation:

$$K(\theta) = K_s\left(\frac{\theta - \theta_r}{\theta_s - \theta_r}\right)^{0.5}\left\{1 - \left[1 - \left(\frac{\theta - \theta_r}{\theta_s - \theta_r}\right)^{1/m}\right]^m\right\}^2 \tag{Eq.8}$$

The water diffusivity $D(\theta)$ was determined using an infiltration method of horizontal columns.

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Results and analysis

Soil hydraulic characteristic parameters

Soil water holding capacity is reflected in the soil water characteristic curves. The Van Genuchten model in Hydrus-1d had good fitting effects for the experimental area (Liu, 2017; Wang, 2016), thus it was used to fit the soil water characteristic curve of every treatments. The fitting results of soil hydraulic characteristic parameters was shown in *Table 1*. The VG model was more effective, the fitting precision was higher than 0.995, the values of RMSE for every treatment ranged from 0.516 to 1.219, thus all the parameters can be used for soil water movement simulation.

With the increase of the amount of biochar applied, the residual soil moisture content and the shape parameter were decreasing, while the saturation moisture content and the reciprocal of air inlet value were increasing. The difference between the saturated and residual water content were getting higher with increasing the amount of biochar applied, which greatly improved the available soil moisture content, a beneficial factor for crop growth.

Table 1. *Parameters for the Van Genuchten model*

Treatment	Residual soil water content θ_r ($cm^3 \cdot cm^{-3}$)	Saturated soil water content θ_s ($cm^3 \cdot cm^{-3}$)	Reciprocal of air inlet value α (cm^{-1})	Shape parameter *n*	Fitting goodness R^2	RMSE
CK	0.10787	0.42188	0.00818	1.66675	0.9956	0.852
C1	0.10712	0.43591	0.00918	1.61491	0.9972	1.295
C2	0.08200	0.45146	0.01166	1.47769	0.9995	0.516
C3	0.08061	0.47913	0.01416	1.45699	0.9996	0.793
C4	0.07313	0.48606	0.01506	1.41372	0.9995	1.141

Model validation

The CK treatment and the rain with 26 mm of total rain-water and 2 h duration occurred on the 22nd of July 2015 were chosen for the model validation. Compare the actual soil water redistribution curves and their simulation results, and calculate their errors to check the model's reliability and accuracy. *Figure 2* shows the soil water redistribution at 20 min, 12 h, 24 h and 48 h after rain and their simulations. The actual value and simulation value were close.

The simulation errors were showed in *Table 2*. The simulation errors were between -5.12% and 9.15% which were within ±10%. The results showed that the simulation model was reliable and accurate, and that the model could be used for soil movement simulation.

Table 2. *Simulated and measured soil water redistribution data and its errors*

Depth (cm)	20 min		Errors (%)	12 h		Errors (%)
	Measured	Simulated		Measured	Simulated	
10	0.3391	0.3540	4.39%	0.3412	0.3261	-4.43%
20	0.311	0.3205	3.05%	0.3021	0.3294	9.04%
40	0.3082	0.3036	-1.49%	0.3381	0.3303	-2.31%
60	0.2913	0.3016	3.54%	0.3083	0.3252	5.48%
80	0.3059	0.2996	-2.06%	0.3278	0.3177	-3.08%
100	0.2921	0.2976	1.88%	0.3010	0.3131	4.02%

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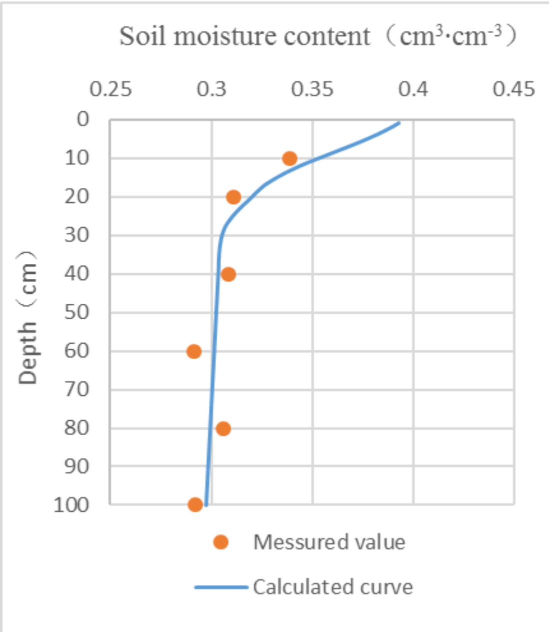
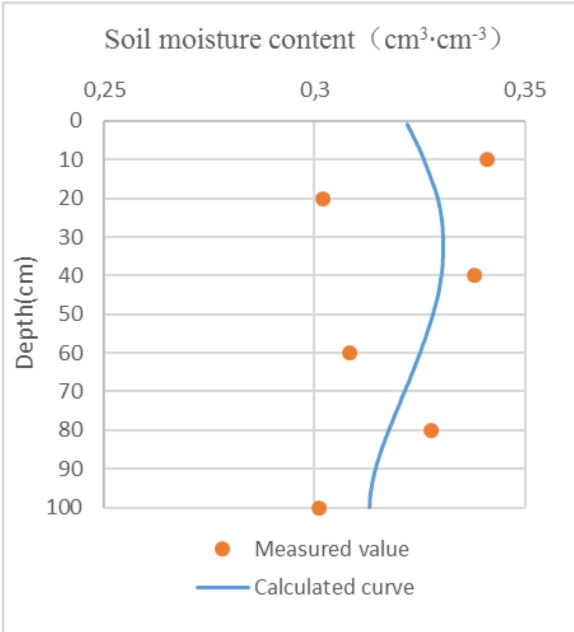
DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

***Table 2. (cont.)** Simulated and measured soil water redistribution data and its errors*

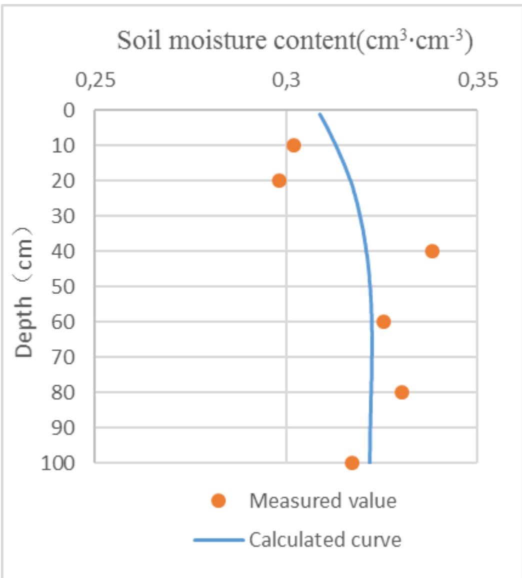
Depth (cm)	24 h		Errors (%)	48 h		Errors (%)
	Measured	Simulated		Measured	Simulated	
10	0.3021	0.3130	3.61%	0.2921	0.301	3.05%
20	0.2981	0.3168	6.27%	0.3221	0.3044	-5.50%
40	0.3381	0.3208	-5.12%	0.2964	0.3099	4.55%
60	0.3253	0.3222	-0.95%	0.3263	0.3139	-3.80%
80	0.3301	0.3221	-2.42%	0.3138	0.3164	0.83%
100	0.3171	0.3217	1.45%	0.2907	0.3173	9.15%



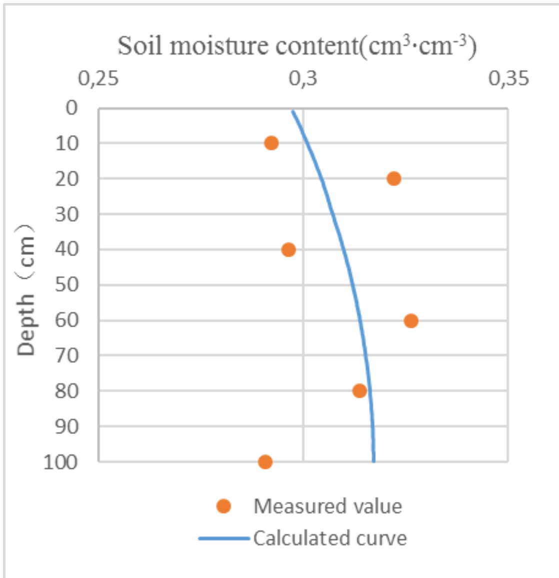
(a) 20 min after rain



(b) 12 h after rain



(c) 24 h after rain



(d) 48 h after rain

***Figure 2.** Calculated and measured soil water redistribution after rain for the CK treatment*

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Simulation results

The rainfall with 19 mm of total rain-water and 1 h duration occurred on the 22nd of July 2015 was chosen to simulate the soil water distribution at 48h after rain. The results was shown in *Figure 3*. The figure showed that with increasing the amount of biochar, the curves right shifted, especially in the case of the soil layer at a 0-50 cm depth, which means that the soil water content in the profile was increasing as well. However, below the soil depth of 50 cm, the soil water content changes were not significant, the reason for which is the fact that the biochar was applied to the upper layer of the soil, which made the soil porosity of the upper layer higher than that of the deeper layers. Thus with the depth increase, the soil water content reduced sharply, and with the increase of the amount of biochar applied, the soil water contents were also increasing by a small increment.

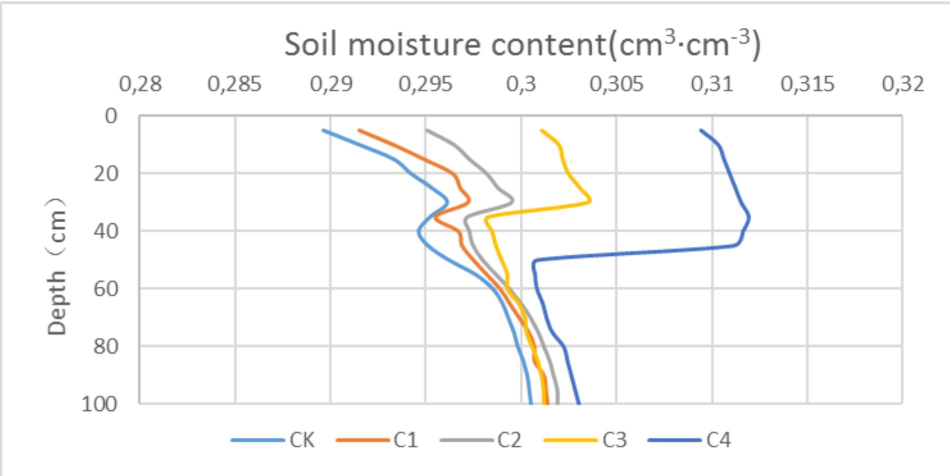

Figure 3. *Simulation results of water content in the soil profile for different treatments*

Conclusions

(1) With the increasing amount of biochar applied, the residual soil moisture content and the shape parameter were decreasing, and the saturated soil moisture content and the reciprocal of air inlet value were increasing. The difference between the saturated and residual water content increased with the amount of biochar, which greatly improved the available soil moisture content. Moreover, biochar treatment can improve soil water redistribution conditions, increasing the soil water holding capability in the cultivated layer, which should be beneficial to the growth of crops.

(2) The errors of soil water movement simulation based on the Hydrus-1d software were between -5.12% and 9.15% which is within ±10%. It could be used for soil water movement simulation under biochar application in the black soil region of Northeast China.

(3) The results above have a great significance for soil and water resources utilization and the protection of the black soil region of Northeast China. However, the research was limited to a farmland with a 3 degree slope gradient, thus it is necessary to do similar researches on lands with a 5 degree gradient, because 3 and 5 degree farmlands are the two main types of sloping farmlands in the region.

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DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

Acknowledgements. Many thanks to the support of the Chinese National Natural Science Foundation Project (50479033) and the Project of Chinese National Key Research and Development Program (2016YFC0400101).

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DOI: http://dx.doi.org/10.15666/aeer/1702_41834192

CHEMICAL COMPONTS OF MOSO BAMBOO *(PHYLLOSTACHYS HETEROCYCLA* VAR. *PUBSCENSE)* CULM AT DIFFERENT AGES AND HEIGHTS

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(Received 9th Dec 2018; accepted 4th Mar 2019)

Abstract. Bamboo is an effective alternative material to alleviate the burden on wood supplies. Chemical components distributed in the gradient structure of bamboo culm are likely to be the determining factors in selecting the optimal application of bamboo. Bamboo culm exhibits a complicated chemical composition of in holocellulose, lignin, pentosan and extractives in the longitudinal direction of its culm cultivated for varying periods of time. In this study, the chemical constituents of moso bamboo were analyzed statistically at different heights cultivated for a minimum of 6 months to as long as 9 years. No significant impact on chemical components was found in the longitudinal direction of the bamboo culm. However, bamboo culm age should be taken into consideration in the trends of holocellulose, pentosan, lignin and extractives located in the inner, middle and outer layers. The moso bamboo cultivated from 3 to 5 years was optimal for industrial application. The yield percentage and difference between layers, in particular, are fundamentals for evaluating and improving the properties for bamboo products.
Keywords: *extractives, holocellulose, lignin, pentosan*

Introduction

Bamboo is mainly distributed in tropical and subtropical areas, the growing stock and yield of which in China is one of the highest worldwide (Mao et al., 2017; FAO, 2010). Bamboo is almost exclusively composed of cellulose, hemicellulose and lignin, which is quite similar to wood in chemical composition. As a fast-growing plant, bamboo is regarded as a huge renewable resource base to reduce stress on wood resources to some extent (Cao et al., 2014; Jiang et al., 2015; Cheng et al., 2015; Kaur et al., 2016). In traditional industries, bamboo utilization only occurs for primary products such as hand-plaited bamboo articles and daily necessities. In high-tech industries, bamboo charcoal fiber, pulp-making, bamboo-based composite lumber and bamboo vinegar present the outstanding value of bamboo (Sulaiman et al., 2005; Ahmad and Kamke, 2011; Sugesty et al., 2015; Han et al., 2017), which is intimately linked to the chemical details of bamboo.

Bamboo is a lignocellulosic biomass with a complicated network structure. Lignocellulosic cell walls in bamboo give it the majority of its properties. Due to the covalent linkages among cellulose, hemicellulose and lignin, the definitive structure of the three natural compositions is almost unfathomable (Guerra et al., 2006; Huang et al., 2016). The chemical constituents of bamboo are known to vary greatly depending on

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species, position within the culm and the age of the culm. The varieties, like *Bambusa arundinacea*, *Dendrocalamus asper, Gigantochloa apus,* and *Phyllostachys heterocycla*, are more common species of bamboo suited to large-scale comprehensive utilization (Jayanetti and Follett, 1998). In very general terms bamboo consists of 50-70% holocellulose and 20–25% lignin (Fengel and Shao, 1984; Liese, 1987). Lignin is a highly complex non-crystalline molecule comprising of a large number of phenyl-propane units. The structure of the lignin present in bamboo is unique, and undergoes changes during the elongation and ageing of the culm (Itoh, 1990). The lignin content is relatively high in the epidermal layer and at the top of bamboo (Jiang et al., 2006). Between 40 and 50 percent of the dry mass of bamboo is in the form of cellulose forming from the anhydrogluclose unit. Cellulose plays a main role in mechanical characteristics of bamboo culm, even for bamboo fiber. The lignification process of bamboo is from the epidermal layer to inner waxy layer, that is, there is less cellulose in the epidermal layer (Lin et al., 2002). Hemicelluloses, therefore, comprise mixtures of polysaccharides manufactured in bamboo from basic sugars such xylose, arabinose, glucomannan and galactose (Balakshin et al., 2011). 90% of the hemicellulose is xylan with a structure intermediate between hardwood and softwood xylans. The distribution of hemicellulose content is in a reversal of that of lignin content (Peng et al., 2012; Peng and She, 2014). Bamboo also has minor amounts of resins, waxes and tannins (Chung and Wang, 2017).

Bamboo industries on chemical components have different requirements. The higher cellulose content the better it is for pulping and papermaking. In general, cellulose is also closely related to the strength and deformation of bamboo-based panels. However, this relationship may be masked by the presence of amounts of extractives. Also, abnormally high lignin fractions in bamboo may influence structures and properties, at least in the longitudinal axis of bamboo (Richard and Harries, 2015; Huang et al., 2017). Therefore, thorough understanding of distributed chemical components in bamboo to optimize the further processing technology is required.

Materials and methods

Preparation

Moso bamboo (*Phyllostachys heterocycla* var. *pubscense*) was obtained from Sanming bamboo stands (27N, 117E) in Fujian province, China. After air drying, bamboo samples (inter node) were collected from 6-month-old to 9-year-old stem-sections at three different heights above ground level (1, 3, and 5 m), and also, on the basis of position which is outer, middle and inner layer of the bamboo stems. Outer layer is the epidermal layer, and inner layer is the waxy layer (*Fig. 1*). Dried samples were ground into powder with a particle size of 40-60 mesh for chemical analysis.

Determination of chemical components

The chemical characteristics of bamboo were determined according to the national standards outlined in GB test methods. Holocellulose content was determined based on GB/T 2677.10-1995 *Fibrous Raw Material* (or FRM for short) – *Determination of Holocellulose* (GB, 1995). The lignin content was determined following the GB/T 2677.8-1994 *FRM – Determination of Acid-insoluble Lignin* (GB, 1994b). Pentosan content was determined in accordance with GB/T 2677.9-1994 *FRM – Determination of*

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Pentosan (GB, 1994c). The determination of extractives was carried out following GB/T 2677.6-1994 *FRM-Determination of solvent extractives* (GB, 1994a) and GB/T 2677.5-1993 *FRM – Determination of One Percent Sodium Hydroxide Solubility* (GB, 1993). The number of replicates was five, and the analyses of variance by Statistica 6 were used.

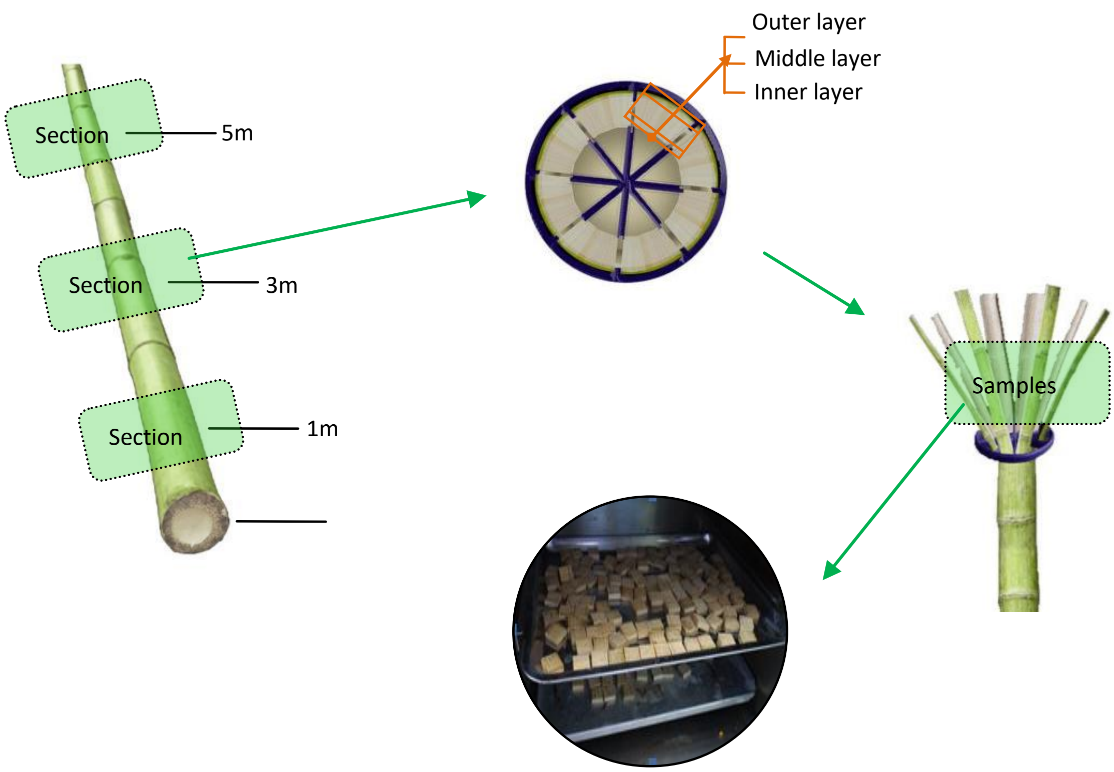

Figure 1. *Preparation of bamboo samples*

Results

Chemical characteristics at different heights

The chemical constitution of inner, middle and outer layer in the moso bamboo stems at different heights was determined respectively. The analysis of variance (ANOVA) on holocellulose, lignin, pentosan and extractives are showed in *Table 1*. There are no significant differences in chemical composition in the longitudinal direction of bamboo stems.

Table 1. *Summary result for analysis of variance for chemical components at different heights*

Position	P-value				
	Holocellulose	Lignin	Pentosan	1% sodium hydroxide extractives	Solvent extractives
Inner	0.750284	0.091319	0.991868	0.841184	0.697808
Middle	0.97058	0.24962	0.987907	0.403769	0.536052
Outer	0.843169	0.335781	0.863107	0.95569	0.509231

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Chemical characteristics at different ages

The chemical constitution of inner, middle and outer layer in the moso bamboo stems at different ages was determined respectively. The analysis of variance (ANOVA) on holocellulose, lignin, pentosan and extractives are showed in *Table 2*. Nearly all the chemical components for different layers shows significant differences among the culm age, except the lignin content in the inner and outer layer. Therefore, it is extremely valuable to perform trend analysis.

Table 2. *Summary result for analysis of variance for chemical components at different ages*

Position	P-value				
	Holocellulose	**Lignin**	**Pentosan**	**1% sodium hydroxide extractives**	**Solvent extractives**
Inner	0.00098	0.289121	0.002851	0.005266	0.004758
Middle	0.002666	0.028988	0.000192	0.017753	6.76E-05
Outer	0.021891	0.251166	0.020283	2.76E-07	0.000511

The yield trends of holocellulose in the inner, middle and outer layer are showed in *Figure 2* from 6-month-old to 9-year-old. The holocellulose content in middle layer is more than that in inner and outer layer in three years. However, holocellulose content in outer layer increases since 5-year-od, even more than that in middle layer. Meanwhile, there is a decreasing trend in percentage difference among the three layers. Considering there is no significance level along the height, the average of each layer at different height is carried out to determine the comparative benefit. The changing trends of holocellulose content for inner and outer layer are the similar, and peak at the fifth year.

The variation of lignin content in the three layers is shown respectively in *Figure 3* from 6-month-old to 9-year-old. Inner layer has a great similarity in lignin content compared to middle layer. However, only the middle layer shows a significant relativity for lignin content. In the middle layer, lignin content increases slightly from the sixth month to the third year, and then it is nearly at a constant level.

Here is that the pentosan content as a whole increases from the outer layer to the inner layer (*Fig. 4*). As such, there is much comparability between the inner layer and middle layer. Pentosan content reaches up to the maximum value in the fifth year and gets the minimum value in the seventh year. In the third year, however, the global minimal value of pentosan content appears in the outer layer.

The 1% sodium hydroxide extractive of bamboo is closely related to decay resistance, which is mainly composed of tannin, pigment, alkaloid, saccharine, starch, degraded hemicellulose and lignin, etc (Jiang et al., 2015). The variation trend in the inner layer is similar to that in the middle layer, but different from that in the outer layer (*Fig. 5*). The yield is ranging from 19.38% to 35.06%. The 1% sodium hydroxide extractive in the inner layer is more than that in other two layers except in the seventh year, and the tendency is almost smooth before the five year and changes suddenly in the seventh year.

The changing trend of solvent extractives in the inner layer is similar with that in the outer layer, and has a little difference from that in the middle layer (*Fig. 6*). The lowest content of solvent extractives is set in the 5-year-old bamboo stems for each layer. The

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variation of solvent extractives is similar between each layer after the third year. In contrast, there is less extractives in the middle layer than that in other two layers.

Figure 2. *Variation of holocellulose content*

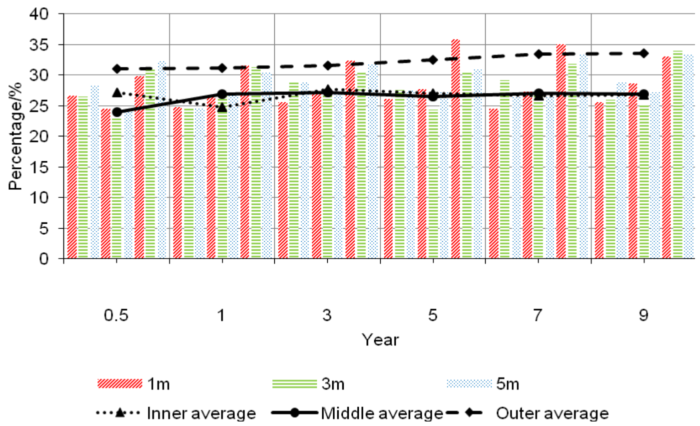

Figure 3. *Variation of lignin content*

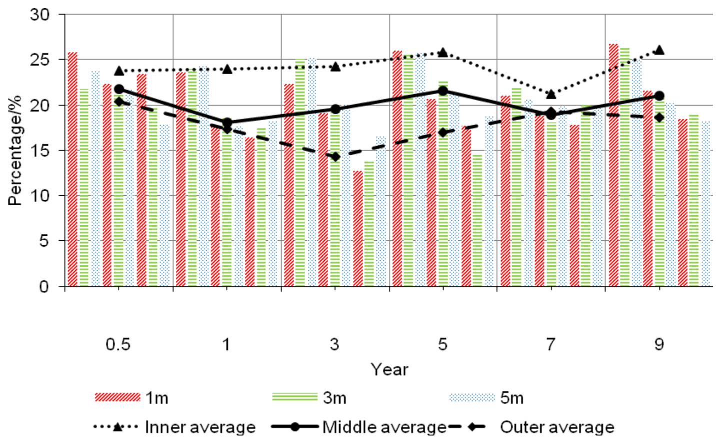

Figure 4. *Variation of pentosan content*

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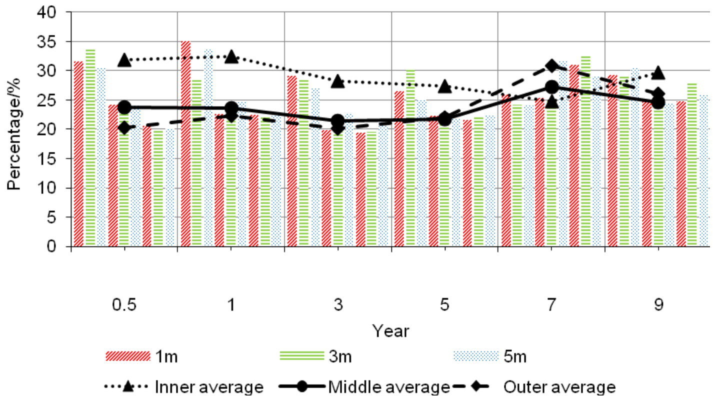

***Figure 5.** Variation of 1% sodium hydroxide extractives*

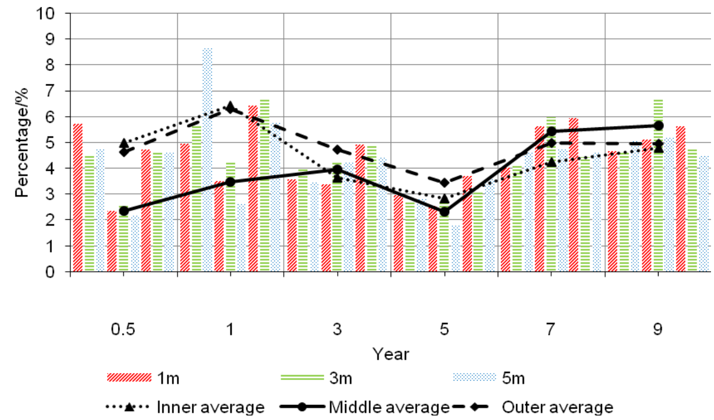

***Figure 6.** Variation of solvent extractives*

Discussion

It should be noted that the –OH groups of the cellulose molecules which give rise to hydrogen bonding are also highly attractive to moisture. Below the fiber saturation point, a subsequent dimensional change was occurred (Anokye et al., 2014). Bamboo strips are liable to distortion such as twist and bow, which has a strong influence with mechanical properties (Saito and Arima, 2002; Kamruzzaman et al., 2008). According to the findings, the distribution of chemical components in the longitudinal direction of bamboo stems should not be the reason to warping. That is, the distortion of bamboo strips may be due to the gradient fibrous structure (Chen et al., 2018).

However, the chemical constituents are fundamental to physical and mechanical properties of bamboo. The holocellulose content in middle layer reaches the maximum of all yield percentage at the third year, which has potential for use as dissolving pulp compared to softwoods (Lovell, 1945). As the panel practice, 3-year-old bamboo is optimum for tangential bamboo sliver, and 5-year-old bamboo is a good choice for radial bamboo sliver in regard of dimensional stability (Sinha and Miyamoto, 2014; Suthon, 2016; Li et al., 2018). A markedly higher proportion of lignin is in the outer layer, which is consistent with the outcome of lignification process (Lin et al., 2002; Tsuyama et al., 2017). For the 3-year-old and 5-year-old bamboo culm, there is obvious

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difference in pentosan content among the three layers. The yield of pentosan in the outer layer is less than the inner layer and middle layer. Pentosan can not only lower moisture absorption of bamboo, it can also improve the toughness of bamboo (Das and Chakraborty, 2008; Banik et al., 2017). Therefore, failure region should be close to the outer layer when a force is applied perpendicular to grain. However, none of studies has been reported from this point at least for now. The extractive contents of moso bamboo are higher than that of softwood and hardwood. Therefore, it is more complicated for preservative treatment (Shah et al., 2018). The high yield percentage of 1% sodium hydroxide extractive indicates that there are more hemicellulose and lignin with lower molecular weight. The solvent extractives consist of wax, fatty acid, essential oil and so on. Higher percentage of solvent extractives has a negative effect on pulping process (Muhammad et al., 2013).

Conclusions

1. Heights have no significant impact on chemical constituents for the inner, middle and outer layer respectively. However, bamboo culm age is closely associated with holocellulose, pentosan, lignin and extractives.
2. The maximum yield of holocellulose is found in the middle layer of the 3-year-old bamboo culm. But it is obvious that the holocellulose contents in the three layers are similar at the fifth year. For lignin content, there is almost no difference in each layer between the third year and the fifth year. There is a clear distinction for percentage of pentosan in the three layers from the third year to the fifth year, which has a potential impact on strength. The lowest percentage of extractives presents in the fifth year. However, the percentage of 1% sodium hydroxide extractives changes hardly from the third year to the fifth year.
3. For the practical application, it is impossible to get a desired percentage of chemical components of moso bamboo in a particular culm age. However, the optimization of culm age could be accomplished in terms of the different performance requirements of bamboo products.

Acknowledgements. This study was supported by National Natural Science Foundation (NSFC) of China (Grant #: 31600455).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41934203

APPENDIX

Appendix 1. *Analysis of variance for chemical components at different heights*

			Sum of squares	df	Mean square	F	P-value
Holocellulose	Inner	Between groups	3.3258544	2	1.629272	0.292877	0.750284
		Within groups	83.44475	15	5.562983		
		Total	86.70329	17			
	Middle	Between groups	1.3279	2	0.66395	0.02992	0.97058
		Within groups	332.8572	15	22.19048		
		Total	334.1851	17			
	Outer	Between groups	2.1411	2	1.07055	0.172543	0.843169
		Within groups	93.06835	15	6.204557		
		Total	95.20945	17			
Lignin	Inner	Between groups	12.03063	2	6.015317	2.819365	0.091319
		Within groups	32.00357	15	2.133571		
		Total	44.0342	17			
	Middle	Between groups	6.374633	2	3.187317	1.524516	0.24962
		Within groups	31.36062	15	2.090708		
		Total	37.73525	17			
	Outer	Between groups	6.415011	2	3.207506	1.174688	0.335781
		Within groups	40.95777	15	2.730518		
		Total	47.37278	17			
Pentosan	Inner	Between groups	0.068011	2	0.034006	0.008169	0.991868
		Within groups	62.4389	15	4.162593		
		Total	62.50691	17			
	Middle	Between groups	0.0673	2	0.03365	0.012176	0.987907
		Within groups	41.4537	15	2.76358		
		Total	41.521	17			
	Outer	Between groups	2.041733	2	1.020867	0.148671	0.863107
		Within groups	102.9993	15	6.866618		
		Total	105.041	17			
1% sodium hydroxide extractives	Inner	Between groups	4.010478	2	2.005239	0.174954	0.841184
		Within groups	171.9231	15	11.46154		
		Total	175.9336	17			
	Middle	Between groups	11.95551	2	5.977756	0.964024	0.403769
		Within groups	93.0126	15	6.20084		
		Total	104.9681	17			
	Outer	Between groups	1.6471	2	0.82355	0.045459	0.95569
		Within groups	271.7434	15	18.11622		
		Total	273.3905	17			
Solvent extractives	Inner	Between groups	1.478633	2	0.739317	0.368582	0.697808
		Within groups	30.08762	15	2.005841		
		Total	31.56625	17			
	Middle	Between groups	2.875233	2	1.437617	0.650175	0.536052
		Within groups	33.16682	15	2.211121		
		Total	36.04205	17			
	Outer	Between groups	1.310978	2	0.655489	0.706147	0.509231
		Within groups	13.92392	15	0.928261		
		Total	15.23489	17			

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DOI: http://dx.doi.org/10.15666/aeer/1702_41934203

Appendix 2. *Analysis of variance for chemical components at different ages*

			Sum of squares	df	Mean square	F	P-value
Holocellulose	Inner	Between groups	68.34063	5	13.66813	8.932118	0.00098
		Within groups	18.36267	12	1.530222		
		Total	86.70329	17			
	Middle	Between groups	249.6375	5	49.9275	7.08631	0.002666
		Within groups	84.54753	12	7.045628		
		Total	334.1851	17			
	Outer	Between groups	59.78872	5	11.95774	04.0511	0.021891
		Within groups	35.42073	12	2.951728		
		Total	95.20945	17			
Lignin	Inner	Between groups	16.29027	5	3.258053	1.409196	0.289121
		Within groups	27.74393	12	2.311994		
		Total	44.0342	17			
	Middle	Between groups	22.92898	5	4.585797	3.71664	0.028988
		Within groups	14.80627	12	1.233856		
		Total	37.73525	17			
	Outer	Between groups	18.47778	5	3.695556	1.534752	0.251166
		Within groups	28.895	12	2.407917		
		Total	47.37278	17			
Pentosan	Inner	Between groups	46.50238	5	9.300476	6.973381	0.002851
		Within groups	16.00453	12	1.333711		
		Total	62.50691	17			
	Middle	Between groups	34.90713	5	6.981427	12.66689	0.000192
		Within groups	6.613867	12	0.551156		
		Total	41.521	17			
	Outer	Between groups	66.5198	5	13.30396	4.144407	0.020283
		Within groups	38.5212	12	3.2101		
		Total	105.041	17			
1% sodium hydroxide extractives	Inner	Between groups	125.6189	5	25.12378	5.991998	0.005266
		Within groups	50.31467	12	4.192889		
		Total	175.9336	17			
	Middle	Between groups	67.42418	5	13.48484	4.310098	0.017753
		Within groups	37.54393	12	3.128661		
		Total	104.9681	17			
	Outer	Between groups	259.1318	5	51.82636	43.61672	2.76E-07
		Within groups	14.25867	12	1.188222		
		Total	273.3905	17			
Solvent extractives	Inner	Between groups	22.70292	5	4.540583	6.147461	0.004758
		Within groups	8.863333	12	0.738611		
		Total	31.56625	17			
	Middle	Between groups	31.24992	5	6.249983	15.65061	6.76E-05
		Within groups	4.792133	12	0.399344		
		Total	36.04205	17			
	Outer	Between groups	12.35763	5	2.471526	10.30781	0.000511
		Within groups	2.877267	12	0.239772		
		Total	15.23489	17			

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_41934203

THE INFLUENCING FACTORS OF INDUSTRIAL CARBON EMISSIONS IN THE CONTEXT OF UNDERTAKING INDUSTRIAL TRANSFER IN ANHUI PROVINCE, CHINA

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(Received 9th Dec 2018; accepted 4th Mar 2019)

Abstract. Industrial development has led to the indisputable fact that high carbon emissions were brought into Anhui Province. This paper calculates the changes in Anhui's carbon emissions from 2000 to 2015 and analyzes the influencing factors. The results prove that carbon emissions show a stable upward trend, but the growth rate has declined after 2005. Carbon emissions produced by three major industrial sectors: mining industry, EGW (electricity, water and gas) and manufacturing industry, show a growing trend, but growth rates decrease successively. According to the STIRPAT model, five factors, including out-of-province investment, energy consumption structure, per capita income, proportion of output value in the secondary industry to GDP and actual use of foreign capital, are positively correlated with Anhui's carbon emissions, while there is a negative correlation between the proportions of the total volume of foreign trade and the industrial added value of enterprises above designated size (two factors) to total industrial output value. As the structure of energy consumption has long been fueled by coal, the transfer of external industry mainly focuses on projects concerning power plants, steel product processing and coal chemical industry, which are highly energy-consuming, which means these are the main factors driving the growth of carbon emissions.
Keywords: *industrial carbon emissions, overall change of carbon emission, STIRPAT model, influencing factors, external investment, economic development, energy structure*

Introduction

Greenhouse gases, especially carbon dioxide, are regarded as a major cause of global warming, thus becoming a widespread concern (Bamminger et al., 2018), while industries, as the main sources of carbon dioxide emissions, have been the focus of the academic scene (Ban et al., 2016; Rahman and Kashem, 2017; Griffin et al., 2018). As the largest developing country in the world, China has seen its economy maintain rapid development for 40 years since it began its reform and opening-up program in 1978 (Liu et al., 2013, 2014). By this time the country has become one of the world's biggest energy consumers (Deng et al., 2012) and the biggest carbon emitter (Zhang et al., 2015; Shao et al., 2016b). Recent academic researches focus on the following aspects:

Changes in carbon emissions and their spatial distribution during urbanization and industrialization

Jiang et al. (2018) have discussed the leading factors influencing carbon emissions of China and the U.S. and differences through comparison and analysis of the carbon emission processes between the two countries (Jiang et al., 2018). Tian et al. (2011) hold that economic growth, industrial sector and technological changes produce much more carbon dioxide than population, energy structure and other sectors after calculating carbon dioxide emissions of 31 provinces in mainland China and then dividing the 30 provinces (excluding Tibet) into seven groups (Tian et al., 2011). The study of Li et al. (2018) finds that from 2000 to 2013, China's carbon dioxide emissions have grown rapidly, with an average growth rate of 8.2% (Li et al., 2017). Olivier et al. (2015) believe that China accounts for 30% of total global emissions in 2014, up 80% from 2004.

In respect to studies on spatial distribution characteristics, Su and Ang (2014) have calculated the total transfer and flow characteristics of embodied carbon emissions in eight regions of China. Feng et al. (2013) find that 80% of China's carbon emissions come from economically developed coastal provinces, but since 2003, the carbon emissions have gradually shifted from eastern to western regions, especially in central ones with a huge increase (Meng et al., 2011). Wang et al. (2018c), based on the input-output data in 2007 and 2012, have established an input-output model, evaluate the characteristics of regional carbon flow in China and changes in carbon emissions of different industries and afterwards hold that carbon emissions see a decline from eastern to western regions; by employing a clustering method to evaluate the changes in the growth of carbon emissions in 30 provinces of China from 1996 to 2013, Jiang et al. (2017) hold that the expression of carbon emission intensity varies from province to province so the stages of development of different provinces should be fully considered when the government formulates emission reduction strategies. Zhou et al. (2018) use SDA model to analyze the characteristics of China's regional carbon emissions and transfer, and believe that China's regional carbon emission transfer is mainly completed through carbon-intensive manufacturing industry. Tian et al. (2018) calculate the periodic change of carbon dioxide emissions in China's manufacturing industry from 1992 to 2012 and conclude three main factors leading to the changes in carbon dioxide emissions: carbon emission intensity, input-output structure and final demand. Chen and Yang (2018), based on China's provincial-level panel data from 2006 to 2015, analyze the spatial-temporal evolutionary trend, spatial correlation and influencing factors of China's carbon emissions and find that the characteristics of carbon emission distribution are affected by various economic and social factors. Population pressure, affluence level, energy intensity, industrial structure, urbanization level and fixed asset investment have significant effects on promoting the growth of carbon emissions. Technological level and government environmental regulation play an obvious role in curbing carbon emissions while foreign direct investment (FDI) does not. Liu et al. (2018) analyze the carbon emission efficiency of ten typical urban clusters in China from 2008 to 2015 by setting up the ideal point cross efficiency (IPCE) model and then put forward four carbon emission effects brought by urbanization. Wang and Ma (2018a) study the influencing factors and regional difference of the emission efficiency of expandable industrial carbon dioxide of 13 cities in Jiangsu Province from 2000 to 2014; by analyzing the panel data of the Yangtze River Delta from 2000 to 2010 and the relationship between urbanization and carbon emissions, Li et al. (2018) believe that a u-shaped curvilinear relationship between carbon dioxide emission efficiency and urbanization indicates that carbon dioxide emission efficiency declines at the initial stage of urbanization and goes up when urbanization reaches a higher level.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

Differences of China's regional carbon emissions and their influencing factors

During the "13th Five-Year Plan" period (2016-2020), China promises that the government will support provincial regions in taking the lead in reaching carbon emission peak value (Shao et al., 2016a). Since 2000, local governments have gradually replaced national ministries and commissions as the main force to implement energy conservation and emission reduction policy (Zhao et al., 2014). China's vast territory and imbalanced social and economic development produce obvious differences in energy consumption, carbon emissions and economic development level in different regions (Wang et al., 2014a; Zhang et al., 2014a), and many scholars begin to pay heed to regional (provincial-level) differences and transfer characteristics of carbon emissions among China's industries. For example, significant differences exist in China's provinces in respect to economic development, industrial structure, energy consumption patterns and other factors (Liu et al., 2013; Shan et al., 2016). Some provinces in eastern China have entered the post-industrial stage, while most of other provinces in central and western China are still at the accelerated stage of industrialization since industry is the main source of GDP for these provinces (Cai et al., 2016; Li et al., 2017; Jia et al., 2018); central and western regions are the major producer and exporter of fossil energy, while most eastern provinces are the main importer of it (Lindner et al., 2013) and in the meantime, some provinces still rely heavily on coal consumption (Wang et al., 2015a).

Consequently, the growth trend of carbon emissions and its driving factors demonstrate the marked features of the differences among provinces in China (Liu et al., 2012a; Guan et al., 2014; Xu et al., 2016). For instance, Su and Ang (2010) analyze the difference in carbon emissions between provinces in China. Meng et al. (2011) find that since 2003, the trend of embodied carbon emissions transfer based on energy products has gradually shifted from eastern to central and western regions, especially in central provinces. Feng et al. (2013) find that 57% of China's emissions is related to the consumption of goods outside the origin provinces. Western regions produce a large number of low value-added but high carbon-intensive products and export them to eastern regions, leading to a rapid increase in carbon emissions in western regions. Zhang et al. (2014b) analyze carbon dioxide emissions in different provinces of China from 1990 to 2011, and the cross-provincial transfer of carbon emissions in different regions and with different phase characteristics by comparing dioxide emission scale, emission growth rate and emission intensity of different provinces over the years. Sun et al. (2016a) also analyze the characteristics of carbon emission transfer in 30 provinces of China in 2007 and calculate the economic spillover effect and emission reduction effect of cross-provincial carbon emission transfer.

The influencing factors and driving forces of China's carbon emissions

At present, the research methods on the relationship between economic growth and carbon emissions mainly include Logarithmic Mean Divisia Index (LMDI) (Zhao et al., 2010; Song et al., 2015), panel models (Ren et al., 2014; Zhou et al., 2013), data envelopment analysis (DEA) (Sun et al., 2015b) and Input-Output models (Tian et al., 2014; Mi et al., 2015). Research scale stretches from regions (Lu et al., 2015; Tian et al., 2014) to cities (Mi et al., 2015; Zhao et al., 2010) and industry sectors (Lin and Moubarak, 2014; Lu et al., 2015).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

For example, STIRPAT model is one of the mainstream means used to conduct research on influencing factors of carbon emissions (Waggoner et al., 2002; York et al., 2003). Ma et al. (2017) analyze the driving forces of carbon emissions in China's public construction industry from 2000 to 2015 based on the expanded STIRPAT model. Zhang et al. (2017) analyze the trend of carbon emissions and energy consumption in Henan Province from 1995 to 2014 with STIRPAT model and believe that energy consumption caused by population growth is the major contributor to carbon emissions in the province. Yang et al. (2018a) analyze the impact of the optimized economic structure of Shanghai City on carbon emissions by employing STIRPAT and NSGA-II models. Wang et al. (2018b) analyze the gross scale, structure and influencing factors of household carbon emissions in 30 provinces and regions in China based on the expanded STIRPAT model.

Two commonly used reverse synthesis methods are employed in existing literature (Liu et al., 2012; Xiao et al., 2016): index decomposition analysis (IDA) and structural decomposition analysis (SDA) (Malla, 2009). Both methods have their own advantages and disadvantages. Generally, SDA needs economic data shown in the form of IO table, while IDA only needs the summary data of each industry category (Cansino et al., 2016; Yang et al., 2016). SDA can only analyze changes between finite years, while IDA can usually analyze those between any year (Cellura et al., 2012). Or the study of the decoupling relationship between economic development and carbon emissions can also be conducted. Decoupling relationship analysis mainly adopts OECD decoupling index method (OECD, 2003) and Tapio elastic decoupling index method (Tapio, 2005). The objects of decoupling relationship analysis are diversified, which can be analyzed from a macro to micro perspective. For example, Yu et al. (2013) employ OECD decoupling model to study the decoupling relationship between carbon dioxide, sulfur dioxide, dust, sewage discharge and economic development in China from 1978 to 2010. Wang et al. (2015c), Zhang and Wang (2013) and Wang et al. (2015b) employ Tapio decoupling model to study the decoupling relationship between the economic development in Tianjin, Jiangsu Provinces and Beijing-Tianjin-Hebei region and pollutant emissions, featuring carbon dioxide. Wang et al. (2014b) combine Tapio decoupling model with LMDI decomposition model to analyze the decoupling situation between China's economic growth and energy consumption from 1991 to 2011.

Study on the impact of changes in industrial structure and industrial transfer on carbon emissions

Industrialization is the dominant driving force of China's economic growth and energy consumption growth. From 1985 to 2011, energy consumption in China's industrial sectors accounts for 70.3% of total energy consumption, showing an upward trend year by year. Energy conservation and emission reduction in China's industrial sectors are the key to China's emission reduction and successful low-carbon transition (Ouyang and Lin, 2015). Carbon emissions, industrial structure change and transfer of different industries in China have gradually become a research field with high attention (Auerbeck, 2001; Lebel et al., 2007). For example, Su et al. (2010) employ input-output analysis framework to analyze industrial agglomeration effect in China and estimate carbon dioxide emissions reflected in economic exports. Yan and Fang (2015) study the historical track, characteristics and influencing factors of carbon emissions in China's manufacturing industry from 1993 to 2011. Cheng et al. (2018) set up total-factor carbon emission efficiency index (TCEI) by employing improved NDDF model and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

estimate the meta-frontier TCEI index of 30 provincial industrial sectors in China from 2005 to 2015. They hold that industrial meta-frontier TCEI in China is relatively low and many provinces still have large room for improving the industrial TCEI. Xian et al. (2018), taking China's electric power industry as an example, put forward a scenario analysis on whether China's electric power industry could achieve the goal of reducing national and provincial-level carbon dioxide emission intensity from 2016 to 2020 by analyzing changes in carbon productivity. Zhao et al. (2017) analyze the decoupling relationship of China's economic growth based on the carbon emissions in five economic sectors, including agriculture and industry.

Some scholars conduct studies on industrial carbon emissions through modeling. For example, Yang et al. (2018a) analyze the relationship between energy consumption and carbon emissions in different industrial sectors in Zhejiang Province from 1995 to 2014 by employing STIRPAT model. Zhang et al. (2017) analyze the influencing factors of carbon emissions in China's construction industry with factor analysis and STIRPAT model. Lindner et al. (2013) employ a bottom-up model to calculate the direct carbon embodied in China's power industry. Zhao et al. (2010) analyze the influencing factors of industrial carbon emissions of Shanghai with LMDI model.

On the other hand, with the development of China's economy, a large number of traditional industries are constantly shifting to central and western regions (Chen et al., 2011; Tian et al., 2014) and industrial transfer, mainly occurring in manufacturing sector, brings the transfer of carbon emissions (Yuan et al., 2009). These studies provide numerous useful information but limitations remain instead. For example, most studies focus on the overall and cross-provincial carbon transfer in China, but existing studies lack of consideration about the inter-industry carbon transfer and leading factors. In addition, most studies only center on short-term changes and lack long-term dynamic studies.

Anhui Province is an underdeveloped province in central China, but it is the main area carrying on industrial transfer in the Yangtze River Delta for being close to the Yangtze River Delta region which is the most economically developed in the country. Although a large number of studies have examined the rapid growth of Anhui's carbon emissions and its influencing factors (Zhang and Wang, 2013), existing studies rarely consider the specific impact of industrial transfer and structural change on Anhui's carbon emissions. Leading industries and technological capabilities vary from region to region, leading to different emission intensity in industrial sectors and there may be differences in the characteristics of carbon dioxide emissions (Tian et al., 2012). At the same time, if the decoupling relationship at the provincial level is not fully understood, the implementation effect of national policies or strategies will decline (Liu et al., 2015). Therefore, only the relationship between industrial transfer and carbon emissions can help to formulate an effective energy conservation and carbon emission reduction policy. Taking Anhui Province as a case study, this paper calculates Anhui's carbon emission model by collecting data of energy consumption and social development in Anhui Province from 2000 to 2015, and employs expanded STRIPAT model to reveal the changes in industrial carbon emissions and its influencing factors in the province in the context of industrial transformation. This study helps to understand the driving factors of industrial carbon emissions in Anhui Province and on that basis, provides reference for reducing industrial carbon emissions.

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Materials and methods

Study area and data

Anhui Province is included in the case study hereof. Located in mid-eastern China, Anhui covers an area of 140,100 km^2 and has 14 prefecture-level cities under its jurisdiction, with a population of 70.27 million in 2016 and an urbanization rate of 52.0%. According to the date of Statistical Bureau of Anhui province (2017), Anhui is in the bottom half of national list of economic development as it ranks 13th among 31 Chinese provinces in GDP growth (from RMB 290.209 billion yuan in 2000 to RMB 2,411.789 billion yuan in 2016, growing by 14.15% annually), and ranks 25th in per capita GDP growth (from RMB 4,800 yuan in 2000 to RMB 39,100 yuan in 2016, growing by 14.04% annually). Anhui economy is dominated by the secondary industry, which accounted for 36.41% of the provincial GDP in 2000, and for about half (48.06%) in 2016, and its second industry is dominated by the Industry, which accounted for 30.04% of the output of second industry in 2000, and this increased to 40.82% in 2016. These indicate that Anhui is still at the mid stage of industrialization, where its economic development is mainly driven by the Industry. Anhui is a close neighbor of Yangtze River Delta (*Fig. 1*), the powerhouse for China's economy, and a critical base for implementation of the state strategy of rise of central China and industrial transference to Wanjiang City Belt, which is a contributor to carbon emission, therefore this study focuses on the carbon emission by Anhui's Industry that can be divided, according to *Industrial Classification for National Economic Activities* (GB/T 4754-2002), into mining, manufacturing and EGW (electricity, gas and water). End-use energy consumption, GDP, added value of industries above a designated scale, total industrial output, total external fund used (including investment from foreign countries, Hong Kong, Macao and Taiwan), output value of secondary industry, population and per capita income are all sourced from *China Energy Statistical Yearbook* and *Anhui Province Statistical Yearbook*, and the data of external investment are from the public data released by Anhui Development and Reform Commission (Anhui Statistical Bureau, http://www.ahpc.gov.cn/).

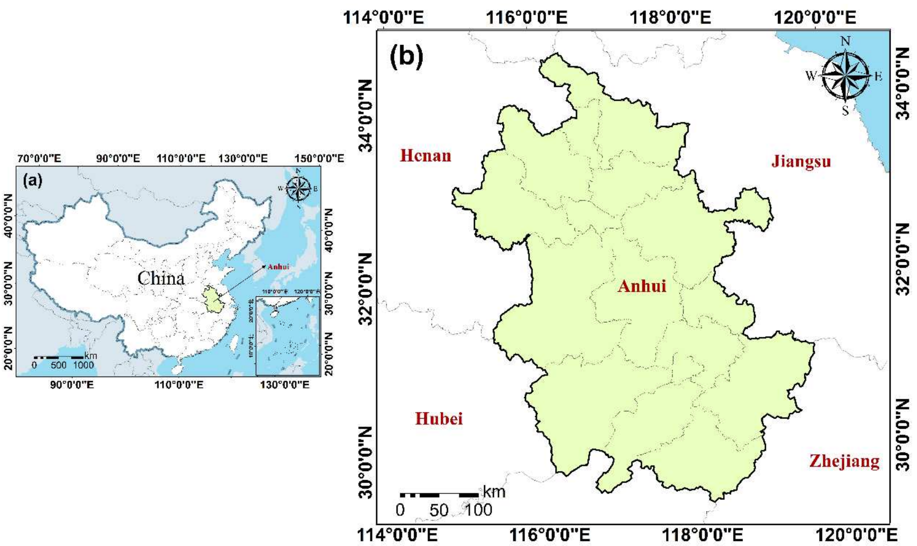

Figure 1. *Location of Anhui province*

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Methods

Calculation of carbon emission

Given that no direct data of CO_2 emission is released by China's statistical organizations, scholars at home and abroad generally make estimation by multiplying the energy consumption with carbon emission coefficient, therefore the industrial carbon emission of Anhui 2000-2005 is calculated by the following model (Yang et al., 2018):

$$c = \sum E_j \times F_j \tag{Eq.1}$$

where, and signify the consumption and emission coefficient of j kinds of fossil fuels respectively. The standard coal conversion coefficient in *China Energy Statistical Yearbook* is adopted, while the emission coefficient is converted based on the statistics of Peng et al. (2013) (see *Table 1*).

Table 1. *Conversion coefficients (by Peng et al., 2013)*

Types of energy	Standard coal conversion coefficient	Carbon emission factor
Raw coal	0.7143 kg·kg^{-1}	0.7599
Washed coal	0.9 kg·kg^{-1}	0.7599
liquefied petroleum gas	1.7143 kg·kg^{-1}	0.5042
Crude oil	1.4286 kg·kg^{-1}	0.5847
Gasoline	1.4714 kg·kg^{-1}	0.5538
kerosene	1.4714 kg·kg^{-1}	0.5714
Diesel	1.4571 kg·kg^{-1}	0.5921
Fuel oil	1.4286 kg·kg^{-1}	0.6185
Coke	0.9714 kg·kg^{-1}	0.855
Gas field	1.2143 kg/m^3	0.4483
Oil field natural gas	1.33 kg/m^3	0.4483
Coke oven gas	0.5714 kg/m^3	0.3548

STIPRAT model

STIRPAT model was built by York et al. (2003) based on IPAT model, with the following formula:

$$\mathrm{I} = \mathrm{a}P^{b}A^{c}T^{d}e \tag{Eq.2}$$

where, I stands for environmental stress, P for population size, A for affluence, T for technical progress, a for the model efficient, b, c, d are the indexes of respective independent variables, and e is the model's error term. b, c and d are introduced to make up for the model's deficiency of I varies with all driving factors in proportion, and can be used for analysis of independent variables' non-proportional impact on environmental stress. After logarithm the model can be converted to:

$$\ln \mathrm{I} = \ln \mathrm{a} + \mathrm{b}\ln \mathrm{P} + \mathrm{c}\ln \mathrm{A} + \mathrm{d}\ln \mathrm{T} + \ln \mathrm{e} \tag{Eq.3}$$

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where, b, c and d embody the elastic relation between independent and dependent variables. Every 1% change of P, A or T will result in b%, c% or d% change of environment impact.

As the regional industrial structure is changed and carbon emission affected by input of extern fund (Chen et al., 2017) during local industrial transference, to truthfully reflect such transference's impact on carbon emission, STIRPAT model for this study is hereby extended as:

$$\ln I = \ln B + \alpha_1 \ln X_1 + \alpha_2 \ln X_2 + \alpha_3 \ln X_3 + \alpha_4 \ln X_4 + \alpha_5 \ln X_5 + \alpha_6 \ln X_6 + \alpha_7 \ln X_7 + \ln e \quad \text{(Eq.4)}$$

where, I signifies the industrial emission, B is the model coefficient and e its error term. There are 7 variables in 5 categories: the factor that embodies the effect of industrial agglomeration, namely X1 – the ratio of the added value of industries above a designated scale to total industrial output value; the 2 factors that embody external capital scale in industrial transference, namely total external fund used X_2 and total external investment X_7; the 2 factors that indicate economic growth, namely X_3 – the proportion of secondary industry in GDP and X_4 – per capita income; X_5- proportion of coal consumption in total energy consumption, the factor that reflects the energy structure; X_6 – the proportion of total import-export volume in total industrial output value and α_i, the elastic coefficients of independent variables, which mean 1% variation of X_i can result in α_i% change of carbon emission.

Results

Anhui's industrial carbon emission

Overall change of carbon emission

Figure 2 shows that, from 2000 to 2015, the value of industrial output and carbon emission in Anhui grew annually by 15.26% and 10.50% respectively, but the 2010-2015 period saw such growths of 11.37% and 4.29% only. The change indicates that Anhui made some achievements in low-carbon transition and restructuring of its industries during the 12th five-year plan, and the rapid growth of carbon emission was more or less contained.

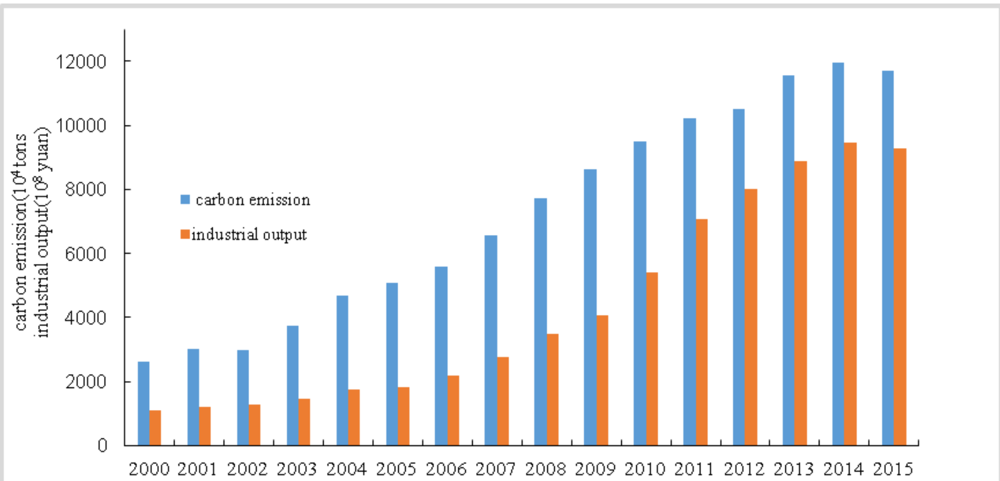

Figure 2. *Change of carbon emissions, industrial production of Anhui province (2000-2015)*

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Change of carbon emission for different industries

(1) In general, 2000-2015 saw increase of carbon emission in indusries of mining, manufacturing and EGW. In growth rate mining > EGW > manufacturing (slope: 217.35, 265.53 and 221.26) (*Fig. 3*), and the trend differs greatly among these three sectors: for manufacturing, the annual growth of carbon emission stood at 11.05% between 2000 and 2005, but by 7.43% only in the period 2005-2014, and this figure further deceased from 2014; For EGW, the carbon emission grew stably by 12.73% annually from 2000-2015, and only from 2015 this growth slowed down. Compared with EGW and manufacturing, mining is at a stage of relatively faster growth, for its growth of carbon emission reached a high level of 19.49% between 2000 and 2005, decreased somehow from 2005 to 2014, but the 12.78% growth rate was still high.

(2) As of proportions of these three industries, the proportion of manufacturing in carbon emission has been decreasing stably. Though EGW saw some narrow marked fluctuation, its proportion is considered stable in long term (about 30%). While the proportion of mining had been rising, its growth rate gradually dropped and reached a plateau after 2010. *Figure 4* shows that in general, the propotions of the three industries all tend to converge to the size of “one third”.

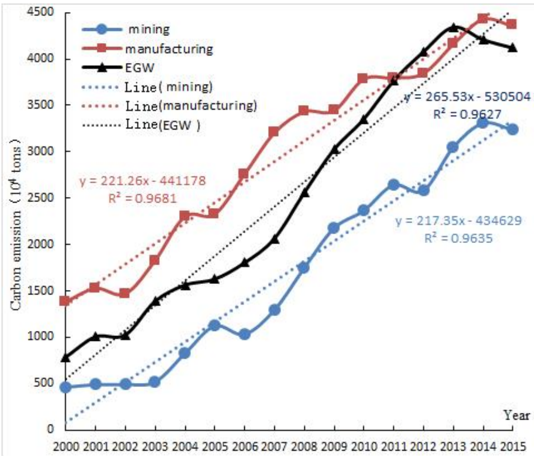

***Figure 3.** Change of carbon emissions of Anhui’s three industrial sectors (2000-2015)*

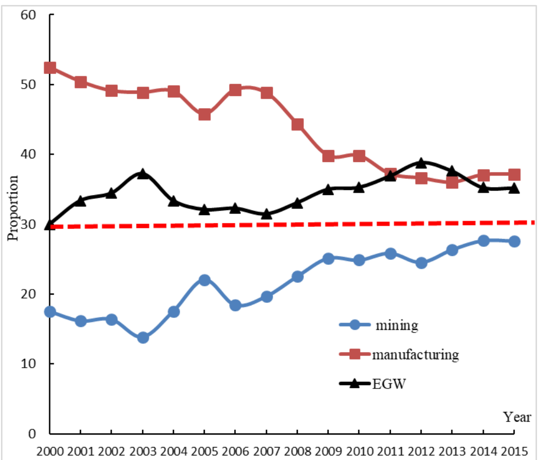

***Figure 4.** Change of carbon emission share of Anhui's three industrial sectors (2000-2015)*

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Calculation results with STIRPAT model

7 factors, namely the proportion of the added value of industries above a designated scale in total industrial output value (X_1), total external fund used (X_2), the proportion of secondary industry in GDP (X_3), per capita income (X_4), energy consumption structure (proportion of coal consumption in total energy consumption) (X_5), the proportion of total import-export volume in total industrial output value (X_6) and total external investment (X_7), are selected for regression analysis with SPSS (a statistical analysis software), to reveal their marginal contribution to carbon emission.

In the first place, least square regression model is used in SPSS for pre-evaluation (*Table 2*). The least square regression model has a fitting goodness of fit $R^2 = 0.995$, and an adjusted goodness of fit $AdjR^2 = 0.991$, both of which pass the F test of 0.001 significant level, indicating that the linear model for independent and dependent variables exists and has a high degree of fitting. But only LnX_1 passes the Student's t test (t test) of 0.005 significant level, and other independent variables fail to pass the significance test (Sig). A study of the correlation matrix of the model shows that the independent variables are closely associated with each other. Variance inflation factor (VIF) is currently used for determination of multiple correlation, while the VIF values of 6 factors calculated are all greater than 10, therefore only when the multicollinearity among independent variables are eliminated can robust results be achieved.

Table 2. *Results of the OLS (ordinary least square) regression model*

	Unnormalized coefficient		**Regression coefficient**	**T test**	**Significance**	**Variance inflation factor**
	Regression coefficient	**Standard error**				
Independent variable	-2.455	2.157		-1.138	0.288	
LnX_1	0.589	0.345	0.124	1.708	0.126	9.05
LnX_2	-0.086	0.049	-0.201	-1.74	0.12	22.859
LnX_3	0.307	0.327	0.116	0.939	0.375	26.204
LnX_4	0.939	0.251	1.242	3.738	0.006	189.191
LnX_5	-1.378	1.836	-0.073	-0.751	0.474	16.08
LnX_6	-0.058	0.201	-0.039	-0.287	0.781	31.123
LnX_7	-0.016	0.105	-0.026	-0.156	0.88	47.071

Partial least square regression model (PLS) is used to address the practical problems of multiple correlations of variables, small sample volume and low degree of freedom, in an analytical process of:

(1) Standardized processing of variables, to rid of the dimensional effects among variables and make them comparable, and the processed data are expressed as $ZLnX_1$, $ZLnX_2$, $ZLnX_3$, $ZLnX_4$, $ZLnX_5$, $ZLnX_6$, $ZLnX_7$ and ZLn_C.

(2) Principal components are extracted from data processed in procedure (1), refer to *Table 3* for the results.

Table 3 shows that the accumulative contribution of FAC_1, FAC_2 and FAC_3 reaches a high level of 95.602%, so 2 principal components are extracted. *Table 4* indicates the following relations between FAC_1, FAC_2 and original variables:

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$$FAC_1 = -0.142 \times ZLnX_1 + 0.155 \times ZLnX_2 + 0.155 \times ZLnX_3 + 0.157 \times ZLnX_4 + 0.149 \times ZLnX_5 - 0.148 \times ZLnX_6 + 0.145 \times ZLnX_7 \quad \text{(Eq.5)}$$

$$FAC_2 = 1.053 \times ZLnX_1 + 0.052 \times ZLnX_2 + 0.114 \times ZLnX_3 + 0.25 \times ZLnX_4 + 0.529 \times ZLnX_5 + 0.78 \times ZLnX_6 + 0.829 \times ZLnX_7 \quad \text{(Eq.6)}$$

Table 3. *Results of the principal component analysis*

Component	Initial eigenvalues			Sums of squared loadings		
	Total	**Variance %**	**Cumulative %**	**Total**	**Variance %**	**Cumulative %**
1	6.33	90.433	90.433	6.33	90.433	90.433
2	0.362	5.169	95.602	0.362	5.169	95.602
3	0.189	2.694	98.297	0.189	2.694	98.297
4	0.046	0.652	98.949			
5	0.038	0.547	99.496			
6	0.031	0.447	99.944			
7	0.004	0.056	100			

Table 4. *Coefficient matrix for each component*

	Component	
	1	**2**
Zscore(X_1)	-0.142	1.053
Zscore(X_2)	0.155	0.052
Zscore(X_3)	0.155	0.114
Zscore(X_4)	0.157	0.25
Zscore(X_5)	0.149	0.529
Zscore(X_6)	-0.148	0.78
Zscore(X_7)	0.145	0.829

(3) OLS (Ordinary Least Square) regression analysis of principal components and total carbon emission

Least square regression analysis is conducted with ZLnC as the dependent variable, FAC_1 and FAC_2 as the independent variables, fitting goodness of fit $R^2 = 0.994$, $F = 313.486$, t test Sig < 0.01, which indicate a good model fitting. Refer to *Tables 5* and *6* for the results of variance analysis and model coefficient respectively.

Table 5. *Results of analysis of variance*

	Quadratic sum	Degrees of freedom	Standard deviation	F test	Significance
Regression	14.695	2	7.348	313.486	0
Residual error	0.305	13	0.023		
Total	15	15			

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Table 6. *Model coefficients*

	Unstandardized coefficients		Regression coefficient	T test	Significance
	Regression coefficient	**Standard error**			
Constant	-2.68E^{-15}	0.038		-7.00E^{-14}	1
FAC1_2	0.981	0.04	0.981	24.829	0
FAC2_2	0.128	0.04	0.128	3.239	0.006

The following equation between dependent variable ZLnC and principal components FAC_1 and FAC_2 can be formed when regression coefficient in *Table 6*'s model is adopted, where constant term is deleted for the Sig value of its test = 1, and Sig values of regression coefficient t test for FAC_1 and FAC_2 are less than 0.01, therefore this model has high authenticity.

$$\mathrm{ZLnC} = 0.981\times \mathrm{FAC}_1 + 0.128\times \mathrm{FAC}_2 \qquad \text{(Eq.7)}$$

Based on standardization formula and standardized variables described in *Table 2,* the above formula can be converted into:

$$\begin{aligned} lnC = -0.0045\times LnX_1 + 0.1587 LnX_2 + 0.1666\times LnX_3 + 0.186 \\ \times LnX_4 + 0.2139\times LnX_5 - 0.0453 LnX_6 + 0.2484\times LnX_7 \end{aligned} \qquad \text{(Eq.8)}$$

The final results are as follows:

$$\mathrm{C} = \mathrm{X}_1^{-0.0045}\mathrm{X}_2^{0.1587}\mathrm{X}_3^{0.1666}\mathrm{X}_4^{0.186}\mathrm{X}_5^{0.2139}\mathrm{X}_6^{-0.0453}\mathrm{X}_7^{0.2484} \qquad \text{(Eq.9)}$$

The above formula shows that, among the driving factors that affect Anhui's carbon emission 2000-2015, 5 are positively correlated, but in degree of influence, external investment > energy consumption structure > per capita income > proportion of secondary industry in GDP > total external fund used. Only 2 factors, namely the proportion of total import-export volume in total industrial output value and the proportion of the added value of industries above a designated scale in total industrial output value are negatively associated with the total carbon emission, but far less influential than the other 5 positively correlated factors.

Discussion

Economic development and carbon emissions

According to previous studies (Yang et al., 2018), there are three inverted U-shaped curves (*Fig. 5*) during the evolution of the relationship between economic development and carbon emissions, namely inverted U-shaped curves of carbon emissions, per capita carbon emissions and carbon emission amount. Specifically, during the evolution process, three curves need to cross their peaks respectively, namely the peaks of the inverted U-shaped curves of carbon emission intensity, per capita carbon emissions and carbon emission amount. During the evolution, dominant driving forces of carbon

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emissions vary from stage to stage. In the period before carbon emission intensity peaks, energy and advance in carbon-intensive technology are the main influencing factors; from the peak of carbon intensity to that of per capita carbon emission amount, economic growth is the main influencing factor; from the peak of per capita carbon emission amount to that of carbon emission amount, advance in carbon emission reduction technology is the main influencing factor but it plays a decisive role after entering the stage in which total carbon emissions decline steadily. As shown in *Figure 6,* Anhui Province's carbon emission intensity reaches its peak in 2005, indicating that energy and carbon-intensive technology advance are the main influencing factors for the growth of carbon emissions before 2005, which is mainly caused by the law of economic development of Anhui Province. Apart from the fact that main economic development remains in the middle stage of accelerated industrialization, Anhui Province (an important area producing coal and iron ore in Central China) has an advanced mining industry in the industrial structure, and most of the industrial types are supported by coal as their main energy and technologies relatively lag behind, so energy and carbon-intensive industries are the important influencing factors for the growth of carbon emissions in this period; from 2005 to 2015, Anhui's carbon intensity continues to decline but per capita carbon emissions keep rising, indicating that economic growth is the leading factor influencing the growth of carbon emissions during the period from 2005 to 2015. This is the golden period for Anhui to take advantage of the industrial transfer, with a large number of foreign capital and traditional industries in the Yangtze River Delta region transferring to Anhui, resulting in rapid industrial growth and further promoting the growth of carbon emissions. It can also be found from the calculation results of STIRPAT model established in this paper that the importance of three coefficients, namely the total amount of out-of-province investment, per capita income and the total amount of actual use of foreign capital, rank the first, third and fifth in positive correlation, which further proves the significance of economic development in carbon emissions. It can be predicted that when the per capita carbon emissions of Anhui Province peak, advance in carbon emission reduction technology will become the main factor influencing the growth of carbon emissions.

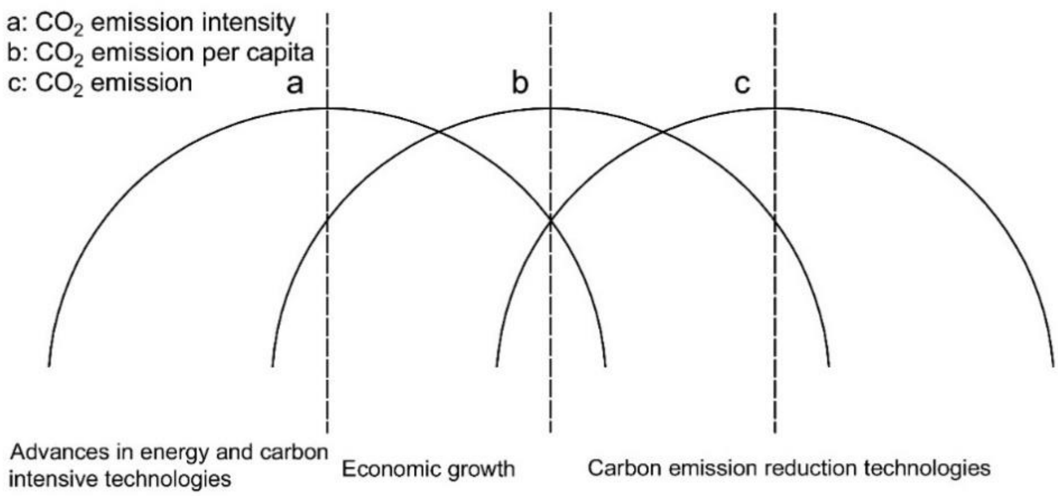

Figure 5. *Three inverted U curves rule of economic development and CO_2 emissions*

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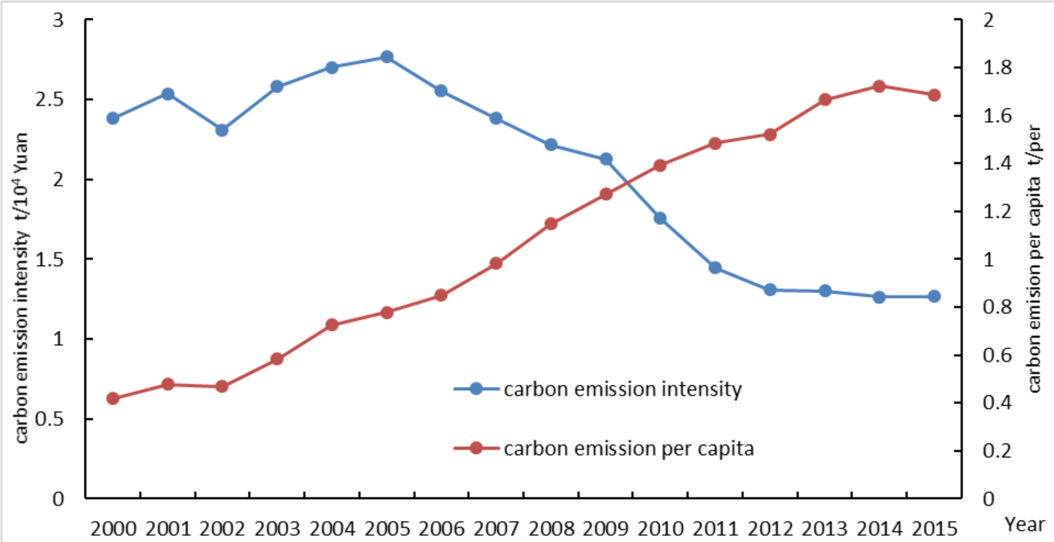

Figure 6. *Change of carbon emission intensity and carbon emission per capita of Anhui province (2000-2015)*

It is worth noting that compared with neighboring developed provinces such as Zhejiang and Jiangsu, Anhui shows an obvious lagged relationship between economic development and carbon emissions. For example, Zhejiang Province has reached the peak of carbon emission intensity as early as 1995 (Yang et al., 2018) and that of Jiangsu has also peaked in 2000 (Zhu et al., 2018). It also reflects that after Zhejiang and Jiangsu implement industrial upgrading and technological transformation, a large number of backward industries move out, thus promoting the reduction in carbon emission intensity. However, as the major industrial undertaking area of the two provinces, Anhui sees an increase in carbon emissions instead, forming a “pollution haven effect”.

Energy structure and carbon emissions

In respect to energy consumption structure, the ratio of coal consumption to total energy promotes the growth of carbon emissions, and the ratio increases by 1% and carbon emission amount will increase by 0.2139%. For a long period of time, fossil energy has been the major resource consumed in Anhui Province, especially coal, which occupies a dominant and gradually consolidated position. The proportion of coal consumption to fossil energy consumption rises from 90.57% in 2000 to 96.08% in 2015 (*Fig. 7*), coal consumption share is 14% higher than the national average and non-fossil energy consumption share is 8.8% lower than the national average. The pressure to reduce the proportion of fossil energy consumption is particularly obvious in the context of China’s increasing emphasis on energy conservation and emission reduction as a way to tackle climate change. There are two main reasons for why coal consumption outshine others: one is the resource endowment of Anhui Province. It has abundant coal resources and is a major coal producer but lacks oil and natural gas, being a typical province with “rich coal but no oil and gas”. In comparison, the coal consumption has taken a dominant position in the province for a long time for its prominent convenience for coal consumption and price advantage; the other is its advanced industrial structure in heavy chemical industries in respect to power, chemical

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industry and steel for the long-term use of coal, which further deepens its dependence on coal, leading to a large demand for the resource. For example, existing research has shown that in 2011, carbon emissions in Anhui's manufacturing industry mainly comes from ferrous metal smelting and rolling processing industry (37.4%-37.9%), non-metallic mineral products industry (28.9%-29.8%), chemical raw materials and products manufacturing industry (14.3%-14.5%), petroleum processing and coking and nuclear fuel processing industry (9.8%-10.0%). Employees in these four industries account for 18% of the total in industry, the proportion to total industrial added value is not more than 1/4 but carbon emissions are more than 90% (Shen et al., 2018). According to the goals of *the Outline of the 13th Five-Year Plan for National Economic and Social Development of Anhui Province* and *China's 13th Five-Year Plan for Energy Development*, by 2020, provincial demand for primary energy consumption will have been limited to 142 million tons of standard coal, non-fossil energy consumption accounted for 5.5% of total energy consumption and the proportion of coal consumption dropped below 75%. The pressure to cut coal capacity remains heavy, which needs to be brought to the forefront.

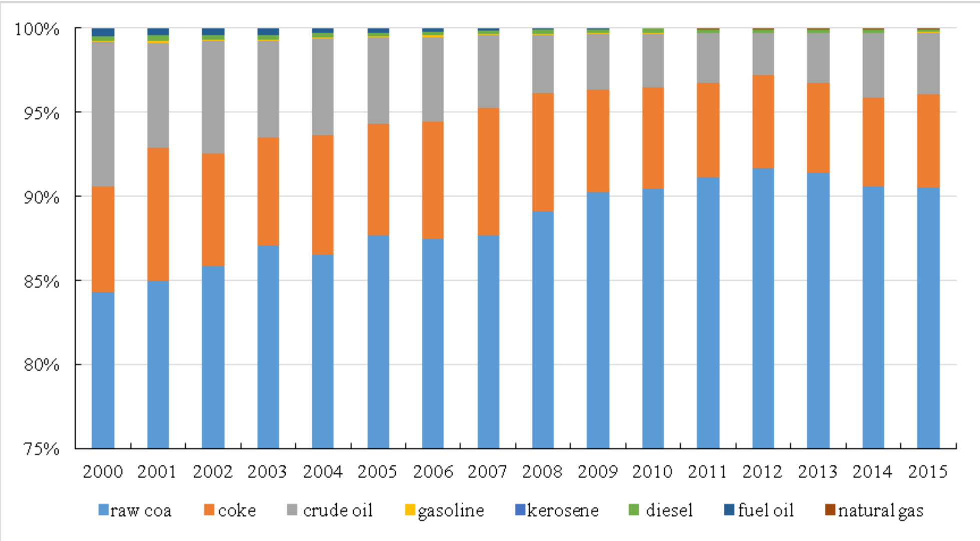

Figure 7. *Change of energy consumption structure changes of Anhui province (2000-2015)*

Industrial agglomeration and carbon emissions

A large number of studies at home and abroad have shown that industrial agglomeration has certain promoting significance for China's carbon emission reduction (Wang et al., 2013). Industrial agglomeration mainly affects carbon emission intensity through scale effect, technology effect and competition effect: firstly, industrial agglomeration can bring scale effect and realize the free flow of production factors within the cluster range so as to improve energy utilization efficiency; secondly, industrial agglomeration can promote the concentration and close connection of industries with upstream and downstream relations in geographical space, so as to save costs such as that of raw materials and transportation, and thus reduce energy consumption intensity; thirdly, agglomeration enables enterprises to communicate more closely with each other, which is conducive to the improvement of technological innovation and competitiveness, and thus promotes the continuous reduction of carbon

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emission intensity by relying on technological advance in respect to energy conservation and utilization (Moreno et al., 2014). However, for every 1% increase in industrial concentration in Anhui Province, the carbon emission amount decrease by only 0.0045%, which has the least influence among the 7 influencing factors, indicating that the industrial agglomeration in Anhui is not obvious and needed to be further strengthened.

External investment and carbon emissions

In respect to Anhui's utilization of out-of-province investment, the investment promotes the growth of carbon emissions, and every 1% rise in such investment will increase carbon emissions by 0.2484%. In 2004, the country proposed a plan for the rise of Central China, and Anhui Province, which is at the intersection of Central and Eastern China, undertook a large number of industrial transfers from China's eastern coastal provinces. From 2005 to 2015, the annual growth rate of out-of-province investment in projects that were above 100 million yuan increased by an average of 31.52%. In the initial stage, industrial projects were mainly introduced. For example, in 2007, the second industry accounted for 60.2% of the actual funds allocated to projects from outside the province with investment of more than 10 million yuan, and among the second production projects, 75.35 billion yuan was allocated to the manufacturing projects, accounting for 47.3% of the projects with investment of more than 10 million yuan. In the external investment of competitive industries in Anhui Province, the investment of power plant projects, steel product processing projects, coal chemical projects and other projects with high energy consumption accounted for 40% of the total investment. Over time, the investment structure of external capital has been optimized. In 2015, among the projects of over 100 million yuan using eternal capital, the growth rate of investment in the low-energy industries, such as modern service industry projects, electronic information projects, pharmaceutical industry projects and other tertiary industry projects was improved, but the secondary industry still accounted for 60.1% of the total investment, indicating that the transfer of external industries in Anhui Province is still dominated by the second industry, and the increase of carbon emission will continue.

Analysis from the perspective of FDI: FDI promotes the growth of carbon emission. For every 1% increase of FDI, carbon emission will increase by 0.1587%, which is consistent with previous research. For example, Zhang et al. (2018) used data from 1982 to 2016 to conclude that the impact of FDI on China's carbon emissions is positive. Gong et al. (2018), based on STIRPAT model research, concluded that the increase of FDI would promote the growth of carbon emissions in the Yangtze River Delta region. Similarly, foreign investment in Anhui Province is still concentrated in traditional industries. For example, from 2000 to 2015, the manufacturing industry and the hydraulic and electric power industry of Anhui Province absorbed 67.19% of FDI, and the carbon emissions of these two industries accounted for 78.16% of the industrial carbon emissions. FDI is mainly invested in industries with high carbon emissions. While stimulating local economy, it still has a large impact on carbon emissions.

Economic openness and carbon emissions

When many domestic and foreign scholars analyze the relationship between China's import and export trade and carbon emissions, most of them believe that China's

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DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

foreign trade mode is also dominated by processing industries with high energy consumption and high pollution. Such a trade structure will result in more emissions as the proportion of China's exports increases. That is, the increase in export trade leads to the rise in carbon emissions, while the import trade will reduce carbon emissions. Anhui is a special case. Although its import and export trade is greater than import, its import and export trade is negatively correlated with carbon emissions, that is, for every 1% increase in the proportion of total import and export in total industrial output value, its carbon emission reduces by 0.0453%. The main reason is that Anhui imports a large number of primary products and energy-consuming products, but when exporting, in addition to primary products, it also exports a certain number of high value-added products. On the contrary, in addition to high-tech products, Anhui's imports also include non-metal and chemical materials and other primary products (*Table 7*). However, objectively speaking, the import and export trade is very limited in reducing carbon emissions, reflecting the low level of regional industrial development.

Table 7. *Comparison of some products of import and export trade in Anhui province in 2015 (10^4 $)*

Primary products	Exports	Imports	Exports-imports	Industrial product	Exports	Imports	Exports-imports
Food and live animals	90668	107868	-17200	Primary form of plastic	12511	53645	-41134
Meat and meat products	600	7384	-6784	Other chemical raw materials and products	13328	15991	-2663
Dairy and egg products	42	8348	-8306	Non-ferrous metal	53624	71059	-17435
Grain and its product	7409	17934	-10525	pharmaceuticals	20704	2241	18463
Vegetables and fruits	23550	25565	-2015	Machinery and transport equipment	1210348	224899	985449
Feed (excluding unground grain)	1930	23683	-21753	Power machinery and equipment	50990	20012	30978
Miscellaneous food	16974	20996	-4022	Special industrial machinery	306321	27507	278814
Non-edible materials (excluding fuels)	36791	643688	-606897	General industrial equipment and parts	170788	46999	123789
Raw rubber (including synthetic rubber and recycled rubber)	625	16173	-15548	Telecom and voice recording and playback equipment	82498	2261	80237
Pulp and waste paper	2152	51948	-49796	Electrical machinery, apparatus and its electrical parts	314048	36220	277828
Natural fertilizers and minerals (except coal, petroleum and precious stones)	1870	2565	-695	Road vehicles (including air cushion)	221586	2865	218721
Metal ore and metal waste	46	528612	-528566	Professional, scientific and control equipment	171277	69442	101835
Coal, coke and coal briquettes	65	10521	-10456	Metalworking machinery	12938	21286	-8348
Petroleum, petroleum products and related raw materials	209	16900	-16691	Office machinery and automatic data processing equipment	25452	66913	-41461
Natural gas and man-made gas	130	768	-638	Photographic equipment, optical objects and clocks	16631	17112	-481

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DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

Conclusion

Taking Anhui Province as an example, this paper calculates the industrial carbon emissions of Anhui Province by using the terminal energy consumption and social statistics data of different industries in the province from 2000 to 2015. Through the expanded STIRPAT model, it analyzes the influencing factors of Anhui's carbon emissions in the context of industrial transformation. The research results show that:

(1) In respect to total carbon emission, Anhui's carbon emission increased steadily from 2000 to 2015 with an average annual growth rate of 10.50%. In respect to growth rate, the average annual growth of carbon emission decreased to 4.29% from 2010 to 2015, indicating that Anhui's rapid growth momentum of carbon emission was restrained to some extent;

(2) In respect to carbon emissions of sub-sectors, the carbon emissions of mining industry, manufacturing industry and EGW from 2000 to 2015 showed an overall trend of growth. In respect to growth rate, it is shown as: mining industry > EGW > manufacturing industry. The proportion of carbon emission in the manufacturing industry shows a stable downward trend, while the proportion of EGW is relatively stable on the whole, and the proportion of the mining industry has been on the rise, showing a certain convergence on the whole and gradually showing a trend of "each accounting for 1/3 share";

(3) According to the extended STIRPAT model analysis, 5 of the driving factors influencing the carbon emissions of Anhui Province from 2000 to 2015 are positively correlated, which is shown as: external investment > energy consumption structure > per capita income > proportion of the second industrial output in GDP > the amount of the actual use of foreign investment. The two factors, the proportion of total import and export in total industrial output value and the proportion of industrial added value above the scale in total industrial output value, are negatively correlated with total carbon emission, but the influence degree is far less than the other 5 factors;

(4) The relationship between Anhui Province's carbon emissions and economic development also shows an obvious inverted U-shaped curve. The carbon emission intensity of Anhui Province reached its peak in 2005, but per capita carbon emissions still kept increasing, indicating that a large number of foreign investment and traditional industries in the Yangtze River Delta region were transferred to Anhui from 2005 to 2015, leading to rapid industrial growth, and thus promoting the growth of carbon emissions;

(5) The energy consumption structure of Anhui Province has been dominated by coal for a long time, and external investment (including that from foreign countries, Hong Kong, Macao, Taiwan and other provinces) mainly invested in projects with high energy consumption, such as power plant projects, steel products processing projects and coal chemical projects, which are the main reasons for the sustainable growth of Anhui's carbon emissions. Although industrial agglomeration and import and export trade can reduce total carbon emissions, the impact is relatively low. Thus it can be seen that policy makers should pay more attention to the introduction of energy-saving and environmentally friendly enterprises when undertaking industrial transfer in Anhui Province, and actively control the increase of carbon emission by introducing technology and encouraging low-carbon technology.

In order to better reflect the spatial characteristics of industrial carbon emissions in Anhui Province, the next research should focus on the micro-level, pay attention to the changes of total carbon emissions and intensity of enterprises.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

Acknowledgements. This study was funding by the Fundamental Research Funds for the Central Universities under grant number 2018ZDPY07.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42054227

EFFECTS OF NITROGEN AND THREE SOIL TYPES ON MAIZE (*ZEA MAYS* L.) GRAIN YIELD IN NORTHEAST CHINA

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(Received 11th Dec 2018; accepted 27th Feb 2019)

Abstract. The study aimed to understand the effects of nitrogen (N) and soil types on maize (*Zea mays* L.). Grain yield (GY) is essential for identifying optimal N fertilizer management practices and agricultural policies. In this study, we report results from an on-farm experiment carried out from 2009 to 2012 with five N levels and three soil types in Northeast China. Results revealed that the GY was affected significantly by soil types, with loam soil having an average GY of 10225 kg ha^{-1}, followed by clay soil (9218 kg ha^{-1}) and sandy soil (6434 kg ha^{-1}). The optimal N rates required to achieve maximum GY were on average 182, 173, and 160 kg ha^{-1}, and the corresponding maximum GYs were 10872, 9999, and 7266 kg ha^{-1} for loam, clay, and sandy soils, respectively. The optimum N treatment (168 kg N ha^{-1}) reduced residual nitrate N content and N losses by 97 and 451 kg N ha^{-1}, respectively, and improved N recovery efficiency (RE_N) by 17%. In conclusion, within-field soil management zones based on soil textural classes could be used to guide soil sampling and establish soil-specific N fertilizer recommendations to achieve high GY with high RE_N in Northeast China.
Keywords: *maize, grain yield, nitrogen recovery efficiency, nitrogen balance, yield stability*

Introduction

The global population is expected to peak at 8.5–10 billion by 2050. Maize (*Zea mays* L.) grain yield (GY) must increase by 101% to feed the livestock industry given the rising meat and poultry consumption, especially in developing countries (Ray et al., 2013). Global maize GY increased slowly and then stagnated for the past 10 years (FAO, 2014), although the GY gap is large, with a 50% GY potential. Grain yield variation among regions, climates, and management practices reduces productivity and contributes to this large GY gap (Liu et al., 2010; Zhang et al., 2013; Tesfaye et al., 2015).

For rain-fed maize production, water is one of the foremost constraints on GY which is dependent on soil water availability at sowing and on rainfall during the cropping season (Calvino et al., 2003; Gang et al., 2019). In a given region with similar climates, soil type often affects the ability to store and use rainfall, which impacts maize GY. Recent research has indicated that GY variability could be as great as 5000-6000 kg ha^{-1}, depending on the soil type and regions in Northeast China (NEC)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42294243

(Lu et al., 2014). However, other research has reported no significant variation in GY between soil types (Tan et al., 2007; Xie et al., 2008).

Nitrogen (N) dynamics and crop responses to N often vary with soil properties, climate, and management (Marjerison et al., 2016; Zhang et al., 2016; Mesbah et al., 2018; Doltra et al., 2019). For example, weather might influence organic N availability, the extent of leaching and denitrification, and crop uptake and residual N at the end of the growing season (Luce et al., 2011; Alotaibi et al., 2018; Bean et al., 2018; Iqbal et al., 2018). Soil texture is another important factor that influences soil productivity and GY by potentially controlling water supply (Cambouris et al., 2006), crop N requirements (Lu et al., 2019), and N mineralization (Luce et al., 2011; Smith, 2018). Variation in the response of maize to N application often leads to poor synchronization between N supply from the soil and fertilizer, and crop requirements resulting in low N-use efficiency (NUE) and low GY (Gao et al., 2012).

NEC is a typical rain-fed maize cultivation region in which the maize planting area was 1121 × 10^4 ha and accounted for 30.4% of the total maize crop in China in 2016 (Chinese Farming Management Department, 2017). Soil conditions, climatic factors, and weather conditions in a given year also influence GY significantly (Benjamin et al., 2003; Yao et al., 2011). A short-term effect could be inconsistent with the results of long-term observations. Agricultural research, as well as other types of research, generally involves short-term studies, but sustainable agriculture requires long-term field and laboratory experiments to understand the complex soil-plant-climate-management interactions (Army, 1991).

In this study, we conducted on-farm experiments with various N levels in three villages in close proximity (< 4 km apart) with the same climatic conditions and differing soil types (loam, clay, and sandy soil) from 2009 to 2012 in Lishu County, Jilin Province. The objectives of this study were: (1) to evaluate GY variation among soil types and years, (2) to evaluate the GY response to N application rate among soil types and years to determine the optimal N rate for different soil types, and (3) to evaluate the soil N balance for different soil types.

Materials and Methods

Study Site

The climate of the study area was warm-temperate, subhumid, and continental monsoon with cold winters and hot summers. Maize was planted at the beginning of May and harvested in early October. The time from maize planting to harvest was 1400–1500 growing degree days (McMaster and Wilhelm, 1997). The annual precipitation was 500–800 mm, with 60–70% of the rainfall occurring during the summer. No irrigation was supplied during the maize growing season. The precipitation and temperature at the experimental site during the maize growing season from 2009 to 2012 are listed in *Figure 1*.

Experimental Design and Management

The experiment was conducted from 2009 to 2012 on three typical soil types (loam, clay, and sandy) in Lishu County, Jilin province. These three soils were located in three nearby villages: Wang-jia-Qiao with loam soil (N43°14’49’’, E124°29’10”), San-Ke-Shu with clay soil (N43°20’17”, E124°00’29”), and Fu-Jia-Jie with sandy soil

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(N43°21’48”, E124°05’02”; FAO 2006) (*Figure 2*). The distance between the three sites was < 4 km.The physical and chemical properties of these three soil types are listed in *Table 1*.

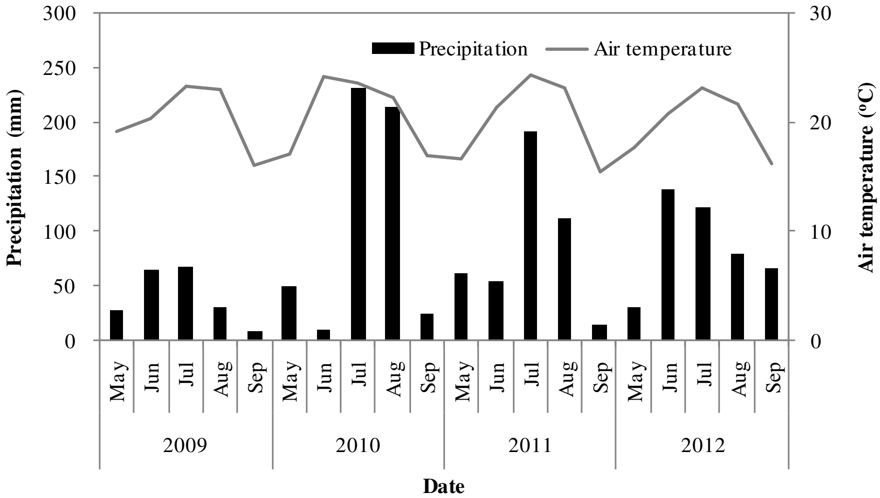

***Figure 1.** Precipitation and temperature at the Li-shu experimental site during the maize growing season from 2009 to 2012*

***Figure 2.** Distribution of experimental site in the Li-shu County, Jilin province*

***Table 1.** Selected soil physical and chemical properties in 2009*

Locations	Texture	Bulk density (mg m^{-3})	Organic matter (gkg^{-1})	Total N (gkg^{-1})	pH	NaOH-N (gkg^{-1})	Olsen-P (gkg^{-1})	NH_4OAc-K (gkg^{-1})
Wang-Jia-Qiao	clay loam	1.32	12.2	1.04	5.15	91.6	29.1	52
San-Ke-Shu	loamy clay	1.42	25.3	1.69	6.16	128.2	43.9	122
Fu-Jia-Jie	sandy loam	1.51	13.2	1.01	6.71	58.8	13.6	43

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42294243

For the three soil types, the experiments from 2009 to 2012 were designed as a randomized block with three replicates of five nitrogen (N) treatments were as follows: control (N0), 70% recommendation N rate (N168), recommendation N rate based on local government recommend fertilization (N240), 130% recommendation N rate (N312), and the typical N dose used by farmers (N270). The N application rates were as follows: 0, 168, 240, 270, and 312 kg N ha^{-1} applied as urea. All N fertilizer was granular urea with one-third applied before planting and the remaining two-thirds applied at the V8 stage. All plots received 100 kg P_2O_5 ha^{-1} as calcium superphosphate and 120 kg K_2O ha^{-1} as K_2SO_4 before planting.

The plot size was 60 m^2 (6 × 10 m). Maize was planted continuously from 2009 to 2012. The planting density was 65,000 plants ha^{-1} with a row spacing of 60 cm. Seeds of the XY335 cultivar were hand-sown on May 5, 8, 5, and 4 during 2009, 2010, 2011, and 2012, respectively. The maize was rain-fed and harvested on October 5 during each of the four years. Weeds were controlled by applying atrazine and acetochlor before seedling emergence, and no obvious pest stress was observed during the maize growing season.

Sampling and Laboratory Procedures

At physiological maturity, when more than 50% of the plants showed a visible black layer at the base of the kernel, plants were removed manually from an area of 18 m^2 (six rows, approximately 3.6-m wide × 5-m long) in the middle of each plot to measure GY. Plant samples were taken at harvest. Aboveground biomass was collected by clipping five plants in three rows near the middle of each plot to measure plant dry matter weight and N concentration. Plants were divided into grain and straw. All plant samples were dried at 70°C in a forced-draft oven to constant weight and then weighed. Subsamples were passed through a 1-mm screen in a sample mill and mineralized using H_2SO_4-H_2O_2, after which the N concentration was determined using the Kjeldahl method (Horowitz et al., 1970).

At least five soil samples were taken in every plot to a depth of 100 cm at 20-cm increments before planting and after harvest. Fresh soil samples were sieved, extracted with 0.01 mol L^{-1} $CaCl_2$ solution and NH_4^+-N and NO_3^--N were analyzed by continuous flow analysis (TRAACS2000; Bran + Luebbe, Norderstedt, Germany) in the laboratory. Soil water content was measured by oven drying at 105°C. Soil samples were collected at the 0–20-cm soil layer before planting, air-dried, and sieved through a 0.2-mm mesh to remove undecomposed plant materials. The sieved samples were used to measure organic material (OM), total N, Olsen-P, and NH_4OAc-K.

Data Analysis

After verifying the homogeneity of error variances, all of the data across soil types, N levels, and years were pooled for analysis of variance using the ANOVA procedure of SAS/STAT (SAS Institute, 1993). Differences were compared using the least significant difference test (LSD) at the 0.05 level of probability.

Three response models were evaluated to describe the relationship between N application and GY: quadratic, quadratic with a plateau, and linear with a plateau (Cerrato and Blackmer, 1990). The models were generated using SAS software (SAS Institute, 1993). The calculated optimum N rate for GY was given with a 95% confidence interval.

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The increase in GY with applied N fertilizer (IY_N) was defined as the difference between the maximum GY and the GY of the zero-N (N0) treatment for each experiment. The agronomic N efficiency (AE_N) and N partial factor productivity (PFP_N) were calculated using *equations (1)* and *(2)* below (Ladha et al., 2005). AE_N was the GY increase per unit of N applied, and PFP_N was the most important index for farmers since it integrates the use efficiency of both indigenous and applied N resources.

$$AE_N = (Y_N - Y_0)/N \quad \text{(Eq.1)}$$

$$PFP_N = Y_N/N \quad \text{(Eq.2)}$$

where Y_N and Y_0 are GY values for N application plots and N_0 plots, respectively, and N is supplied by the applied fertilizer.

Results

Grain YieldVariance among Soil Types and Years

Considering all four years and five N treatments, GY was affected significantly by soil type (*Table 2*). The rank order of the GYs for the three soil types was loam > clay > sandy soil with GY values of 10225, 9218, and 6434 kg ha^{-1}, respectively (*Table 2*).

Table 2. *Analysis of variance of maize grain yield, ear number, grain number,and 100 grain weight for various nitrogen treatments in three soil types across 4 years*

Treatments	Yield (kg ha^{-1})	Earnumber (ha^{-1})	Grain number (ear^{-1})	100 Grain weight (g)
Soil				
Loam soil	10225a	58951a	522.6a	33.3a
Clay soil	9218b	54942b	526.3a	31.6b
Sandy soil	6434c	49251c	398.7b	28.9c
Year				
2009	7276c	48787d	421.9c	30.2c
2010	9522a	56287b	513.8a	33.0a
2011	8366b	54119c	515.8a	29.8c
2012	9339a	58330a	478.5b	32.0b
Nitrogen				
0	5732c	51938b	389.8c	26.9b
168	9257b	55019a	492.1b	32.5a
240	9554a	54827a	516.6a	32.4a
270	9379ab	55262a	505.0ab	32.3a
312	9207b	54859a	509.1ab	32.2a
Source of variation				
Soil(S)	**	**	**	**
Year (Y)	**	**	**	**
Nitrogen(N)	**	**	**	**
Y×S	**	**	**	**
Y×N	**	**	**	**
S×N	**	NS	**	NS
Y×S×N	**	*	**	NS

Within soil, nitrogen or year, numbers followed by different letters indicate significant differences ($P < 0.05$).
NS: not significant ($P > 0.05$).
*Significant at $P < 0.05$.
**Significant at $P < 0.01$

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Plant density was uniform for all three soil types. The numbers of ears at maturity were significantly different with a rank order of loam > clay > sandy soil (*Table 2*). Similar results were obtained for 100 grain weight among the soils, with a rank order of loam > clay > sandy soil. The grain number was essentially the same for the loam (523 per ear) and clay (526 per ear) soils, which were approximately 31.6% higher than the 399 grains per ear for sandy soil (*Table 2*).

Considering all three soil types and the five N treatments, GY was affected significantly by year (*Table 2*). The highest GYs were observed in 2010 and 2012, which might be explained by greater precipitation during those years (583 and 437 mm for 2010 and 2012, respectively; *Fig. 1*). The lowest GY in 2009 might be explained by rainfall during the maize growth period of only 207 mm and drought during the flowering and early grain filling stages between July and August (*Fig. 1*). Meanwhile, the average GYs in the loam and clay soils were 9574 and 10142 kg ha^{-1}, respectively, which were significantly higher than the GY in sandy soil (981 kg ha^{-1}, *Fig. 3*).

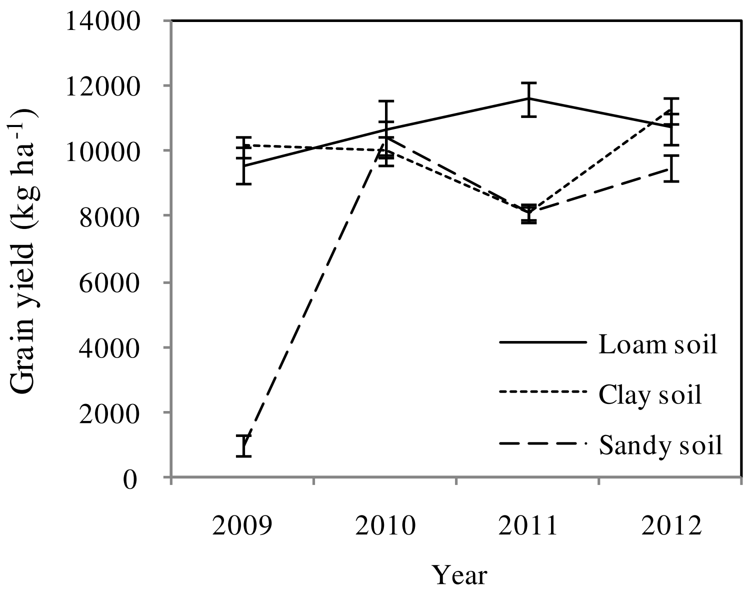

Figure 3. *Yield stability in three soil types with under optimal nitrogen application. Data were pooled across four years (n = 36)*

The low GY in 2011 might have been due to lodging between July 30 and August 1. Due to different lodging rates among the three soil types, with a rank order of clay > loam > sandy soil and values of 29.2, 13.1, and 8.2%, respectively (*Table 3*), the GY for clay and sandy soils averaged 8089 and 8126 kg ha^{-1}, respectively, which was significantly less than the GY in loam soil (11600 kg ha^{-1}; *Fig. 3*).

The effect of year × soil interaction on GY was significant, indicating that the effects of soil on GY differed among years. During the four years (2009–2012), GYs for the N168 treatment in the loam, clay, and sandy soils averaged 10638 kg ha^{-1} with a range of 8960–12021 kg ha^{-1}, 9882 kg ha^{-1} with a range of 7861–11713 kg ha^{-1}, and 7252 kg ha^{-1} with a range of 695–10913 kg ha^{-1}, respectively (*Fig. 3*).

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Table 3. *Analysis of variance of lodging rates for three soil types under nitrogen application in 2011*

Treatments	Soil type		
	Loam soil	**Clay soil**	**Sandy soil**
N0	3.7c	9.1c	1.4e
N168	11.1b	24.6b	4.7d
N240	12.0b	25.5b	8.5c
N270	12.9b	29.6b	11.7b
N312	25.6a	57.3a	14.7a

Means followed by the same lowercase letter within a column for a given year are not significantly different

Grain Yield Response to N Application Rate among Soil Types and Years

Grain yield response to increasing N rate differed among the soil types and years as indicated by a significant soil type × year × N interaction effect. A linear-plateau GY response to increasing N rate was the most appropriate model for the three soil types. Across the four years, the optimal N rate required to achieve maximum GY averaged 182, 173, and 160 kg ha^{-1} for loam, clay, and sandy soils, respectively. The corresponding maximum GYs for loam, clay, and sandy soils were 10872, 9999, and 7266 kg ha^{-1}, respectively.

The GY in loam soil increased quadratically with increasing N rate in 2010, while a linear-plateau model was most appropriate for 2009, 2011, and 2012. The minimum N rates needed to achieve maximum GY were 270, 180, 150, and 186 kg ha^{-1} for 2009 (*Fig. 4a*), 2010 (*Fig. 4d*), 2011 (*Fig. 4g*), and 2012 (*Fig. 4j*), respectively. Grain yields for the N0 rate were 8810, 9560, 6538, and 6397 kg ha^{-1} in 2009 (*Fig. 4a*), 2010 (*Fig. 4d*), 2011 (*Fig. 4g*), and 2012 (*Fig. 4j*), respectively.

Similar results were observed for clay soil during the experiment years. The linear-plateau GY responses to N were the most appropriate models for clay soil (*Fig. 4*). Grain yield increased from 9539 kg ha^{-1} for the N0 rate to a maximum GY of 10521 kg ha^{-1} obtained with 320 kg N ha^{-1} in 2009 (*Fig. 4b*). Maximum GYs for 2010 (*Fig. 4e*), 2011 (*Fig. 4h*), and 2012 (*Fig. 4k*) were 10148, 8241, and 11256 kg ha^{-1}, respectively, at minimum N rates of 175, 174, and 155 kg N ha^{-1}, respectively.

A linear-plateau GY response to increasing N rate was the most appropriate model for the sandy soil from 2010 to 2012, while there was no relationship in 2009 (*Fig. 4c*). The minimum N rates needed to achieve maximum GY were 158, 90,and 175 kg ha^{-1} for 2010 (*Fig. 4f*), 2011 (*Fig. 4i*), and 2012 (*Fig. 4l*), respectively. Maximum GYs for 2010, 2011, and 2012 were 9944, 8108, and 9829 kg ha^{-1}, respectively. Grain yields for the N0 rate in 2010 (*Fig. 4f*), 2011 (*Fig. 4i*), and 2012 (*Fig. 4l*) were 4296, 5387, and 1272 kg ha^{-1}, respectively.

Under drought conditions in 2009 (206 mm rainfall during the maize growing season) (*Fig. 1*), a linear-plateau GY response to increasing N rate was the most appropriate model for the loam (*Fig. 4a*) and clay soils (*Fig. 4b*), while there was no relationship in sandy soil (*Fig. 4c*). The total rainfall values during the maize growing seasons in 2010 and 2012 were 583.2 and 437.2 mm, respectively, which were greater than for 2009 (*Fig. 1*). Despite the relatively small GY for the N0 rate in sandy soil, the maximum GY in sandy soil (9944 and 9829 kg ha^{-1} for 2010 and 2012, respectively)

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was similar to the maximum GY observed for loam soil (10428 and 11170 kg ha^{-1} for 2010 and 2012, respectively) and clay soil (10148 and 11256 kg ha^{-1} for 2010 and 2012, respectively).

Across the experiment years, the optimal N rate required to achieve maximum GY was 160–180 kg ha^{-1} for the three soil types. The PFP_N ranged from 57.0 to 69.0 kg kg^{-1} in loam soil and from 48.0 to 67.1 kg kg^{-1} in clay soil. In contrast, the PFP_N ranged from 5.8 to 61.9 kg kg^{-1} in sandy soil, indicating that N efficiencies in loam and clay soils were stable compared with sandy soil, for which the PFP_N of 5.8 kg kg^{-1} was affected by drought in 2009.

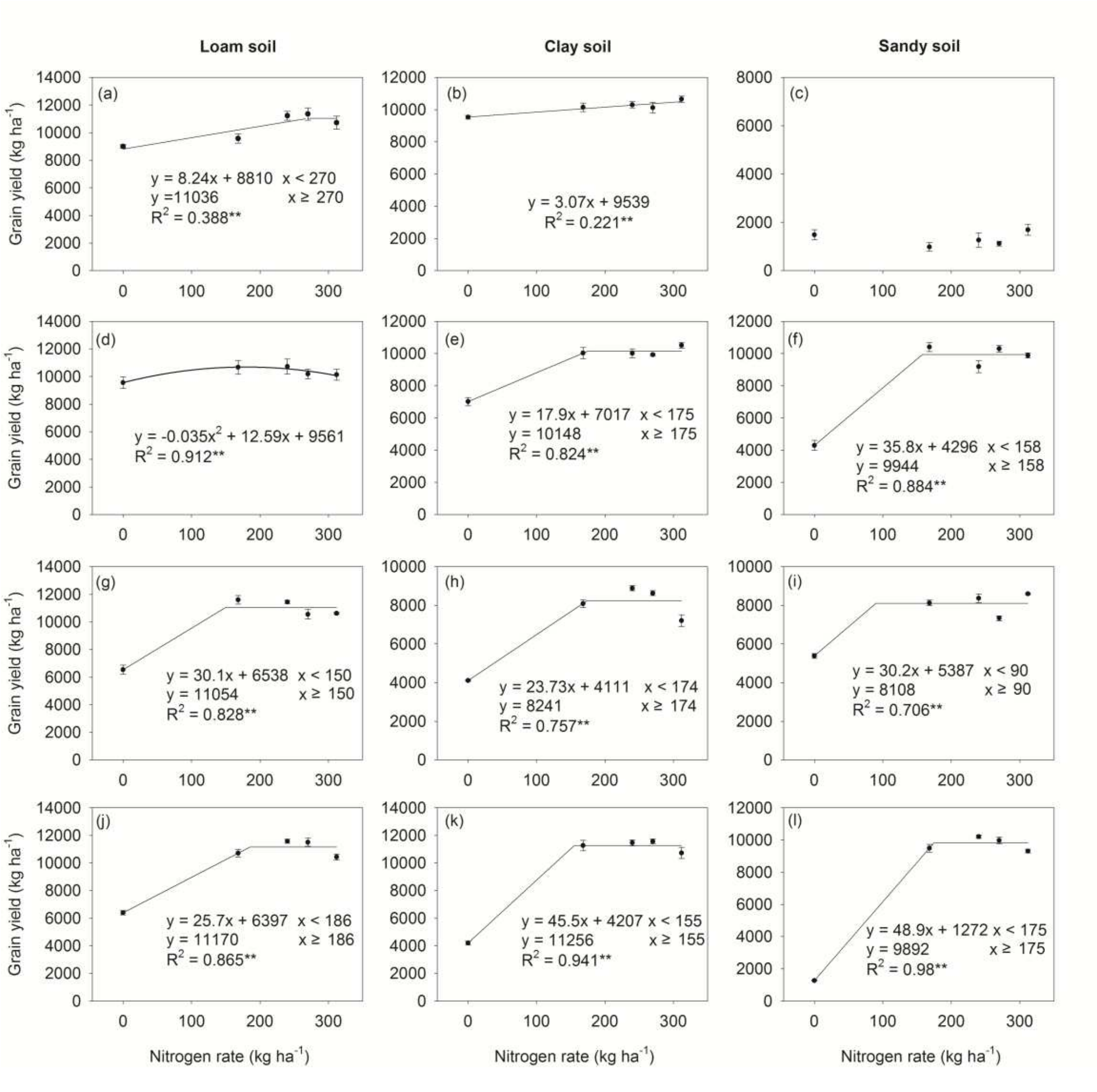

Figure 4. *Effects of nitrogen application on grain yield (at 14% moisture) on loam (left), clay (middle), and sandy soils (right) from 2009 to 2012*

Considering all four years and three soil types, GY was affected significantly by N treatments. Nitrogen fertilizer increased the maize GY significantly, with the GYs of the N168, N240, N270,and N312 treatments increased by 3525 (62%), 3822 (67%), 3647 (64%), and 3475 kg ha^{-1} (61%), respectively, compared with the N0 control ($P < 0.05$, *Table 2*). Compared with the N312 treatment, the N240 treatment increased the maize

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GY significantly (347 kg ha^{-1}, $P < 0.05$), despite applying an average of 72 kg less N ha^{-1}. The average maize N uptake was affected significantly by the N treatments. The average maize N uptake values for the N240, N270, and N312 treatments were 178, 178, and 176 kg ha^{-1}, respectively, which were significantly higher than for the N0 and N168 treatments (100 and 169 kg ha^{-1}, respectively). The average PFP_N was affected significantly by the N treatments with a rank order of N312 < N270 < N240 < N168 and values of 30, 35, 40, and 55 kg kg^{-1}, respectively (*Table 4*).

Table 4. *Analysis of variance of nitrogen partial factor productivity (PFP_N) and agronomic efficiency (AE_N) on three soil types across four years*

Treatments	PFP_N(kg kg^{-1})	AE_N(kg kg^{-1})
Soil		
Loam soil	46.0a	12.5c
Clay soil	42.4b	16.0b
Sandy soil	30.9c	17.7a
Nitrogen		
168	55.1a	21.0a
240	39.8b	15.9b
270	34.7c	13.5c
312	29.5d	11.1d
Source of variation		
Soil(S)	**	**
Nitrogen(N)	**	**
S×N	**	ns

Within soil, nitrogen, numbers followed by different letters indicate significant differences ($P < 0.05$).
NS: not significant ($P > 0.05$).
*Significant at $P < 0.05$.
**Significant at $P < 0.01$

Residual Nitrate-N and Soil N Balance

Across the experiment years (2009–2012), residual minimum soil N_{min} data showed an average of 87.2 kg N ha^{-1} with a range of 61.7 to 126.8 kg N ha^{-1} and an average of 183.7 kg N ha^{-1} with a range from 76.5 to 321.3 kg N ha^{-1} in the 0–100-cm soil profile for the N168 and N312 treatments, respectively, after harvest (*Table 5*). Presumably, some of the residual soil N_{min} would be subject to environmental loss, particularly in the period after maize harvest. Soil types affected the residual soil N_{min} significantly, with residual soil N_{min} in the 0–100-cm soil layer of clay soil being significantly higher than that in the loam and sandy soils (*Table 5*). Compared to sandy soil (73.2 and 76.5 kg N ha^{-1} for the N168 and N312 treatments, respectively) and loam soil (61.7 and 153.4 kg N ha^{-1} for the N168 and N312 treatments, respectively), more residual soil nitrate-N was present in clay soil (126.8 and 321.3 kg N ha^{-1} for the N168 and N312 treatments, respectively). Similar results were observed for residual soil NO_3^--N in the 0–100-cm soil layer. The residual soil NO_3^--N in the 0–100-cm soil layer in sandy soil was 26.0 kg ha^{-1} as compared with 74.2 and 57.2 kg ha^{-1} for clay and loam soils, respectively. We attributed these differences to soilfertility and soil texture in addition to high soil silt and clay content (*Table 1*).

The calculated total apparent N losses across the experiment years (2009–2012) ranged from 315 to 481 kg N ha^{-1} with a mean of 372 kg N ha^{-1} and from 678 to 965 kg N ha^{-1} with a mean of 823 kg N ha^{-1} for the N168 and N312 treatments, respectively

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DOI: http://dx.doi.org/10.15666/aeer/1702_42294243

(*Table 5*). The calculated total apparent N losses across the experiment years were affected significantly by the soil type. The calculated total apparent N losses in loam and sandy soils increased to 965 and 827 kg N ha^{-1}, respectively, compared with 678 kg N ha^{-1} in clay soil for the N312 treatment.

Table 5. *Calculated N balances for three soil types from 2009–2012 (kg N ha^{-1})*

Treatments	N0			N168			N312		
	Loam	**Clay**	**Sandy**	**Loam**	**Clay**	**Sandy**	**Loam**	**Clay**	**Sandy**
Overall summary (2009-2012)									
A. N Input									
1. N fertilizer	0	0	0	672	672	672	1248	1248	1248
2. 0–100 cm N min before sowing	222.4a	178.3a	70.0b	222.4a	178.3a	70.0b	222.4a	178.3a	70.0b
3. Apparent N mineralization†	447.9a	328.1b	146.3c	447.9a	328.1b	146.3c	447.9a	328.1b	146.3c
Total input: 1+2+3	670.3a	506.4b	216.3c	1342.3a	1178.4b	888.3c	1918.3a	1754.4b	1464.3c
B. N output									
4. N removed by grain and straw	616.1a	409.6b	178.7c	799.1a	731.7a	500.0b	800.4a	755.3a	561.1b
5. 0–100 cm N min after harvest	54.2b	96.8a	37.6c	61.7b	126.8a	73.2b	153.4b	321.3a	76.5c
Total output: 4+5	670.3a	506.4b	216.3c	860.9a	858.5a	573.2b	953.7b	1076.6a	637.6c
Apparent N losses‡:A-B				481.4a	319.9b	315.0b	964.6a	677.8b	826.7a
Nitrogen-use efficiency§ (%)				27.2b	47.9a	47.8a	14.8b	27.7a	30.6a

† Apparent N mineralization was calculated as the difference between the N output (plant N uptake plus residual soil Nmin) and the N input (initial soil Nmin in0–100cm soil layers) in no N treatment (Meisinger, 1984).
‡ Apparent N losses were calculated as the difference between the N input (initial soil Nmin plus apparent N mineralization and N fertilizer) and the N output(plant N uptake plus residual soil Nmin) in N-applied treatments (Zhao et al., 2006).
§ N recovery = (N uptake in N fertilization plot - N uptake in no N fertilization plot)/the amount of N fertilizer ×100.
*Different letters indicate significant difference at $P = 0.05$.

Considering all four years and the three soil types, RE_N was affected significantly by the N treatments compared with the N312 treatment. RE_N for the N168 treatment increased significantly by 17%, from 24 to 41% (*Table 5*). The soil types affected RE_N significantly for loam soil (27.2 and 14.8% for N168 and N312, respectively) and RE_N was higher in clay soil (48 and 28% for N168 and N312, respectively) and sandy soil (48 and 31% for N168 and N312, respectively).

Discussion

Across the experimental years, grain yield was significantly affected by soil types, especially in 2009 and 2011. In 2009, the rainfall during the maize growth period was only 207 mm, and there was a drought in May and early grain filling stage (*Fig. 1*). Drought in seedling stage affected emergence resulted lower seedling emerge rate (Fu et al., 2008), drought stress in flowering and early grain filling stage limited photosynthesis and reduced the flux of assimilates to the developing ears and filling

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grain (Saini and Westgate, 2000; Beyene et al., 2016; Tao et al., 2016; Kim et al., 2017), and the higher evaporation on sandy soil (Lu et al., 2014), which resulted lower ear number and 100 grain weight, further decreased the GY in sandy soil (*Table 2*). Due to differing degrees of drought vulnerability of the loam, clay, and sandy soils (Egamberdiyeva, 2007), the average GYs in the loam and clay soils were significantly higher than the GY in sandy soil (*Fig. 3*). Root lodging can reduce harvestable yield of many crops including maize (Brune et al., 2018). Root lodging tends to be associated with environmental factors such as heavy rains coinciding with wind (Farkhari et al., 2013), such as in 2011, there was a heavy rain (80.7 mm) with wind in July 30-31. Higher lodging rate in clay soil might be explained by a plow pan which prevent the root grow into deep soil (He, 2006; Qin, 2008).

Grain yield for the zero-N treatment in loam soil, clay soil and sandy soil averaged 7873, 6216 and 3110 kg ha^{-1} respectively, indicated that greater soil N availability supply contributed to greater GY. Additional soil characteristics, especially soil texture (Ziadi et al., 2013), soil water content (Cambouris et al., 2006), and N mineralization (Luce et al., 2011; Smith, 2018), appeared to have contributed to variation in soil N availability, soil productivity and yield potential among in-field location (Tolk et al., 1999).

In general, sandy soil had lower productivity due to lower soil fertility (soil organic matter content), nutrient preserved capability and water retaining capacity, compared with loam and clay soil (Egamberdiyeva, 2007). Such as in 2012, grain yield for the zero-N treatment in sandy soil averaged 1272 kg ha^{-1}, which was significantly lower than that in loam soil (6397 kg ha^{-1}) and clay soil (4206 kg ha^{-1}). The grain yield stability in loam and clay soil soil is significantly higher than that in sandy soil, the coefficients of variation for GY over the four years were 7.8, 13.4, and 59.1% for the loam, clay, and sandy soils, respectively.

In rain-fed maize system, yield response to N fertilizer may various, which has been attributed to differences in soil N supply, N use efficiency, and environments (Meisinger, 1984; Lory and Scharf, 2003). In this study, the GY response to nitrogen was various among the three soil type. The linear-plateau model of GY response to increasing N rate was showed that maximum GY of three soil type followed by loam soil (10872 kg ha^{-1}) > clay soil (9999 kg ha^{-1}) > sandy soil (7266 kg ha^{-1}). However, the IY_N followed by sandy soil (4156 kg ha^{-1}) > clay soil (3783 kg ha^{-1}) > loam soil (2999 kg ha^{-1}). Across the experimental years, the optimal N rate required to achieve the maximum GY maintained 160-180 kg ha^{-1} for the three soil types. Nitrogen partial factor productivity maintained from 57.0 to 69.0 kg kg^{-1} in loam soil, from 48.0 to 67.1 kg kg^{-1} in clay soil. In contrast, the nitrogen partial factor productivityvaried from 5.8 to 61.9 kg kg^{-1} in sandy soil, indicated that nitrogen efficiency in loam soil and clay soil were stable compared with sandy soil, such as nitrogen partial factor productivity just 5.8 kg kg^{-1} affected by drought in 2009.

To achieve satisfactory agronomic performance while minimizing negative environmental impact, N fertilization recommendations must consider the dynamics between N supply from the soil and N demand by the crops (Ayoub et al., 1995; Cassman et al., 2002; Cui et al., 2009). However, in China, pursuing high grain yield has been the top priority in policy and in practice. The typical N rate applied by maize farmers in the Northeast China (NEC) is excess (Chen et al., 2014). As expected, the RE_N in Northeast China maize production systems is low, the potential environmental impact from over fertilization and low N recovery can be substantial. In this study, the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42294243

optimum N treatment (N168) reduced residual nitrate N content in the top 100-cm soil layer and N losses by 97 and 451 kg N ha^{-1}, respectively, compared with the excessive N treatment (N312).

In the current corn production system, the big difference in IY_N (increased yield for applied N fertilizer) was attributed to the differences among soil types, suggesting that soil type led to a high degree of field-to-field variability in the yield response to applied N fertilizer. Consequently, the big challenge is how to reduce variation among different soil types and decrease yield gaps.

Conclusions

The challenge of meeting food demand in China during the next 50 years must be met by simultaneously increasing GY and NUE; however, the rain-fed maize GY differed among soil types, indicating a conflict between high GY and improved NUE under current maize production practices. An improved fundamental understanding of GY and NUE in response to management practices and soil types is needed to rectify this situation. Our study demonstrated that higher GY was obtained in loam and clay soils compared to sandy soil, which implies that recommended N fertilizer rates need to be adjusted and take into account soil type. Across the four years, the optimal N rates required to achieve the maximum GY averaged 182, 173, and 160 kg ha^{-1} for loam, clay, and sandy soils, respectively. The corresponding maximumGYs for loam, clay, and sandy soils were 10872, 9999, and 7266 k g ha^{-1}, respectively. The PFP_N averaged 46.0, 42.4, and 30.9 kg kg^{-1} for loam, clay, and sandy soils, respectively. The average RE_N, AE_N, and PFP_N values under the optimum N treatment (N168) were 41%, 21 kg kg^{-1}, and 55 kg kg^{-1}, respectively, which were all significantly higher than for N312 (RE_N, 24%; AE_N, 11 kg kg^{-1}; and PFP_N, 30 kg kg^{-1}). As a result, the optimum N treatment (N168) reduced residual nitrate N content in the top 100-cm soil layer and N losses by 97 and 451 kg N ha^{-1}, respectively, compared withthe excessive N treatment (N312). Such knowledge could be used todevelop robust N management practices to provide effective N management practice recommendations over a wide range of soil-climate combinations.

Acknowledgements. This study was supported by the National Key Research and Development Program of China (2017YFD0201801).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42294243

BROWN BEAR (*URSUS ARCTOS*) HABITAT SUITABILITY MODELLING AND MAPPING

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(Received 20th Dec 2018; accepted 27th Feb 2019)

Abstract. Today, the biggest threats for mammal predators are habitat losses, humans, and other factors. Although brown bears are not under threat in Turkey, there is still risk for fragmented and isolated populations. It is necessary to carry out habitat suitability analysis in order to determine these risks. The study area is the Western Mediterranean Region and the modelling was carried out using Maximum Entropy method with presence data collected from 56 sample areas in Antalya – Akseki. MAXENT method was used since it reveals reliable and valid models in larger areas with little local data. Cross-validation was done separating 90% of data for training and 10% for testing to validate test data in the modelling process. AUC values of the training and test data were found to be 0.956 and 0.909, respectively. The model was also evaluated according to Receiver Operating Characteristic value and Jackknife test. Environmental variables contributing to the model were Ruggedness Index, Elevation, Slope, Normalized Difference Vegetation Index, Solar Illumination Index, and Roughness Index. Habitat suitability map of the brown bear was created following the modelling process and the usability of the model and the map were evaluated for brown bear management plans. To conclude, Brown bear is a significant mammal species the habitat of which must be preserved.
Keywords: *environmental factor, jackknife, MAXENT, maximum entropy, ROC*

Introduction

Populations and habitats of large predators are decreasing at a worrying rate due to anthropogenic effects, habitat fragmentation, and hunting activities. Brown bear, an important large mammal, are at a risk of disappearing as well (Nellemann et al., 2007; Roellig et al., 2014; Støen et al., 2015; Fernández-Gil et al., 2016). Brown bears having few individuals in isolated populations are stated to be under threat for spreading out in large areas, even though their total population is large and their IUCN red list category and criteria is LC (McLellan et al., 2017). Today, the majority of the brown bear population is isolated in mountainous regions under the effect of conservation activities (Swenson et al., 2000; Zedrosser et al., 2001). Although brown bears are stated to live in areas far from human pressure, a large part of the areas occupied by humans provide potentially optimal habitat conditions for the distribution of this species (Roellig et al., 2014). Despite this situation, populations living in the same space or being close to residential areas are under further threat and are more heavily isolated, as interaction among these populations are hindered due to many factors (McLellan et al., 2017). Thus, it is necessary to make conservation plans revealing actual and potential habitats for brown bears and other species.

Distributions, biology, and ecological needs of species should be known for their conservation status and action plans (Pacifici et al., 2015). Modelling and mapping of species habitat within a good planning is of great importance. MAXENT is known to be one of the most appropriate methods in habitat modelling studies of wild animal species. This method works well with presence data and gives correct results with little data. It is also based on modelling of relationships with environmental factors in areas where the species exists (Elith et al., 2006; Hernandez et al., 2006; Phillips et al., 2006).

In Turkey, brown bears live in deciduous forests, conifer forests, and mix forests up to a height of 3500 to 4000 m, and in scrublands, shrubs, valleys being close to seas, alpine valleys, and cliffs (Başkaya et al., 2008; Ambarlı, 2012). Habitat variables of brown bears are height, anthropogenic factors, slope, roughness, roughness index, heat index, solar illumination index, and vegetation index as well as forests, open spaces, closedness, and ecological factors such as roads and climate (Apps et al., 2004; Ambarlı, 2012; Bojarska and Selva, 2012). Habitat type and quality play an important role in brown bear distribution (Can and Togan, 2004) and it is necessary to use environmental factors and to determine their effects on brown bear distribution in studies. Brown bears spread in the Black Sea and East Anatolian regions of Turkey densely (Ambarlı et al., 2016). It is known that the species live in the Mediterranean region and (Turan, 1984) have intact and natural habitats in Antalya district (Can and Togan, 2004). When the distribution map of brown bears prepared in accordance with the IUCN data is examined, it is clearly seen that the species has disappeared in many areas and information regarding with their reproduction in their living areas are insufficient in the Mediterranean region (McLellan et al., 2017). In addition, populations of brown bear are fragmented and isolated in the Western Mediterranean region. This is seen to be a big threat for this species and it is important to determine the factors affecting its distribution and to reveal potential regions to maintain its continuity in the region. In other words, studies involving latest information regarding with the actual distribution and potential appropriate habitats of this species in the region should be done. This study aims to do suitability modelling and mapping to determine the distribution of brown bears in the Western Mediterranean region.

Materials and method

Data collection

West Mediterranean region in the south-west of Turkey was picked as the study area (*Fig. 1*). Inventory study was conducted in Akseki district to represent this region. West Taurus Mountains are surrounded by Bey Mountains, Elmalı Mountains, Katrancık Mountains, and Boncuk Mountains in the west and Akçalı Mountains, Geyik Mountains, Dedegöl Mountains, Kuyucak and Erenler Mountains in the east. There are many karstic shapes in this topography since this area is formed by limestone bedrock (Öztürk et al., 2018). The area is used extensively by locals for transhumance practices (Sarı, 2013). In this study, indirect observation technique was used in the collection of land data. Presence data of indirect inventory was obtained belonging to tracks, feces, and other signs of brown bears in 56 points Akseki district in 2016.

Environmental variables

Slope, Aspect, and Elevation maps of the region were created using Digital Elevation Model (DEM) on ArcMap 10.2 software. Topographic Position Index (TPI) was created using ArcMap 10.2 software with "Topography Tools "extension developed by Jennes (2016). Also, Topographic Convergence Index (TWI), Landform Classification (Jenness), Solar Illumination Index (6 am, 8 am, 10 am, noon, 2 pm, 4 pm, 6 pm, and 8 pm) (Tagil and Jennes, 2008) were created. Terrain Ruggedness Index (Ruggedness)

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DOI: http://dx.doi.org/10.15666/aeer/1702_42454255

was created via Terrain Tools extension (Riley et al., 1999). Roughness Index, (Roughness) and Heat load index (HLI) were created with the help of Geomorphometric and Gradient Metrics Toolbox. Beers Aspect (BA) (*Eq. 1*) (Beers et al., 1966) and topographic radiation index (TRASP) (*Eq. 2*) (Moisen and Frescino, 2002) values were calculated and maps were created using different equations, respectively.

$$BA = \cos(Amax\text{-}A) + 1 \quad \text{(Eq.1)}$$

$$TRASP = [1 - \cos((\pi/180)(\Theta - 30))]/2 \quad \text{(Eq.2)}$$

Normalized Difference Vegetation Index (NDVI) was obtained from MOD13Q1 module, one of the satellite data of MODIS VI, monthly. (Didan et al., 2015). NDVI values range between -2000 (-0, 2) and 10000 (1, 0). Negative values of NDVI show water bodies; values approximate to 0 show bare soil); values 0.1 and lower show barren areas of rock, sand, or snow; values between 0.2 and 0.3 show shrub and grassland, and values between 0.6 and 0.8 show temperate and tropical rainforests (Karaburun, 2010; Xei et al., 2010; Bhandari et al., 2012).

Climate data was downloaded on the following website (http://www.worldclim.org). Nineteen pieces bio-climate data were prepared (Hijmans et al., 2005). This climate data were cut according to the study area size and prepared according to 19 climatic data.

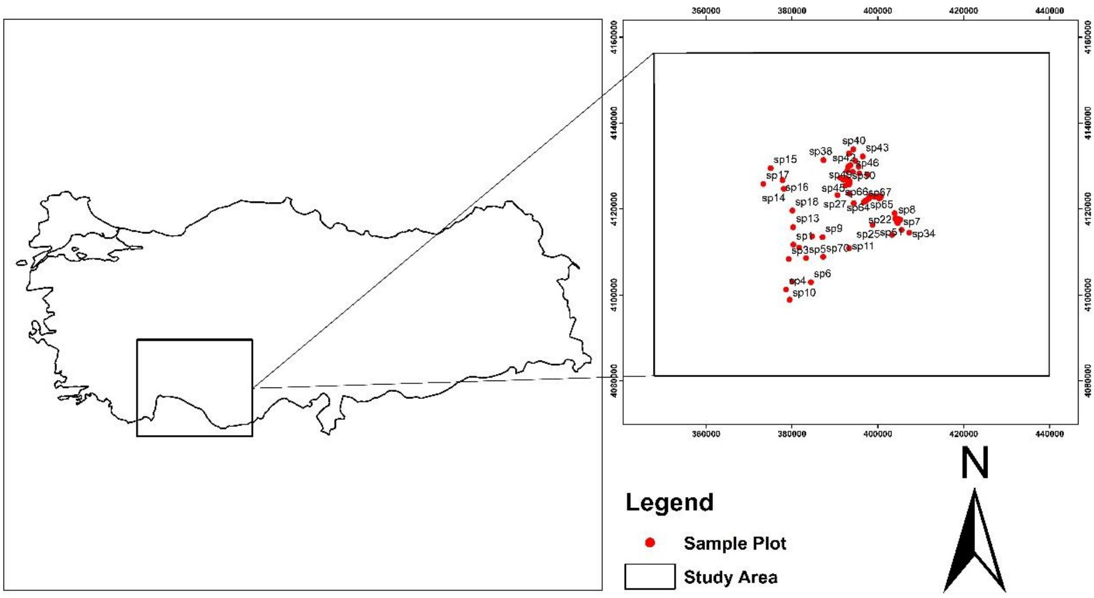

Figure 1. *Study area and map of sample areas*

Modelling and mapping processes

Factor analysis between elevation and nineteen bio-climate variables was applied in the modelling process in order to prevent independent variables from causing any bias. As a result of the analysis, elevation was chosen as representative variable since strong relationship between elevation and nineteen bio-climate variables might cause bias (Mert and Kıraç, 2017; Suel et al., 2018). After this process, potential habitat suitability modelling process was completed using MAXENT method.

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This method creates suitability model evaluating the data collected in the study area where the species exists and areas with similar features together (Baldwin, 2009). When compared to other modelling processes of presence data, MAXENT enables to create more realistic distribution and suitability models with less data (Phillips et al., 2004, 2006; Hernandez et al., 2006; Wisz et al., 2008; Baldwin, 2009; Elith et al., 2010).

3.4. lk version of MAXENT is used for habitat suitability analysis. 90% of the brown bear presence data was allocated as training value and 10% was allocated as test value and modelled with environmental variables. The success of MAXENT model was assessed using ROC (Receiver Operating Characteristic) curves; AUC values of training-test data and their contribution to model levels were assessed with jackknife graphs (Deleo, 1993; Fielding and Bell, 1997; Phillips et al., 2006; Baldwin, 2009; Monterroso et al., 2009). Potential distribution map ranging between 0 and 1 was illustrated in ArcMap 10.2 software (Özkan et al., 2015)

Results

A successful and valid model with the ROC value of 0.956 was obtained in this study (*Fig. 2*). AUC value of the test data is 0.909 whereas AUC value of training data is higher than test data, yet the discrepancy is not high. However, the area under the ROC curve is higher than the area under the random prediction line. These results show that the model can be used as it gives realistic results.

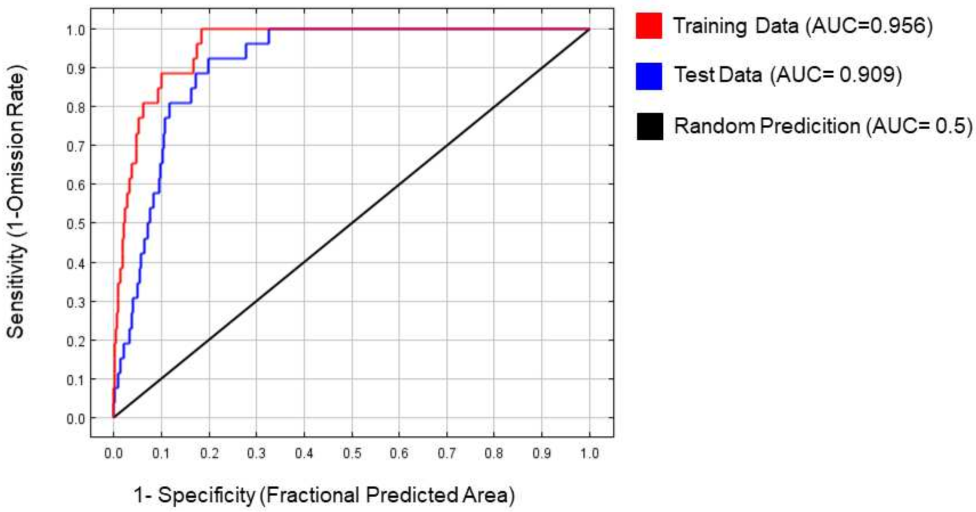

Figure 2. *ROC and AUC MAXENT values of the model*

Environmental variables are 2 pm, 6 am, Elevation, Slope, NDVI (September), Roughness, and Ruggedness (*Table 1*). Variables that contribute most to the model are roughness, elevation, and NDVI (*Table 1*).

When the results of jackknife statistics are examined, it is seen that ruggedness makes the most contribution to the model. Although the contribution level of roughness is high, excluding it would not cause a great loss in the model, in which case it can be said that elevation is more descriptive compared to roughness and thus is the second highest contributor of the model. Another significant variable in terms of model gain is

NDVI. 6 am and slope also play a role in the model's success. 2 pm has the lowest contribution alone and in terms of total model gain (*Fig. 3*).

Table 1. *Variables contributing to the model and their contribution levels*

Environmental variable	Contribution %
Ruggedness	59.9
Elevation	23
NDVI	9.2
6 am	3.1
Slope	2.5
Roughness	1.6
2 pm	0.8

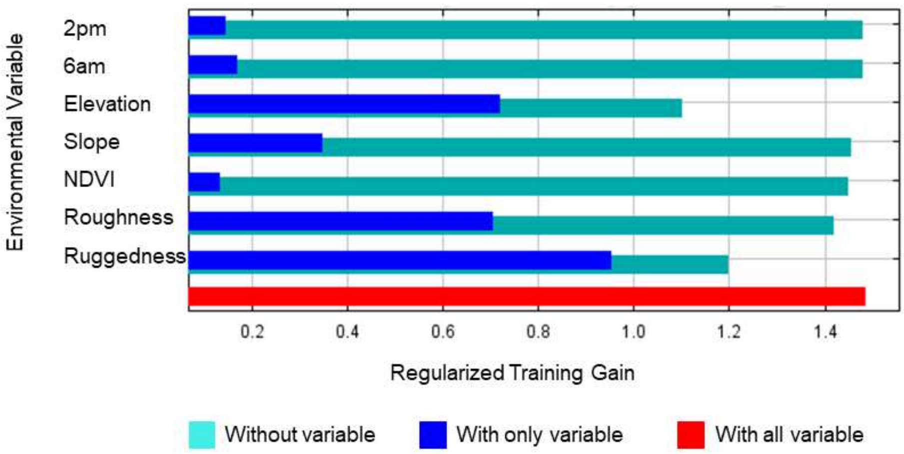

Figure 3. *Jackknife test for evaluating the relative importance of environmental variables*

Three variables making the most contribution to the model in percentages are ruggedness, elevation, and NDVI and when examined together with roughness, the highest contributor according to jackknife results, it is seen that brown bears prefer rugged areas up to a specific point and avoid extremely rugged terrains topographically (*Fig. 4*).

Elevation has a positive effect on the habitat of this specific species up to about 2000 m whereas this effect becomes negative in higher locations. In terms of NDVI results, it is seen that brown bears prefer natural, open areas and forests and avoid dense forests. Finally, these species prefer rough areas up to a point meaning it avoids flat spaces (*Fig. 4*).

The scale regarding with the potential habitat suitability of brown bear on the habitat suitability map ranges between 0 and 0.97. Red areas are the most suitable habitats potentially whereas blue areas show unsuitable terrains for brown bears (*Fig. 5*). Currently, brown bear populations are available in Kuyucak, Civikli, Geyik and Gidengelmez Mountains and their surroundings. Although there is no brown bear population apart from these areas such as Akçal, Cehennem, Aygır, Çamurlu and Gelincik mountains, model results reveal these as potential habitats for brown bears.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42454255

Akcal Mountain and the area in its east part are suitable for brown bears, but there is no sufficient data regarding with whether brown bears exist in this area or not.

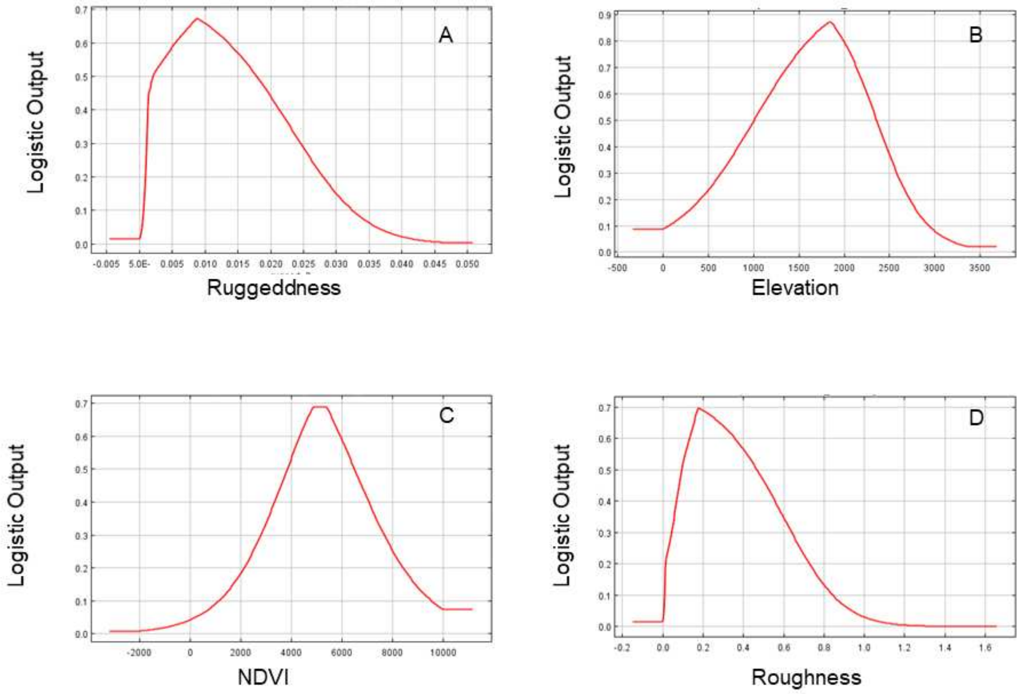

Figure 4. *Important factor response curve: (A) ruggedness index, (B) elevation, (C) NDVI; (D) roughness index*

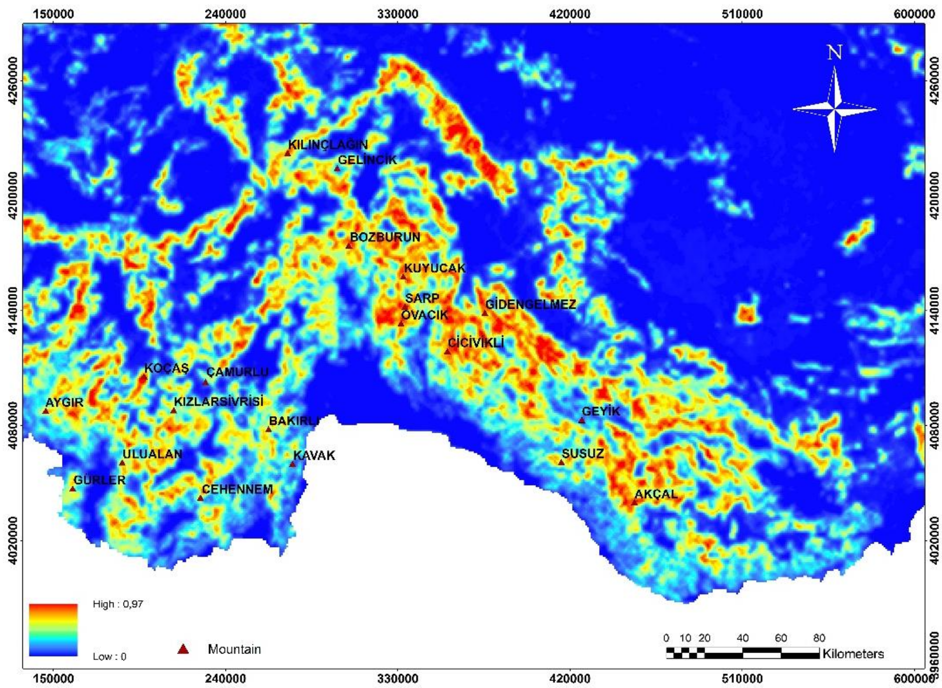

Figure 5. *Habitat suitability map of brown bears*

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Discussion

Modelling studies provide significant guide for potential distribution and habitat suitability maps of animal and plant species. In terms of its topographic structure, forests, and geographical location, West Mediterranean Region deserves its potential to be revealed (Özkan et al., 2015). In this study, habitat suitability modelling and mapping of brown bears were performed. Although the data is relatively little and involves a local area, successful and real results were obtained thanks to the MAXENT method. The model was verified via ROC graph, difference between training (AUC: 0.956) and test values (AUC: 0.909), and jackknife tests (Phillips et al., 2006; Wisz et al., 2008; Baldwin, 2009; Elith et al., 2010; Tekin et al., 2018). Both researchers and executors can use the habitat suitability map.

Variables forming the MAXENT model are Elevation, Slope, Solar Illuminations Index, Ruggedness Index, Roughness Index, and NDVI according to their contribution percentages and the results of jackknife test. These results show that rugged areas play an important role in brown bear distribution and brown bears prefer areas with specific slope degrees. In previous studies, slope is stated to be a significant environmental factor that affects habitat preferences of brown bears (Štofík, and Saniga, 2012). When topographic structure of West Taurus Mountains is considered, it is understood that it is an expected situation for slope to contribute to the model in especially cliffy areas and that brown bears do not prefer excessively sloped areas. Ruggedness is also another important variable in habitats potentially suitable for brown bears and previous studies point out that brown bears prefer rugged terrains for they are not affected by human activities and rich in terms of vegetation (Apps et al., 2004; Nielsen et al., 2004; Nellemann et al., 2007.).

It was observed in field studies that rugged areas with their local vegetation in West Taurus Mountains, which form the topography in the West Mediterranean Region, overlap with areas where there are brown bears. Field studies carried out in Akseki district show that brown bears choose rugged areas with rich vegetation. Moreover, Brown bears use rugged areas where there is Juniperus drupacea for nutrition and use some other rugged fields for shelter. As for roughness index, brown bears are seen to avoid flat and extremely rough surfaces and use rough areas for daily activities. Although there is not clear information about this in the literature, roughness index overlaps and shows similarities with other variables such as ruggedness and slope.

The natural forests in altitudes between 500 and 2000 m in Western Mediterranean have been found to be ideal habitats for brown bears. Can and Togan (2004) stated the terrains with same features are potential habitats for brown bears. In addition, the reason why brown bears prefer altitudes between 800 and 1000 m as habitat is related to vegetation, human impact and land structure (Posillico et al., 2004). The information here overlaps with the interpretations related to ruggedness as mentioned above and the effects of this can be clearly seen in brown bear distribution map.

NDVI was determined as a significant variable that contributes to habitat suitability model of the brown bear- an omnivorous living being and this reveals that brown bears prefer to live in areas where there are dense natural forests in the Western Mediteranean region. This overlaps directly with the living spaces of brown bears where there are coniferous trees or mix forests. The relationship between habitat preferences of brown bears and NDVI was revealed in previous studies, brown bears have been found to prefer green vegetation (Can and Togan, 2004; Bojarska and Selva, 2012).

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This study found out that Solar Illuminations index contributes to the habitat suitability model, though this contribution is small. It is therefore understood that brown bears prefer noon to early morning hours and avoid extensive sunlight. It was pointed out in the literature that brown bears are more active during day in shorter days of the year (Ordiz et al., 2012) and that there are differences in their daily activities by seasons and light period (Ware et al., 2018). Hence, all these results show that brown bears react to sunlight, yet this situation might differ by seasons.

The habitat suitability map shows that our map is consistent with the distribution maps presented in the literature (Ambarlı et al., 2016; McLellan et al., 2017). Besides, the habitat suitability map shows potential areas for brown bears. Suitable areas for brown bears in the Western Mediterranean region were presented on the map.

Conclusion

In conclusion, habitat suitability modelling and mapping study was carried out successfully in the Western Mediterranean region and supported with the literature. The actual state and potential distribution areas of the species under the effect of basic environmental variables that affect habitats of brown bears were revealed. In fact, brown bears are not under the threat of extinction; however, they are large mammals and their populations are divided, they are also in the risky group. This study determined habitat factors of and potential areas for brown bears and this is of great importance for action plans to be prepared. Thus, the findings of this study are expected to provide guidance for action plans to be created for this species.

Acknowledgements. The author would like to express special thanks to Serkan ÖZDEMİR, Murat ERCAN and Ahmet MERT practical support of the work. We thank to project 4752-YL1-16 supported by the Coordination Unit for Scientific Research Projects, Süleyman Demirel University.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42454255

CO-ORDINATION OF PHOTOSYNTHESIS AND STOMATAL RESPONSES OF MONGOLIAN OAK (*QUERCUS MONGOLICA* FISCH. EX LEDEB.) TO ELEVATED O_3 AND/OR CO_2 LEVELS

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(Received 25th Dec 2018; accepted 26th Feb 2019)

Abstract. Four-year-old trees grown in open-top chambers (OTCs) were exposed to elevated O_3 (80 nmol/mol, EO) or/and elevated CO_2 (700 μmol/mol, EC) levels. The object is to study the responses of photosynthesis and stomata in leaves of Mongolian oak (Quer*cus mongolica*) to EO and/or EC for two successive growing seasons in an urban area. The experiment was conducted in the arboretum located at the center of Shenyang city. The results show that: (1) EO decreased P_n (Light-saturated net photosynthesis rate), g_s (stomatal conductance) and T_r (transpiration rate). The stomata closure maybe associated with the direct effect of EO on guard cells. The decreases of F_0, F_m and F_v/F_m indicated that EO limits the capability of the plant to use photon energy and thus alters photosynthesis. (2) EC increased P_n and C_i (intercellular CO_2 concentration) of leaves, but decreased g_s and T_r slightly. Stomatal density decreased significantly at the end of the second season. Perhaps, EC was detected by mature leaves in the first season which signaled to the developing leaves in the next season, the stomatal developments of which altered accordingly. (3) Under EC+EO, EC relieved most of the O_3-induced adverse effects to P_n. The decreases of g_s and T_r were mainly caused by EO. There was no significant difference of F_v/F_m detected compared to the control, EC can largely relieve the adverse effects of EO on the PSII reaction center. Changes of stomatal parameters were just like the changes under EC. Perhaps the morphological changes of the stomata were mainly caused by EC.
Keywords: *photosynthetic CO_2 exchange, chlorophyll fluorescence, stomatal density, stomatal area*

Abbreviations: C_a**:** concentration of ambient CO_2; C_i**:** concentration of intercellular CO_2; F_m**:** fluorescence maximum; F_0**:** fluorescence origin; F_v/F_m**:** maximum quantum yield of PSII photochemistry; g_s**:** stomatal conductance; P_n**:** light-saturated net photosynthesis rate; T_r**:** transpiration rate

Introduction

Since the industrial revolution, accumulating greenhouse gas (O_3 and CO_2) levels have increased over two-fold (Guo et al., 2017). The atmospheric concentrations of CO_2 have increased since 1750. In 2011, the concentration of CO_2 was 391 μmol/mol and exceeded the pre-industrial levels by about 40%. The rise primarily resulted from emissions of fossil fuel burning and secondarily from net land use change (IPCC, 2013). This rise has been accompanied by the increase in troposphere O_3 concentrations, which is not only a significant greenhouse gas but is also recognized as a serious phytotoxic air pollutant that damages plants (Serengil et al., 2011). As concentrations of NOx and VOCs increase through industrialization and vehicle exhausts, the daily surface O_3 levels in industrialized countries have increased remarkably from 10 nmol/mol prior to the industrial revolution to 60 nmol/mol in the current summer seasons, and are predicted to increase by another

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20% by 2050 (IPCC, 2007). Ground-level O_3 concentrations have significantly increased over the past decades, especially in northeast Eurasian region (Akimoto et al., 2015; Feng et al., 2015; Verstraeten et al., 2015).

These two co-occurring pollutants are quite interesting as they act in diametrically different directions. Stomatal aperture and density influence the rate of photosynthesis and transpiration of plant directly. Regulation of stomatal development and stomatal conductance is a key factor for plant in its adaptation to the environment changes and plants have evolved sophisticated mechanisms to control stomata density and behavior (Engineer et al., 2014). The stomatal control is achieved by the regulation of stomatal aperture through changes in guard cell turgor, and by alteration of stomatal density through modification of stomatal initiation and leaf expansion during leaf development (Haworth et al., 2013). Research of Frey et al. (1996) showed that stomatal density of birch could be increased under a consistently O_3 exposure. A reduction in stomatal conductance is commonly observed in response to elevated CO_2 (Woodward, 1987). Reported changes in stomatal density with growth at elevated CO_2 include increases, decreases, and no change (Drake and Gonzàlez-Meler, 1997). Those species with little or no control of stomatal aperture were more likely to exhibit a reduction in stomatal density under elevated CO_2 (Haworth et al., 2013). But how can stomatal response to both elevated O_3 and elevated CO_2? The stomatal development and stomatal conductance of leaves would how to be regulated to adapt to the combinational environments? The objective of this study was to measure photosynthetic and stomatal parameters of leaves of *Q. mongolica* (*Quercus mongolica*) trees grown in open-top chambers (OTCs) for two growing seasons to evaluate the photosynthesis and stomata responses of *Q. mongolica* to elevated concentrations of O_3 and/or CO_2. Cities are with concentrated industrial plants and heavy traffic. Trees grown in urban regions are more likely affected by elevated O_3 and CO_2, so the OTCs of this experiment were built in the center of the city to simulate the O_3 and CO_2 effects on leaves of *Q. mongolica*.

Research design and methods

Site and OTCs

The experiment site is located in Shenyang Arboretum of Chinese Academy of Sciences (41°46′ N, 123°26′ E and 41 m above sea level), which is located in the metropolitan area of Shenyang, Liaoning province, China). In this area, average annual precipitation is 755.4 mm and average annual temperature is 7.4 °C. The mean daytime temperature and the mean relative humidity in the OTCs showed no significant differences among treatments (Tmean = 26 °C Hmean = 70%). Ambient rainfall was occasionally supplemented to minimize water stress. The soil in the OTCs was loamy, supplemental fertilizer was provided and weeds were removed regularly. The factorial design of the OTCs has already been reported, they are 4 m in diameter and 3 m in height with a 45° sloping frustum, and distance among them is at least 4 m (He et al., 2006). There are twelve OTCs provided for four treatments, each with 3 replicates (in 3 OTCs randomly). The treatments were (1) control (ambient air, CK), (2) elevated O_3 (80 nmol/mol O_3 + ambient CO_2, EO), (3) elevated CO_2 (ambient O_3 + 700 μmol/mol CO_2, EC), (4) elevated O_3 + elevated CO_2 (80 nmol/mol O_3 + 700 μmol/mol CO_2, EO+EC).

O_3 was produced from bottled pure oxygen with an ozone generator (GP-5J, China) and pure CO_2 was injected into the chambers from cylinders. In order to monitor O_3 and CO_2 concentrations, O_3 transducer (S-900, Aeroqual, New Zealand) and CO_2 infrared

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transducer (SenseAir, Sweden) were used inside the chambers. Elevated CO_2 was applied for 24 h/day. Elevated O_3 was applied for 9 h/day (08:00-17:00).

Plant material

In April, uniform and healthy *Q. mongolica* trees (four-year-old) from local nursery were randomly planted in the soil of twelve OTCs (three OTCs were used as replicates for each treatment), 20 trees per chamber. These young trees were exposed to CK, EO and/or EC from 18 June to 10 October in the first season, overwintered in the OTCs and then re-exposed from 18 June to 10 October in the second season.

Gas exchange measurements

One leaf per plant and three plants per OTC (three OTC_S per treatment, n = 9) were measured by a portable photosynthesis system (LI-6400, Li-Cor Inc., Lincoln NE, USA) to attain a mean value every 15 days. The same method of gas exchange measurements has been reported (Wang et al., 2009).

Chlorophyll a fluorescence measurements

Chlorophyll a fluorescence was measured once every 15 days at ambient temperature in the OTC's in the second year, using a portable fluorometer (Handy-PEA, Hansatech, England). One leaf per plant and three plants per OTC (three OTC_S per treatment, n = 9). The environmental conditions were similar to gas exchange measurement. The same method of Chlorophyll a fluorescence measurement has been reported (Wang et al., 2009).

Stomatal parameters measurements

Q. mongolica leaves were collected after 0, 60 days (in the first season) and 210 days exposure (in the second season). Three leaf per plant, three plants per OTC and three OTC_S per treatment (n = 27). The top, middle and bottom of leaves were painted with transparent nail polish, and then the films were torn off after it dried. The temporary microscopic slides of dried nail polish were observed and photographed with picture pick-up system of bright-field microscope (Olympus BX-50, 1.3 million pixels), and image processing software (Motic Images Advanced 3.0) was used to measure and calculate the stomatal parameters including stomatal density, stomatal length, stomatal width, stomatal perimeter and stomatal area.

Statistical analysis

ANOVA was carried out using SPSS 13.0 computer package to analyze all sets of data and the means were compared by the Tukey test at 5% probability Levels. Sample variability is given as the standard deviation (S.D.) for presentation.

Results

Effects of elevated O_3 and/or CO_2 on Pn, gs, Ci and Tr of Q. mongolica leaves

EO suppressed P_n of *Q. mongolica* leaves significantly ($p < 0.05$) over two growing seasons compared to the control. The maximum difference (about 72%) was recorded

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after 45 days exposure in the first season, while EC increased it generally. P_n of leaves exposed to EO+EC was generally significantly higher than that exposed to EO alone, but lower than control except the last measurement (*Fig. 1a* and *b*).

In the first season, there was decrease of C_i detected in the EO environment compared to the control. While in the second season, there was almost no significant difference between EO and control treatments. However, EC and EO+EC treatments increased C_i of leaves compared to the control, and it was higher under EC than EO+EC (*Fig. 1c* and *d*).

EO and EO+EC generally decreased g_s of leaves significantly ($p < 0.05$), compared to the control and EC alone. However, there was no significant difference between EO+EC and EO treatments except after 60 days exposure in the second season. Compared to the control, EC slightly decreased g_s of leaves over the two growing seasons (*Fig. 1e* and *f*).

Similar change was shown between T_r and g_s of *Q. mongolica* leaves (*Fig. 1g* and *h*).

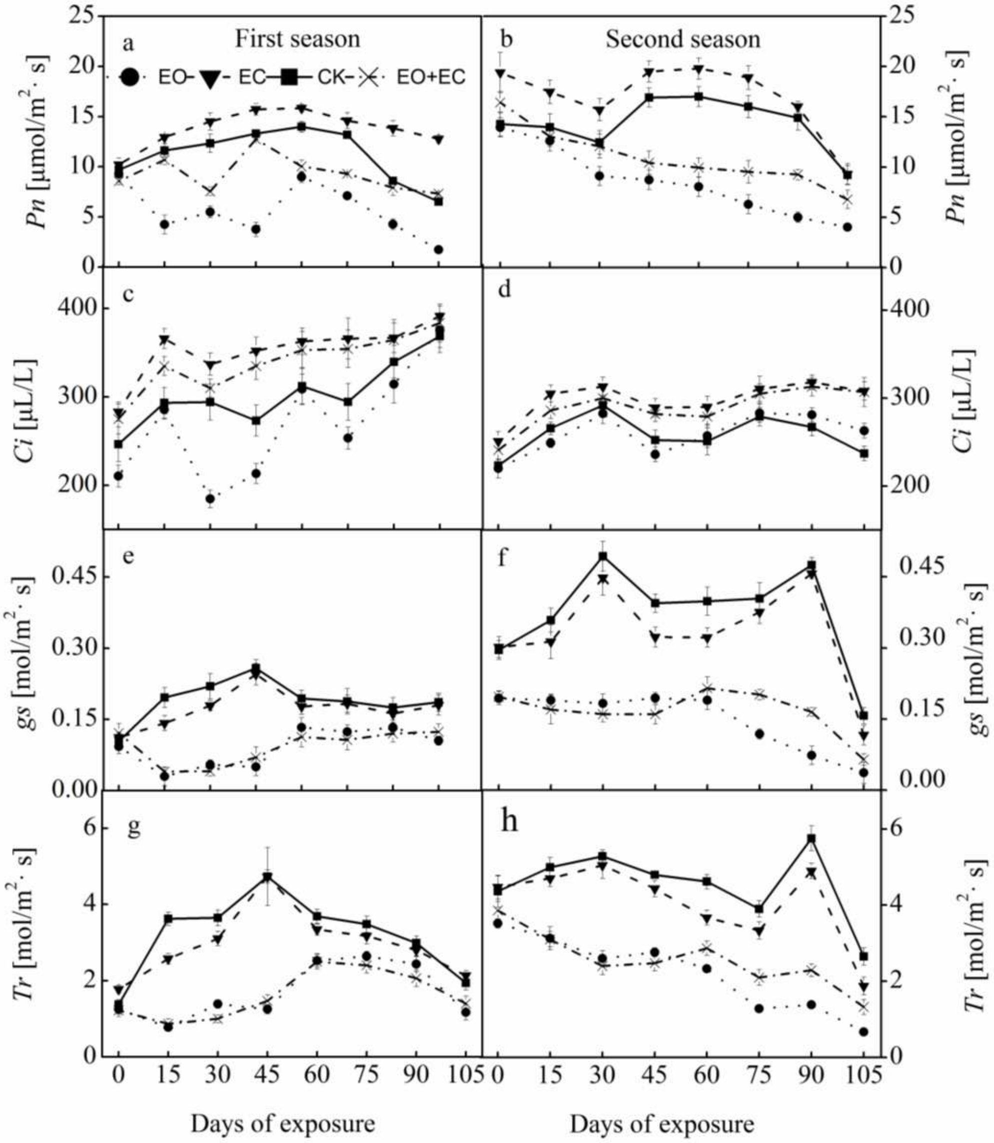

Figure 1. *Effect of elevated CO_2 and/or O_3 on the seasonal changes of P_n, g_s, C_i and T_r of Q. mongolica leaves. Each value represents the mean ± SD*

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DOI: http://dx.doi.org/10.15666/aeer/1702_42574268

Effects of elevated O_3 and/or CO_2 on chlorophyll fluorescence parameters of Q. mongolica leaves

EO decreased F_0, F_m and F_v/F_m of *Q. mongolica* leaves after 75 days in the second season compared to the control. The differences were significant in the later dates ($P < 0.05$), but there was no significant differences detected in other treatments (*Fig. 2*).

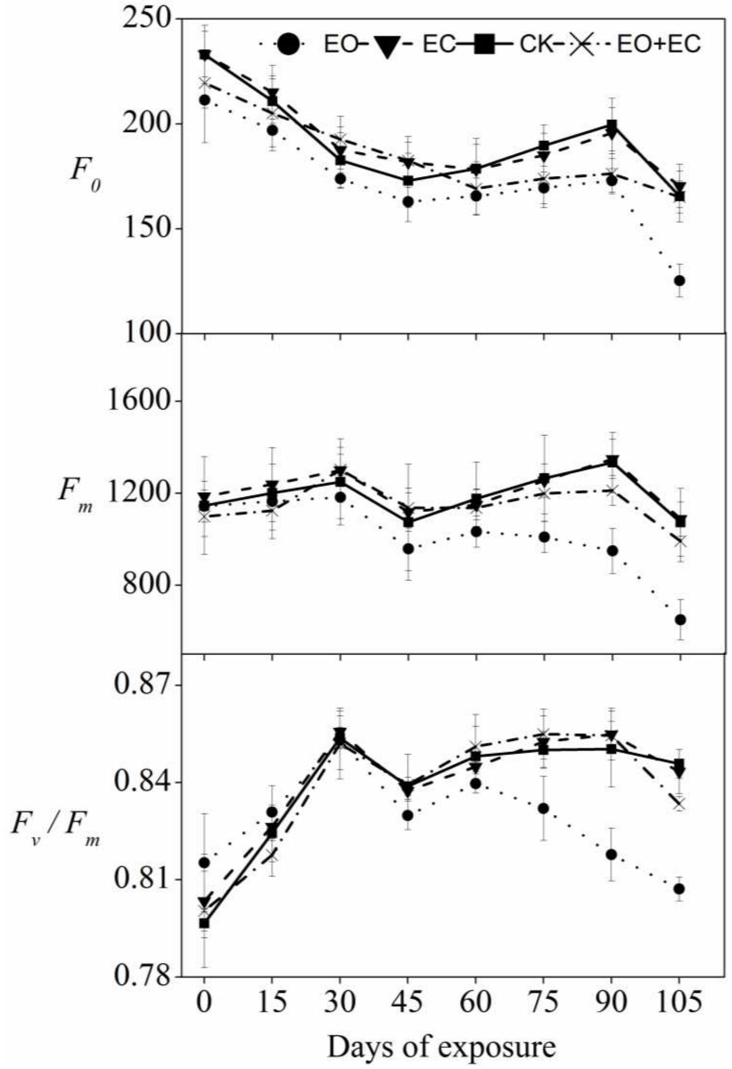

***Figure 2.** Effect of elevated CO_2 and/or O_3 on the seasonal changes of F_0, Fm and Fv/Fm (the maximum quantum yield of PSII photochemistry) of Q. mongolica leaves. Each value represents the mean ± SD*

Effects of elevated O_3 and/or CO_2 on stomatal parameters of Q. mongolica leaves

There was no difference of stomatal density of leaves detected in all the treatments in fist season (0th, 60th day in the first season), but in the end of the second season (210th day), EC and EO+EC significantly decreased it compared to the control ($P < 0.05$). There was only a slight increase of stomatal width of leaves by EC compared to the control on 60th day ($P < 0.05$). There were no significant differences of stomatal length, stomatal perimeter and stomatal area of leaves in the all treatments (*Table 1).*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42574268

Table 1. *Effect of elevated CO_2 and/or O_3 on stomatal density, length, width, perimeter and area of Q. mongolica leaves.*

Days of exposure	Treatments	Stomatal density (No/mm²)	Stomatal length (μm)	Stomatal width (μm)	Stomatal perimeter (μm)	Stomatal area (μm²)
0 days	CK	293.00±22.03^a	72.72±5.21^a	23.20±1.42^a	204.91±18.80^a	1553.1±197.1^a
	EO	293.05±19.01^a	73.91±4.01^a	23.51±1.73^a	205.70±16.51^a	1555.0±173.6^a
	EC	293.01±20.16^a	74.13±5.72^a	24.05±1.91^a	206.27±11.59^a	1548.3±178.4^a
	EO+EC	293.00±18.05^a	73.50±6.51^a	23.47±1.58^a	206.05±25.62^a	1545.5±113.0^a
60 days	CK	293.05±25.02^a	76.06±7.22^a	21.05±1.53^b	203.04±16.72^a	1541.0±179.7^a
	EO	293.00±19.01^a	76.11±4.03^a	22.21±1.66^{ab}	204.72±12.12^a	1597.3±166.2^a
	EC	291.47±10.10^a	77.10±6.20^a	23.58±1.31^a	205.41±20.01^a	1646.6±105.8^a
	EO+EC	284.86±24.32^a	76.04±7.14^a	22.02±2.15^{ab}	203.60±18.88^a	1597.0±102.7^a
210 days	CK	366.00±16.90^a	65.92±6.82^a	18.09±1.11^a	174.21±19.12^a	913.8±167.5^a
	EO	350.43±22.60^a	66.07±4.38^a	18.22±1.44^a	175.83±13.64^a	923.3±108.4^a
	EC	325.67±12.00^b	67.03±4.31^a	18.53±1.62^a	181.11±16.63^a	967.2±117.2^a
	EO+EC	319.09±15.70^b	67.31±5.90^a	18.61±1.40^a	181.21±11.21^a	968.6±134.9^a

Each value represents the mean ± SD. (Different letters indicate significant differences between treatments at the same time at $P < 0.05$)

Discussion

Effects of elevated O_3

Many authors have found that photosynthetic processes are very sensitive to high O_3, and a decrease in the rate of light-saturated net photosynthesis as a result of exposure to elevated O_3 has been reported (Farage and Long, 1999). In our experiment, from the apparent decreases in P_n of *Q. mongolica* leaves under EO fumigation appear in early of the growing season, It can be concluded that EO has a severe impact on P_n of *Q. mongolica* leaves and *Q. mongolica* is an O_3-sensitive tree species. Since Bortier et al. (2000) suggested that faster growing species tend to be more sensitive than slower growing species. Also compared with mature trees, young trees are more susceptible to O_3 stress because of their high metabolic activity (Laurence et al., 1994). The sensitivity of *Q. mongolica* to EO in our experiment may be related to their young age. It has been correlated observed that photosynthesis decreased with a reduction in stomatal conductance under O_3 exposure (Mansfeld, 1998). In other cases it was assumed that the decline in photosynthetic rate was the result of the interaction of O_3 with photosynthetically active mesophyll cells (Farage and Long, 1995).

Stomatal conductance plays an important role in the photosynthetic efficiency of O_3 stressed plants by controlling the amount of pollutant entering the plants through the stomata (Matyssek et al., 2004). It is well known that species with high stomatal conductance are more prone to injury because of enhanced pollutant uptake (Matyssek et al., 2004). Our results showed that g_s of *Q. mongolica* leaves were significantly decreased by EO at the beginning of the experiment, while the similar decrease was appeared in T_r of leaves. These results indicate that the partial closure of stomata can be induced by EO to avoid the further ozone uptake. Stomatal limitation is mainly attributed to stomata closure and, subsequently, to the decrement of Ci under normal conditions (Salazar-Parra et al., 2015), however, many authors suggested

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42574268

that stomata closure is not a direct response to O_3 insult, but a reaction to an increased intercellular CO_2 concentration resulted from the inhibition of carbon assimilation (Weber et al., 1993; Heath and Taylor, 1997). In our study, there was nearly no increase in C_i of leaves under EO, compared to the control at 15 days when g_s of leaves had decreased to minimum, so we propose that the stomata closure maybe associated with the direct effect of EO on guard cells of *Q. mongolica* leaves.

In low O_3 environments, photosynthesis and stomatal conductance are typically tightly coupled because the stomatal aperture equivalently controls the exchange of CO_2 and water at the leaf surface (Lombardozzi et al., 2012). However, from our study we can see that the typical coupling of stomatal conductance and photosynthesis does not always hold under EO and the decreases in P_n can be independent (in some extent) of g_s, suggesting that decreases in carboxylation, rather than g_s, are responsible for decreases in P_n.

The reduction in photosynthesis may also occur due to structural damage of thylakoids, which affects the photosynthetic electron transport and is indicated by the reduction in F_v/F_m ratio (Rai and Agrawal, 2012). F_v/F_m ratio is an indicator of the photoinhibition to PS II complexes. Under O_3 exposure, there are reports on wheat and rice showing increase in F_0 and a parallel decrease in F_m, suggesting impairment of PS II activity (Feng et al., 2011; Ishii et al., 2004). In our experiment, in the end of season, under EO exposure (after 75 days), there were decreases of F_0, F_m and F_v/F_m. Decreases of F_0 and F_v/F_m are the characteristics of thermal dissipation which depend on xanthophyll cycle and that dissipation reduces the photon energy to the photochemical reaction center (Xu, 2002). This can be seen as an important protection mechanism to avoid the destruction of PSII reaction centre. The decreases of F_0, F_m and F_v/F_m indicate that O_3 can limit the capability of the plant to use photon energy and thus alter photosynthetic processes. Decrease in the F_v/F_m is generally attributed to damage to the PSII reaction centre, apart from the down-regulation of the capacity of PSII electron transport (Chaumont et al., 1995).

Increased stomatal density seems to be a consistent effect related to ozone exposure and has been reported by Matyssek et al. (1991), but the same result was not found in this study, there were nearly no significant differences of stomatal parameters under EO compared to the control.

Effects of elevated CO_2

Rising CO_2 will impact plants through two processes, reducing g_s and increasing P_n (Ainsworth and Rogers, 2007). It has been reported that elevated CO_2 stimulated light-saturated photosynthesis in C_3 plants (Ainsworth and Rogers, 2007; Lahive et al., 2018; Panigrahi et al., 2016). In our experiment, EC also increased P_n of *Q. mongolica* leaves generally in two growing seasons until in the late of the second season (after 90 days).

Decreases in g_s of some plants induced by high CO_2 have been measured (Gao et al., 2012). However, the CO_2-sensing mechanism in guard cells that is responsible for the short-term sensitivity of g_s to elevated CO_2 is still unknown (Ainsworth and Rogers, 2007). In our experiment, EC decreased g_s slightly. Interestingly, unstressed plants tend to express a rather conservative range of C_i during steady-state photosynthesis, C_i is generally maintained at 0.7 C_a (concentration of ambient CO_2) even when C_a is varied (Drake and Gonzàlez-Meler, 1997). In our experiment, C_i of leaves under EC was generally higher than under ambient CO_2 treatments, that means perhaps change in C_i is closely related to C_a, and it also may be the reason of why g_s was decreased by EC.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Shifts in stomatal function and morphological response to CO_2 are likely to affect transpiration rates under rising atmospheric CO_2. In our study, reduction of stomatal aperture and conductance (g_s) explains the reduction in T_r observed in plants grown in EC.

Stomata display a wide range in short-term behavioral and long-term morphological responses to atmospheric CO_2 concentration (Haworth et al., 2013). In the short term, stomatal aperture generally decreases in response to high CO_2, as described earlier. In the long term, decreases in g_s can be caused by changes in stomatal density, as well as stomatal aperture (Ainsworth and Rogers, 2007), in the other hand, changes in leaf stomatal densities which in turn can control maximum values of stomatal conductance.

Ainsworth and Rogers' study (2007) showed that elevation of CO_2 in FACE experiments reduced stomatal conductance by 22%, yet this reduction was not associated with a similar change in stomatal density. However, some researchers believe that plants adapt to the elevated CO_2 by reducing their stomatal density (that is, the number of stomata per unit of epidermal surface area) (Engineer et al., 2014). One expectation at increased C_a is that fewer stomata are required because the rate of CO_2 diffusion into the leaf will be a decreasing limitation to photosynthesis as C_a rises (Drake and Gonzàlez-Meler, 1997). While in our experiment, there were no difference in stomatal density of mature *Q. mongolica* leaves detected among all treatments on 60th day (in the first season), but at the end of the second season (210th day), a significant decrease in stomatal density in response to EC was shown. Perhaps high CO_2 was detected by mature leaves in the first year and signaled to developing leaves in the next year, whose stomatal developments altered accordingly (Lake et al., 2001). Researchers believed that those species with little or no control of stomatal aperture (termed passive) to C_a were more likely to exhibit a reduction in stomatal density than species with active stomatal control when grown in atmospheres of elevated CO_2 (Haworth et al., 2013), that is perhaps why there was only a slight decrease in g_s of leaves detected in our experiment.

Modification to stomata abundance and size as a stress response is also stated an important pollutant absorption-controlling mechanism, and these parameters mutually affected each other. An inverse relationship between stomatal pore length or size and stomatal density has been observed in the gas exchange responses of plants in response to increased CO_2. However, a similar pattern was not observed in the species analyzed in Haworth's study (2013). While in our study, we can only see a slight increase in stomatal width of leaves in EC on 60th day ($P < 0.05$).

Effects of elevated CO_2 and O_3

It is concluded that season-long exposure to elevated CO_2 had a greater effect on photosynthetic parameters than elevated O_3 (Alison et al., 2001), while this result is opposite to ours. When high O_3 and high CO_2 were given in our experiment, P_n of leaves was still lower than control in the whole growing season except at the end of the season. It is also found that the combination of elevated O_3 and CO_2 can led to a significant decrease in the light-saturated rate of photosynthesis, but compared to elevated O_3 alone, the level of decrease was lower (Kellomäki and Wang, 1997). The study of the combined effects of elevated CO_2 and O_3 on photosynthesis show that elevated CO_2 compensated most of the adverse effects of O_3 (Reid and Fiscus, 1998). In our study, P_n of *Q. mongolica* leaves under EO+EC were nearly all significantly higher than EO in the whole seasons ($p < 0.05$). This suggests that O_3-induced adverse effects

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42574268

to photosynthesis can be ameliorated, at least partly, by high CO_2, but the complete mechanism behind this interaction is, however, still unclear.

There was no significant difference of g_s between the combination and EO treatments except after 60 days in the second season, which was mainly caused by EO but not EC.

It is found there was a significant interaction between elevated CO_2 and increased O_3, elevated $O_3 + CO_2$ led to a 14% decrease in F_v/F_m compared to the control (Kellomäki and Wang, 1997). While in this experiment, there was no significant difference in F_v/F_m detected under the combined treatment, compared to the control. High CO_2 can largely relieve the adverse effects of high O_3 to PSII reaction center.

Changes in stomatal parameters of *Q. mongolica* leaves under EO+EC were just like the changes of them under only EC exposure. Perhaps the effect of stomatal morphology was mainly caused by EC but not EO.

Conclusion

Q. mongolica is an O_3-sensitive tree species and EO had a severe impact on P_n of *Q. mongolica* leaves because of their young age. g_s and T_r of leaves was significantly decreased by EO at the beginning of the experiment, which indicates that the partial closure of stomata can be induced by EO to avoid the further ozone uptake. The stomata closure maybe associated with the direct effect of EO on guard cells. Under EO, there were decreases in F_0, F_m and F_v/F_m at the end of the season (after 75 days). This indicates EO can limit the capability of the plant to use photon energy and thus alter photosynthetic processes; EC generally increased P_n of *Q. mongolica* leaves, but decreased g_s slightly. The decrease of g_s is thought to maintain the ratio of C_i and C_a, and regulate the WUE of trees. A significant decrease in stomatal density in response to EC was shown only at the end of the second season (210th day) but not in the first season. Maybe, EC was detected by mature leaves in the first growing season and signaled to developing leaves in the next growing season, whose stomatal developments altered accordingly; Under EO+EC, P_n of leaves were still lower than that control, but nearly all significantly higher than EO in the whole seasons ($p < 0.05$). This suggests that O_3-induced adverse effects to photosynthesis can be ameliorated, at least partly, by CO_2. The decrease in g_s was mainly caused by EO but not EC. There was no significant difference in F_v/Fm detected under EO+EC, compared to control. EC can largely relieve the adverse effects of EO to PSII reaction center. Changes in stomatal parameters of leaves under EO+EC were just like the changes of them under only EC. Perhaps the effect of stomatal morphology was mainly caused by EC, but not EO.

Acknowledgements. The authors wish to thank Prof. Tao for his help in revision of manuscript. This work was founded by the National Natural Science Foundation of China (31600314) and Major Incubating Project of Shenyang Normal University.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42574268

EFFECTS OF DIFFERENT WATER MANAGEMENT METHODS ON SEEDING RATE, PHENOLOGICAL AND YIELDING PROPERTIES OF DIFFERENT RICE CULTIVARS (*ORYZA SATIVA* L.)

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(Received 25th Dec 2018; accepted 8th Feb 2019)

Abstract. Flooding management is an effective and environmental-friendly way to reduce weed populations in paddy fields. However, flooding would also cause waterlogging in direct seeding rice production. The present study was conducted to explore the effect of different water management methods at the germination stage on rice growth and yield, with six rice varieties as materials. 0, 1, 2, and 3 cm water layer treatments at germination stage were set and named as I0, I1, I2 and I3, respectively. The result showed that I1 and I2 treatments significantly reduced seedling rate and seedling quality compared to I0. The highest yield was recorded during I0, while the yield in the case of I3 treatment was lower than the former but higher than I1 and I2. Similar trends were also recorded in dry matter weight at heading stage and maturity stage.
Keywords: *rice, water condition, seedling rate, seedling quality, yield, dry matter accumulation*

Introduction

Rice (*Oryza sativa* L.), as one of the main stable food crops, feeds more than half of the world's population (Ashraf et al., 2017). Pan et al. (2016) demonstrated that more than 60% of the population live on rice in China while rice makes up 40% of the country's total grain. It is very important to maintain the rice productivity and improved the yield potential of rice in China.

In Chinese history, transplanted rice production system has been the main rice production modern for a very long time (CabangonTuong and Abdullah, 2002). Traditionally, rice seedlings were transplanted in puddling condition. However, with the severe labor scarcity and low efficiency due to laborious nature of work and lower net returns, transplanted rice production system is becoming more and more unsuitable for Chinese agriculture (Tao et al., 2016). Direct-seeded rice, as a substitutive rice growing technique, has the benefit to reduced production costs, labor and extra efforts for nursery raising, seedling uprooting and transplanting (Liu et al., 2015). Pan et al. (2017) indicated that this new rice production system not only favors earlier crop establishments, but also provides an opportunity to make better use of early season rainfall, and finally leading

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_42694279

towards increase the grain yield. Nevertheless, direct-seeded rice production system still has many parts no perfect enough.

Water management is an important part in rice production which could greatly affect rice yield and quality. The study of Kong et al. (2017) revealed that short term water management at filling stage was able to improve antioxidant enzyme activity and alleviate the damage from heat stress to rice. Ren et al. (2017) also demonstrated that feebly arid conditions (water potential of - (15 ± 5) kPa) at booting stage could increase 2-acetyl-1-pyrroline content in fragrant rice grains significantly. Normally, pre-germinated seeds would be hill-seeded in paddy filed in direct-seeded rice production system. The water management during the germination stage would be important for seedling growth and development. The research of Luo et al. (2018) showed that water layer higher than 4.5 cm would significantly inhibit the germination and seedling growth and decreased the antioxidant enzyme activity. However, there was no much report about the water management during the germination stage in direct-seeded rice production.

Therefore, present study was conducted in Experimental Research Farm, College of Agriculture, Yangtze University in order to investigate the effect of different water conditions at germination stage on rice growth and development.

Materials and methods

Seed materials

Seeds from six rice cultivars, *Wandao107*, *Liangyou168*, *Fengliangyouxiang-1*, *Fengliangyou-2*, *Guofeng-1*, *Quanyou801* were used as the experimental materials in this pot experiment. Those rice varieties were all widely grown in Middle China.

Treatment description and growth conditions

Before sowing, the seeds were soaked in water for 24 h, germinated in manual climatic boxes for another 10 h (33 °C), then shade-dried. Then, seeds were direct-seeded into soil containing plastic pots (30 cm in height and 450 cm^2 in surface area) and 50 seeds for each pot in triplicate. The different water managements were applied after the sowing and sustained for 25 days. The water layer treatment description is as below:

I0: Maintenance of 0 cm water layer at soil surface throughout day and night.

I1: Maintenance of 1 cm water layer at soil surface throughout day and night.

I2: Maintenance of 2 cm water layer at soil surface throughout day and night.

I3: Maintenance of 3 cm water layer at soil surface throughout day and night.

15 days after the sowing, the seedling rate was recorded and at 25 days after the sowing, twenty seedlings were collected randomly from each pot for the estimation of dry matter, seedling length, leaf surface area and chlorophyll content. Chlorophyll content was determined by using the SPAD meter ‘SPAD-502’ (Konica Minolta, Japan). Leaf area of all green leaf blades was measured with Li-Cor area meter (Li-Cor Model 3100, Lincoln, NE). Then, ten seedlings were retained for each pot and every pot in the experiment was remained at 3 cm water layer at soil surface until harvest. The experiment soil was sandy loam consisting 23.78 g/kg organic matter, 1.61 g/kg total nitrogen, 78.66 mg/kg available nitrogen, 0.87 g/kg total phosphorus, 21.79 mg/kg available phosphorus, 17.64 mg/kg total potassium, 151.09 mg/kg available potassium and 6.48 soil pH. The environment of climatic box were set as: 33 °C days and 27 °C nights, under 1200X yellow light intensity and 75% humidity.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_42694279

Estimation of dry matter accumulation transportation

At heading stage and maturity, ten rice plants were collected for the determination of dry matter weight. The leaves, stem-sheaths and grains were separated from the plants and dried under the condition of 80 °C respectively in order to get the estimation of dry matter. Some parameters were calculated as below:

$$\text{Transportation rate in leaf} = \frac{\text{leaf weight at heading stage} - \text{leaf weight at maturity stage}}{\text{leaf weight at heading stage}}$$

$$\text{Transportation rate in stem} - \text{sheath} = \frac{\text{stem} - \text{sheath weight at heading stage} - \text{stem} - \text{sheath weight at maturity stage}}{\text{grain weight at maturity stage}}$$

$$\text{Contribution rate in leaf} = \frac{\text{leaf weight at heading stage} - \text{leaf weight at maturity stage}}{\text{grain weight at heading stage} - \text{grain weight at maturity stage}}$$

$$\text{Contribution rate in stem} - \text{sheath} = \frac{\text{stem} - \text{sheath weight at heading stage} - \text{stem} - \text{sheath weight at maturity stage}}{\text{grain weight at heading stage} - \text{grain weight at maturity stage}}$$

Determination of yield and yield related attributes

At maturity, twenty random rice plants were harvested and threshed manually and sun dried (adjusted to ~14% moisture contents) to get the grain yield per pot and expressed in grams per plant (g $plant^{-1}$). Grains were separated manually from each panicle to count total number of grains and number of filled grains per panicle. To record 1000-grain weight, six random samples from filled grains were counted, weighed and averaged. Panicle number per pot was measured by counting the panicle numbers of each plant in three pots in each treatment and averaged.

Statistical analysis

This study was managed as a randomized complete block design with three replicates (n = 3). Data were analyzed on Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) at the probability level of 5% ($P < 0.05$). Differences among means were separated by using least significant difference (LSD) test.

Results

Seedling rate

As shown in *Table 1*, different water management affected seedling rate of different rice varieties differently. The highest seedling rate was recorded in I3 condition. Compared to other rice varieties, *Wandao107* had the highest seedling rate which was 67.50%. The lowest seedling rate was recorded in I1 treatment while the seedling rate in *Liangyou168* was lower than other rice varieties.

Seedling quality

There were some differences in seedling quality of different varieties under different water managements (*Table 2*). Compared to I0, I1 treatment significantly reduced leaf area and SPAD values while there was no remarkable difference among I0, I2 and I3 in leaf area and SPAD values. The highest plant height and shoot dry weight were both

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

recorded in I3 and the lowest plant height, root length, shoot dry weight and root dry weight were all recorded in I1.

Dry matter accumulation

As shown in *Table 3*, different water managements at germination stage affected dry matter accumulation differently. At heading stage, the trend of leaf dry weight at heading stage was recorded as I0 > I3 > I2 > I1. Similar conditions were also observed in stem-sheath weight and grain weight. At maturity, the highest dry weight of leaf, stem-sheath and grain were all recorded in I0. There was no significant difference between I2 and I3 in stem-sheath dry weight and total dry weight. Moreover, the lowest dry matter weight was recorded in I1 at both heading stage and maturity.

Table 1. *Differences in survival rates of different varieties under different water layers*

Treatment	Variety	Seedling rate (%)
I0	*Fengliangyou-2*	26.67c
	Fengliangyouxiang-1	35.00b
	Liangyou168	26.67c
	Wandao107	60.00a
	Guofeng-1	45.83a
	Quanyou801	35.83b
	Mean	**36.95**
I1	*Fengliangyou-2*	32.50a
	Fengliangyouxiang-1	39.17a
	Liangyou168	18.33b
	Wandao107	66.67a
	Guofeng-1	38.33a
	Quanyou801	22.50b
	Mean	**31.39**
I2	*Fengliangyou-2*	34.17bc
	Fengliangyouxiang-1	43.33ab
	Liangyou168	27.50c
	Wandao107	40.83ab
	Guofeng-1	49.17a
	Quanyou801	33.33bc
	Mean	**38.06**
I3	*Fengliangyou-2*	40.83bc
	Fengliangyouxiang-1	48.33b
	Liangyou168	33.33c
	Wandao107	67.50a
	Guofeng-1	45.83b
	Quanyou801	20.83d
	Mean	**42.78**

Means in the same column followed by different lower case letters for the same variety differ significantly at $P < 0.05$ by T-test, the same as below

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

Table 2. *Differences in seedling quality of different varieties under different water layers*

Treatment	Variety	Plant height	Root length	Leaf area	SPAD value	Shoot dry weight	Root dry weight
I0	*Fengliangyou-2*	32.66ab	13.89b	19.43ab	35.95b	0.239b	0.081bc
	Fengliangyouxiang-1	36.73ab	14.99ab	18.15ab	39.84a	0.266b	0.099bc
	Liangyou168	28.70b	14.38b	13.05b	39.23ab	0.204b	0.073c
	Wandao107	37.20a	18.17ab	19.47ab	39.26ab	0.311ab	0.099bc
	Guofeng-1	39.62a	20.28a	18.39ab	37.76ab	0.394ab	0.124ab
	Quanyou801	40.40a	15.12ab	22.56a	39.23ab	0.462a	0.147a
	Mean	**35.89b**	**16.14a**	**18.51a**	**38.54a**	**0.313b**	**0.104a**
I1	*Fengliangyou-2*	19.23c	8.14a	7.60c	31.98a	0.067c	0.021b
	Fengliangyouxiang-1	19.81c	7.59a	7.46c	32.64a	0.076c	0.019b
	Liangyou168	19.26c	6.84a	3.48d	32.57a	0.054c	0.012b
	Wandao107	35.12ab	12.61b	11.5b	36.95a	0.234ab	0.051b
	Guofeng-1	39.66a	13.87b	16.87a	34.18a	0.320a	0.100a
	Quanyou801	26.74bc	12.51b	10.59bc	32.57a	0.170bc	0.047b
	Mean	**26.64c**	**10.26c**	**9.58b**	**33.48b**	**0.153c**	**0.042c**
I2	*Fengliangyou-2*	40.16a	13.54ab	21.70a	38.3a	0.443a	0.091a
	Fengliangyouxiang-1	38.84a	10.47b	18.83ab	36.94a	0.286a	0.062a
	Liangyou168	32.78a	11.5ab	14.31b	35.71a	0.252a	0.056a
	Wandao107	39.09a	13.4ab	17.03ab	38.82a	0.320a	0.067a
	Guofeng-1	38.97a	16.04a	17.28ab	35.72a	0.334a	0.091a
	Quanyou801	41.24a	14.16ab	21.93a	35.71a	0.484a	0.108a
	Mean	**38.51ab**	**13.18b**	**18.51a**	**36.87a**	**0.353a**	**0.079b**
I3	*Fengliangyou-2*	40.13a	13.76a	19.11ab	39.22a	0.439a	0.076a
	Fengliangyouxiang-1	42.99a	14.86a	24.41a	36.94ab	0.437a	0.076a
	Liangyou168	36.06a	13.01a	20.28ab	39.75a	0.342a	0.060a
	Wandao107	44.61a	16a	23.83a	37.07ab	0.497a	0.098a
	Guofeng-1	35.36a	15.44a	14.10b	32.33b	0.317a	0.069a
	Quanyou801	44.82a	15.31a	22.4ab	39.75a	0.473a	0.097a
	Mean	**40.66a**	**14.73ab**	**20.69a**	**37.51a**	**0.418a**	**0.079ab**

Matter transportation and contribution during the filling stage

As shown in *Table 4*, different water managements during the germination stage affected the matter transportation during the filling stage differently. The leaf transportation rate in I0 was significantly higher than I2 and I3 which were 0.394, 0.374 and 0.372 while there was no significant difference among I0, I1, I2 and I3 in leaf contrition. The lowest stem-sheath transportation rate was recorded in I1 and there was no significant difference among I0, I2 and I3. Furthermore, the highest photosynthesis contribution rate was recorded in I1 and the lowest was recorded in I0.

Yield and related attributes

Different water management during the germination stage affected rice yield by regulating the yield related attributes (*Table 5*). The highest yield was recorded in I0 treatment as well as panicle number, grain number, seed-setting rate and grain weight.

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

There was no significant difference between I2 and I3 in yield, 1000-grain weight, seed-setting rate and grain number. Furthermore, I1 treatment had the lowest yield due to the lowest panicle number, grain number and 1000-grain weight.

Table 3. *Differences in dry matter accumulation of different varieties under different water layers*

Treatment	Variety	Heading stage				Maturity			
		Leaf dry weight	Stem-sheath dry weight	Grain dry weight	Total dry weight	Leaf dry weight	Stem-sheath dry weight	Grain dry weight	Total dry weight
I0	*Fengliangyou-2*	1.023d	1.830e	0.750e	3.603e	0.633cd	1.723d	2.140c	4.497e
	Fengliangyouxiang-1	1.112c	1.937d	0.770d	3.819d	0.667c	1.820c	2.203bc	4.690d
	Liangyou168	0.826e	1.743f	0.677d	3.246f	0.517e	1.620e	2.013d	4.150f
	Wandao107	1.124c	2.127c	0.813c	4.064c	0.620d	2.003b	2.303b	4.927c
	Guofeng-1	1.203b	2.237b	0.920b	4.360b	0.740b	2.080ab	2.407ab	5.227b
	Quanyou801	1.324a	2.330a	1.097a	4.751a	0.830a	2.140a	2.500a	5.470a
	Mean	**1.102a**	**2.034a**	**0.838a**	**3.974a**	**0.668a**	**1.898a**	**2.261a**	**4.827a**
I1	*Fengliangyou-2*	0.626e	1.243d	0.517d	2.386d	0.387d	1.223d	1.523bc	3.133d
	Fengliangyouxiang-1	0.631e	1.147e	0.413e	2.191e	0.393d	1.100e	1.393c	2.887e
	Liangyou168	0.670d	1.107e	0.400e	2.177e	0.347e	1.047e	1.407c	2.800e
	Wandao107	0.832b	1.677b	0.620b	3.129b	0.533b	1.567b	1.593b	3.693b
	Guofeng-1	1.246a	2.080a	0.850a	4.176a	0.767a	2.050a	2.093a	4.910a
	Quanyou801	0.725c	1.530c	0.590c	2.845c	0.487c	1.473c	1.507bc	3.467c
	Mean	**0.788d**	**1.464d**	**0.565d**	**2.817c**	**0.486d**	**1.410c**	**1.586d**	**3.482c**
I2	*Fengliangyou-2*	0.942c	1.777b	0.727ab	3.446b	0.623a	1.650ab	2.107a	4.380ab
	Fengliangyouxiang-1	1.172a	1.327f	0.577c	3.075d	0.613a	1.280c	1.703d	3.597c
	Liangyou168	0.758e	1.473c	0.627c	2.858f	0.510c	1.407c	1.803cd	3.720c
	Wandao107	0.726e	1.523d	0.690b	2.939e	0.500c	1.433bc	1.903bc	3.837c
	Guofeng-1	0.879d	1.637c	0.700b	3.216c	0.570b	1.537b	2.003ab	4.110bc
	Quanyou801	1.136b	1.890a	0.787a	3.812a	0.637a	1.747a	2.107a	4.490a
	Mean	**0.936c**	**1.604c**	**0.684b**	**3.224b**	**0.576b**	**1.509b**	**1.938b**	**4.022b**
I3	*Fengliangyou-2*	0.940d	1.520b	0.610b	3.070d	0.603b	1.463b	1.800b	3.867
	Fengliangyouxiang-1	1.325a	1.420b	0.560c	3.305c	0.743a	1.387b	1.750b	3.88
	Liangyou168	0.744e	1.307c	0.530c	2.581f	0.503c	1.223c	1.703b	3.43
	Wandao107	1.123b	1.807a	0.753a	3.683a	0.657b	1.777a	2.103a	4.537
	Guofeng-1	0.737e	1.500b	0.603b	2.840e	0.503c	1.400b	1.787b	3.69
	Quanyou801	1.024c	1.790a	0.760a	3.574b	0.637b	1.713a	2.117a	4.467
	Mean	**0.982b**	**1.557b**	**0.636c**	**3.175b**	**0.608c**	**1.494b**	**1.877c**	**3.978b**

Table 4. *Differences in matter transportation of different varieties under different water layers*

Treatment	Variety	Transportation rate in leaf	Contribution rate in leaf	Transportation rate in stem-sheath	Contribution rate in stem-sheath	Contribution rate of photosynthesis
I0	*Fengliangyou-2*	0.381b	0.280b	0.058a	0.076b	0.643ab
	Fengliangyouxiang-1	0.401b	0.312ab	0.060a	0.082ab	0.606ab
	Liangyou168	0.375b	0.232c	0.071a	0.092ab	0.676a
	Wandao107	0.448a	0.338a	0.058a	0.082ab	0.579bc
	Guofeng-1	0.384b	0.311ab	0.070a	0.105ab	0.584b
	Quanyou801	0.373b	0.352a	0.082a	0.135a	0.512c
	Mean	**0.394a**	**0.304a**	**0.067a**	**0.095a**	**0.600b**

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

I1	*Fengliangyou-2*	0.382b	0.240b	0.016a	0.019b	0.742a
	Fengliangyouxiang-1	0.377b	0.244b	0.041a	0.050ab	0.707a
	Liangyou168	0.483a	0.322a	0.054a	0.061ab	0.618b
	Wandao107	0.359bc	0.307bc	0.066a	0.113a	0.580b
	Guofeng-1	0.385b	0.385b	0.014a	0.025b	0.589b
	Quanyou801	0.329c	0.263c	0.037a	0.066ab	0.671ab
	Mean	**0.386ab**	**0.294a**	**0.038b**	**0.055b**	**0.651a**
I2	*Fengliangyou-2*	0.338bc	0.231c	0.071a	0.092a	0.677b
	Fengliangyouxiang-1	0.477a	0.500a	0.035a	0.041a	0.459a
	Liangyou168	0.327bc	0.211c	0.046a	0.056a	0.733a
	Wandao107	0.311c	0.186c	0.059a	0.074a	0.740a
	Guofeng-1	0.352b	0.238c	0.061a	0.083a	0.679a
	Quanyou801	0.439a	0.383b	0.076a	0.114a	0.503b
	Mean	**0.374b**	**0.292a**	**0.058ab**	**0.077ab**	**0.632ab**
I3	*Fengliangyou-2*	0.358cd	0.283c	0.037a	0.048a	0.670a
	Fengliangyouxiang-1	0.439a	0.489a	0.024a	0.028a	0.484b
	Liangyou168	0.324d	0.208d	0.064a	0.073a	0.720a
	Wandao107	0.415ab	0.347b	0.017a	0.023a	0.630a
	Guofeng-1	0.317d	0.199d	0.066a	0.089a	0.712a
	Quanyou801	0.378bc	0.287c	0.041a	0.064a	0.649a
	Mean	**0.372b**	**0.302a**	**0.041ab**	**0.054b**	**0.644ab**

Table 5. *Differences in yield and related attributes of different varieties under different water layers*

Treatment	Variety	Panicle number per plant	Grains number per panicle	Seed-setting rate (%)	1000-grain weight (g)	Yield (g plant^{-1})
I0	*Fengliangyou-2*	15.30ab	298.55a	0.93a	26.61c	32.50c
	Fengliangyouxiang-1	11.20b	172.19b	0.90a	25.59d	24.65f
	Liangyou168	13.50ab	225.05b	0.92a	27.13b	27.17d
	Wandao107	17.40a	195.43b	0.93a	27.11b	40.12a
	Guofeng-1	11.10b	204.41b	0.91a	26.46c	26.67e
	Quanyou801	14.10ab	190.78b	0.91a	27.61a	35.13a
	Mean	**13.80a**	**214.40a**	**0.92a**	**26.7506a**	**31.04a**
I1	*Fengliangyou-2*	6.90a	221.11a	0.92ab	26.24c	12.32d
	Fengliangyouxiang-1	8.00a	117.74d	0.92ab	25.49d	14.36bc
	Liangyou168	8.20a	145.29c	0.84c	26.67b	13.98c
	Wandao107	8.80a	158.46bc	0.88bc	26.63b	18.46a
	Guofeng-1	7.00a	172.74b	0.9ab	25.98c	12.70d
	Quanyou801	7.20a	177.78b	0.93a	27.57a	15.15b
	Mean	**7.70c**	**165.52c**	**0.9ab**	**26.4317c**	**14.50c**
I2	*Fengliangyou-2*	7.30a	250.54a	0.9a	26.20c	15.8967a
	Fengliangyouxiang-1	7.80a	139.59c	0.84a	25.89d	13.20d
	Liangyou168	7.40a	147.11bc	0.88a	26.94b	13.39d
	Wandao107	8.00a	160.45bc	0.85a	26.78b	15.22b
	Guofeng-1	7.10a	186.85b	0.89a	26.26c	14.15c
	Quanyou801	7.70a	180.85bc	0.86a	27.39a	16.11a
	Mean	**7.60c**	**177.57bc**	**0.87b**	**26.5772b**	**14.66b**

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

I3	*Fengliangyou-2*	13.30a	269a	0.84a	26.43c	22.65a
	Fengliangyouxiang-1	9.90ab	192.15b	0.86a	25.29d	15.82d
	Liangyou168	9.90ab	147.09cd	0.88a	26.93b	15.79d
	Wandao107	8.20b	127.7d	0.89a	26.93b	14.80e
	Guofeng-1	8.70b	180.22bc	0.91a	26.25c	19.91c
	Quanyou801	12.50ab	182.39bc	0.85a	27.73a	21.25b
	Mean	**10.40b**	**183.09b**	**0.87b**	**26.5928b**	**18.37b**

Correlation analysis

As showed in *Table 6*, the rice yield had significant positive correlation with root length, leaf area, SPAD value and root dry weight of seedling. There also exited a significant positive correlation between panicle number and SPAD value of seedling. Furthermore, the dry matter weight of leaf, stem-sheath and grain at both heading stage and maturity had significant positive correlation with seedling quality (height, root length, leaf area, SPAD value, shoot dry weight and root dry weight).

Table 6. *Relationship among seedling quality, yield and dry matter weight*

		Height	Root length	Leaf area	SPAD value	Shoot dry weight	Root dry weight
Panicle number per plant		0.155	0.281*	0.260*	0.371**	0.132	0.220
Grains number per panicle		0.107	0.210	0.205	0.164	0.172	0.218
Seed-setting rate (%)		-0.218	0.110	-0.121	-0.162	-0.118	0.066
1000-grain weight (g)		0.060	0.141	0.077	0.141	0.125	0.153
Yield (g plant^{-1})		0.148	0.453**	0.259*	0.341**	0.152	0.412**
Heading stage	Leaf dry weight	0.616**	0.492**	0.658**	0.307**	0.529**	0.605**
	Stem dry weight	0.485**	0.6320**	0.482**	0.329**	0.440**	0.694**
	Grain dry weight	0.499**	0.592**	0.509**	0.331**	0.459**	0.685**
	Total dry weight	0.569**	0.623**	0.584**	0.347**	0.508**	0.715**
Maturity	Leaf dry weight	0.664**	0.558**	0.688**	0.357**	0.577**	0.659**
	Stem dry weight	0.476**	0.643**	0.462**	0.313**	0.427**	0.669**
	Grain dry weight	0.531**	0.635**	0.557**	0.417**	0.477**	0.663**
	Total dry weight	0.558**	0.658**	0.567**	0.383**	0.498**	0.699**

As shown in *Table 7*, there exited a significant positive correlation between seed-setting rate and stem-sheath dry weight. Both panicle number and yield had significant positive correlation with dry weight of leaf, stem and grain. Moreover, there was a significant positive correlation between grain number and stem-sheath dry weight.

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DOI: http://dx.doi.org/10.15666/aeer1702_42694279

Table 7. *Relationship between dry matter weight at heading stage and yield and yield related attributes*

	Panicle number per plant	**Grain number per panicle**	**Seed-setting rate (%)**	**1000-grain weight (g)**	**Yield (g plant^{-1})**
Leaf dry weight	0.311**	0.137	-0.043	-0.056	0.396**
Stem-sheath dry weight	0.416**	0.261*	0.233*	0.314**	0.657**
grain dry weight	0.361**	0.241*	0.156	0.354**	0.592**
Total dry weight	0.399**	0.234*	0.139	0.223	0.603**

Discussion

It is well known that water condition is one of the most important factors affecting the seed germination. Ismail et al. (2009) demonstrated water layer at 10 cm would greatly inhibit the germination and early seedling growth of rice. The study of Prakash et al. (2016) had evidenced that flooding treatments not only could significantly influenced the survival rate of rice seedling, but also was able to affect all the parameters of seedling quality such as shoot, root and total dry matter production. Present study also showed the similar conditions. Compared to I0, I1 treatment reduced the seedling rate significantly whilst there was no remarkable difference I0 and I3 in seedling rate. The possible reason might be the flooding stress at I3 level could stimulate the mechanisms which were associated with tolerance to flooding for some rice varieties (Zhang et al., 2017). For example, the lowest seedling rates of both *Wandao107* and *Fengliangyouxiang-1* were recorded in I2 while there was no significant difference between I0 and I3. Furthermore, we noticed that there was no much significant difference between I0 and I3 in seedling quality. Some parameters (seedling height and shoot dry weight) in I3 were even higher than I0. It might because the 3 cm water layer conditions would also be suitable for early seedling growth. Similar discovery was reported by Fan et al. (2017) who demonstrated shallow water irrigation during the seedling stage could significant improved seedling quality of fragrant rice.

Seedling quality is one of the factors which could greatly affect the rice yield. Previous study had shown that different seedling age significantly affected yield and economics of hybrid rice by influencing the growth and chlorophyll content (Pramanik and Bera, 2013). The study of Xia et al. (2000) revealed that magnetic treatment was able to up-regulate the rice yield by improving the seedling growth. Normally, it is generally recognized that good quality of rice seedling would be the base for high yield. Our study emphasized this point by showing the significant positive correlation between seedling quality and dry matter accumulation at heading stage and maturity while there exited a significant positive correlation between dry matter production and rice yield. Our founding was also consistent with previous study (Farooq et al., 2006) which stated that seedling establishment is a key part in rice production and good seedling quality could improve the potential of rice yield.

Furthermore, the highest yield was recorded in I0 and the yield in I3 treatment was lower than I0 but higher than I1 and I2. Luo et al. (2018) demonstrated that water layer higher than 3 cm would inhibit germination and growth of weed and volunteer rice. Thus, 3 cm water irrigation might be a management which is able to reduce the weed and volunteer without causing a great yield loss.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_42694279

Conclusion

Compared to I0, I1 and I2 treatments significantly reduced seedling rate, seedling quality and the grain yield. I3 treatment was lower than I0 but higher than I1 and I2. Similar trends were also recorded in dry matter weight at heading stage and maturity stage. Therefore, the 3 cm water irrigation might be considered as a management which is able to reduce the weed and volunteer without causing a great yield loss. More studies should be conducted at the field trials or at molecular level before the real application.

Acknowledgements. This study was supported by Innovation and Application of Key Technologies for Light Simplified Direct Sowing of Rice in the North of the Middle and Lower Reaches of the Yangtze River (2017YFD0301403-06), Research and Demonstration on the Common Key Technologies of Changing Rice into Japonica in Jianghan Plain (2015Z20007) and Key Technological Tackling of Common Characters in Japonica Rice (2015Z20021).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_42694279

INTEGRATING PHOSPHORUS FERTILIZER RECOMMENDATION AND ENVIROMENTAL RISK INDEX OF SOIL TOTAL-P

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(Received 26th Dec 2018; accepted 25th Feb 2019)

Abstract. Phosphorus (P) fertilizer application and increasing soil total P can cause environmental issues. Therefore, in the present study an experiment was carried out with optimal P-fertilizer recommendation based on the environmental aspects for two years (2015 & 2016). In 2015, 90 soil samples were collected from agricultural lands of Iran and their environmental P index (EPI) was estimated. Also, through maize greenhouse cultivation, the available P critical level was determined. Then, P requirement for 90 fields was assessed based on EPI and soil available P. In 2016, through an onsite field experiment, the effects of P fertilizer levels on yield were estimated. The results revealed that the farmers are permitted to apply P fertilizer only at 8 fields out of 90 according to EPI. While based on the soil available P, 36 farms of 90 need to receive P-fertilizer, and the farmers can apply P fertilizer only at 4 fields out of 36 according to EPI. Considering EPI with no P application farmers faced a 23% yield reduction. If the farmers are not satisfied with this reduction, increasing EPI with P application should reduce soil erosion. Using a 50 kg ha^{-1} P_2O_5 application should reduce soil erosion from 7 to 2 ton ha^{-1}.
Keywords: *maize, P-accumulation, total phosphorus, P index, P-fertilizer*

Introduction

Phosphorous (P), one of the macro elements essential for plant growth. Therefore, P-fertilizer application is crucial to reach the optimum economical yield. For decades, soil testing has been used for proper fertilization recommendation. In this method, the critical level of available P is considered as the criteria of P-fertilization (Malakouti, 2016). However, based on soil testing, P-fertilizer application may lead to P-accumulation, precipitation and increasing total P that may lead to environmental problems. It has been proven that most of the P-fertilizers (~80%) were precipitated in acidic soils (due to fixation by iron and aluminum oxides) and in calcareous soils (due to fixation by calcium carbonate) (Leytem and Mikkelsen, 2005). Recent studies have shown that soil total P is increased by long term P-fertilizer application in many countries (Li et al., 2015; Wei-Xia et al., 2015; Wang et al., 2015; Powers et al., 2016). For instance, Sun et al. (2015) reported that on average about 1.1 mg P-available per kg soil had been added to some Chinese soils annually in the 1997-2002 period. They also showed that the total P increased from 702 to 840 mg kg^{-1} from 1983 to 2002. Xiaoying et al. (2015) showed that changes in soil Olsen-P follow the application of P fertilizer, so that each 100 kg P ha^{-1} surplus increase the Olsen-P by 1~6 mg P kg^{-1}. Their results also indicated that soil organic matter and pH have important effects on the changes in soil Olsen-P by 1 kg ha^{-1} of P balance.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

When soils' P increases, soil water erosion and runoff may able to transfer it into the surface water, rivers, lakes, etc. This may lead to bio-environmental issues such as cultural eutrophication (Sharpley et al., 2003; Bolster et al., 2014). Cultural eutrophication is the process that speeds up natural eutrophication because of human activity. Natural eutrophication is when a body of water becomes overly enriched with minerals and nutrients that induce excessive growth of plants and algae (Schindler, 2012). Surveys showed that 54% of lakes in Asia are eutrophic; in Europe, 53%; in North America, 48%; in South America, 41%; and in Africa, 28% (Ansari et al., 2010). On top of that based on scientific reports, in the region with enriched drinking water by P, the health of habitants is in risk e.g. neurological disorders (Yang et al., 2008). Fataei (2010) reported results from a laboratory experiment designed to study pollution condition of the Aras River in Moghan. Agricultural lands around the Aras River in Moghan are intensively farmed, and P pollution to surface waters and Aras River via surface runoff resulting from application of P-fertilization is a major concern. He has reported that the concentration of phosphorus has increased from 0.10 mg L^{-1} at the beginning of the river to 0.44 mg L^{-1} at the end of the river. Stephen et al. (2016) investigate long-term accumulation and transport of anthropogenic phosphorus in three river basins. They presented an analysis of P fluxes in three rivers, including harvested crops, published data on P-fertilizer, sewages and river fluxes. Their analysis show that the magnitude of P accumulation has varied over the past 40-70 years in mixed agricultural–urban landscapes of the Thames Basin, UK, the Yangtze Basin, China, and the rural Maumee Basin, USA. They demonstrated that human-dominated river basins may undergo a prolonged but finite accumulation phase when P inputs exceed agricultural demand.

Heretofore environmental scientists of developed countries have been used and established indexes and tough rules and regulations for environmental protection purposes (Bolster, 2011). Phosphorus sorption index (PSI) (Sims et al., 2000), the simple soil test (STP) (Nelson and Shober, 2012), the degree of Phosphorus saturation (DPS) (Ige et al., 2005) and environmental P index (EPI) (Nelson and Shober, 2012) have been successfully applied for this aim.

Among these indicators, EPI is a more accurate and complete index due to incorporate into a single index, factors related to soil management and to P-transport (Sharpley et al., 2003). The most recent EPI was presented by Bolster et al. (2014) which was categorized in three levels as low (less than 40), moderate (between 40 to 100) and high (more than 100). Based on this index categories, P-fertilizer application is not allowed if the total P equal or higher than 800, 600 and 400 mg kg^{-1}, respectively. In this method, EPI influenced by various parameters such as the amount of P fertilizer, available P, total P, soil erosion and method of P fertilizer application.

In this index risk, the agricultural yield was not considered and the reduction in biomass has not been taken into account. In contrast, the environmental index risk is not considered by the agricultural engineers and their fertilization recommendations are based on crop yield and response to fertilizer. This indicates the existence of the gap between environmental and agricultural scientist's perspectives.

Overall, as mentioned above, traditional P fertilizer application can cause environmental issues in all over of the world and continuing this trend may increase crop yield and production but may destroy and damage the environment. So, it seems necessary to carry out the research to fill the gap between the two mentioned aspects in which the optimum yield with low environmental risk is achieved.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

The main aim of this study, that is conducted the first time till now, is assessing and investigating P fertilizer recommendation based on the combination of agricultural and environmental aspects. Within this research we are trying to answer the following questions “how would be the yield reduction if the farmers consider environmental risk index comparing to considering only soil testing analysis in P-fertilizer application?” and “if the yield reduction is not the favor of farmers and so they tend to apply maximum P fertilizer for maximum economical yield, what should they do to reduce environmental risk index?”

Materials and methods

At the present study, three experiments including determination of EPI, determination of available-P critical level for maize and field experiment were carried out to optimal P-fertilizer recommendation based on the environmental aspects in two years (2015 & 2016).

The Study area description

This experiment was carried out in 90 fields of Moghan Agricultural lands. Moghan is located at latitudes 39^{O}20'N and 47^{O}30'E with 40-50 m above sea level, in the North-West of Iran (*Figure 1*). The climate of this region is considered as semi-arid with average annual precipitation of 271.2 mm. This region with more than 62024 hectares of cultivated land is surrounded by the Aras river. The river has got thousands of branches, so the eroded soils, sediments and runoff from these lands flow into this river. Aras river provides drinking water to tens of cities and villages and irrigation water to thousands of hectares of land (Nasrabadi et al., 2009).

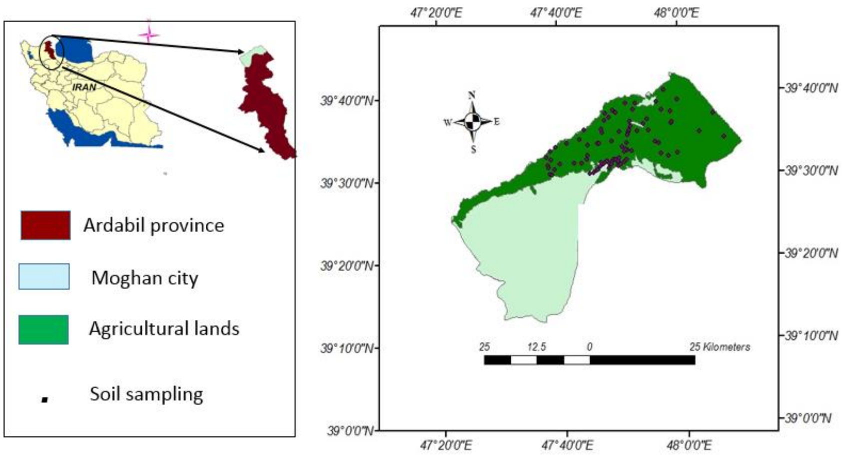

Figure 1. *The location, layout and sampling locations of the study site*

First experiment: determination of EPI for 90 fields and assessment of the P-fertilizer recommendation

In this part of the study, some soil factors such as soil total-P, available-P was obtained from laboratory analysis of soil samples. A number of other factors, including the method and amount of phosphate and organic fertilizers, were asked from farmers, and factors such as soil erosion were estimated from the defined models.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

The EPI, used in this study, was estimated based on the method presented by Bolster et al. (2014). In this section, the generalities are explained briefly. The general formula (*Eq. 1*) for EPI and how to calculate the sub-formulas can be written as:

$$\mathbf{EPI=18\ [(DP_{Soil}+ DP_{Fert}+ DP_{Man})\ BMP_{DP}+ P_{Sed}\ (BMP_{Psed})]} \quad \text{(Eq.1)}$$

DP: Dissolved P; **BMP**: Best management practice; **P_{sed}**: Particulate P loss from erosion; **Fert**: inorganic fertilizers; **Man**: organic fertilizers.

Where all the components are explained in detail as follow:

DP_{Soil} estimation method: DP_{Soil} refers to the part of the dissolved P in runoff released from the soil. The following equation (*Eq. 2*) was used to calculate the DP_{soil}:

$$\mathbf{DP_{Soil}} = 0.1 \times STP \times PX \times RQ \quad \text{(Eq.2)}$$

where STP is the amount of soil P (mg kg^{-1}). **PX** refers to the coefficient of P extraction (µg L^{-1}), obtained from the relation, Y = 1.8X + 78.9, where Y and X are PX (µg L^{-1}) and soil P (mg kg^{-1}), respectively (Vadas et al., 2005; Bolster et al., 2014). RQ is the average annual runoff (cm).

DP_{fert} estimation method: This identifies the role of chemical fertilizers used in runoff dissolved P. The following equation (*Eq. 3*) was used to calculate this factor.

$$\mathbf{DP_{Fert}} = 0.5 \times 0.43 \times P_F \times (RQ \div PT) \times AF_F \quad \text{(Eq.3)}$$

where DF is Amount of fertilizer added (Kg $_{P2O5}$ ha^{-1}). AF_F is related to the time and method of using the fertilizer. It is obtained from the tables provided by Bolster et al. (2014). **PT** refers to the average annual sediment (cm) and is calculated from the following formula (*Eq. 4*).

$$\mathbf{PT} = 38.77\ e^{0.0353\ R} \quad \text{(Eq.4)}$$

in which, R is degree of sedimentation (the total sum of the scores considered in the MPSIAC model), **PT** is annual deposition rate (m^3 km^{-2}) and P_{sed} estimation method: In fact, this factor estimates the particulate P transferred by soil erosion. The following formula (*Eq.5*) was used to calculate it.

$$\mathbf{P_{Sed}} = 10^{-6} \times TP \times SED \times PER \times SDR \quad \text{(Eq.5)}$$

where, **TP** is related to soil total P (mg kg^{-1}) which was obtained in this experiment by alkaline melting method for all the samples. **SED** refers to the annual average soil erosion (kg ha^{-1}), which was estimated by the MPSIC method. **PER** refers to the ratio of soil particle enrichment, which means that fine particles are much richer in P than coarse particles. **SDR** refers to the proportion of sediment delivery and part of the amount of erosion soil that goes to the runoff.

SDR=1 slope length < 3 (m), **SDR** = -0.17* in (slope length) + 0.9 slope length ≥ 3 (m).

In general, in this study, soil erosion estimated by MPSIC method. Modified Pacific Southwest Inter-Agency Committee (MPSIC) uses nine factors including geology, soil, climate (including isotherm and isohyets), runoff, topography (including elevation and land slope), land cover, land use, upland erosion, and channel erosion. The values of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

these factors are ranked based on the corresponding tables. Each of these factors is to be ranked based on a visual appraisal of the watershed. Generally, this model has been widely applied at a watershed scale in arid and semiarid regions of the USA and was assessed for its applicability to Iranian watershed environments (Bagherzadeh and Daneshvar, 2013). In *Figure 2*, a part of the water erosion is observed in the studied area.

Figure 2. *Flooding of river and eroded soils from the studied area*

BMP_{DP} and **BMP_{Psed}**: are based on tables provided by Bolster et al. (2014). In fact, these are related to any land conservation operation that can reduce DP (DP_{Soil}+DP_{Fert}+DP_{Man}) and P_{sed}.

DP_{fert}: Considering that the use of organic fertilizers in this area is negligible, this sector was considered insignificant.

Based on EPI explained above, P-fertilizer recommendation is summarized in *Table 1*.

Table 1. *Recommendation of P-fertilizer based EPI (Bolster et al., 2014)*

Range	Category	Interpretation
0-40	**Low**	If the soil total P is ≥800 (mg kg^{-1}), P- fertilizer should not be used
40-100	**Moderate**	If the soil total P is ≥600 (mg kg^{-1}), P- fertilizer should not be used
>100	**High**	If the soil total P is ≥400 (mg kg^{-1}), P- fertilizer should not be used

In this section of the study, for calculation and better interpenetrating the DOP (Deviation from optimal percentage) equation (*Eq. 6*) (Montanes et al., 1993) can be used as follow:

$$DOP = \left(\frac{Soil\ total\ P}{Maximum\ acceptable\ P} * 100\right) - 100 \quad \text{(Eq.6)}$$

In this equation soil total P and maximum acceptable P were driven based on EPI according to *Table 1*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

Second experiment: determination of available-P critical level for maize and spatial assessment of P-fertilizer requirement

In order to determine a critical level of available-P for maize (SC 704, (MO_{17}×B_{73}), in maturity group of late hybrid with a growth period of 274 days), the greenhouse experiment was carried out. The experimental design was randomized completely plots with two treatments and three replications. Initially, 30 soil samples with significant variation of available and total P were selected and P-fertilizer treatments were applied at two levels of 0 and 100 kg ha^{-1} from super phosphate [$CaH_4(PO_4)_2$ H_2O]. To do so, 180 pots of 10 kg soil were used. The similar amount of other nutritious elements (100 mg kg^{-1} N from urea [$CO(NH_2)_2$] in two times, 50 mg kg^{-1} K of potassium sulfate (K_2SO_4), 20 mg kg^{-1} Mg from magnesium sulfate ($MgSO_4$), 10 mg kg^{-1} Fe from Iron sulfate [$Fe_2(SO_4)_3$], 2 mg kg^{-1} Cu from copper sulfate ($CuSO_4.5H_2O$) and 2 mg kg^{-1} boron from boric acid (H_3BO_3)) were applied to all pots equally.

One day after soil amendment, six corn seedlings (pre-germinated by placing pre-soaked corn seeds on wet paper towels for five days) were transplanted per pot, and then thinned to two after one week. Pots were periodically weighed to maintain soil moisture at 80% field capacity. Corn was harvested after 185 days by cutting above-ground biomass at the soil surface, which was weighed. Then, critical level of available–P was calculated based on Cate and Nelson method (Cate and Nelson, 1971). Finally, the P requirement of 90 fields was compared and assessed with soil available P and measured critical level.

Third experiment: field experiment and yield reduction estimation due to integrating environmental index and fertilizer recommendation

The field experimental design was a randomized complete block design and three replications. Treatments were 0 (the control without P application), 100, 150, 200 and 250 kg ha^{-1} of super phosphate. As mentioned above other nutrients were applied to all plots equally. The size of each plot was 3×4 m in which 300 seeds were planted in each (in 20 cm interval). After harvesting, in addition to measuring P in the plant, the shoot yield of each treatment and environmental index of different treatments were calculated. In *Figure 3*, field experiment of corn cultivation is observed in the studied area.

Figure 3. *Field experiment of corn cultivation*

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Soil Samples laboratory analysis

Soil samples were air-dried and sieved through 2 mm sieve. Soil properties were determined according to Page et al. (1982). Particle size analysis was made using the hydrometer method (Gee and Bauder, 1986). The pH of the soil samples was measured in a 1:1 (w/v) soil to water ratio suspension (McLean, 1982). Organic matter was measured using Walky and Black method (Nelson and Sommers, 1982). The available-P and total-P were measured using the Olsen method (Olsen, 1954; Pasricha et al., 2002) and alkaline melting (Jackson, 1958), respectively.

Investigating spatial variation and the mapping of variables

To assess the spatial variability, first, the most important statistical description and distribution indexes were performed for all soil samples. The mean, min, max, variance, mod, skewness and coefficient of variation of available-P, total-P and EPI were calculated. The out of ranged data has been removed and some properties were logarithmically transformed to have a lognormal distribution. After all, the semivariogram was plotted and the correlation coefficient was evaluated. Statistical and geostatistical analysis have been performed using IBM SPSS (version 20) and GS+(version 5). For the statistical analysis, Analysis of variance (ANOVA) was used in combination with Duncan method at 0.05 significant different tests. The distribution maps of soil properties in the study area were mapped using ArcGIS (version 10.4).

Results and discussion

Results of the first experiment

Determination of EPI for 90 fields and assessment of the P-fertilizer recommendation

The EPI status of 90 fields of the study area is presented in *Figure 4*. The minimum and maximum and average values of EPI were 40, 72 and 55, respectively. According to categories and the recommendation of this index (*Table 1*), most of the fields are classified in medium level in view of risk assessment. In the fields with total-P more than 600 mg kg^{-1}, application of P fertilizer is limited (*Table 1*).

Considering *Eq. 6* shows that if DOP is positive it means soil total P was higher than total P of the environmental index and thus P fertilizer cannot be used in that field (and vice versa). Results of this part of study are shown in *Figure 5*. Results showed the farmers are permitted to apply P fertilizer only at 8 fields of 90 fields according to EPI. In other words, in case of plant requirement, P fertilizer application in current condition causes environmental degradation.

It should be noted that EPI has been introduced for decades in which the first one was presented by Lemunyon and Gilbert (1993). Reports and assessments based on EPI were presented by many environmental scientists. Heathwaite and Sharpley (1999) used the soil available P between 75 to 200 mg kg^{-1} as the critical level for environmental protection and P fertilizer application. This critical level was obtained based on significant statistical relation between available and soluble P (r^2= 0.58 – 0.98). Nair et al. (2004) reported the critical environmental index based on P saturation ratio (molar ratio of P to Fe+Al). Based on statistical relation between dissolved P in runoff and soil P saturation ration, they observed if P saturation ratio become more than a value (normally 0.1), the P concentration in runoff increased by applying P fertilizer.

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These methods are under critics by agricultural scientists. Because these recommendations are based only on environmental parameters and plant growth is not considered. Therefore, in such cases, a method that considers both the agricultural and environmental aspects is worthwhile (Malakouti, 2016).

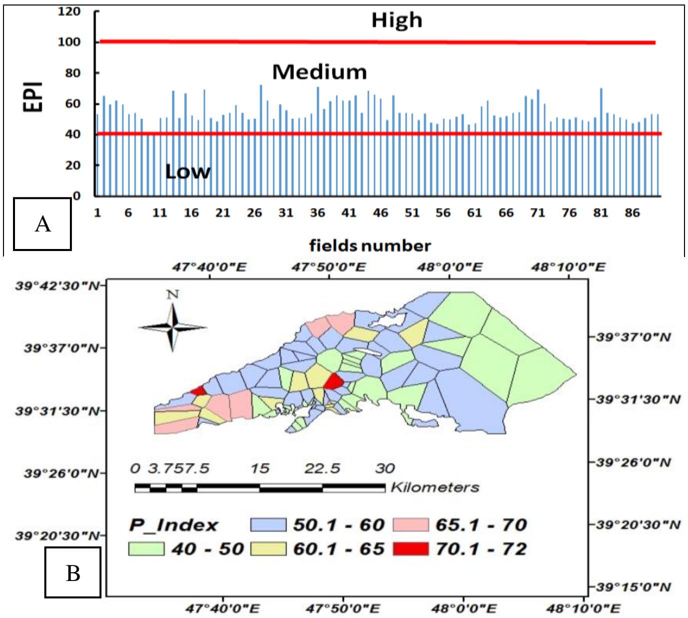

Figure 4. *EPI values in the fields (A) and zoning of agricultural fields based on EPI (B).* ***Low****: 0<EPI<40; If the soil total P is ≥800 (mg kg^{-1}), P-fertilizer should not be used.* ***Medium****: 40<EPI<100; If the soil total P is ≥600 (mg kg^{-1}), P-fertilizer should not be used.* ***High****: 100<EPI; If the soil total P is ≥400 (mg kg^{-1}), P- fertilizer should not be used (Bolster et al. 2014)*

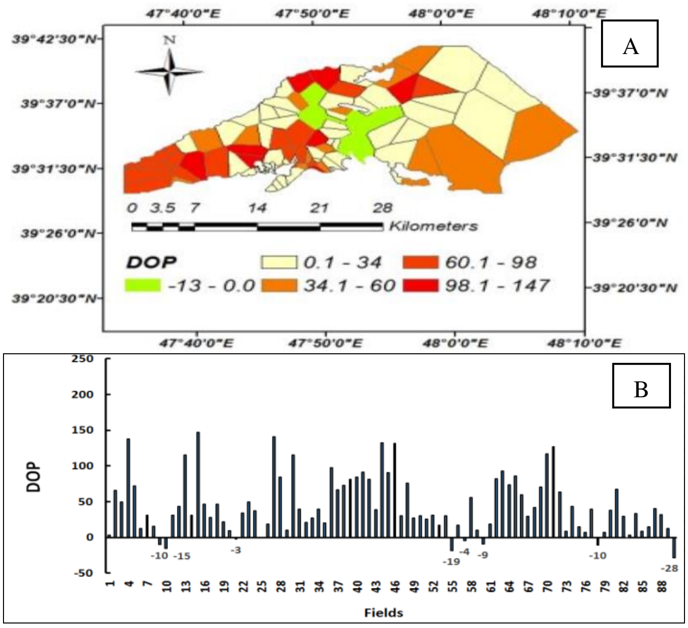

Figure 5. *DOP values in the fields (A) and zoning of agricultural fields based on DOP (B). If* ***DOP*** *<0, farmer can use P-fertilizer; If* ***DOP*** *>0, farmer cannot use P-fertilizer*

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The effects of soil total P and soil water erosion on EPI are indicated in *Figure 6*. According to scientific reports, soil water erosion is very effective in the runoff enrichment potential. So, deflation and some environmental indexes like phosphorus sorption index and degree of phosphorus saturation (because of ignoring the importance of soil erosion) cannot be accurately used to assess the potential of P losses from fields (Sims et al., 2000). Although in the studied area, the annual amount of rainfall is low, but a large part of the annual precipitation occurs in four months when the land is free of vegetation cover. This factor and other factors such as heavy texture, low organic matter, and low permeability caused high soil water erosion. Also, in this area, wind erosion (deflation) is less important because of the low wind speed. In general, the amount of phosphorus in the river is more affected by water erosion. Because, in the water erosion, all the eroded soil and both forms of phosphorus (dissolved and particulate P) enters the river. The significant correlation between EPI and soil total P and soil erosion indicates that EPI is strongly influenced by these parameters. t is obvious that when soil total P is high, more P can be removed from the field with particles of soil. In this regard, scientists have criticized the accuracy of EPI in which soil total P is not involved (Bolstre et al., 2014).

Overall, from this part of the study, fields that EPI does not allow farmers to use P-fertilizers, was identified. It was also found that a farmer can reduce EPI by controlling soil erosion or reducing soil total P. The important thing is that we must combine these finding with agricultural finding, which we have tried to do with the next experiments of this study.

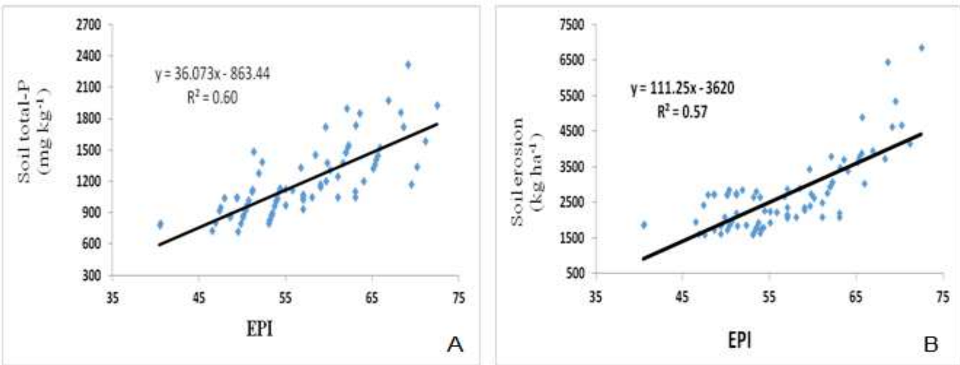

Figure 6. *Relationship between EPI and soil total P (A) and soil erosion (B)*

Investigation of spatial distribution of EPI and total P

Some of the descriptive statistics of data and semivariogram of total P and EPI are shown in *Table 2* and *Figure 7*, respectively. In this kind of experiments, understanding the variation of factors affecting soil total P and EPI are important whether from soil interstice properties (parent material or topography) or management practice (fertilizing) are affecting them. In geostatistics, it can be achieved by calculating spatial dependence of variables. Spatial dependence can be calculated by the effect of the part over the threshold level. If this ration is less than 25, or between 25-75 and higher than 75 so, spatial dependence is considered as high, moderate and week respectively (Chien et al., 1997). In most cases, high spatial dependence is affected by internal processes (parent material, hydrological situation, etc.) and week spatial dependence is affected by

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management practice and fertigation (Cambardella et al., 1994). According to variograms of variables (*Figure 7*), it can be noticed that both semivariograms of soil total P and EPI have the limits and are moderate spatial dependence. So, they are influenced by interstice soil properties and management practices.

***Table 2.** Descriptive statistics of soil total-P and environmental P index data*

Factors	Minimum	Maximum	Average	Middle	Standard deviation	Variance	Skewness	Kurtosis
Soil total-P (kg ha^{-1})	618	2315	1186	1107	331	1.1	0.36	-0.74
EPI	40	72	55.5	53.4	7.2	51.9	0.30	-0.48

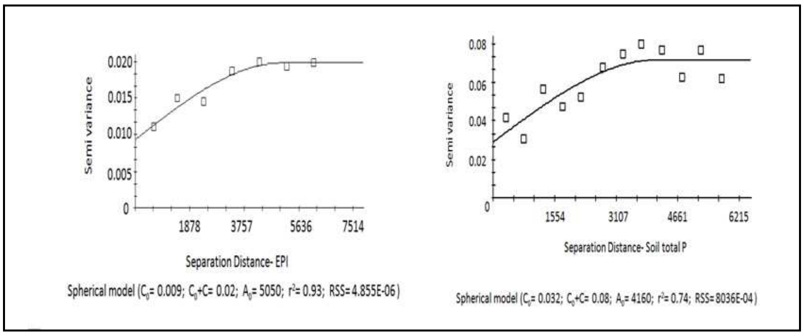

***Figure 7.** Semivariogram models fitted to studied variables (EPI and soil total P)*

Results of the second experiment

Determination of available-P critical level for maize and assessing P requirement for 90 fields

Figure 8 shows available P critical level of study soils (the data for this figure is provided in the *Appendix*). Critical level indicates field requirement to P-fertilizers but does not show the amount of P to be applied. As it is obvious, the critical level of soil available P was 13 mg kg^{-1} using Olsen method. For maize in various locations, different values were reported (Fernandes et al., 2000; Malakouti, 2016). The reasons for the differences may be climate conditions, soil texture, crop yield potential and etc. Results of assessing P requirement for 90 fields based on soil available P critical level are presented in *Figure 9*. Generally, 36 fields have got available P less than 13 mg kg^{-1}. Many years ago, this method was one of the most principled methods of fertilizer recommendation in agriculture and many countries still use it.

Recently, the use of this method has been challenged due to increasing EPI and soil total-P (Gou et al., 2000; Ma et al., 2009; Malakouti, 2016). This method and phosphorus chemistry in the soil causes its accumulation in the soil. Thus, about 20% of the fertilizer that is consumed is absorbed by the plant and the remaining is deposited in acidic (high Al^{2+} and Fe^{3+}) and calcareous (high Ca^{2+}) soils (Marschner, 2011). Many researchers have reported that the use of P fertilizer based on this fertilization method, causes P accumulation in most regions of the world and in the future will restrict farmers in the use of P-fertilizers (Johnston and Steen, 2000; Li et al., 2015; Keke et al., 2016). In this regard, it has been reported that change in the current fertilizers recommendation is necessary because P accumulation subject (increasing EPI) is in contrast to sustainable agriculture (Malakouti, 2016).

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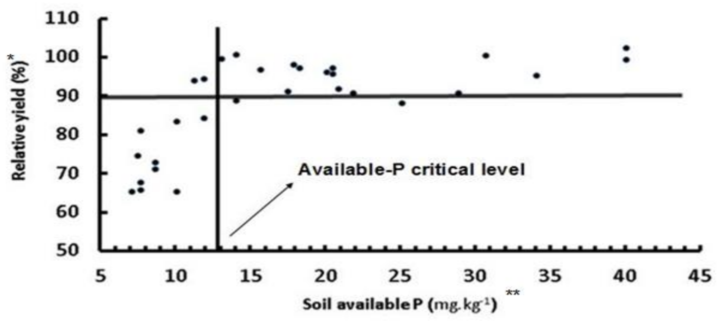

Figure 8. *Determination of available-P critical level by Cate and Nelson Method (1971).*
** Relative yield (%) = (yield of control treatment/yield of fertilizer treatment)*100.*
*** Soil available-P is measured by Olsen method (Olsen 1954)*

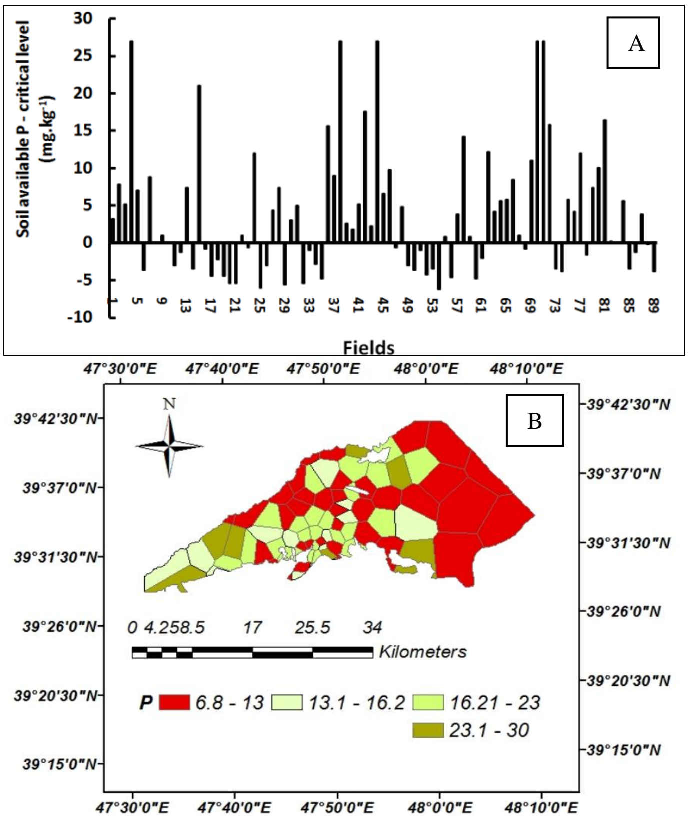

Figure 9. *Available P status of fields as compared to critical level (A) and zoning of agricultural fields based on soil available P (B). If (Soil available P - critical level) <0, P-fertilizer should be used*

Using the results of the first experiment and its match with the results of this part of study can help us.

Considering *Figure 5* in which field were assessed based on EPI, it can be noticed that the farmers can apply P fertilizer only at 4 fields where their DOP are negative. In other fields, P application can causes environmental problems while they need P-fertilizer. Therefore in this case, the fields can be divided into 3 groups. First, the regions where do not need any P fertilizer; second the regions where need P-fertilizer

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and do not have any limitation for P application; and third the region where need for P-fertilizer but the application of P is limited by EPI. When a farmer cannot use P-fertilizer (based on EPI), he can take one of the following decisions:

- The farmer doesn't apply P-fertilizer and accept yield reduction. In this case, the state or the environmental organization must pay subsidies to farmers. Otherwise, it would be hard to recommend environmental indicators and acceptance of financial compensation for farmers.
- The farmer provides possibility of P-fertilizer by reducing EPI.

This issue can be examined in the next results of the present study.

Third experiment

In the field, experiment, available-P and total P were 8.6 and 1025 mg kg^{-1}, respectively. In this field the EPI is 26, in case of without P application and with erosion rate of 7 ton ha^{-1} y^{-1}. According to EPI (*Table 1*) P fertilizer is limited while based on low available P (<13 mg kg^{-1}) P application is needed.

Effects of P fertilizer level on soil total P

According to *Table 3* and the soil P balance (amount of P extract by crop - applied P by fertilization ignoring runoff), 3.8, .24, 1.26, 2.7 and 6.02 mg P was added to each hectare while using 0, 15, 20, 25 and 35 kg P_2O_5 per hectare respectively during growing season. However, these values are not comparable to soil total P. In other words, in the same condition the differences between total P and these values do not change EPI during a growing season. But for longer period fertilization can significantly effect on total P and EPI.

It should be mentioned that reports related to P application on increasing soil total P are based on long period i.e. 20 years. For instance, Sun et al. (2015) in their geostatistics study, investigated long period (20 years) of P accumulation in Chinese agricultural fields. They reported that P was added from 60 to 159 kg ha^{-1} during 1982 until 2002 and soil total P increased from 0.72 to 0.84 gr kg^{-1} (16.7%). Li et al. (2015) investigated long-term (20 years) P fertilizers application on soil available and total P and of rice. Their results showed P application increased soil total P considerably and changed it from 600 to 719 mg kg^{-1}.

Effects of P- fertilizer on yield, EPI and reduce EPI by controlling erosion

Based on *Table 3*, considering EPI and no P application farmers faced with 23% yield reduction. If the farmers with any reason are not satisfied with this reduction, the minimum things to be done is considering the management practice in which EPI is not increased. In other words, increasing environmental index from P application is reduced by other factors like soil total P and soil erosion.

Based on short-term results there is not any option to reduce total P but based on *Table 4* controlling soil erosion can be one of the immediate and effective solutions in protecting environment for farmers. According to *Table 4*, the farmers can reduce EPI by reducing erosion rate from 7 to 4 ton ha^{-1} y^{-1} so they can apply 25 Kg ha^{-1} P_2O_5 without increasing EPI. In the aim of this protocol, for using 50 kg ha^{-1} P_2O_5 the applicant should reduce soil erosion from 7 to 2 ton ha^{-1} where increasing EPI will not occur.

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Bolster (2014) showed that reducing water erosion by conservation practices can reduce 50% of EPI. In this study, in six fields with excessively high (>2.24×1000 kg ha^{-1}) soil erosion rates, soil erosion was reduced by conservation practices such as buffer/filter strip, sediment basin, and water control structure and effect of soil erosion on environmental P index was investigated.

Table 3. *Effects of P-fertilizer level on yield and bill of P in soil*

Treatment	Added-P to soil	Yield (dry weight)	P concentration in plant	Removed-P by plant	Soil P balance	Reduced yield
	(kg ha^{-1})		%	(kg ha^{-1})		%
T_0	0	7381^1	0.15^1	11.32^1	11.32-	-23
T_1	15	8737^2	$0.16^{1,2}$	14.27^2	0.73	-8.8
T_2	20	9359^3	$0.17^{2,3}$	16.22^3	3.78	-2.3
T_3	25	9564^4	0.18^3	16.90^3	8.1	-1.9
T_4	35	9582^4	0.18^3	16.93^3	18.07	0

In each column, similar numbers indicate that meanings do not differ significantly in the 5% level
Soil P balance= amount of P extract by crop - applied P by fertilization ignoring runoff

Table 4. *Interaction of soil erosion and P-fertilizer on P environmental index*

P-fertilizer (kg P_2O_5 ha^{-1})	Soil erosion (kg ha^{-1})							
	0	1000	2000	3000	4000	5000	6000	7000
0	1	6	10	14	17	20	23	**26**
25	9	15	19	22	**25**	28	31	34
50	17	23	**27**	31	34	37	40	43
75	**26***	32	36	40	43	47	50	53
100	35	41	45	48	51	54	57	60

* Bold numbers are the minimum amount of EPI that farmer should avoid increasing it by appropriate ratio of P-fertilizer and soil erosion

Conclusions

Results of this study showed that minimum, maximum and mean of the EPI are 40, 72 and 55, respectively. According to categories and recommendations of the environmental index used in this study, only 8 fields of 90 fields are allowed to apply P fertilizers.

The soil available P of 36 fields was less than 13 mg kg^{-1} (critical level of available P) and P fertilizer application is crucial. But based on EPI, P fertilizer can be applied only at 4 fields of 36 fields. P application can be depredated environment for other fields.

In the meanwhile, without P application, yield reduction will be about 23%. If the farmers with any reason are not satisfied with this reduction, the minimum things to be done is considering the management practice in which EPI is not increased. In the other words, increasing EPI from P application is reduced by other factors like soil total P and soil erosion.

Results showed controlling soil erosion can be one of the immediate and effective solutions in protecting the environment for farmers.

It should be noted that the cumulative increasing of soil total P cannot be ignored. Because in short-term (one growing season) P application may not increase total P significantly, but in the longer period it can be influenced on to EPI.

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Finally, the uncertainty of the results for other region is obvious, but each researcher by applying this methodology can recommend fertilizer application more precisely and multi-purposes considering agro-environmental.

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APPENDIX

Table A1. *Physical and chemical characteristics of soils used in potting and the effective of P treatments on yield*

Soil	Total P	Ava. P	pH	O.C	Clay	Yield		Relative yield %
	mg kg^{-1}		(1:2)	%		T_0	T_1	
1	800	7.0	8.1	1.47	40	35.0	53.7	65
2	883	7.4	7.8	0.57	35	36.1	48.5	74
3	800	7.6	7.8	2.42	50	35.9	53.2	67
4	875	7.6	7.8	1.27	43	35.2	53.6	66
5	1248	7.6	7.7	0.80	51	37.7	46.7	81
6	1025	8.6	7.8	1.72	38	37.9	53.4	71
7	1025	8.6	7.8	1.72	38	38.5	52.9	73
8	1050	10.0	7.8	1.94	43	35.3	54.1	65
9	1175	10.0	7.9	1.47	48	48.0	57.7	83
10	1150	11.8	7.8	0.87	25	55.2	56.6	84
11	1275	10.2	7.8	1.52	38	53.4	56.6	94
12	1300	11.4	7.7	3.11	40	51.1	54.5	94
13	780	13.0	7.7	1.25	45	52.1	52.0	99
14	825	14.0	7.6	1.48	53	49.0	55.2	89
15	925	14.0	7.5	1.48	55	52.1	51.8	101
16	1448	15.6	7.8	0.88	20	53.8	55.7	97
17	1925	17.4	7.8	1.19	33	52.3	57.5	91
18	1405	17.8	7.9	1.09	35	55.8	57.0	98
19	1200	18.2	7.6	1.55	55	54.5	56.2	97
20	1375	20.0	7.1	1.45	50	54.2	56.4	96
21	1475	20.4	7.8	1.33	35	51.2	53.6	96
22	1725	20.4	8.0	1.07	38	52.3	53.9	97
23	1325	20.8	7.6	1.86	50	50.2	54.8	92
24	1050	21.8	7.7	2.45	45	47.6	52.5	91
25	1100	25.0	7.7	2.04	38	48.2	54.7	88
26	1310	28.8	7.7	2.41	48	47.4	52.3	91
27	1450	30.6	7.9	1.13	33	52.3	52.1	100
28	1975	34.0	7.9	7.01	45	47.5	49.9	95
29	1738	40.0	7.7	2.84	35	54.3	53.1	102
30	1900	40.0	8.2	1.98	35	54.3	54.7	99

Ava.: *Available;* **O.C:** *Organic carbon;* **T.N.V** ; **T_0:** *Control treatment;* **T_1:** *Fertilizer treatment*
Relative yield (%) = (yield of control treatment/yield of fertilizer treatment)*100

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42814296

EFFECT OF PARACETAMOL ON ANTIOXIDANT SYSTEM AND OSMOPROTECTANTS OF MUNG BEAN (*VIGNA RADIATA)*

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(Received 26th Dec 2018; accepted 20th Feb 2019)

Abstract. There is no report on panadol mediated changes in antioxidant system and osmoprotectants of *Vigna radiata* plants. An experiment was carried out to dissect the response of activities of antioxidant enzymes, stress biomarkers and proline accumulation in the presence of panadol in *Vigna radiata* plants. 10 days after germination, a range of panadol concentration (0.0005, 0.01, 0.1 or 1.0 ppm) was administered to the foliage of *Vigna radiata* plants, at the end of germination process (30 days from the beginning of germination) the plants were sampled to evaluate stress biomarkers, leaf water potential, antioxidant enzymes and proline content. The results demonstrate that the highest concentration of panadol was 1.0 ppm that induced oxidative stress that was noticeable via increasing levels of lipid peroxidation, and H_2O_2 accumulation in *Vigna radiata* plants. On the other hand, 0.1 ppm of PD treatment modified the antioxidant system and the range of osmolytes to make the plants tolerate unfavorable conditions and helped to increase gross production through increasing photosynthetic activity.
Keywords: *panadol, proline, mung bean, biomarker, stress physiology*

Introduction

In the recent years, researchers have dissected out the role of pharmaceutical drugs and how they can affect target organs in the human body and how to make the drugs insistent so to maintain their chemical structure long enough to do their remedial work (Heberer, 2002; Kümmerer, 2004). However, the possible physiological and ecological influence of those compounds on non-target species or the environment are neglected (Hilton and Thomas, 2003). Therefore, the release of pharmaceuticals compounds in the environment is going to emerge as a great concern for both the ecosystem and human health (Kümmerer, 2004; Picó et al., 2017). Many prescribed drugs for humans are not completely absorbed by patients' body following intake, and approximately more than fifty percent of these are excreted as human waste to sewage systems in a non-biodegradable form or degraded to only a certain extent (Breton and Boxall, 2003; Hilton and Thomas, 2003).

Panadol (Paracetamol/Acetaminophen; PD) which represents one of the most widespread pharmaceuticals in surface waters and sediments even after wastewater treatment in the world, has negative effects on humans, animals, microbes, and plants (Kümmerer, 2004; An et al., 2009). Kolpin et al. (2002) found that 10 USA streams are contaminated with PD at maximum concentrations up to 10 ppm. In Madinah City-Saudi Arabia alone, tons of PD had been dispensed by public hospitals and medical clinics in the year of 2009 (Shraim et al., 2017). Even though wastewater treatment plant may remove 80% of PD in the water, they are far from the negligible amount (Ternes, 1998, 2000; Heberer, 2002). Increased human population in Saudi Arabia also may increase the consumed and unused PD, which lead to steady continual increase of PD in the wastewater. Continual use of sewage sludge and reclaimed waters in irrigation may lead to increased concentration of Panadol in agricultural lands (Kümmerer, 2004; Alyemeni et al., 2014).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

Mung bean, *Vigna radiata*, Fabaceae, *Vigna* genus include about 150 species. One of the most important crop plants among these species is *Vigna radiata* with green seeds. Mung bean contains about 23.9% protein; rich in lysine which is generally low or deficient in cereals. Mature seeds are rich in proteins. The tender pods of mung bean are also consumed as vegetable (Agrawal et al., 2006). Its fast growth and sensitivity to abiotic stress make it suitable as a test subject to understood the phytotoxic effect of PD toward crop plant (Fariduddin et al., 2014).

In our previous study, we have found that varied concentrations of Panadol influences the photosynthetic efficiency of *Vigna radiata* plants in a concentration dependent manner (Al-Muwayhi, 2018; Almohisen, 2018). Therefore, it is essential to dissect out the behavior of antioxidant enzymes and osmoprotectants to unravel the panadol mediated changes in antioxidant systems and osmoprotectants.

Materials and Methods

Growth conditions and analysis of various parameters

Seeds source of *Vigna radiata* was from National Seed Corporation Ltd., New Delhi, India. Healthy seeds were decontaminated with 1% Chlorox (NaOCl) for ten minutes, and then washed twice with distilled water. Panadol was used as the source of Paracetamol. It was acquired from Sigma Aldrich India Ltd. The stock solution of PD (10 ppm) was obtained by dissolving the required amount (0.0000005, 0.00001, 0.0001 and 1 mg) of paracetamol in distilled water in a volumetric flask and the final volume was made up to the required volume. The required concentrations (0.0005, 0.01, 0.1 or 1.0 ppm) of PD were prepared by diluting stock solution and the concentrations of PD was based on the screening of various concentrations (Data unpublished).The seeds were sown in washed sand media, and allowed to germinate in growth chamber. The sand media kept moist using deionized water. When germination completed, the seedlings were irrigated with a solution of nutrients (Hewitt, 1966) every other day during the experimental period. At day 10^{th}, the seedlings sprayed with deionized water (control), and 0.0005, 0.01, 0.1 or 1.0 ppm of PD for 10 days. Each seedling was speckled. The sprayer nozzle was adjusted to push out approximately 1 mL in one spray. Thus, each seedling received 3 mL of deionized water or PD treatement. The plants were grown under controlled conditions that is the average temperature ranged between 28/22°C day/night, maximum 75% relative humidity (RH) at day and minimum RH was 65% at night, and the photoperiod was 14 hour. Irrigation was applied with deionized water and nutrient solution on every other day. Harvesting of plants began at 30 days stage to estimate the chlorophyll content, net photosynthetic rate, maximum quantum yield of PSII and related attributes. The experimental design was completely randomized block design (CRB). Total cups were 25, cup size was 350 mL, each cup contained three plant and replicated five times. The replication of treatment was five times. The leaf water potential (LWP) was measured in the fully expanded leaves of the plant using Psypro Water Potential System (Wescor, Inc. 370 West 1700 South Logan, Utah 84321, USA).

Estimation of Lipid peroxidation rates was performed with the measurement of the malondialdehyde equivalents as described by Hodges et al. (1999). 80% ethanol was used to homogenize 0.5 g of the leaf. Then the homogeneous sample was centrifuged at 3000g for 10 minutes under 4°C temperatures. The pellet was extracted twice with the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

same solvent. 1 mL of supernatant was added to a test tube containing the same volume of a solution of 20% trichloroacetic acid, 0.01% butylated hydroxy toluene and 0.65% thiobarbutyric acid.

Samples were heated for 25 min at 95°C, and then cooled down to 24±1°C. Wavelengths of 440, 532 and 600 nm were used to measure absorbance of the samples. The formula of Hodges et al. (1999) was used to calculate Lipid peroxidation rates equivalent (nano mole malondialdehyde mL^{-1}).

The accumulation of hydrogen peroxide was estimated according to the method explained by Jana and Choudhuri (1982). 500 mg from samples was homogenized in 3.0 mL of phosphate buffer (50 mM and pH 6.8). Then the homogeneous sample was centrifuged for 25 minutes at 6000g.

3.0 mL from the extract was mixed with 0.1% titanium chloride in 20% (v/v) sulphuric acid, the mixture was centrifuged at 6000g for 15 minutes. A spectrophotometer was used for the measurement of color absorbance at 410 nm, and the color absorbance was compared with that of the calibration curve. The standard curve of known concentration of H_2O_2 was used to compute H_2O_2 content on fresh mass basis. 0.5 g from leaf was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone, for the assessment of antioxidant enzymes. The homogeneous sample was centrifuged for 10 minutes at 27600 g under a temperature of 4°C, the supernatant was used as the source of catalase, peroxidase and superoxide dismutase enzymes. The procedure described by Chance and Maehly (1955) was followed to assay Peroxidase and catalase. Catalase was measured by titrating the reaction mixture consisting of phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract and 2% H_2SO_4, against solution of 0.1 N potassium permagnate. The reaction mixture for peroxidase consisted of pyragallol, phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract. Change in absorbance caused by catalytic conversion of pyragallol to purpurogallin, was noticed at an interval time of 20 second and 2 minutes, at wavelength 420 nm on a spectrophotometer. Control set was prepared by using distilled water as an alternative of enzyme extract. The activity of superoxide dismutase was computed by estimating its capability to inhibit the photochemical reduction of nitroblue tetrazolium by following Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 0–50 mL enzyme extract and was situated under 15W fluorescent lamp. The reaction began by switch on the light and run for 10 minutes. The reaction was stopped by switching off the light. 50% inhibition by light was considered as one enzyme unit. The estimation of proline content in fresh leaves samples was carried out according to Bates et al. (1973). Sulphosalicylic acid was used for sample extraction. Equivalent volume of glacial acetic acid and ninhydrin solutions were added to the extracted sample. The mixture was boiled at 100°C after adding 5 mL of toluene. The toluene layer absorption was recorded at 528 nm wavelength on a spectrophotometer.

Statistical analysis

Data were analyzed by using SPSS, 17.0 for Windows (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was achieved to examine vitiations between treatments at $P<0.05$, least significant difference (LSD) was used for comparison of the means.

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DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

Results

Lipid peroxidation (LPO), H_2O_2 content and leaf water potential

It is evident from *Table 1* that LPO and H_2O_2 accumulation in plant leaves significantly increased with increasing of PD concentration. Plants treated by 1.0 ppm of PD accumulated 27.96 and 24.95% LPO and H_2O_2, respectively, as compared to control plants. However, plants treated by 1.0 ppm of PD accumulated higher content of LPO than H_2O_2. The plants treated by 0.005, 0.01, 0.1 or 1.0 ppm of PD had varied leaf water potential compared with control plants in a concentration dependent manner and maximum increase was noted at 0.1 ppm of PD (*Table 1*) whereas the minimum increase was noted at 1.0 ppm of PD.

Table 1. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on lipid peroxidation, H_2O_2 content and leaf water potential of Vigna radiata 30 days after sowing*

Treatment	**Lipid peroxidation (n mol g^{-1} FM)**	**H_2O_2 content (n mol g^{-1} FM)**	**Leaf water potential (-MPa)**
Control	8.01 ± 0.11 c	5.01 ± 0.05 c	- 0.84 ± 0.01 b
PD (0.005)	8.15 ± 0.10 c	5.12 ± 0.06 c	- 0.77 ± 0.01 c
PD (0.01)	8.97 ± 0.14 b	5.81 ± 0.05 b	- 0.69 ± 0.02 d
PD (0.1)	9.21 ± 0.15 b	5.61 ± 0.06 b	- 0.63 ± 0.01 e
PD (1.00)	10.25 ± 0.09 a	6.26 ± 0.05 a	- 0.92 ± 0.02 a
LSD	0.42	0.27	0.03
Significannce level	***	***	**
F- value	117.13	119.99	137.77

** P <0.01
*** P < 0.001

Proline and sugar content

The leaf proline and sugar content significantly increased with increasing concentrations of PD (0.005, 0.01, 0.1 or 1.0 ppm) but 1.0 ppm showed the highest increase of proline and sugar content by 29.95% and 25.96%, respectively, in comparison to control plant contents. Also catalase activity was increased by 31.87% and 24.49% in plants treated by 0.1 and 1.0 ppm PD, respectively, as compared to control plants (*Table 2*).

Table 2. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on proline content, sugar content and catalase activity of Vigna radiata 30 days after sowing*

Treatment	**Proline content (µ mol g^{-1} FM)**	**Sugar content (mg g^{-1} FM)**
Control	8.98 ± 0.10 e	25.11 ± 0.04 d
PD (0.005)	9.96 ± 0.14 d	28.12 ± 0.03 c
PD (0.01)	10.68 ± 0.15 c	29.12 ± 0.05 bc
PD (0.1)	11.67 ± 0.07 a	31.63 ± 0.04 a
PD (1.00)	11.13 ± 0.08 b	30.13 ± 0.02 b
LSD	0.52	1.04
Significance level	***	**
F- value	132.65	154.12

** P <0.01
*** P < 0.001

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

Activities of antioxidants enzymes CAT, POX, and SOD

PD significantly affect the antioxidant enzyme activities (catalase/CAT, peroxidase/POX, and super oxide dismutase/SOD) in all plants treated by the PD, and the three enzyme activities increased with the increasing level of PD. The highest enzyme activities were recorded in plants treated by 0.1 ppm PD (*Table 3*). The highest activity rate for CAT, POX, and SOD were recorded at 0.1 ppm of PD in *Vigna radiata* plants demonstrating increases of 24.94%, 21.92%, and 17.51%, respectively, in comparison to the control plants.

Table 3. *Effect of panadol (PD; 0.005, 0.01, 0.1, or 1.0 ppm) induced changes on catalase, peroxidase activity, and superoxide dismutase activity of Vigna radiata 30 days after sowing*

Treatment	Catalase activity (m mol L^{-1} H_2O_2 decomposed g^{-1} FM)	Peroxidase activity	Superoxide dismutase activity
Control	298 ± 1.10 d	10.90 ± 0.10 e	177 ± 1.75 d
PD (0.005)	327 ± 2.10 c	12.20 ± 0.12 d	196 ± 1.95c
PD (0.01)	357 ± 2.22 b	12.82 ± 0.11c	205 ± 1.92 bc
PD (0.1)	393 ± 2.59 a	13.95 ± 0.13 a	221 ± 2.00 a
PD (1.00)	371 ± 3.09 b	13.29 ± 0.14 b	208 ± 1.99 b
LSD	11.9	0.46	9.09
Significance level	***	***	**
F- value	111.11	124.76	121.21

** P <0.01
*** P < 0.001

Discussion

Lipid peroxidation and H_2O_2 accumulation are considered biochemical markers for the free radical attributed injury under different environmental cues and external stimuli (Verma and Dubey, 2003). Furthermore, these biochemical processes are commonly defined by increasing formation of reactive oxygen species (ROS) (Foryer and Noctor, 2000; Gill and Tuteja, 2010). The results of this study, confirmed and verified this phenomenon, as our results showed an increasing accumulation level of lipid peroxidation, and H_2O_2 at the highest concentration (1 ppm) of PD (*Table 1*).

Therefore, it could be assumed that higher concentration would be toxic to *Vigna radiata* plants and lead to excess production of reactive oxygen species (ROS). ROS in plant tissues are regulated by plant antioxidant systems or antioxidant enzymes such as Catalase, Peroxidase, and Superoxide dismutase (Schutzendubel, 2002) as well as osmoprotectants i.e. proline (Szabados and Savouré, 2010). The present study revealed that treatment of plants with 0.1 ppm of PD boosted the activity of antioxidant enzymes (CAT, POX and SOD; *Tables 2 and 3*) along with the level of proline accumulation (*Table 2*). In the similar line with An et al. (2009) who found that the activities of peroxidase and superoxide dismutase significantly increased in the wheat plant when exposed to paracetamol in a duration dependent manner. Proline increase plant stress tolerance by maintaining NADPH/$NADP^+$ balance, GSH levels, and during infection, drives the oxidative rupture of the hypersensitive response (Miller et al., 2009; Ben Rejeb et al., 2014). As Szabados and Savouré (2010) confirmed proline as a signaling molecule to modest mitochondrial functions, and it activates specific gene expression that could be

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

necessary for plant recovery in stress conditions. The findings of this study are in consistence with these previous studies, as this study showed that presence of PD significantly increased the proline accumulation and sugar content (*Table 2*). With cumulative effort of enhanced antioxidant system and proline accumulation under exogenously sourced PD i.e. paracetamol enhanced tolerance capacity of *Vigna radiata* plants to withstand various environment cues and helped in increasing their gross production.

In this study, we determined that the limit of PD in rivers and agricultural irrigation systems should be less than 1 ppm to decrease the negative effect of PD on mung bean plantation. Environmental Protection Agency and Saudi Government may use this limit as one of the allowable pharmaceutical water quality standard for the protection of the plant.

Conclusions

The highest concentration of panadol that induced oxidative stress was 1.0 ppm that was obvious through increased levels of lipid peroxidation, and H_2O_2 accumulation in *Vigna radiata* plants. On the other hand, 0.1 ppm of PD treatment improved the antioxidant system and the level of osmolytes to make the plants tolerate unfavorable conditions and helped in the increase of gross production through increasing the photosynthetic activity.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_42974303

STUDY OF THE LICHENS OF THE MOROCCAN ATLANTIC COAST SAFI-ESSAOUIRA: BIOINDICATION OF AIR QUALITY AND LIMITING FACTORS

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(Received 23rd Dec 2018; accepted 22nd Feb 2019)

Abstract. In order to estimate the global air pollution of the Moroccan Atlantic coast Safi-Essaouira, we have used a qualitative approach based on the spatial distribution of saxicolous-calcicolous lichens that reflect special ecological and pollution conditions. The measurement of the beta diversity by the calculation of the Jaccard index makes it possible to quantify the similarity between the studied sites and has made it possible to measure the gradient of change of this diversity along an increasing gradient of the pollution supposed high in the industrial zone of the Safi city and decreasing going north or south. The hierarchical clustering has made it possible to partition the seven studied sites into two different classes and differentiate four groups of indicator species of poleotolerance and eutrophication. The identification of 36 species of lichens including indicator species of SO_2 and NOx pollutions, the study of the impact of climate and pollution on the lichens biological spectrum, and the exploitation of the poleotolerance index and eutrophication index were necessary to elucidate the global air quality of the region.
Keywords: *air pollution, beta diversity, poleotolerance index, eutrophication index, biological spectrum*

Introduction

Since the beginning of the industrial era, human activities have been transforming the atmosphere of the globe more and more rapidly. Because of its geographical situation and its socio-economic development, Morocco is among the most vulnerable countries to the disturbances atmospheric. The cost of air degradation and its impacts has been estimated at 370 million USD per year (Worldwide air quality, 2018), which represents about 1.03% of Morocco's GDP in 2014. Ambient air pollution is responsible for nearly three quarters of this cost (Croitoru et al., 2017). In May 2016, World Health Organization in a report on urban air quality around the world, decreed Casablanca as the most polluted city of Morocco with a concentration level of small particles in the air of 61 $\mu g/m^3$ (the maximum annual threshold tolerated by the WHO is 20 $\mu g/m^3$); in the city of Safi, the concentration of small particles in the air is 21 $\mu g/m^3$.

The use of lichens is one of the biological assessment approaches to environmental changes. At the regional scale, the lichens distribution is explained by the sensitivity to pollution (Bobbink et al., 2010; van denBerg et al., 2011), climate variations (Werth et al., 2005; Giordani et al., 2008; Hickling et al., 2006; Gottfried et al., 2012) and the continuity of the habitat (Selva, 1994; Hauck, 2011). Temporal and spatial variation in lichen diversity could be evaluated using beta diversity and similarity. This information could then be used to quantify anthropogenic impacts in a given region (Giordani et al., 2018).

Without root system, protective cuticle or regulating mechanisms of matter flow, nutrients and toxic substances dissolved in the atmosphere can be absorbed over the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43054323

entire surface of the lichen thallus (Tyler, 1989; Bačkor et al., 2009). Since the early works of Nylander (1866), lichens are widely used as bioindicators for assessing air pollution (Garrec et al., 2002). Bioindication is an ecological approach that seeks to evaluate possible air disturbances from the presence/absence of key species in a specific lichenic survey.

The first catalog of Moroccan lichens was published by Werner and Gattefossé (1931) and includes 542 species. An important scientific study of Moroccan lichenic flora was conducted by Werner between 1931 and 1976. Egea (1996) in his catalog of lichens in Morocco reviews the studies of Moroccan lichen flora between 1879 and 1990 and gives a commented list of lichens in our country. In 2013, a synthesis on the lichens of the Rabat herbarium (RAB) was published by Ajaj et al. (2013).

The lichenic bioindication approaches used and still mostly use corticolous lichens. Comparatively, there are no bioindication studies of air quality using saxicolous lichens and especially saxicolous-calcicolous species. The reason we chose the calcicolous lichens is the ecological nature of the biotope of this study area harboring few corticolous and terricolous lichens, but showing a remarkable presence of calcicolous lichens.

The present study aims to study the lichen flora of the Atlantic coastal fringe of the Safi-Essaouira regions over 120 km, to use the beta diversity measure to quantify the similarity between the studied sites, to study the climate impact and air pollutants (SO_2 and NOx) on this diversity and to exploit the poleotolerance index and the eutrophication index to highlight air pollution and map the overall air quality of the region.

Materials and methods

The Moroccan Atlantic coast Safi-Essaouira includes two urbanized areas (the city of Safi and the city of Essaouira) and less urbanized areas. Since the 1960s, it is home to a major industrial phosphate processing plant located in the south of the Safi, and accused of being one of the contributors to the deterioration of the air quality. In the near future, a coal-fired power plant, already installed 12 km south of Safi, will be operational by the end of 2018; it aims to cover 25% of Morocco’s electricity needs. The impacts of its construction and operation are expected to have an impact on the air quality caused by the dust generated during construction and the emissions that will be associated with coal combustion (sulfur, trace metals, radioactive elements, etc.).

The Atlantic coast Safi-Essaouira is characterized by a semi-arid climate with rains fall regularly in autumn and winter. The average annual rainfall is estimated at 350 mm. Temperatures are moderate with an average annual value of 18 °C. In August, the average temperature is M = 25.7 °C; it is the hottest month of the year. The coldest month is January with an average temperature of m = 8.9 °C. The drought period is 7 months, from April to October. The relative humidity of the air is on average between 70 and 80%. The winds are particularly strong in the coastal zone. They are predominantly northwest and are frequent towards the end of the afternoon.

Seven sites were selected assuming an increasing air pollution gradient from non-urban areas to urban areas (*Fig. 1*). Lichen sampling focused on saxicolous species on limestone rock formations with homogeneous lichenic cover exposed to the air while avoiding sheltered rocky surfaces and surfaces directly exposed to the sea. Sometimes the species are taken with their support using a hammer and a chisel.

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The sampling campaigns covered the period from February 2016 to June 2016 and were updated in 2017 and 2018.

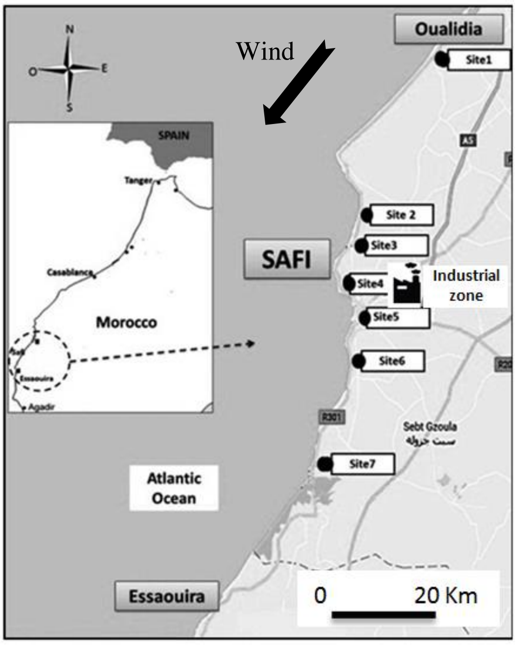

Figure 1. *Location of the Safi-Essaouira coastline and the seven sites. The arrow indicates the general direction of the wind*

Species determination was performed using a binocular loupe and an optical microscope for thallus sections. Different bibliographic sources were used in the determination, especially "Likenoj of Okcidenta Eŭropo" by Clauzade and Roux (1985): "Lichens: Biological study and illustrated flora", by Ozenda and Clauzade (1970) and "Guide of the lichens-350 species of lichens of Europe" (Thievant, 2001).

The following chemicals have been used in the determination of species: saturated potash for reactions on the thallus and medulla; chlorine in the form of sodium hypochlorite solution and paraphenylenediamine in stabilized solution.

Lichen diversity is also used for biomonitoring (Nimis et al., 1990; Loppi et al., 2004; Giordani, 2007; Giordani et al., 2014; Matos et al., 2017). To compare the studies sites in pairs, we considered the β diversity that corresponds to the importance of species replacement along environmental gradients (Whittaker, 1972; Podani et al., 2013). The comparison of the number of taxa that are unique to each site makes it possible to measure the gradient of change of diversity along the increasing gradient of the supposed pollution from the industrial zone and going to the north or the south.

As bioindication approach, the method of the index of atmospheric purity (IAP) has not been used; this method requires the determination of the entire lichenic flora. The method of Hawksworth and Rose, established in the British Isles (1970), is inapplicable in the Mediterranean (Semadi et al., 1995). The method of Van Haluwyn and Lerond (1986), although adaptable to all the study areas, has been validated only for the corticolous species.

To overcome this situation, and considering the available means, we have selected among recent methods the studies of Nimis and Martellos (2017) which attributed to

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DOI: http://dx.doi.org/10.15666/aeer/1702_43054323

different species of lichens a poleotolerance index and an eutrophication index. This work done in Italy has the advantage of being interested in lichens from Mediterranean region whose ecological characteristics are close to those of Morocco, It is also a solution in the absence of cartographic studies of the overall air quality using saxicolous lichens and especially saxicolous-calcicolous and facing the absence of similar works on the Moroccan territory.

To illustrate the arrangement of sites and species of lichens studied in terms of ecological parameters, we have processed the databases using the SPSS statistics 20.0 software and the R software using the FactoMineR and factoextra package that divide datasets, containing n observations, into a set of k groups (ie clusters) in which each cluster is represented by the center or the means of the data points belonging to the cluster (K-means clustering).

Results

Beta diversity and spatial bioindication of air quality

It is evident that the exhaustive study of all lichens in the region cannot be established because of the difficulty of identifying all species in particular crustose saxicolous calcicolous lichens. The number of taxa identified in the study area is 36 saxicolous lichens (*Table A1* in the *Appendix*). As we move further away from the industrial zone (Site 4), the number and diversity of lichens increases.

Morphologically, fruticose forms such as Roccella phycopsis and Ramalina pollinaria disappear 20 km far from the S4 site. The foliose forms penetrate up to around 4 km from the S4 site, only a few crustose forms settle in the S4 site. Of the 36 calcicolous species there is a diversity of 22 different genera. This diversity is between 36 and 3 species per station. There are 30 species of crustose, 4 foliose species and 2 fruticose species.

Among the indices used to measure beta diversity, we used the Jaccard index (1908) to quantify the similarity between the studied sites. The similarity increases with the value of the index which takes the value 0 when two sites have no similarity (no species in common) and 1 when the similarity is maximal (all the species are in common). The equation of this index is (*Eq. 1*):

$$Sj = \frac{c}{a+b+c} \tag{Eq.1}$$

c = number of common species

a = species of the site a

b = species of the site b

Table 1 illustrates the similarity matrix between sites taken two by two: The two most similar sites are S1 and S7 (Sj = 1), the least similar are S4 and S1 (Sj = 0). A hierarchical K-means clustering has allowed partitioning the sites according to their degree of similarity: On the dendrogram (*Fig. 2*), a cutoff passing through the value combination distance 5 individualizes three groups of sites: group 1 “S1, S2, S6 and S7”, group 2 “S3 and S5” and group3 “S4”.

These results reflect the grouping of these sites according to the habitat quality they represent. The study of beta diversity via the similarity index and the hierarchical

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ascending classification (*Fig. 2*) show a differentiation of these sites into three groups of lichenic biodiversity.

Table 1. *Similarity matrix of the studied sites. (Performed by IBM SPSS statistics 20.0 software)*

	Site1	Site2	Site3	Site4	Site5	Site6	Site7
Site1	1.000	0.926	0.556	0.000	0.333	0.815	1.000
Site2	0.926	1.000	0.670	0.032	0.333	0.835	0.830
Site3	0.556	0.670	1.000	0.111	0.661	0.761	0.689
Site4	0.000	0.032	0.111	1.000	0.222	0.049	0.022
Site5	0.333	0.333	0.661	0.222	1.000	0.453	0.362
Site6	0.815	0.835	0.761	0.049	0.453	1.000	0.921
Site7	1.000	0.830	0.689	0.022	0.362	0.921	1.000

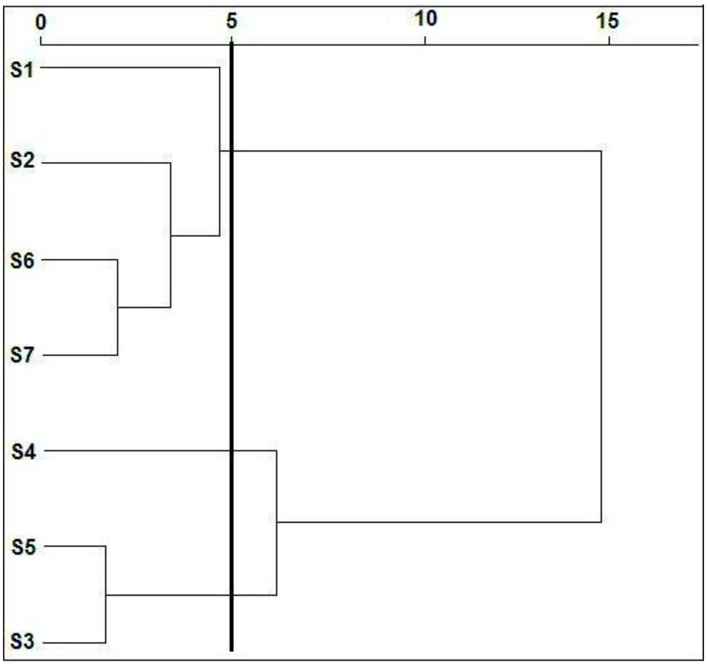

Figure 2. *Cluster dendrogram of inter sites similarity. (Performed by IBM SPSS statistics 20.0 software)*

Use of the poleotolerance index and the eutrophication index

Nimis and Martellos (2017) have attributed to a variety of lichen species an index of poleotolerance: This value points to the tendency of a lichen to occur in areas with different degrees of human disturbance. It is expressed on 4 classes, as follows:
3 = species occurring in heavily disturbed areas, including. large towns.
2 = species occurring in moderately disturbed areas (agricultural areas, small settlements etc.).
1 = species occurring in natural or semi-natural habitats.
0 = species which exclusively occur on old trees in ancient, undisturbed forests. Contrary to the other values, this one has been assigned to epiphytic species only, since it is useful to point out indicators of long ecological continuity of forests.

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DOI: http://dx.doi.org/10.15666/aeer/1702_43054323

The authors also proposed an eutrophication index: this value expresses the degree of nitrogen pollution, including deposition of dust and nitrogen compounds, there are 5 values:
1 = no eutrophication
2 = very weak eutrophication
3 = weak eutrophication
4 = rather high eutrophication
5 = very high eutrophication (*Table A2* in the *Appendix*).

The species with a low eutrophication index are confined in the S1, S2, S6 and S7 sites (*Table A2*): This is the case of Acrocordia conoidea and Parabagliettoa cyanea. Although its poleotolerance index is low to medium (1-2), others species with an eutrophication index of 2-3 like Coscinocladium gaditanum can grow in sites near industrial zone (Sites S3 and S5). Only species with an index of 4-5 such as Caloplaca citrina and Lecania erysibe seem to support the air quality of the S4 site (industrial zone).

Discussion

Due to their close dependence on the atmosphere for their metabolic processes (Rundel, 1988; Nash, 1996), lichens are strongly influenced by environmental variables, which affect their richness and distribution. The species composition and its richness is often used for spatial analysis in ecosystems (Brunialti et al., 2003; Kapusta et al.,2004; Giordani, 2006; Will-Wolf et al., 2006). Numerous studies have shown that different disturbances of the biotope have a limiting effect on lichen communities: SO_2 in the 1970s in Great Britain (Hawksworth and Rose1970), dust (Loppi and De Dominicis, 1996,), fire (Longán et al., 1999) in the Mediterranean regions, and nitrogen compounds in the Netherlands (van Dobben et al., 2001; van Herk, 2002). The differences in species richness between the studied sites (S1 to S7) can be correlated with the action of environmental fluctuations (Seaward1977; Nash, 1996).

Bioindication of climate as a limiting factor

The study of the biological spectrum of calcicolous lichens harvested in unpolluted rural sites (S1 and S7) shows that crustose forms reach a frequency of 80% (*Fig. 3*). This value reflects the bioclimatic nature of the region. According to the climatic data we can calculate the aridity Martonne index (I) (*Eq. 2;* De Martonne, 1923) and the pluviometric Quotient of Emberger (Q) (*Eq. 3;* Emberger, 1955):

$$I = \frac{P}{T + 10} \tag{Eq.2}$$

where I = 12.07.

$$Q = \frac{2000P}{M^2 - m^2} \tag{Eq.3}$$

where Q= 57.57.

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To correlate the biological spectrum of lichens with the ecological conditions of the biotope Renaut et al. (1968) reported, in a study conducted in Morocco, that a biological spectrum with 50% crustose forms corresponds to Q = 80, with 70% crustose forms Q is close to 60. Finally with 80% crustose forms Q is less than 60.

In our study Q = 57.57 and the biological spectrum of harvested lichens is dominated by crustose forms (80%) which corresponds to the observations made by Renaut et al. (1968). On the other hand, the regional semi-arid climate (I = 12.07 and Q = 57.57) imposes a steppe-type vegetation which explains a relatively small number of trees that can support corticolous lichens. The dominance of limestone rocks combined with a semi-arid climatic is such that our study biotope is colonized by calcicolous lichens.

Bioindication of the pollution as limiting factors

The closer we get to the industrial zone (site S4), the more the lichenic biological spectrum is dominated by crustose forms (*Fig. 3*). Fruticose lichens are more sensitive to pollution than foliose and crustose forms.

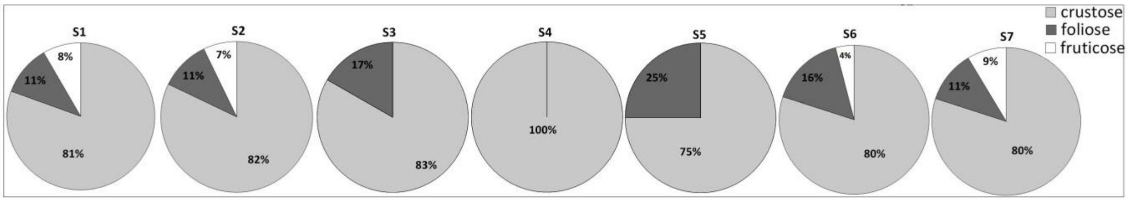

Figure 3. *Spatial variation of the biological spectrum of harvested lichens, according to the distance to Safi industrial zone (site S4)*

Studies have shown a relationship between the lichen growth form and its resistance to pollution. Maizi et al. (2017) reported that the Fruticose lichen Ramalina is more sensitive to metal trace element than the foliose lichen Xanthoria parietina and the absorption capacity of MTE is different depending on the type of thallus (foliose or fruticose).

Gauslaa et al. (2002) reported that in polluted sites, the medullary metabolites in Ramalina have been reduced and lichen is less protected. Weissman et al. (2006) reported that under conditions of low pollution, occur in Ramalina antioxidant activities as protective mechanisms. However, after prolonged exposure, these protective mechanisms are overwhelmed and damage to cellular components begins to accumulate. It follows a decrease in antioxidant activities and degradation of lichen. The disappearance of the fruticose forms from the site S3 and S4 can be related to the death of these lichens as a result of damage to their biological components caused by air pollution.

Bioindication of SO_2 as a limiting factor

To give credibility to the bioindication approaches adopted in this work, the results of a research project on the bioaccumulation of metallic trace elements by lichens (Essilmi et al., 2018) made it possible to quantify and characterize air pollution trends in the seven studied sites. These results and other measurements of air quality, shown in *Table 2*, present exact values of air pollution and its distribution in space, which must be verified and supplemented by lichen bioindication.

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Table 2. *Some measurements of air pollution in the Safi-Essaouira region*

Measurement approaches	Pollutants	S1	S2	S3	S4	S5	S6	S7	References
Measuring instruments	SO_2 µg/m³	<5	5	35	50	35	NA	NA	OCP (2011)
	NO_X µg/m³	NA	NA	53.62	53.62	NA	NA	NA	Ministry of Energy (2014)
Lichenic bioaccumulation in Xanthoria calcicola	Cd	0.000	0.178	0.301	0.532	0.210	0.191	0.154	Essilmi et al. (2018)
	Pb	1.719	6.171	15.756	30.187	5.192	7.579	2.443	
	Cu	1.390	2.547	4.586	21.634	1.995	1.978	2.322	
	Zn	14.546	35.840	66.019	165.093	47.364	45.096	14.055	

NA = not available

The "Office chérifien des phosphates" group (OCP, 2011), owner of the chemical industry "Maroc Phosphore", reported in its 2011 report, the measurements of SO_2 of the air (*Table 2*). In its 4th report of 2014, the Moroccan Ministry of Energy (2014) reported an annual average NO_2 of 53.62 µg/m³, in Safi city, which exceeds the annual norm of 50 µg/m³.

In the absence of studies on the susceptibility of calcicolous lichens to pollution, the cortical form of Xanthoria parietina is an example of nitrophytic lichen (NO2) and sulfur toxitolerant (SO_2) which disappears at a high concentration of SO_2 sulfur dioxide ≥67 µg/m³ (Wetmore, 1983) (*Table 3*). In 1970 Hawksworth and Rose reported that Ramalina pollinaria disappear at 38.78 µg/m³, in 2014 Duman et al., reveal that Ramalina pollinaria is sensitive to pollution stresses as evernia prunastri. The disappearance of Xanthoria parietina and Ramalina Pollinaria from the S4 site confirms the rate of 50 µg/m³ of SO_2 reported by the measurements. On the other hand, the disappearance of Physcia adscendens from S4 may be due to its oligotrophy for NOx.

Table 3. *Examples of SO2 and NOx tolerance threshold in lichens*

	SO_2 tolerance threshold	NOx tolerance threshold
Xanthoria parietina	Absent at 67 µg/m³ (Wetmore, 1983)	Eutroph with a high N requirement, peak detection frequency occurs at 6.1 kg N per ha per year (McCune and Geiser, 2009)
Ramalina pollinaria	Absent at 38.78 µg/m³ (Hawksworth and Rose, 1970)	Oligotroph with a low N requirement, peak detection frequency occurs at 1.1 kg N per ha per year (McCune and Geiser, 2009)
Physcia adscendens	Absent at 87.24 µg/m³ (Wetmore, 1983)	Oligotroph with a low N requirement, peak detection frequency occurs at 1.1 kg N per ha per year (McCune and Geiser, 2009)

The Hawksworth and Rose scale showed absolute SO_2 concentrations as the only factor influencing lichen species rather than the acidity of their support. Several studies have tried to find an explanation of the physiological impact of SO_2 on lichens. The sensitivity of lichens living on acid bark, tested in the laboratory, showed the same physiological symptoms as in the field. However, for species living on alkaline supports

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the correlation appeared to be negative (Baddeley et al., 1972; Ferry et al.,1979). Most nitrophytic species appear to have low sensitivity to the toxic effects of SO_2; their only requirement is a high pH of the support. Van Herk (2001) reported that an increase in the pH of the substrate appears to be the main cause of the considerable increase in the number of nitrophytic species and the

disappearance of acidophyte species during the 1990s in the Netherlands. Other studies suggest that species tolerant to SO_2 are sensitive to NH_3 and vice versa (Van Dobben, 1999).

It has been found that, with considerable SO_2 pollution in an area, the first loss of the same pH-sensitive lichens occurs on birches and conifers (acid bark and low buffering capacity); the next loss on oaks and sycamore (intermediate acidity and buffering capacity); the last on trees like elm (alkaline bark and high buffering capacity). In general, an alkaline substrate such as basic bark or limestone counteracts the acidity of SO_2 pollution (New York State's Urban Forestry council 2018).

Methods using the diversity of lichen species to estimate SO_2 air pollution should be used with caution. This is the case of calcicolous lichens studied in this work and living on a limestone support whose pH is alkaline. The decrease in lichen species richness in the S3 and S4 sites can be correlated with an increase in the pH of the support; the disappearance of oligotrophic species such as Ramalina pollinaria and Physcia adscendens indicates that the increase in pH is due to an increase in NOx levels in the air, this indication is confirmed by the annual rate of 53.62 $\mu g/m^3$ of NO_2 which exceeds the annual norm of 50 $\mu g/m^3$ (Ministry of Energy (2014). Current evidences suggest that increases in substrate pH due to NH_3 may be a driving force of change of the lichenic communities (Wolseley et al., 2006).

Bioindication of human disturbance and nitrogen pollution

The use of the poléotolérance index and the eutrophication index show that species with a low poleotolerance index (PI = 1) and a low eutrophication index (EI = 1) are absent in the S3 and S4 sites; Species with low poleotolerance index and low eutrophication index are restricted to sites S1, S2, S6 and S7 indicating low levels of anthropogenic disturbance and low eutrophication; this is the case of Acrocordia conoidea and Parabagliettoa cyanea. Only species with high value indices, such as Caloplaca citrina and Lecania erysibe, can withstand high levels of eutrophication and anthropogenic disturbances and are able to settle in site S4 indicating high pollution.

The HCPC statistical analysis (Hierarchical Clustering on Principal Components) (Husson et al., 2010), allows to group lichens according to 4 ecological variables: Poléotolérance index (PI), eutrophication index (EI), the biological spectrum and the specific présence in each site (*Figs. 4*, *5* and *6*).

There are 4 types of species profile indicator of poleotolerance and eutrophication (*Table 4*):

- Species in natural habitats without disturbance and without eutrophication (Cluster 1)
- Species present in moderately disturbed habitats and support low eutrophication (Cluster 2)
- Nitrophytic species present in moderately disturbed areas (Cluster 3)
- Highly nitrophytic species present in heavily disturbed areas (Cluster 4)

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Depending on the value of these indices we can classify the sites S1 to S7 into 3 classes (*Table 5*).

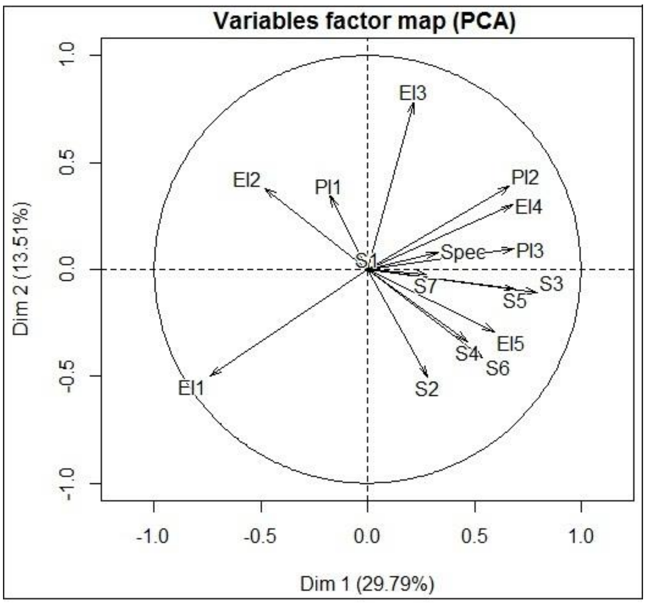

***Figure 4**. Circle of correlation between 4 variables: Poléotolérance index (PI), eutrophication index (EI), the biological spectrum and the specific presence/absence in each site. (Performed by R software)*

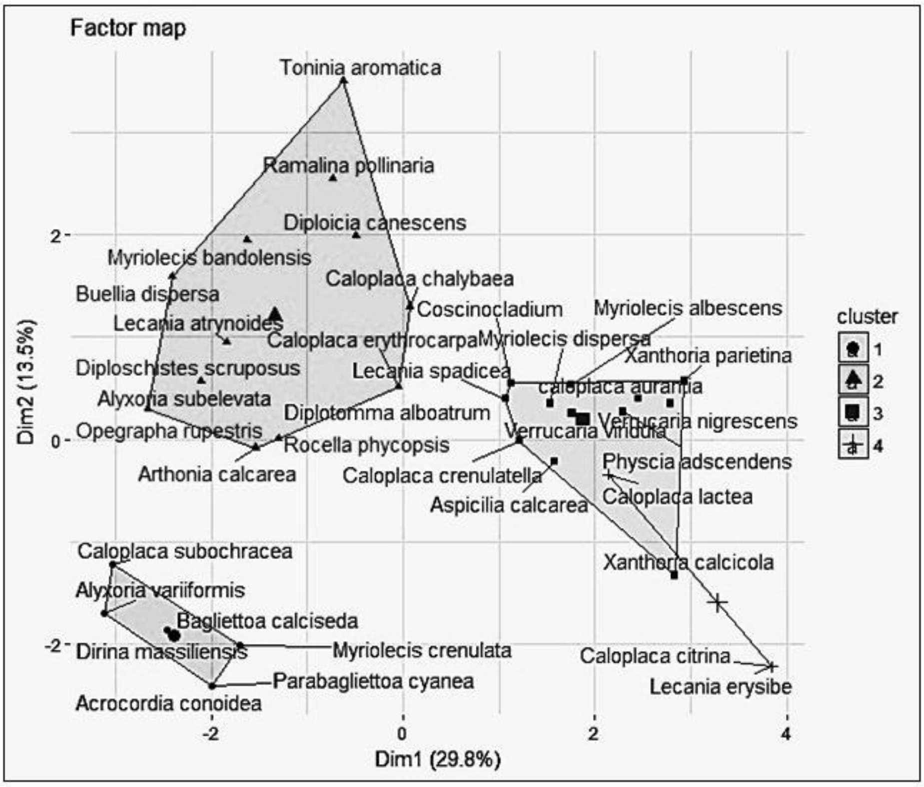

***Figure 5.** Differentiation of four species profile indicator of poleotolerance and eutrophication. (Performed by R software)*

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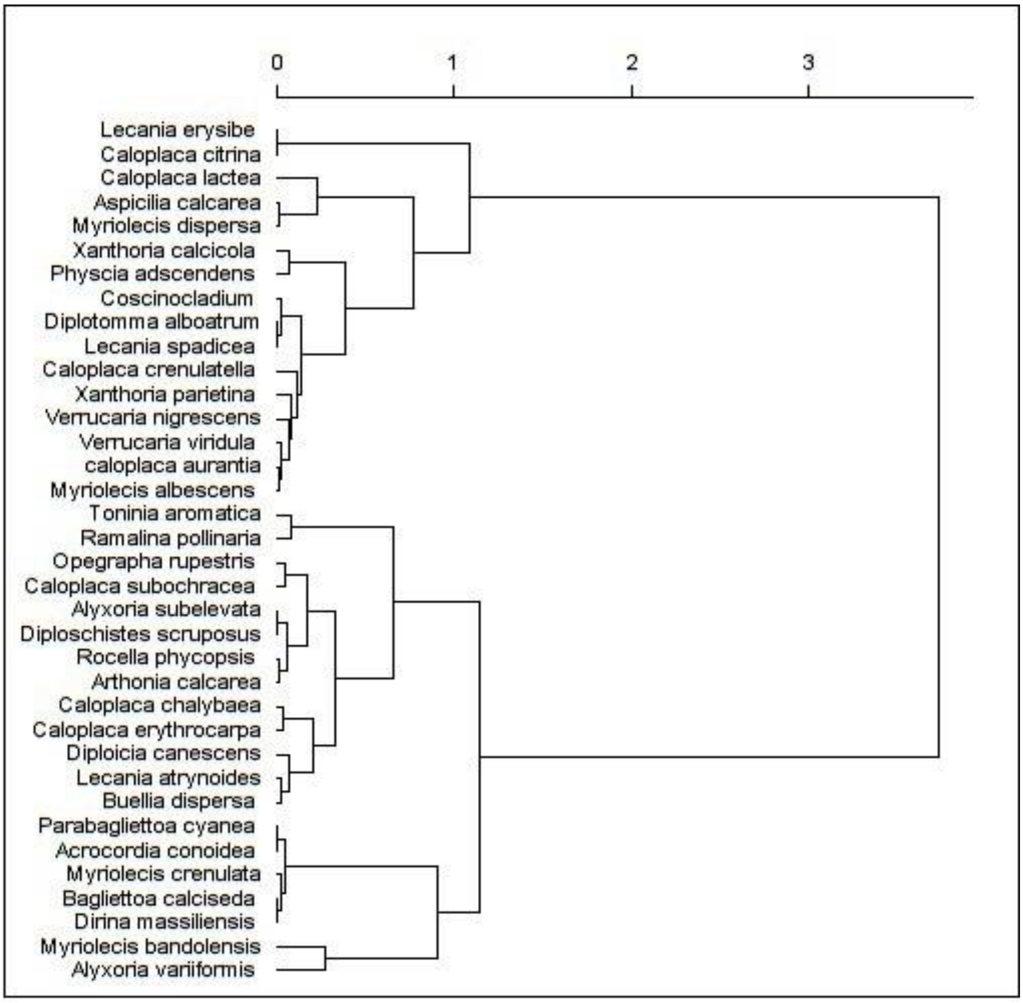

***Figure 6.** Hierarchical clustering of studied lichens according to their, PI, EI, Biological spectrum and specific presence/absence in each site. (Performed by R software)*

***Table 4.** Differentiation of four groups of Poléotolérance and eutrophication species indicator*

Cluster 1	Cluster 2	Cluster 3	Cluster 4
Caloplaca subochracea	Alyxoria subelevata	Aspicilia calcarea	Caloplaca lactea
Alyxoria variiformis	Arthonia calcarea	Caloplaca aurantia	Lecania erysibe
Dirina massiliensis	Buellia dispersa	Caloplaca crenulatella	Caloplaca citrina
Bagliettoa calciseda	Caloplaca chalybaea	Coscinocladium gaditanum	
Myriolecis crenulata	Caloplaca erythrocarpa	Diplotomma alboatrum	
Parabagliettoa cyanea	Diploicia canescens	Lecania spadicea	
Acrocordia conoidea	Diploschistes scruposus	Myriolecis albescens	
	Lecania atrynoide	Myriolecis dispersa	
	Myriolecis bandolensis	Physcia adscendens	
	Opegrapha rupestris	Verrucaria nigrescens	
	Ramalina pollinaria	Verrucaria viridula	
	Rocella phycopsis	Xanthoria calcicola	
	Toninia aromatica	Xanthoria parietina	

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Table 5. *Classification of sites according to the poleotolerance index and the eutrophication index of harvested lichens*

Classes	Sites	Species indicator	PI	EI	Degrees of disturbance
1	S1 S2 S6 S7	*Acrocordia conoidea* *Dirina massiliensis* *Parabagliettoa cyanea*	1 1 1	1 1-2 1	- Natural or semi-natural habitats - Very low eutrophication
2	S3 S5	*Caloplaca erythrocarpa* *Diplotomma alboatrum* *Lecania spadicea*	1-2 1-2 1-2	2-3 3-4 3-4	- Areas with moderate human disturbance - Low eutrophication
3	S4	*Caloplaca citrina* *Lecania erysibe* *Xanthoria calcicola* *Caloplaca crenulatella*	1-3 1-3 1-2 1-2	4-5 4-5 4-5 4	The disappearance of all the species and the presence of these 2 species indicates that they are zones with strong disturbance of anthropic origin and strong eutrophication

Despite its low poleotolerance index (PI = 1-2), Xanthoria calcicola has been identified at the periphery of the S4 site characterized by a high degree of human disturbance: the explanation can be approached according to the following considerations:

- The effect of pollution on lichen depends on the pH of the substrate. Soils containing calcium and limestone are better able to neutralize sulfuric and nitric acid deposits than siliceous soils. The calcareous substrate moderates the effects of an acid pollution by SO_2 and NOx. The sulfuric acid reacts with the limestone in a neutralization reaction.
  Limestone: $CaCO_3 + H_2SO_4 \dashrightarrow CaSO_4 + H_2CO_3$
  The calcium sulfate is soluble in water and hences the limestone dissolves and crumbles.
  $H_2CO_3 \dashrightarrow CO_2 + H_2O$
  The original acid (hydrogen ions) have been converted to water in these reactions.
- Thanks to a high tolerance to eutrophication, species with low poleotolerance manage to settle in heavily polluted areas; Lachat et al. (2011) report that eutrophication promotes the differential development of some lichen species that resist the effects of pollution. Recently it has been shown that lichen community composition near motorways is shifting towards species associations with more nitrophytic lichens (Madl, 2009). Generally, changes in N-deposition affect the composition of plant diversity and may induce a dominance of nitrophytic species over those species that are more susceptible to nitric-enrichment in nutrient-poor habitats (Frati et al., 2007; Bowman et al., 2006; Riddell et al., 2011). One mechanism leading to this extreme sensitivity of some lichens to excessive supply of nitrogen compounds may be the ability to bind such compounds within cell walls until the concentrations become toxic by causing electrolyte leakage as was shown for *Evernia prunastri* by Munzi et al. (2009a, b).
- In the dried condition the lichens were clearly more resistant. Xerophytic species may appear to be more tolerant to SO_2 pollution only because they are

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dry for longer periods of time during which they are quite tolerant (Beekley et al., 1981). Under low relative humidity conditions, lichens are more resistant to high SO_2 concentrations.

Sites S1 and S2 are characterized by the development of crustose, foliose and fruticose lichens, the fruticose form is represented by only two species: Ramalina pollinaria and Roccella phycopsis which disappear from sites S3, S4 and S5.

Foliose species such as Xanthoria calcicola and Coscinocladium gaditanum were collected at the S3 and S5 sites but absent at S4. Only crustose forms such as Lecania erysibe and caloplaca citrina persist in S4. Overall, there is a decrease in the abundance and diversity of lichens with urbanization and industrialization.

Three categories of calcicolous lichens can be distinguished:

- Species “resistant” to air pollution, encountered in the S4 site
- Moderately resistant species that disappear from S4
- Poleophobe species whose presence is limited to sites S1 S2, S6, and S7

It can be concluded that there is a pollution gradient whose maximum in the S4 site and which decreases as we moves north or south. The results of the two bioindication approaches “Presence/absence of key species” and the “Use of the poleotolerance index and the eutrophication index” have led to the conclusion that in the industrial zone (site S4) the air quality is disturbed by urbanization and industry, the further we get from the S4 site, the better the air quality, and 20 km from the S4 site we have good air quality. Thanks to these results it is possible to model a global map of air pollution in the study area (*Fig. 7*).

It is clear that in nature lichen responds to a mixture of pollutants that may have synergistic, protective or harmful effects on it. The lichens are different in their sensitivity to pollutants and their response to pollutant mixtures (Hyvärinen et al., 1992; Gilbert, 1986; Farmer et al., 1992).

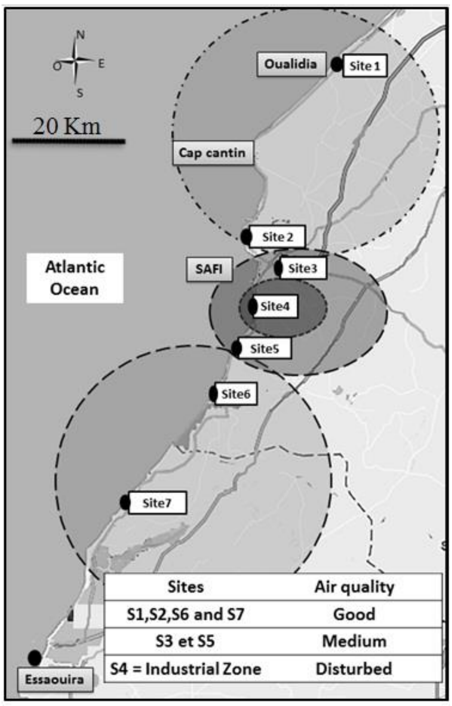

Figure 7. *Delineation of the air quality of the studied sites*

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Conclusion

The study of beta diversity via the similarity index revealed a gradual decrease in beta diversity due to an increasing gradient of pollution, while the hierarchical clustering has allowed a partitioning of the sites studied into three groups of lichenic biodiversity. The bioindication study using the calcicolous lichens has allowed to deduce that sites S3, S4 and S5 S5 are in a circle of 20 km diameter whose center is the industrial zone and where air quality is more or less disrupted by urbanization and industry; while sites S1, S2, S6 and S7 are outside this circle indicating good air quality. The further away from the industrial site (S4), the greater the number and diversity of lichens: The fruticose and foliose forms disappear at 20 km and 4 km respectively around site S4. Only a few crustose forms inhabit the S4 site, however the lichenic desert is not observed.

By using the poleotolerance index and the eutrophication index we conclude that the S4 site suffers a strong anthropogenic disturbance and a strong eutrophication: Further we get from this site, more the environment becomes natural and its rate of eutrophication decreases. This approach has shown that medium poleotolerance combined with high eutrophication resistance allows some calcicolous lichen species, such as Xanthoria Calcicola, to withstand areas of high pollution.

The present study confirms that lichens are good indicators of climatic parameters: the abundance of crustose forms and the low presence of fruticose and foliose forms, indicates an arid character of the regional climate.

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APPENDIX

Table A1. *Inventory of lichens harvested and identified in each site ("+" means species present)*

Site	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7
Distance to the industrial zone in km	**60**	**20**	**10**	**0**	**15**	**33**	**60**
Libellée	Oualidia	North of Safi	City center	Industrial zone	Safi power plant	Souiria	Bhibah
Coordinates	32°43'N 9°60'W	32°20'N 9°16'W	32°17'N 9°14'W	32°15'N 9°15.4'W	32°10'N 9°16'W	32°02'N 9°19'W	31°46'N 9°34'W
Aspicilia calcarea (L.) Mudd	+	+	+		+	+	+
Caloplaca citrina (Hoffm.) Th. Fr.	+	+	+	+	+	+	+
Caloplaca lactea (A. Massal.) Zahlbr.	+	+	+	+	+	+	+
Lecania erysibe (Ach.) Mudd	+	+	+	+	+	+	+
Xanthoria parietina (L.) Th. Fr.	+	+	+		+	+	+
Caloplaca aurantia (Pers.) Hellb.	+	+	+		+	+	+
Coscinocladium gaditanum (Clemente) A. Crespo, Llimona	+	+	+		+	+	+
Myriolecis dispersa (Pers.) Sliwa, Zhao Xin & Lumbsch	+	+	+			+	+
Verrucaria nigrescens Pers.	+	+	+		+	+	+
Verrucaria viridula (Schrad.) Ach.	+	+	+		+	+	+
Xanthoria calcicola Oksner	+	+	+	+	+	+	+
Caloplaca crenulatella (Nyl.) H. Olivier	+		+		+	+	+
Caloplaca erythrocarpa (Pers.) Zwackh	+	+	+			+	+
Diplotomma alboatrum (Hoffm.) Flot. (éco. saxicole-calcicole)	+	+	+			+	+
Lecania spadicea (Flot.) Zahlbr.	+	+	+			+	+
Myriolecis albescens (Hoffm.) Sliwa, Zhao Xin & Lumbsch	+	+	+			+	+
Myriolecis crenulata (Hook.) Sliwa, Zhao Xin & Lumbsch	+	+	+			+	+
Acrocordia conoidea (Fr.) Körb.	+	+				+	+
Arthonia calcarea (Sm.) Ertz & Diederich	+	+				+	+
Baglietto a calciseda (DC.) Gueidan & Cl. Roux	+	+				+	+

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Parabagliettoa cyanea (A. Massal.) Gueidan et Cl. Roux	+	+				+	+
Physcia adscendens (Fr.) H. Olivier	+	+				+	+
Alyxoria subelevata (Nyl.) Ertz & Tehler	+	+					+
Buellia dispersa A. Massal.	+						+
Caloplaca chalybaea (Fr.) Müll. Arg.	+	+	+				+
Caloplaca subochracea (Wedd.) Werner	+	+					+
Diploicia canescens (Dicks.) Massal	+					+	+
Lecania atrynoide M. Knowles	+					+	+
Myriolecis bandolensis. Bertrand, Cl. Roux et Nimis	+				+		
Rocella phycopsis Ach.	+	+				+	+
Dirina massiliensis (A. Massal.) Tehler	+	+				+	+
Alyxoria variiformis (Anzi) Ertz	+	+					
Opegrapha rupestris Pers.	+						+
Ramalina pollinaria (Westr.) Ach.	+	+					+
Toninia aromatica (Sm.) A. Massal.	+						+
*Diploschistes scruposus (*Schreb.) Norman	+	+					+
Total number of species per site	**36**	**30**	**18**	**3**	**12**	**25**	**34**

Table A2. *Poleotolerance indice (PI) and eutrophication indice (EI) of calcicolous species harvested*

Identified species	**PI**	**EI**
Acrocordia conoidea (Fr.) Körb.	1	1
Alyxoria variiformis (Anzi) Ertz	1	1
Alyxoria subelevata (Nyl.) Ertz & Tehler	1-2	1-3
Arthonia calcarea (Sm.) Ertz & Diederich	1-2	1-3
Aspicilia calcarea (L.) Mud*d*	2-3	2-3
Bagliettoa calciseda (DC.) Gueidan & Cl. Roux	1	1-2
caloplaca aurantia (Pers.) Hellb.	1-3	3-4
Caloplaca chalybaea (Fr.) Müll. Arg.	1-3	2-3
Caloplaca citrina (Hoffm.) Th. Fr.	1-3	4-5
Caloplaca crenulatella (Nyl.) H. Olivier	1-2	4
Caloplaca erythrocarpa (Pers.) Zwackh	1-2	2-3
Caloplaca lactea (A. Massal.) Zahlbr.	1-3	2-3
Caloplaca subochracea auct.	1	1-2
Coscinocladium gaditanum (Clemente) A. Crespo, Llimona & D. Hawksw.	1-2	2-3
Diplotomma alboatrum (Hoffm.) Flot. (éco. saxicole-calcicole)	1-2	3-4
Dirina massiliensis (A. Massal.) Tehler	1	1-2
Lecania erysibe (Ach.) Mudd	1-3	4-5
Lecania spadicea (Flot.) Zahlbr.	1-2	3-4
Myriolecis albescens (Hoffm.) Sliwa, Zhao Xin & Lumbsch	1-3	3-4
*Myriolecis crenulata (*Hook.) Sliwa, Zhao Xin & Lumbsch	1	1-2
Myriolecis dispersa (Pers.) Sliwa, Zhao Xin & Lumbsch	2-3	2-4
Parabagliettoa cyanea (A. Massal.) Gueidan et Cl. Roux	1	1
Physcia adscendens (Fr.) H. Olivier	1-3	3-5
Ramalina pollinaria (Westr.) Ach.	1-2	2-4
Rocella phycopsis Ach.	1-2	1-3
Toninia aromatica (Sm.) A. Massal.	1-3	2-4
Verrucaria nigrescens Pers.	1-3	2-5
Verrucaria viridula (Schrad.) Ach.	1-2	3-4
Xanthoria calcicola Oxsner	1-2	4-5
Xanthoria parietina (L.) Th.Fr.	1-3	3-4
Buellia dispersa A. Massal.	1	2-3
Diploicia canescens (Dicks.) Massal	1-2	2-4
Diploschistes scruposus (Schreb.) Norman	1-2	1-3
Lecania atrynoides M. Knowles	1	2-3
Myriolecis bandolensis (B. de Lesd.) Bertrand, Cl. Roux et Nimis	1	2-4
Opegrapha rupestris Pers.	1-2	1-2

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DOI: http://dx.doi.org/10.15666/aeer/1702_43054323

EFFECTS OF FOLIAR UREA, POTASSIUM AND ZINC SULPHATE TREATMENTS BEFORE AND AFTER FLOWERING ON GRAIN YIELD, TECHNOLOGICAL QUALITY AND NUTRIENT CONCENTRATIONS OF WHEAT

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(Received 30th Dec 2018; accepted 27th Feb 2019)

Abstract. This study was carried out under field conditions to determine the effects of foliar urea, potassium sulphate (K_2SO_4) and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) treatments at vegetative and generative periods on yield, technological quality and mineral element concentrations of wheat (*Triticum aestivum* L. cv. Altinbasak). Experiments were conducted in two experiment groups (foliar treatments of 0% (Control); 0.5% Urea; 1% Urea; 0.5% $ZnSO_4.7H_2O$; 0.5% Urea+0.5% $ZnSO_4.7H_2O$; 1% Urea+0.5% $ZnSO_4.7H_2O$; 0.5% K_2SO_4 and 1% K_2SO_4 were performed in the first experiment and foliar 0%; 0.5% Urea+0.5% K_2SO_4; 0.5% Urea+1% K_2SO_4; 1% Urea+0.5% K_2SO_4; 1% Urea+1% K_2SO_4 were performed in the second experiment). All treatments increased yield and sedimentation values as compared to the control treatments. In the first experiment, 0.5% urea treatment was prominent for yield, nitrogen and protein and urea+$ZnSO_4.7H_2O$ treatments for grain zinc and iron concentrations. In the second experiment, 1% urea+0.5% K_2SO_4 treatments were prominent for protein and grain nitrogen concentrations and 1% urea+1% K_2SO_4 and 0.5% urea+0.5% K_2SO_4 treatments for yield.
Keywords: *nitrogen, sulphur, micronutrients, gluten, Zeleny sedimentation*

Introduction

Cereals are staple foods, and are significant sources of nutrients in both developed and developing countries. Therefore, nutritional quality of cereals should be improved. Foliar fertilization seems to be a practical way to eliminate nutritional problems and to improve product quality. In this sense, foliar nitrogen (N) treatments had significant effects on yield and quality parameters (Shah et al., 2003; Rahman et al., 2014). Especially, foliar application of urea was demonstrated to be an efficient method of N fertilization in grain cereals in 1950's (Finney et al., 1957). Foliar applications of potassium (K) (Hamouda et al., 2015) and zinc (Zn) (Habib, 2009) had also positive effects on grain yield and quality of wheat. It was recommended by Wang et al. (2017) that foliar N and K treatments could be used to maximize plant respond to Zn treatments.

Prevention of sulphur (S) release to atmosphere with environmental measures, high-cost and resultant decreasing uses of S fertilizers, again decreasing uses of S-containing N and phosphorus (P) fertilizers, excessive nitrogenous fertilizer uses, breeding of high yield varieties and similar factors have made S deficiency in soils and plants a widespread problem. Insufficient S levels prevent grain cereals from reaching their actual potential in yield, quality and protein content. Insufficient S also hinders efficient use of N applied to soils (Sahota, 2006). It was reported that N and S nutrition had significant effects on grain yield and protein concentration of wheat. Wheat S need is less than N need. However, in sulphur deficiency, non-protein N compounds (free amides: asparagine, glutamine) are accumulated, and synthesis and accumulation of S-containing amino acids (cysteine, methionine) in grains decreased (Zhao et al., 1999a;

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Granvogl et al., 2007). Under S deficiency, these N forms increase and signal is passed from the shoots to roots as if there was sufficient N, thus less N is up taken by the plants from the nutrient ambient. When the plants are nourished with sufficient S, protein N, organic S and SO_4-S concentrations increase, but nitrate and soluble organic acid concentrations decrease (Marschner, 1995). Such findings revealed that S-deficiency destroyed plant N metabolism (McGrath and Zhao, 1996). Nitrogen and S are the basic components of proteins, therefore a balance between N and S is a critical issue for bread making quality of wheat (Randall and Wrigley, 1986). Sulphur not only influences N use and protein quality, but also plays a significant role in baking quality (Ryant and Hřivna, 2004).

Recent studies revealed that cereal grains were poor in mineral element concentrations. For instance, Zn concentrations of wheat grains (generally 25-30 mg kg^{-1}) were quite below the requirements for healthy human nutrition or the recommended levels (Erdal et al., 2002; Cakmak, 2008). Previous studies also indicated that grain Zn and Fe concentrations could be improved with N treatments, and Zn and N treatments had a synergic effect in increasing Zn concentrations of wheat grains (Kutman, 2010; Shi et al., 2010). In another study, increasing Zn and Fe concentrations of wheat grains grown under field conditions were reported with increasing N treatments (Cakmak et al., 2010). When the sufficient Zn levels were supplied to plants, both soil and foliar N treatments were reported to increase grain Zn concentrations (Kutman et al., 2010). Analyses for grain mineral nutrient concentrations revealed that there were significant correlations between grain Zn and Fe concentrations and S concentrations (McDonald and Mousavvi, 2009).

The objective of the present study was to evaluate the effects of urea, K_2SO_4 and $ZnSO_4.7H_2O$ treatments on yield parameters, technological quality and grain nutrient concentrations of Altinbasak bread wheat cultivar.

Materials and methods

Seed and soil materials

Altinbasak bread wheat cultivar commonly grown in Çukurova region was used as the seed material of the present study. Field experiments were conducted over the experimental fields of Eastern Mediterranean Agricultural Research Institute. Experiments were carried out at Dogankent station (36°85′ N, 35°34′ E) during the wheat growing seasons of the years 2014-2015 and 2015-2016. Soil physical and chemical characteristics of the experimental site are provided in *Table 1*.

Table 1. *Soil physical and chemical characteristics of the experiment site for 2014-2015 and 2015-2016 growing seasons*

Growing Season	Texture	pH	Salinity	$CaCO_3$	Org.M.	P	K	SO_4-S	Zn	Fe	Cu	Mn
		(1:2.5)	(mmhos cm^{-1})	%		(mg kg^{-1})						
2014-15	CL	7.85	0.25	15.2	1.45	5.60	426	10.2	0.35	4.60	2.30	4.81
2015-16	CL	8.01	0.02	14.2	1.50	4.95	310	11.2	0.36	4.76	0.67	2.57

CL: clay-loam

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Field experiments

Experiments were conducted in randomized blocks – split plots experimental design with 4 replications. Experiments were set up on 7 November 2014 in the first year and on 14 November 2015 in the second year. Treatment periods (before flowering – BF, after flowering – AF) were placed in the main plots and foliar treatments were placed in sub-plots (1.4 x 5 = 7 m^2). Sowing density was arranged as 450 seed per m^2. At sowing, 70 kg ha^{-1} phosphorus and 160 kg ha^{-1} nitrogen fertilizers were also applied (in DAP and urea forms). Nitrogen was applied at sowing and tillering stages. Weed control was practiced at tillering stage with 200 ml ha^{-1} Terdok 240 EC. At the end of tillering period, 2-4 D Amine was applied against broad-leaf weeds.

Before flowering (BF) period; foliar treatments were performed once at stem elongation stage (Zadoks 34-36) and once at booting stage (Zadoks 47-49). After flowering period; foliar treatments were performed once at early milk stage (Zadoks 73-74) and once at early dough stage (Zadoks 83-84) (Zadoks et al., 1974). Any surfactant was not used in the fertilizer solutions (w/v). Generally, a typical foliar Zn fertilizer solution contains 2-5 g zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) per liter (Cakmak and Kutman, 2018). In present study, foliar treatments of the first experiment were: 0% (Control); 0.5% Urea; 1% Urea; 0.5% $ZnSO_4.7H_2O$; 0.5% Urea+0.5% $ZnSO_4.7H_2O$; 1% Urea+0.5% $ZnSO_4.7H_2O$; 0.5% K_2SO_4 and 1% K_2SO_4. Foliar treatments of the second experiment were: 0% (Control); 0.5% Urea+ 0.5% K_2SO_4; 0.5% Urea+1% K_2SO_4; 1% Urea+0.5% K_2SO_4; 1% Urea+ 1% K_2SO_4. Only water was applied to control plots. In the other plots, 1000 ml solution was used for each one of 7 m^2 plots as to homogeneously wet the entire plot at stem elongation and booting stages before flowering and at early milk and early dough stages after flowering.

Plots were harvested by a combine, and total grain yield was expressed in kg per hectare at 11% moisture basis. Harvest date was 29 May 2015 in the first year and 13 June 2016 in the second year. Following harvests from 5 m x 1.4 m (7 m^2) plots, grain yields per hectare were determined. The 1000-grain weights were determined in accordance with Ozkaya and Ozkaya (2005). Grain samples were ground and wet-digested in a microwave digester using 2 ml of 35% H_2O_2 and 5 ml of 65% HNO_3. After the digestion, K, S, Zn and Fe were analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES; Varian-Vista Pro). LECO TruSpec C/N Analyzer (Leco Corp. St Joseph, MI, USA) operating in accordance with Dumas method was used to determine nitrogen concentrations. Reference leaf samples from National Institute of Standards and Technology (Gaithersburg, MD, USA) were used to check the related element (S, K, Zn and Fe) measurements. Total protein concentration of the grain was calculated as 5.7 x N concentration (Zhao et al., 1999b). Wet gluten concentration was measured in a FOSS NIRS 6500 System according to standard method ICC 155 (International Association for Cereal Chemistry, 1994). Zeleny sedimentation was determined by using ICC method 116/1 (International Association for Cereal Chemistry, 1994).

Soil analysis

Available soil Zn, Fe, manganese (Mn) and copper (Cu) concentrations were determined according to Lindsay and Norvel (1978), available P concentration in accordance with Olsen et al. (1954). Soil K concentrations were measured in accordance with the ammonium acetate (pH: 7, 1N) method of Carson (1980). Soil pH was detected

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

according to Jackson (1959). Soil organic matter content was determined following the Walkey-Black wet-etching method (Jackson, 1959). Soil texture was determined according to Bouyoucus (1951). Soil lime content was determined according to Allison and Moodie (1965) and soil salinity was determined from saturation paste extracts according to Wheatstone bridge method (U. S. Salinity Laboratory Staff, 1954). For soil soluble SO_4-S analyses, dried and sieved (< 0.18 mm) 5 g soil sample was placed into 250 ml Erlenmeyer flasks, supplemented with 50 ml 0.1 M LiCI solution and shaken for 30 minutes. Soil suspension was then filtered through filter papers and resultant extract was read at 182.037 nm wave length of an ICP device for soluble SO_4-S quantity (Arkley, 1961).

Weather conditions

A total of 687 mm rainfall was received in the 2014-2015 growing season and 348 mm in the 2015-2016 growing season. While the precipitations of the first year were above the long-term averages (about 25% greater than the averages), precipitations of the second year were quite below the long-term averages (about 38% less than the averages). Temperatures of the experimental years were close to long-term averages (*Table 2*).

Table 2. *Weather conditions of experimental site for 2014-2015 and 2015-2016 growing seasons and long-term averages (1978-2016)*

Parameters	Time	Nov. 2014	Dec. 2014	Jan. 2015	Feb. 2015	Mar. 2015	Apr. 2015	May 2015	Jun. 2015	Total
Rainfall (mm)	Long-term	86.3	115.6	97.4	80.7	60.5	47.7	43.8	16.8	549
	2014-2015	72.5	102.5	153.0	160.0	91.0	19.0	58.0	30.5	687
Temperature (°C)	Long-term	14.7	10.4	9.3	9.9	12.8	17.3	21.5	25.3	
	2014-2015	15.0	13.0	9.0	11.0	14.0	16.0	22.0	24.0	
		Nov. 2015	**Dec. 2015**	**Jan. 2016**	**Feb. 2016**	**Mar. 2016**	**Apr. 2016**	**May 2016**	**Jun. 2016**	**Total**
Rainfall (mm)	Long term	86.3	115.6	97.4	80.7	60.5	47.7	43.8	16.8	549
	2015-2016	0.0	0.0	105.0	64.0	98.0	5.0	71.0	5.0	348
Temperature (°C)	Long term	14.7	10.4	9.3	9.9	12.8	17.3	21.5	25.3	
	2015-2016	13.1	9.1	7.3	12.3	14.0	18.5	19.8	23.0	

Statistical analysis

JUMP software was used for statistical analyses. Following ANOVA, significant factor means were compared by TUKEY's multiple range tests. The significance levels were taken as $P<0.05$ (*) and $P<0.01$ (**).

Results and discussion

Effects of treatments on grain yield, 1000-grain weight, protein, wet gluten and Zeleny sedimentation

There were significant differences in the yields of the years because of climate factors (*Table 3*). Rainfalls and temperatures are the most significant climate factors influencing plant growth and development. Total precipitations of the second year (348 mm) were quite lower than the precipitations of the first year (687 mm) (*Table 2*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Table 3. *Analysis of variance for investigated parameters*

Source of variation	d.f.	Grain Yield	1000 Grain Weight	Protein	Wet Gluten	Zeleny Sed.	Grain N	Grain K	Grain S	Grain Zn	Grain Fe
					Ist Experiment						
Year (A)	1	<.0001**	<.0001**	<.0001**	0.0555	0.0015**	<.0001**	<.0001**	0.0054**	0.0048**	0.0114*
Error 1	6	0.4507	0.9987	0.7636	0.9012	0.7267	0.7672	0.5821	0.9405	0.6595	0.9987
Application Time (B)	1	0.5819ns	0.7290ns	0.3378ns	0.6061ns	0.8653ns	0.3407ns	0.2529ns	0.3918ns	0.6281ns	0.8007ns
A x B	1	0.9842ns	0.7237ns	0.5931ns	0.3854ns	0.4843ns	0.5926ns	0.8254ns	0.9394ns	0.7842ns	0.9800ns
Error 2	6	0.0105	<.0001	0.0564	<.0001	0.3030	0.0552	0.5931	<.0001	0.0071	<.0001
Applications (C)	7	0.0041**	0.8750ns	<.0001**	0.0507ns	0.0205*	<.0001**	0.0068**	0.0615ns	<.0001**	0.0020**
A x C	7	0.0921ns	0.7622ns	0.0055**	0.1209ns	0.2446ns	0.0053**	0.9935ns	0.2177ns	0.2241ns	0.1259ns
B x C	7	0.0020**	0.7639ns	0.0898ns	0.0191*	0.1328ns	0.0867ns	0.7819ns	0.1521ns	0.5063ns	0.4446ns
A x B x C	7	0.9795ns	0.5557ns	0.0003**	0.2436ns	0.3321ns	0.0003**	0.9989ns	0.4522ns	0.0020**	0.8867ns
General	84										
C. Total	127										
					IInd Experiment						
Year (A)	1	<.0001**	0.0042**	0.0031**	0.0814ns	0.0026**	0.0030**	<.0001**	0.0011**	<.0001**	0.0006**
Error 1	6	0.8896	0.6189	0.3242	0.7276	0.9771	0.3218	0.0129	0.796	0.4032	0.9991
Application Time (B)	1	0.6087ns	0.4619ns	0.0746ns	0.2460ns	0.2698ns	0.0718ns	0.3427ns	0.3155ns	0.2919ns	0.3185ns
A x B	1	0.2722ns	0.4599ns	0.0411*	0.4927ns	0.7885ns	0.0420*	0.6947ns	0.5212ns	0.5940ns	0.4196ns
Error 2	6	0.0235	<.0001	0.0178	0.0002	0.0143	0.0182	0.8969	0.0131	0.7854	0.0013
Applications (C)	4	0.0335*	0.2004ns	0.0002**	0.2359ns	<.0001**	0.0002**	0.1854ns	0.1817ns	0.0463*	0.3065ns
A x C	4	<.0001**	0.3736ns	0.0154*	0.0301*	0.9519ns	0.0150*	0.9801ns	0.8747ns	0.2447ns	0.0718ns
B x C	4	0.3588ns	0.6439ns	0.1211ns	0.6182ns	0.2951ns	0.1236ns	0.2147ns	0.9896ns	0.3313ns	0.1020ns
A x B x C	4	0.7770ns	0.6757ns	0.5963ns	0.1620ns	0.5572ns	0.6122ns	0.9950ns	0.2286ns	0.2415ns	0.1291ns
General	48										
C. Total	79										

*: p<0.05; **: p<0.01; NS: not significant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Therefore, yields of the second year were lower than the yields of the first year in both experiments (*Table 4*). Considering the treatment periods, the differences in yields of before flowering (BF) and after flowering (AF) periods were not significantly different (*Table 4*).

In the first experiment, effects of treatments on yields were found to be significant (*Table 3*). With regard to general averages of treatments, yields of 0.5% Urea+0.5% $ZnSO_4.7H_2O$ and 0.5% $ZnSO_4.7H_2O$ treatments were placed in the same statistical group with the control treatment (*Table 5*). The greatest yield (974 kg da^{-1}) was obtained from 0.5% Urea treatments (*Table 5*). Jakhro et al. (2000) reported that urea treatments increased plant height, number of tillers, spike lengths, harvest index, grain yield, hay yield and protein content of wheat grains. Khan et al. (2009) applied 4% urea at 6 different concentrations and at consecutive 3 stages (tillering, stem elongation and booting) and reported 32% increase in wheat grain yield. Considering the general averages of treatments of the first experiment, it was observed that foliar zinc treatments did not result in significant differences in yields. Foliar urea and zinc combined treatment (1% Urea+0.5% $ZnSO_4.7H_2O$) slightly increased yields as compared to the control treatment (*Table 5*). With regard to general averages of the treatments of the second trails, 0.5% Urea+0.5% K_2SO_4 and 1% Urea+1% K_2SO_4 treatments resulted in significant increases in yields as compared to the control treatments (*Table 5*). In the first experiment, foliar K_2SO_4 treatments (0.5% K_2SO_4, 1% K_2SO_4) before flowering slightly increased grain yields as compared to the control treatment (*Table 5*). Such a positive impact probably resulted from both K and S. Thusly, foliar potassium treatments had positive effects on physiological parameters especially in grain-fill period (Zareian et al., 2013) and yield values (Hamouda et al., 2015). Similarly, Gupta et al. (2004) reported significant increases in yield and yield components with S treatments. It was reported in a study carried out in Germany, yield increase rates improved by 17% with S treatments and the yield increase supplied by S treatments varied between 5-30% (Zhao et al., 2002). Zinc sulphate and K_2SO_4 sources are commonly preferred in foliar fertilization practices (Singh et al., 2013).

While the treatments did not result in significant differences in 1000-grain weights of the first and second experiments, years created significant differences (*Table 3*). In the first experiment, 1000-grain weight of the first year (45.8 g) was greater than the second year (33.9 g). Similar decrease trends were also observed in the second experiment (*Table 4*). The differences between the treatment periods (BF and AF) were not found to be significant in both experiments (*Table 3*). General averages of 1000-grain weights in before and after flowering periods were not significant in both experiments (*Table 5*).

In the first experiment, treatments resulted in significant differences in grain protein concentrations of both periods. The highest protein concentration was found in the second year with 1% urea application of BF period. Together with decreasing 1000-grain weights, grain protein concentrations increased in the second year (concentration effect) as compared to the first year. Similar findings were also observed in the second experiment (*Table 4*). With regard to averages of treatments, the greatest grain protein concentration in the first experiment was obtained from 0.5% urea treatment (12.9%) and the lowest value was observed in control treatment (12.1%) (*Table 5*). In the first experiment, as the averages of treatments, all treatments increased grain protein concentrations as compared to the control treatments (*Table 5*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Table 4. *Effects of different foliar fertilizer treatments, applied before and after flowering, on grain yield, 1000-grain weight and protein content of Altınbaşak bread wheat cultivar*

Appl. Time	**Applications**	**Grain Yield (kg ha^{-1})**			**1000 Grain Weight (gr)**			**Protein (%)**		
		1st Year	**2nd Year**	**Mean**	**1st Year**	**2nd Year**	**Mean**	**1st Year**	**2nd Year**	**Mean**
				Ist Experiment						
Before Flowering	Control	10248	8340	9294 ab	44.40	34.02	39.21	11.94 ıjk	12.10 h-k	12.02
	Urea 0.5 %	10430	9030	9730 a	46.46	33.79	40.12	12.36 d-k	13.43 a	12.89
	Urea 1 %	10503	8660	9581 ab	45.90	33.63	39.77	12.23 f-k	12.67 b-ı	12.45
	$ZnSO_4$ 0.5 %	9863	8273	9068 b	45.72	34.19	39.96	11.87 jk	12.84 a-h	12.35
	Urea 0.5 % +$ZnSO_4$ 0.5 %	9820	8533	9176 ab	43.94	33.83	38.88	12.27 e-k	13.12 abc	12.70
	Urea 1 % +$ZnSO_4$ 0.5 %	9993	8237	9115 ab	44.66	34.30	39.48	11.90 jk	13.21 abc	12.55
	K_2SO_4 0.5 %	10313	9110	9711 ab	45.85	34.04	39.94	12.24 f-k	13.01 a-e	12.63
	K_2SO_4 1 %	10593	8837	9715 ab	45.94	33.50	39.72	12.02 ıjk	12.67 b-ı	12.34
	Mean	**10220**	**8628**	**9424**	**45.36**	**33.91**	**39.64**	**12.10**	**12.88**	**12.49**
After Flowering	Control	10233	8453	9342 ab	46.46	34.04	40.25	11.81 k	12.57 c-j	12.19
	Urea 0.5 %	10472	9028	9750 a	46.92	33.93	40.43	12.62 c-j	13.09 a-d	12.85
	Urea 1 %	10450	8818	9634 ab	46.36	33.97	40.17	12.24 f-k	12.97 a-f	12.61
	$ZnSO_4$ 0.5 %	10433	8653	9543 ab	45.79	34.25	40.02	12.24 f-k	12.86 a-g	12.55
	Urea 0.5 % +$ZnSO_4$ 0.5 %	9868	8790	9329 ab	46.59	34.25	40.42	12.09 ıjk	13.01 a-e	12.55
	Urea 1 % +$ZnSO_4$ 0.5 %	10563	8818	9690 ab	46.45	32.96	39.70	12.17 g-k	12.89 a-g	12.53
	K_2SO_4 0.5 %	10020	8585	9302 ab	45.59	34.63	40.11	12.51 c-k	12.51 c-k	12.51
	K_2SO_4 1 %	10270	8388	9329 ab	45.60	33.21	39.41	12.04 ıjk	13.41 ab	12.73
	Mean	**10288**	**8691**	**9489**	**46.22**	**33.9**	**40.06**	**12.21**	**12.91**	**12.56**
	General mean	**10254 a**	**8659 b**		**45.8 a**	**33.9 b**		**12.16 b**	**12.90 a**	
	CV (%)	**3.92**			**3.84**			**2.16**		
				IInd Experiment						
Before Flowering	Control	9800	8030	8915	42.08	31.91	37.00	11.90	12.97	12.43
	Urea 0.5 % + K_2SO_4 0.5 %	9893	8430	9161	43.31	31.73	37.52	11.74	13.78	12.76
	Urea 0.5 % + K_2SO_4 1 %	9450	8548	8999	43.07	32.80	37.94	11.91	13.08	12.50
	Urea 1 % + K_2SO_4 0.5 %	9923	8313	9118	40.58	33.31	36.94	12.47	13.92	13.20
	Urea 1 % + K_2SO_4 1 %	10423	8413	9418	43.09	33.37	38.23	11.66	13.43	12.55
	Mean	**9898**	**8347**	**9122**	**42.43**	**32.62**	**37.53**	**11.94 b**	**13.44 a**	**12.68**
After Flowering	Control	9595	8398	8996	37.11	31.55	34.32	13.13	13.08	13.10
	Urea 0.5 % +K_2SO_4 0.5 %	9865	8598	9231	40.60	33.18	36.89	12.04	13.26	12.65
	Urea 0.5 % +K_2SO_4 1 %	9540	9068	9304	39.89	33.22	36.56	13.01	13.51	13.26
	Urea 1 % +K_2SO_4 0.5 %	9938	8365	9151	39.29	33.29	36.29	14.02	13.67	13.84
	Urea 1 % +K_2SO_4 1 %	10155	8338	9246	40.63	31.91	36.27	12.31	13.23	12.77
	Mean	**9819**	**8553**	**9186**	**39.50**	**32.63**	**36.07**	**12.90 ab**	**13.35 a**	**13.13**
	General mean	**9858 a**	**8450 b**		**40.9 a**	**32.6 b**		**12.42b**	**13.39 a**	
	CV (%)	**3.50**			**5.98**			**4.15**		

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

***Table 5.** Effects of different foliar fertilizer treatments and combinations on general mean of grain yield, 1000-grain weight, protein, wet gluten and Zeleny sedimentation of Altınbaşak bread wheat cultivar*

	Applications	General Mean				
		Grain Yield	1000 G. Wt.	Protein	Wet Glu.	Zeleny Sed.
		(kg ha^{-1})	(g)	(%)		(ml)
Ist Experiment	Control	9318 b	39.7	12.1 c	25.6	50.1 b
	Urea 0.5 %	9740 a	40.3	12.9 a	26.7	54.6 ab
	Urea 1 %	9607 ab	40.0	12.5 b	26.7	55.6 a
	$ZnSO_4$ 0.5 %	9305 b	40.0	12.5 b	25.6	52.7 ab
	Urea 0.5 % + $ZnSO_4$ 0.5 %	9253 b	39.7	12.6 ab	26.6	54.1 ab
	Urea 1 % + $ZnSO_4$ 0.5 %	9403 ab	39.6	12.5 b	26.2	55.1 ab
	K_2SO_4 0.5 %	9507 ab	40.0	12.6 b	26.5	55.7 a
	K_2SO_4 1 %	9522 ab	39.6	12.5 b	26.1	55.5 a
IInd Experiment	Control	8956 b	35.7	12.8 b	27.7	48.5 b
	Urea 0.5 % + K_2SO_4 0.5 %	9196 a	37.2	12.8 b	27.3	56.5 a
	Urea 0.5 % + K_2SO_4 1 %	9151 ab	37.3	12.9 b	27.7	54.5 a
	Urea 1 % + K_2SO_4 0.5 %	9134 ab	36.6	13.5 a	29.0	59.5 a
	Urea 1 % + K_2SO_4 1 %	9332 a	37.3	12.7 b	27.8	56.8 a

In the second experiment, the greatest grain protein concentration was obtained from 1% Urea + 0.5% K_2SO_4 treatments (13.5%) and the other treatments had similar protein concentrations with the control treatments (*Table 5*). Present protein concentrations (*Table 4*) were all within 11-14% limit values specified by Mailhot and Patton (1988) for bread making. Grain protein concentrations of the 23 bread wheat genotypes varied between 7.99-13.31% (Soboka et al., 2017).

In the first experiment, the greatest wet gluten concentration (27.09%) was obtained from 1% urea treatment of AF period (*Table 6*). Similarly, increasing wet gluten levels were reported with increasing nitrogen doses (Erekul et al., 2012). In present study, the lowest wet gluten concentration (24.85%) was obtained from 0.5% $ZnSO_4$ treatment of BF period (*Table 6*). In the second experiment, wet gluten concentrations did not change with treatments (*Table 6*). In both experiments, greater wet gluten concentrations were observed in the second year, but the differences in wet gluten concentrations of the years were not significant (*Table 6*). Considering the general averages of the treatments, effects of treatments on wet gluten concentrations were not found to be significant in both experiments (*Table 5*).

The sedimentation values of the wheat cultivar in this research, characterizing the swelling capacity of gluten, exceeded the nominal value (20 ml) for bread wheat in both years (*Table 6*). In both experiments, Zeleny sedimentation values of treatment periods were not found to be significant (*Table 3*), but the treatments were found to be significant (*Table 3 and 5*). In the first experiment, the greatest Zeleny sedimentation value was obtained from 1% Urea, 0.5% K_2SO_4 and 1% K_2SO_4 treatments and the lowest value was obtained from the control treatments (*Table 5*). Slightly greater Zeleny sedimentation values were observed from single $ZnSO_4.7H_2O$ and combined urea treatments as compared to the control treatment (*Table 5*). The increases in Zeleny sedimentation values with the separate urea and K_2SO_4 treatments of the first experiment as compared to the control treatments were also observed with urea and K_2SO_4 combined treatments of the second experiment (*Table 5*). Zeleny sedimentation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

values should be above 37 ml for a quality bread making. The greater the values over this value, the greater the quality will be. While 43 ml is assessed as moderately well, the values over 50 ml were assessed as the 1st Class wheat. In another study, Zeleny sedimentation (ml) values were classified as; > 30 ml very well; 25-30 ml well; 20-25 ml moderate and <20 ml poor (Unal, 2002). In present study, all treatments of both experiments yielded Zeleny sedimentation values above 50 ml (*Table 5*).

It was reported in a previous study that urea treatments applied at flowering period significantly increased protein, Zeleny sedimentation and wet gluten values of bread wheat cultivars (Varga and Svečnjak, 2006). Late period urea treatments were also reported to increase protein and wet gluten contents of wheat cultivars (Peltonen, 1992).

Table 6. *Effects of different foliar fertilizer treatments, applied before and after flowering, on wet gluten and Zeleny sedimentation of Altınbaşak bread wheat cultivar*

Appl. Time	Applications	Wet Gluten (%)			Zeleny Sedimentation (ml)		
		1st Year	2nd Year	Mean	1st Year	2nd Year	Mean
			Ist Experiment				
Before Flowering	Control	25.83	25.85	25.84 ab	48.25	50.88	49.56
	Urea 0.5 %	25.34	27.69	26.51 ab	50.50	59.75	55.13
	Urea 1 %	26.10	26.46	26.28 ab	52.25	61.13	56.69
	$ZnSO_4$ 0.5 %	24.28	25.41	24.85 b	47.25	56.50	51.88
	Urea 0.5 % + $ZnSO_4$ 0.5 %	25.72	27.80	26.76 ab	49.25	60.00	54.63
	Urea 1 % + $ZnSO_4$ 0.5 %	25.67	26.03	25.85 ab	50 25	55.00	52.63
	K_2SO_4 0.5 %	26.24	27.65	26.95 ab	53.75	54.88	54.31
	K_2SO_4 1 %	24.03	26.11	25.07 ab	57.50	58.50	58.00
	Mean	**25.40**	**26.63**	**26.01**	**51.13**	**57.08**	**54.10**
After Flowering	Control	23.74	27.16	25.45 ab	49.00	52.25	50.63
	Urea 0.5 %	26.01	27.85	26.93 ab	52.00	56.19	54.09
	Urea 1 %	26 07	28.11	27.09 a	53.00	56.00	54.50
	$ZnSO_4$ 0.5 %	24.99	27.82	26.41 ab	47.50	59.63	53.56
	Urea 0.5 % + $ZnSO_4$ 0.5 %	24.96	27.85	26.41 ab	53.00	54.13	53.56
	Urea 1 % + $ZnSO_4$ 0.5 %	25.30	27.99	26.64 ab	56.00	59.25	57.63
	K_2SO_4 0.5 %	25.23	26.73	25.98 ab	56.25	58.00	57.13
	K_2SO_4 1 %	24.22	29.60	26.91 ab	49.25	56.88	53.06
	Mean	**25.07**	**27.89**	**26.48**	**52.00**	**56.54**	**54.27**
	General mean	**25.23**	**27.26**		**51.56 b**	**56.81 a**	
	CV (%)	**4.67**			**8.96**		
			IInd Experiment				
Before Flowering	Control	25.16	28.50	26.83	41.75	52.75	47.25
	Urea 0.5 % + K_2SO_4 0.5 %	23.22	30.09	26.66	49.75	62.88	56.31
	Urea 0.5 % + K_2SO_4 1 %	26.14	27.92	27.03	46.50	55.38	50.94
	Urea 1 % + K_2SO_4 0.5 %	26.80	31.08	28.94	54.25	65.00	59.63
	Urea 1 % + K_2SO_4 1 %	27.13	26.15	26.64	51.25	59.88	55.56
	Mean	**25.69**	**28.75**	**27.22**	**48.70**	**59.18**	**53.94**
After Flowering	Control	27.28	29.97	28.62	44.00	55.38	49.69
	Urea 0.5 % + K_2SO_4 0.5 %	27.19	28.88	28.03	53.50	59.88	56.69
	Urea 0.5 % + K_2SO_4 1 %	27.07	29.64	28.36	52.50	63.75	58.13
	Urea 1 % + K_2SO_4 0.5 %	28.73	29.32	29.02	56.50	62.25	59.38
	Urea 1 % + K_2SO_4 1 %	29.11	28.96	29.03	52.00	64.00	58.00
	Mean	**27.88**	**29.35**	**28.61**	**51.70**	**61.05**	**56.38**
	General mean	**26.78**	**29.05**		**50.20 b**	**60.11 a**	
	CV (%)	**7.45**			**9.39**		

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Effects of treatments on grain nitrogen (N), potassium (K), sulphur (S), zinc (Zn) and iron (Fe) concentrations

With regard to effects of different foliar fertilizer treatments applied before and after flowering on grain N, K, S, Zn and Fe concentrations, it was observed that years created significant differences (*Table 3*). Because of greater precipitations of the second year (*Table 2*), nutrients concentrated in the grains and greater values were observed in the second year (*Table 7 and 8*).

Treatment periods did not have significant effects on grain N, K, S, Zn and Fe concentrations in both experiments (*Table 3*). Treatment periods did not have significant effects on grain K concentrations in both experiments (*Table 3*). In the first experiment, the greatest grain K concentration was obtained from 0.5% K_2SO_4 treatment. In the second experiment, treatments did not have significant effects on grain K concentrations (*Table 9*).

With regard to effects of general averages of treatments on N concentrations, it was observed that all treatments increased N concentrations as compared to the control treatment (*Table 9*). In the first experiment, the greatest N concentration (2.26%) was observed in 0.5% urea treatment and the lowest N concentration (2.12%) was observed in control treatments (*Table 9*). In the second experiment, the greatest N concentration was obtained from 1% Urea + 0.5% K_2SO_4 treatments (*Table 9*). Especially in the second experiment, AF treatments were more effective on mean N (*Table 7*) and protein concentrations (*Table 4*) than BF treatments. Thusly, N treatments close to flowering were reported to influence N uptake and protein contents (Banziger et al., 1994).

The critical extractable S level is commonly reported as 10 mg S kg^{-1}, but is known to vary from 8 to 25 mg kg^{-1} (Mukhopadhyay and Mukhopadhyay, 1995) and 8 to 12 mg kg^{-1} as reported by Tandon (1991) depending on soil, crop, extractant and laboratory procedures. For wheat, the critical level for soil available S concentration was reported as 12 mg kg^{-1} (Rodríguez et al., 2001; Alfaro et al., 2006). According to the survey study results in Ankara, Turkey, more than 50% of the soil, plant straw and grain samples contained lower S than the critical limits (Inal et al., 2003). As the average of the first and the second year, there was 10.7 mg/kg available S in soil (*Table 1*). This value did not result in S deficiency. In both experiments, treatment periods, treatments and interactions did not have significant effects on S concentrations (*Table 3*). Grain S concentrations lower than 1.2 mg g^{-1} and grain N:S ratios higher than 17:1 appear to be critical values for S deficiency (Randall et al., 1981). In present study, S concentrations of both experiments were greater than 1.2 mg g^{-1} and N:S ratios were lower than 17:1. Sulphur plays a significant role in methionine (21% S) and cysteine (27% S) formation, chlorophyll and protein synthesis, seed oil contents and nutritive quality accumulation (Tandon, 1986; Jamal et al., 2005). Sulphur status of wheat grain also influences quality parameters (Marschner, 1997; Zhao et al., 1999a; McGrath, 2003; Honermeier and Simioniuc, 2004). Grain N:S ratio influences bread-making quality and rheological characteristics of the dough.

In recent studies, a new strategy called as "*agronomic biofortification*" have come into prominence. In this strategy, selection, breeding and molecular methods are employed and improvement of grain micro nutrient contents and bio-availabilities are targeted. Such a strategy gained a great support because of sustainability and widespread impacts (Ortiz- Monasterio et al., 2007; Cakmak, 2008).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Table 7. *Effects of different foliar fertilizer treatments and combinations, applied before and after flowering, on grain K, N, S concentrations and N/S ratio of Altınbaşak bread wheat cultivar*

Appl. Time	Applications	K (%)			N (%)			S (%)			N/S
		1st Year	2nd Year	Mean	1st Year	2nd Year	Mean	1st Year	2nd Year	Mean	Mean*
						Ist Experiment					
Before Flowering	Control	0.339	0.365	0.352	2.10 ıjk	2.12 h-k	2.11	0.139	0.156	0.148	0.139
	Urea 0.5 %	0.346	0.375	0.361	2.17 d-k	2.36 a	2.26	0.131	0.152	0.142	0.131
	Urea 1 %	0.332	0.360	0.346	2.15 f-k	2.22 b-ı	2.18	0.140	0.154	0.147	0.140
	$ZnSO_4$ 0.5 %	0.327	0.355	0.341	2.08 jk	2.25 a-h	2.17	0.123	0.153	0.138	0.123
	Urea 0.5 % + $ZnSO_4$ 0.5 %	0.333	0.355	0.344	2.15 e-k	2.30 abc	2.23	0.138	0.157	0.148	0.138
	Urea 1 % +$ZnSO_4$ 0.5 %	0.338	0.363	0.351	2.09 jk	2.32 abc	2.20	0.131	0.162	0.146	0.131
	K_2SO_4 0.5 %	0.348	0.373	0.361	2.15 f-k	2.28 a-e	2.21	0.132	0.154	0.143	0.132
	K_2SO_4 1 %	0.341	0.363	0.352	2.11 ıjk	2.22 b-ı	2.16	0.142	0.154	0.148	0.142
	Mean	**0.338**	**0.363**	**0.351**	**2.12**	**2.26**	**2.19**	**0.135**	**0.155**	**0.145**	**15.2**
After Flowering	Control	0.338	0.363	0.351	2.07 k	2.21 c-j	2.14	0.141	0.161	0.151	14.2
	Urea 0.5 %	0.338	0.365	0.352	2.21 c-j	2.30 a-d	2.25	0.146	0.157	0.152	15.0
	Urea 1 %	0.328	0.358	0.343	2.15 f-k	2.27 a-f	2.21	0.134	0.154	0.144	15.4
	$ZnSO_4$ 0.5 %	0.332	0.360	0.346	2.15 f-k	2.26 a-g	2.20	0.133	0.155	0.144	15.3
	Urea 0.5 %+ $ZnSO_4$ 0.5 %	0.331	0.355	0.343	2.12 ıjk	2.28 a-e	2.20	0.138	0.168	0.153	14.5
	Urea 1 %+ $ZnSO_4$ 0.5 %	0.332	0.353	0.343	2.14 g-k	2.26 a-g	2.20	0.131	0.160	0.146	15.2
	K_2SO_4 0.5 %	0.353	0.380	0.367	2.20 c-k	2.20 c-k	2.20	0.149	0.164	0.156	14.1
	K_2SO_4 1 %	0.332	0.355	0.344	2.11 ıjk	2.35 ab	2.23	0.138	0.163	0.150	14.9
	Mean	**0.336**	**0.361**	**0.349**	**2.14**	**2.27**	**2.20**	**0.139**	**0.160**	**0.150**	**14.8**
	General mean	**0.337b**	**0.362a**		**2.13 b**	**2.26 a**		**0.137 b**	**0.158 a**		
	CV (%)		**4.62**			**2.16**			**5.77**		
						IInd Experiment					
Before Flowering	Control	0.357	0.388	0.373	2.09	2.28	2.18	0.147	0.171	0.156	13.8
	Urea 0.5 % +K_2SO_4 0.5 %	0.341	0.368	0.355	2.06	2.42	2.24	0.134	0.161	0.149	15.2
	Urea 0.5 % + K_2SO_4 1 %	0.360	0.398	0.379	2.09	2.29	2.19	0.142	0.169	0.152	14.2
	Urea 1 % + K_2SO_4 0.5 %	0.351	0.383	0.367	2.19	2.44	2.31	0.138	0.166	0.155	15.3
	Urea 1 % + K_2SO_4 1 %	0.345	0.373	0.359	2.05	2.36	2.20	0.139	0.163	0.149	14.6
	Mean	**0.351**	**0.382**	0.367	**2.09 b**	**2.36 a**	**2.23**	**0.140**	**0.164**	**0.152**	14.6
After Flowering	Control	0.354	0.385	0.370	2.30	2.29	2.30	0.149	0.165	0.16	14.7
	Urea 0.5 % + K_2SO_4 0.5 %	0.359	0.390	0.375	2.11	2.33	2.22	0.148	0.164	0.154	14.3
	Urea 0.5 % + K_2SO_4 1 %	0.355	0.385	0.370	2.28	2.37	2.33	0.144	0.162	0.156	15.2
	Urea 1 % + K_2SO_4 0.5 %	0.354	0.383	0.369	2.46	2.40	2.43	0.149	0.173	0.157	15.2
	Urea 1 % + K_2SO_4 1 %	0.348	0.373	0.361	2.16	2.32	2.24	0.143	0.158	0.153	14.9
	Mean	**0.354**	**0.383**	0.369	**2.26 ab**	**2.34 a**	**2.30**	**0.146**	**0.166**	**0.156**	14.8
	General mean	**0.352**	**0.382**		**2.18 b**	**2.35 a**		**0.143 b**	**0.165 a**		
	CV (%)		**4.88**			**4.16**			**6.23**		

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

Table 8. *Effects of different foliar fertilizer treatments and combinations, applied before and after flowering, on grain Zn and Fe concentrations of Altınbaşak bread wheat cultivar*

Appl. Time	Applications	Zn (mg kg^{-1})			Fe (mg kg^{-1})		
		1st Year	2nd Year	Mean	1st Year	2nd Year	Mean
Ist Experiment							
Before Flowering	Control	19.3 e	23.6 e	21.5	22.6	33.8	28.3
	Urea 0.5 %	21.6 e	23.0 e	22.2	27.2	33.0	30.0
	Urea 1 %	21.8 e	22.2 e	22.0	30.5	30.8	30.6
	$ZnSO_4$ 0.5 %	33.9 cd	41.4 abc	37.7	27.1	37.4	32.3
	Urea 0.5 % + $ZnSO_4$ 0.5 %	38.0 abc	42.3 abc	40.2	32.2	38.3	35.2
	Urea 1 % + $ZnSO_4$ 0.5 %	35.2 c	45.0 a	40.1	29.6	40.2	35.1
	K_2SO_4 0.5 %	18.7 e	22.7 e	20.7	27.1	33.2	30.2
	K_2SO_4 1 %	22.9 e	23.2 e	23.0	24.6	30.1	27.4
	Mean	**26.4**	**30.4**	**28.4**	**27.6**	**34.7**	**31.1**
After Flowering	Control	19.2 e	24.3 e	21.9	26.3	34.5	30.5
	Urea 0.5 %	17.5 e	23.2 e	20.4	27.1	31.2	29.2
	Urea 1 %	19.7 e	22.8 e	21.4	27.0	28.4	27.8
	$ZnSO_4$ 0.5 %	40.7 abc	38.7 abc	39.8	29.1	35.0	32.1
	Urea 0.5 % + $ZnSO_4$ 0.5 %	35.5 bc	44.0 ab	39.8	27.4	36.4	32.0
	Urea 1 % + $ZnSO_4$ 0.5 %	37.4 abc	39.6 abc	38.5	27.6	37.3	32.4
	K_2SO_4 0.5 %	17.1 e	25.5 de	21.3	28.2	37.6	33.0
	K_2SO_4 1 %	18.0 e	23.1 e	20.6	23.2	32.2	28.1
	Mean	**25.7**	**30.2**	**27.9**	**27.0**	**34.2**	**30.6**
	General mean	**26.0 b**	**30.3 a**		**27.3 b**	**34.4 a**	
	CV (%)		**10.97**			**15.08**	
IInd Experiment							
Before Flowering	Control	18.0	27.0	22.5	25.4	37.5	31.4
	Urea 0.5 % + K_2SO_4 0.5 %	19.3	28.3	23.8	21.6	35.3	28.4
	Urea 0.5 % + K_2SO_4 1 %	20.0	28.5	24.3	22.0	35.6	28.8
	Urea 1 % + K_2SO_4 0.5 %	19.4	27.5	23.5	24.3	40.9	32.6
	Urea 1 % + K_2SO_4 1 %	18.1	27.6	22.8	21.5	33.8	27.7
	Mean	**19.0**	**27.8**	**23.4**	**22.9**	**36.6**	**29.8**
After Flowering	Control	18.2	25.8	22.0	23.4	38.6	31.0
	Urea 0.5 % + K_2SO_4 0.5 %	18.3	31.7	25.0	23.3	38.3	30.8
	Urea 0.5 % + K_2SO_4 1 %	17.9	26.8	22.3	32.3	36.0	34.2
	Urea 1 % + K_2SO_4 0.5 %	19.1	27.5	23.3	24.9	37.8	31.3
	Urea 1 % + K_2SO_4 1 %	18.4	26.1	22.2	28.6	34.5	31.6
	Mean	**18.4**	**27.6**	**23.0**	**26.5**	**37.0**	**31.8**
	General mean	**18.7 b**	**27.7 a**		**24.7 b**	**36.8 a**	
	CV (%)		**8.98**			**12.72**	

Considering the effects of different foliar fertilizer treatments before and after flowering periods on grain Zn and Fe concentrations, it was observed that both the years and the treatments were found to be significant (*Table 3*). Treatment periods (BF and AF) were not found to be significant (*Table 3*). In *Table 8*, 0.5% $ZnSO_4.7H_2O$, 1% Urea+0.5% $ZnSO_4.7H_2O$ and 0.5% Urea+0.5% $ZnSO_4.7H_2O$ treatments resulted in significant increases in grain Zn concentrations as compared to the other treatments for both years and BF-AF periods. Similarly, higher grain Zn concentration results were obtained from the general averages of treatments (*Table 9*). Zinc sulphate heptahydrate and urea combination was more effective on grain Zn concentrations as compared to single $ZnSO_4.7H_2O$ treatment (*Table 8 and 9*).

Table 9. *Effects of different foliar fertilizer treatments and combinations on general mean of grain N, K S, Zn and Fe concentrations of Altınbaşak bread wheat cultivar*

	Applications	**General Mean**				
		K	**N**	**S**	**Zn**	**Fe**
		(%)			**(mg kg^{-1})**	
Ist Experiment	Control	0.352 ab	2.12 c	0.149	21.7 b	29.4 ab
	Urea 0.5 %	0.357 ab	2.26 a	0.147	21.3 b	29.5 ab
	Urea 1 %	0.345 b	2.20 b	0.146	21.7 b	29.2 ab
	ZnSO4 0.5 %	0.344 b	2.18 b	0.141	38.7 a	32.2 ab
	Urea 0.5 % + $ZnSO_4$ 0.5 %	0.344 b	2.21 ab	0.150	39.9 a	33.6 a
	Urea 1 % + $ZnSO_4$ 0.5 %	0.347 ab	2.20 b	0.146	39.3 a	33.7 a
	K_2SO_4 0.5 %	0.364 a	2.21 b	0.149	21.0 b	31.6 ab
	K_2SO_4 1 %	0.348 ab	2.20 b	0.149	21.8 b	27.7 b
IInd Experiment	Control	0.371	2.24 b	0.158	22.2 b	31.2
	Urea 0.5 % + K_2SO_4 0.5 %	0.365	2.23 b	0.151	24.4 a	29.6
	Urea 0.5 % + K_2SO_4 1 %	0.375	2.26 b	0.154	23.3 ab	31.5
	Urea 1 % + K_2SO_4 0.5 %	0.368	2.37 a	0.156	23.3 ab	31.9
	Urea 1 % + K_2SO_4 1 %	0.360	2.22 b	0.151	22.5 ab	29.1

It was stated in previous studies that when the sufficient Zn levels were supplied to the growth ambient for plants, both soil and foliar N treatments increased grain Zn concentrations (Kutman, 2010; Kutman et al., 2010; Cakmak et al., 2010). Yılmaz et al. (1997) indicated that soil, foliar, seed and combined Zn treatments yielded significant increases in plant and grain Zn concentrations. It was also reported that foliar zinc treatments improved Zn concentrations of the grains and especially of the endosperm (Jiang et al., 2007; Cakmak et al., 2010; Xue et al., 2012; Zhang et al., 2012).

As compared to control treatments in the second experiment, combined urea and K_2SO_4 treatments yielded significant increases in grain Zn concentrations (*Table 9*). Especially, the effects of 0.5% Urea + 0.5% K_2SO_4 treatments were more remarkable. Such a value was still behind the effects of urea+ $ZnSO_4.7H_2O$ treatments of the first experiment on Zn concentrations.

Orman and Ok (2012) reported that S treatments did not have significant effects on grain Zn concentrations under sufficient Zn conditions, but increased grain Zn concentrations under insufficient Zn conditions. In the same study, S treatments did not have significant effects on grain Fe concentrations under both sufficient and insufficient Zn conditions. Considering the general averages of the treatments of the first experiment, the greatest Fe concentration was obtained from combined urea and $ZnSO_4.7H_2O$ treatments and the lowest Fe concentration was obtained from 1% K_2SO_4 treatment (*Table 9*). Similar findings were also reported by the other researchers (Cakmak et al., 2010; Kutman, 2010; Kutman et al., 2010; Shi et al., 2010). In a study conducted by Kutman (2010), increasing N treatments increased Zn and Fe concentrations by up to 100%. Such an impact of N on Zn concentrations disappeared

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DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

under insufficient Zn conditions; on the other hand, combined high N and Zn treatments resulted in synergic effect. Significant correlations were reported between the N transported to the grain by remobilization and Zn and Fe supplied to the grain by remobilization (Kutman et al., 2011). There are also some genetic findings about the close relationships between the nitrogen transported from aged leaf tissues into the grain and grain Zn and Fe concentrations (Uauy et al., 2006; Distelfeld et al., 2007; Waters et al., 2009).

Conclusion

It was concluded based on present findings that foliar fertilizer treatments generally had positive impacts on quality of wheat grains. In this sense, it was observed in both experiments that all treatments increased yield and sedimentation values as compared to the control treatments. In the first experiment, 0.5% urea treatment was prominent for yield, nitrogen and protein and urea+$ZnSO_4.7H_2O$ treatments for grain zinc and iron concentrations. In the second experiment, 1% urea+0.5% K_2SO_4 treatments were prominent for protein and grain nitrogen and 1% urea+1% K_2SO_4 and 0.5% urea+0.5% K_2SO_4 treatments for yield. Further research is recommended to be conducted with greater number of wheat genotypes about the effects of combined urea, K_2SO_4 and $ZnSO_4.7H_2O$ treatments on wheat yield, quality and grain Zn and Fe levels.

Conflict of interests. The author has not declared any conflict of interests.

Acknowledgements. This research work was financially supported by Republic of Turkey, Ministry of Food, Agriculture and Livestock (Project Code No: TAGEM/TSKAD/15/A13/P04/05).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43254342

LIFE CYCLE ASSESSMENT OF INTEGRATED EXPLOITATION TECHNOLOGY FOR TAILINGS IN BAYAN OBO MINE, CHINA

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(Received 12th Feb 2019; accepted 6th Mar 2019)

Abstract. Bayan Obo mine in China is the largest iron-niobium-rare-earth symbiotic and associated mine in the world. Its exploitation has brought great economic benefit over the years. At the same time, many environmental problems arise. One of the most serious problems is tailings. Based on the theory and method of life cycle assessment, the environmental problems caused by integrated exploitation technology for tailings in Bayan Obo mine have been studied. Environmental impact assessment method TRACI 2.1 developed by Environmental Protection Agency of the United States has been chosen in this study. Through the study of resource consumption, energy consumption, pollution input by raw materials, pollution from industrial emissions etc. of each process, 10 types of environmental impacts have been studied and their values have been calculated. Results show that the main environmental impact types are Human toxicity non-cancer, Human toxicity cancer, Ecotoxicity, Global warming air, Human health particulate air, respectively. And the pollution input by raw materials is much greater than that from industrial emission, which provides a typical case and support for green purchasing, green manufacturing, process improving etc.

Keywords: *life cycle impact assessment; life cycle inventory; environmental load; rare-earth; green manufacturing*

Introduction

Bayan Obo mine, the largest iron-niobium-rare-earth symbiotic and associated deposit in the world, is located 149 km north of Baotou city, Inner Mongolia Autonomous region, China (*Fig. 1*). Beside rare-earth resources, the mine also contains other elements of interest, including iron, niobium, scandium, fluorite, sulphur and potassium etc. These resources also have an extremely high integrated exploitation value (Smith et al., 2015). Bayan Obo tailings ponds accumulated a large amount of iron tailings and rare-earth tailings due to the large scale mining for half a century. The integrated exploitation technology is a comprehensive method including beneficiation method and metallurgical method, which can extract the remaining useful elements from the tailings of Bayan Obo mine.

Six million tons oxidized ores are mined by Bayan Obo mine each year and 3.8618 million tons tailings are produced (Li et al., 2015a). These tailings bring a series of environmental problems including land occupation, radiation, dust pollution and groundwater pollution etc. The remaining elements in tailings such as iron, rare-earth, sulphur, fluorine, niobium, scandium etc. still have an extremely high recoverable

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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value. Recycling these elements not only can generate considerable economic benefits, but also can greatly reduce environmental hazards. Currently, environmental problems caused by tailings generally are used some methods called “End-of-pipe Treatment”. These methods cure the symptoms, not the disease-temporary medical relief. However, using the Life Cycle Assessment Method to analyze environmental problems produced in the process of integrated exploitation of tailings, can calculate their environmental pollution values in advance from the beginning of technology designing and achieve the goal of green production from the origin.

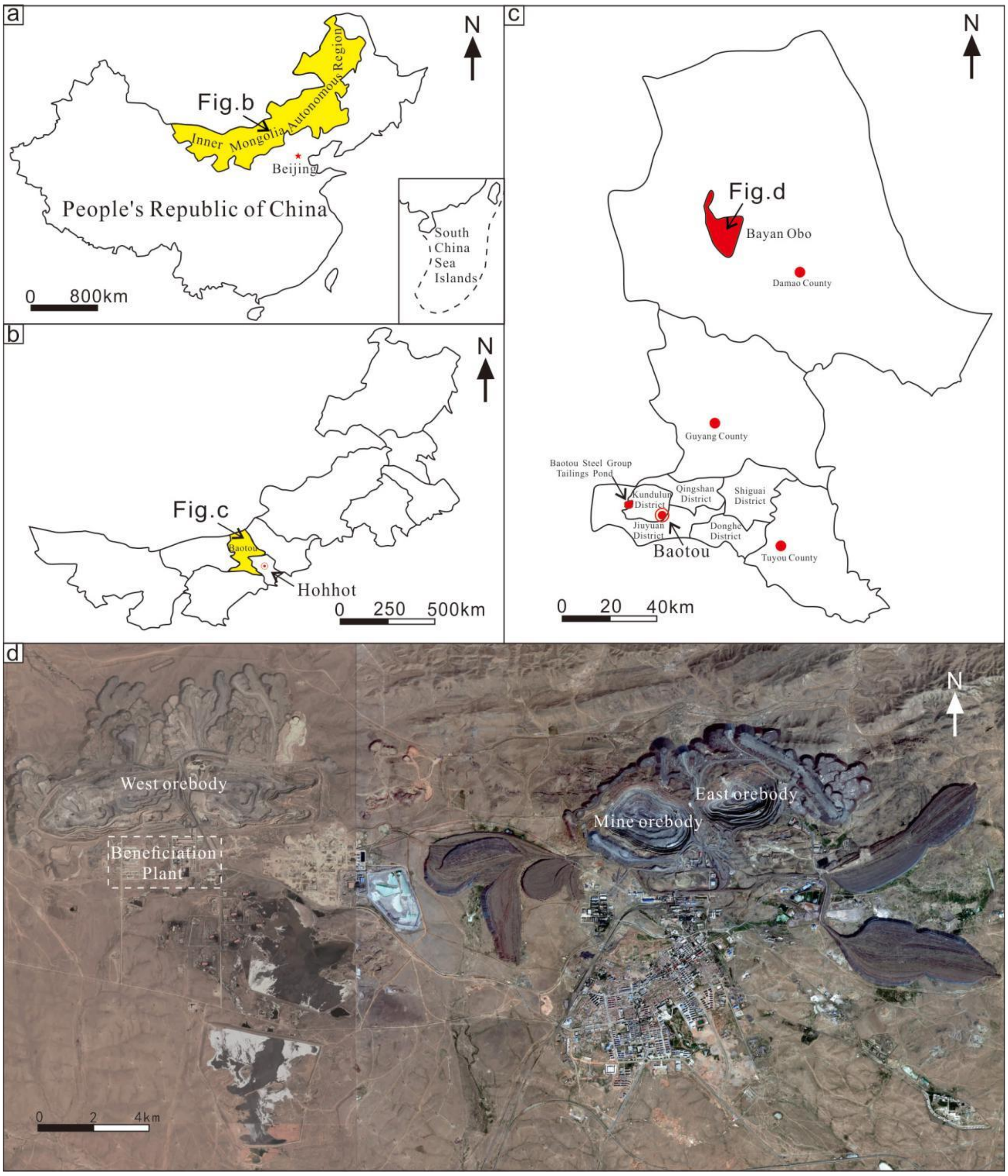

Figure 1. *Location map of Bayan Obo mine.* ***a*** *Location of Inner Mongolia Autonomous Region.* ***b*** *Location of Baotou City.* ***c*** *Location of Bayan Obo mine.* ***d*** *Satellite image map of Bayan Obo mine (Base map from Google Earth)*

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There are a lot of assessment methods at present (Ni et al., 2018; Zhai et al., 2018; Zhao et al., 2018; Zhou et al., 2018; Li et al., 2017; Yang et al., 2017; Jin et al., 2015; Li et al., 2015b; Song et al., 2015; Yan et al., 2014). Most of the methods are based on the current situation, and only the Life Cycle Assessment (LCA) starts from the source. LCA method assesses the entire life cycle of a product, technique, or activity in relation to its environmental impact, including the initial collection of the raw material, the production, transportation, sale, use, recycling, maintenance, and disposal of the product. The life cycle assessment method is an advanced method used to determine the comprehensive environmental impact of a process. It has been widely applied as an advanced method in modern environmental management (Mazzi et al., 2017).

At present, life cycle assessment of rare-earth is mainly on rare-earth oxide, neodymium-iron-boron (NdFeB) permanent magnet material and related products containing rare-earth elements at home and abroad (Vahidi and Zhao, 2017, 2018; Zapp et al., 2018; Lima et al., 2018; Weng and Mudd, 2017; Schulze et al., 2017; Vahidi et al., 2016; Weng et al., 2016; Jin et al., 2016; Zaimes et al., 2015; Li et al., 2015c; Koltun and Tharumarajah, 2014; Navarro and Zhao, 2014; Sprecher et al., 2014). However life cycle assessment for tailings integrated exploitation of Bayan Obo mine is rare. This study can quantitatively analyze the environmental influence of useful elements in tailings in the process of integrated exploitation, which is benefit for improving mineral processing technology and achieve green purchasing (Liobikienė et al., 2017) and green manufacturing (Ahn et al, 2013). Meanwhile, it provides the basis for improvement and optimization of integrated exploitation technology of Bayan Obo mine.

Study methods

Assessment method

At present, the LCA methods mainly include CML (Centrum voor Milieukunde Leiden), EDIP (Environmental Design of Industrial Products), EI (Eco-Indicator), EPS (Environmental Priority Strategy), ES (Eco-Scarcity), ReCiPe, BEES (Building for Environment and Economic Sustainability), NOGEPA (Netherlands Oil and Gas Exploration and Production Association), TRACI (Tool for the Reduction and Assessment of Chemical and other Environmental) and so on. In CML method, weight of environmental impact factor mainly is the global average value, which will bring some error if it is used in a specific area. EDIP method is similar to CML method, which will has some error if a specific country is studied. Meanwhile, due to the early development, this method lacks timely update. EI and EPS methods have few environmental impact types, which cannot cover overall environmental impact. Using ES method requires some adjustments according to the change of country or area. ReCiPe method is not perfect in some calculation methods. BEES and NOGEPA are two methods that are developed on TRACI. The improved parts of these methods are limited in use and not widely applicable.

Based on the reasons mentioned above, this study selected TRACI 2.1 assessment method developed by Environmental Protection Agency of the United States (USEPA) in 2002 to assess the environmental influence of tailings integrated exploitation technology for Bayan Obo mine in China. This assessment method was developed for sustainable indexes, LCA, industrial ecology and impact assessment of process design, which was a kind of impact assessment methods for treating chemical emissions,

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resource utilization and environmental load source etc. Environmental impact factors considered by TRACI 2.1 were 10 types: AD (Acidification), EP (Eutrophication), GWA (Global Warming Air), ODA (Ozone Depletion Air), HHPA (Human Health Particulate Air), HTC (Human toxicity cancer), HTNC (Human toxicity non-cancer), ET (Ecotoxicity), SA (Smog Air) and RFF (Resources, Fossil fuels). The characterization unit, normalization references and weight factors of TRACI 2.1 are seen in *Table 1*.

Table 1. *Classification, normalization references and weight factors of TRACI 2.1*

Environmental impact type	Characterization unit	Normalization reference/(kg·person^{-1}·a^{-1})	Weight factor
ET	CTUe	1.1E+04	6.7
HTC	CTUh	4.8E-05	7.8
HTNC	CTUh	1.0E-03	7.8
RFF	MJ surplus energy	1.9E+04	6.2
HHPA	kg PM2.5-Equiv	3.0E+01	6.6
ODA	kg CFC 11-Equiv	1.5E-01	5.2
SA	kg O_3-Equiv	1.5E+03	6.9
GWA	kg CO_2-Equiv	2.4E+04	8.9
AD	kg SO_2-Equiv	9.5E+01	5.8
EP	kg N-Equiv	2.0E+01	6.6

Data from USEPA (Norris, 2002) https://nepis.epa.gov/Adobe/PDF/P100HN53.pdf

Data quality

The data related to integrated exploitation technology of Bayan Obo tailings were mainly acquired from patents (Zhang et al., 2015), statistical yearbooks (Baotou steel group, 2016; Baotou steel group, 2014), published books and literatures (Yang, 2017; Lu and Liu, 2016; Hu, 2014; Ji, 2013; Zhang et al., 2010; Xuan et al., 2009; Cheng et al., 2007; Che and Yu, 2006; Mei et al., 2006; Zhang et al., 2002; Xie et al., 2000; Tian and Lu, 1999), and real production data of enterprises. The data acquisition time is from 2014-2018 (The application of integrated exploitation technology to Bayan Obo tailings starts from 2014). The data also had been logged by Baotou Iron & Steel (Group) Co. Ltd.

The data can be divided into four types: energy, material, emission and front-end data. The first two data had been acquired from beneficiation plant of Bayan Obo mine, whose GPS coordinates is 41°47′30″N and 109°51′51″E. The data of pollutant emission were acquired from environmental impact assessment report of integrated exploitation project for Bayan Obo tailings in 2016 and the People’s Republic of China National Standard “Emission Standards of Pollutants from Rare Earths Industry” (Liu et al., 2015; Hu et al., 2013; Chen et al., 2012; Xiao et al., 2003).

Front-end data used in the study were from GaBi database. GaBi covers more than 12000 life cycle inventory data, which is the largest life cycle inventory data set, covered more than 40 industries in the world so far (the database has been updated to 2018). Data of electricity, steam and water were from the China mean value; input of other materials were from global mean value, European Union (EU) mean value, or Germany mean value. Because of the similar energy structure between China and

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Germany whose energy supplies mostly have been dominated by coal (Bonatz et al., 2019), the environmental impact of material production in Germany can substitute that of China. If GaBi database lacks China mean value, global mean value and EU mean value, Germany data were chosen, which can approximately substitute China data (Mo and Zhang, 2003).

For materials not in the database, we created some new data models or used some similar materials in database to substitute the original materials (Cao, 2017).

Goal and scope

Based on the integrated exploitation technology of tailings in Bayan Obo mine as the research goal, according to the collected data and materials, this study conducts a study on LCA of this technology. The purpose was to analyze environmental load, environmental impact types in each production stage and identify the key processes causing pollution and environmental impact types, which can provide basic data for life cycle assessment of integrated exploitation technology of tailings in Bayan Obo mine. Meanwhile, it can promote green purchasing, green manufacturing and sustainable development of rare-earth industry and tailings integrated exploitation industry.

In this study of LCA, system functional unit has been chosen as3.8618 million tons of rare-earth tailings, which is an average quantity of tailings annual output. System boundary was from rare-earth tailings to scandium concentrate (500 ppm) (*Fig. 2*), which included processes of rare-earth flotation, iron flotation, sulfur flotation, fluorite flotation, niobium flotation and scandium magnetic separation etc. namely comprehensively recycling rare-earth concentrate, iron concentrate, sulfur concentrate, fluorite concentrate, niobium concentrate and scandium concentrate from rare-earth tailings.

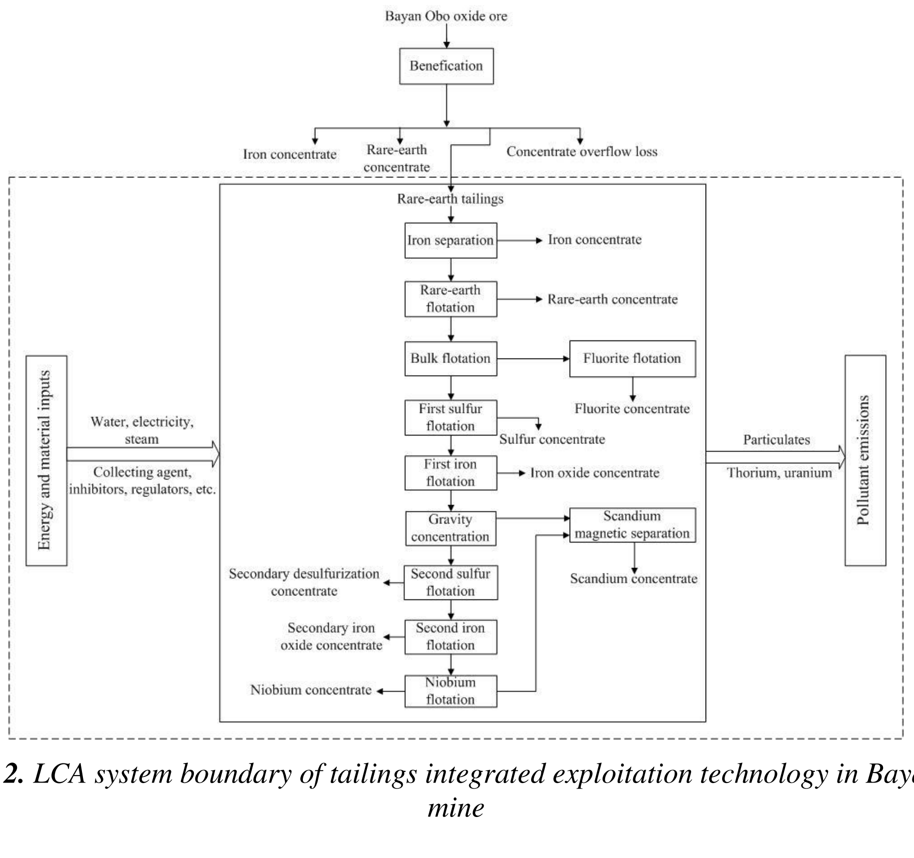

Figure 2. *LCA system boundary of tailings integrated exploitation technology in Bayan Obo mine*

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Life cycle inventory (LCI)

Integrated exploitation technology of tailings in Bayan Obo mine is a kind of process which can comprehensively recycle rare-earth concentrate, iron concentrate, sulfur concentrate, fluorite concentrate, niobium concentrate and scandium concentrate from Bayan Obo mine. In this technology, wastewater had achieved zero discharge. Types of energy included electricity, water and steam. Raw materials consumption mainly included collecting agents, inhibitors, regulators etc. in each beneficiation process. Pollutant discharge mainly included thorium, uranium, sulfur dioxide, carbon dioxide etc. The LCI data is shown in *Figure 3*.

Life cycle impact assessment (LCIA)

LCIA was the key procedure of LCA. This stage needs to quantitatively assess potential environmental impacts. At present, the methods used internationally are mainly qualitative analysis and quantitative calculation, which generally include three stages: characterization, normalization and weighting.

Characterization

Characterization was quantitatively analysis contributions of each environmental factor on specific environmental impact types, and then converted them into unified unit. Characterization results are shown in *Table 2*.

Table 2. *Characterization results of LCI data*

Types	Iron separation	Rare-earth flotation	Bulk flotation	Fluorite flotation	First sulfur flotation	First iron flotation
AD/kg	3.48E+05	5.98E+05	9.70E+05	1.07E+06	2.05E+04	1.55E+06
ET/CTUe	3.41E+08	4.06E+08	1.56E+08	1.19E+08	1.41E+07	3.39E+08
EP/kg	1.34E+05	1.44E+05	7.45E+04	1.17E+05	1.17E+04	1.30E+05
GWA/kg	9.24E+07	1.46E+08	2.40E+08	2.53E+08	7.92E+06	3.32E+08
HHPA/kg	1.42E+05	1.91E+05	3.18E+05	3.54E+05	1.83E+04	4.60E+05
HTC/CTUh	1.32E+00	1.53E+00	6.17E-01	4.81E-01	6.98E-02	1.34E+00
HTNC/CTUh	1.90E+01	2.91E+01	3.37E+01	3.56E+01	8.17E-01	5.03E+01
ODA/kg	2.85E+00	3.48E+00	9.87E-01	6.82E-01	6.04E-02	1.23E+00
RFF/MJ	7.53E+07	8.40E+07	5.98E+07	6.18E+07	5.51E+06	6.34E+07
SA/kg	4.10E+06	7.07E+06	1.21E+07	1.33E+07	2.74E+05	1.77E+07
Types	**Gravity concentration**	**Second sulfur flotation**	**Second iron flotation**	**Niobium flotation**	**Scandium magnetic separation**	**Total value**
AD/kg	1.16E+04	3.12E+03	7.68E+05	2.25E+05	5.78E+03	5.58E+06
ET/CTUe	8.59E+05	3.43E+06	1.32E+08	8.24E+07	2.97E+05	1.59E+09
EP/kg	4.16E+03	2.50E+03	5.44E+04	3.43E+04	9.58E+02	7.07E+05
GWA/kg	6.29E+06	1.11E+06	1.72E+08	4.58E+07	2.10E+06	1.30E+09
HHPA/kg	2.43E+04	1.68E+03	2.34E+05	6.38E+04	1.14E+04	1.82E+06
HTC/CTUh	1.91E-02	1.55E-02	5.24E-01	3.29E-01	4.32E-03	6.25E+00
HTNC/CTUh	4.09E-01	1.42E-01	2.49E+01	7.19E+00	1.89E-01	2.01E+02
ODA/kg	1.84E-05	1.47E-02	4.53E-01	3.27E-01	3.87E-06	1.01E+01
RFF/MJ	6.32E+06	5.88E+05	3.20E+07	1.43E+07	1.43E+06	4.04E+08
SA/kg	1.82E+05	3.84E+04	9.07E+06	2.44E+06	8.16E+04	6.64E+07

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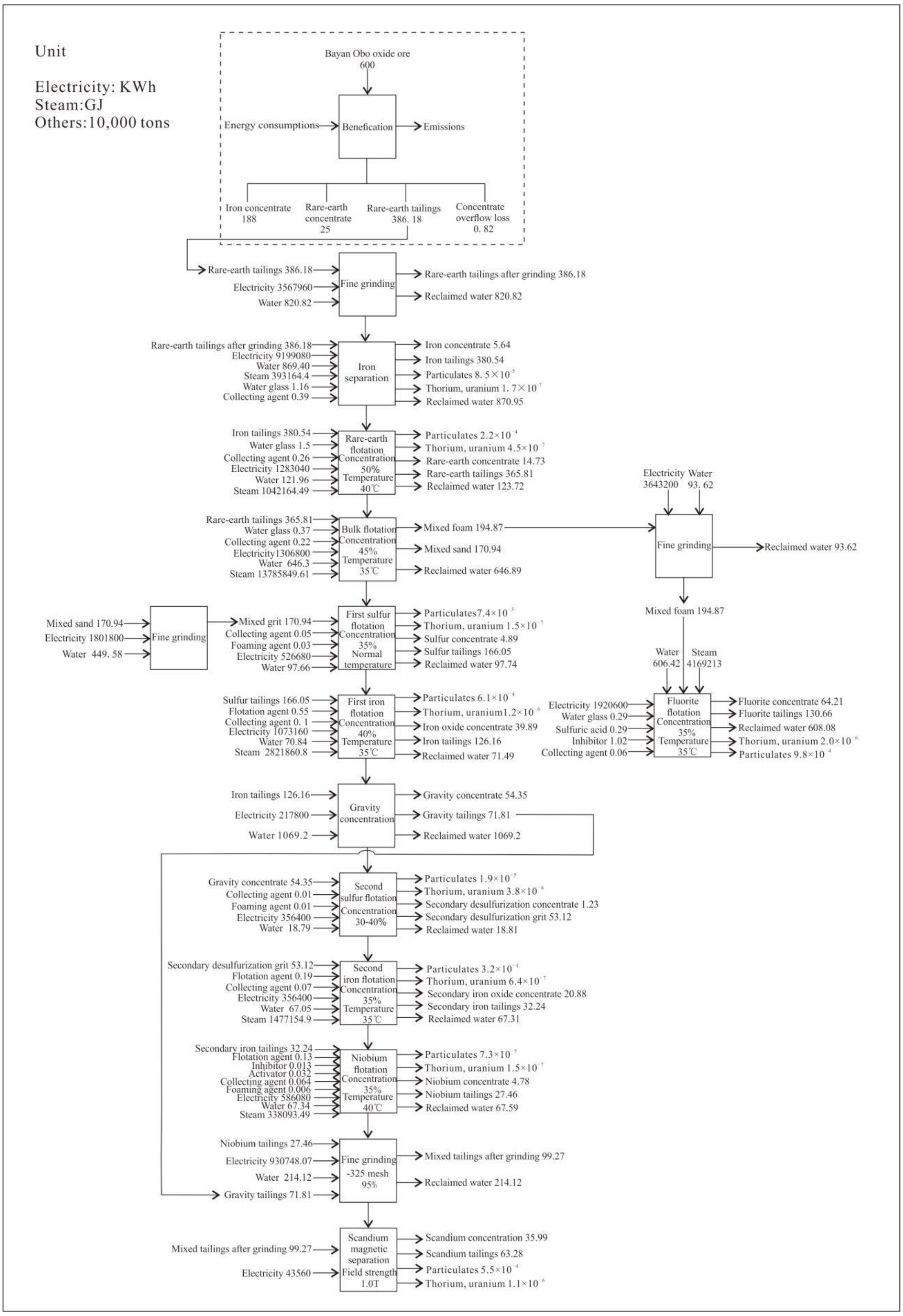

Figure 3. *LCI data of integrated exploitation technology for tailings in Bayan Obo mine*

Normalization

Normalization is a calculating process that using the acquired characterization values divided corresponding normalization reference values, respectively. In the end, this calculation can get some data without unit. After normalization process, these environmental impact types are comparable due to the removal of previous units. Normalization reference value of each environmental impact type is closely related to the severity of itself. Relative levels of various environmental impact types can be compared preliminarily after this procedure.

Normalization reference values of this study are provided by TRACI 2.1 in GaBi 8.0 software. The calculation results are shown in *Table 3*.

Table 3. *Normalization results of LCI data*

Types	Iron separation	Rare-earth flotation	Bulk flotation	Fluorite flotation	First sulfur flotation	First iron flotation
AD	3.67E+03	6.32E+03	1.03E+04	1.13E+04	2.17E+02	1.64E+04
ET	3.11E+04	3.70E+04	1.42E+04	1.08E+04	1.29E+03	3.09E+04
EP	6.48E+03	6.96E+03	3.61E+03	5.70E+03	5.64E+02	6.28E+03
GWA	3.84E+03	6.06E+03	1.00E+04	1.05E+04	3.29E+02	1.38E+04
HHPA	4.78E+03	6.43E+03	1.07E+04	1.19E+04	6.16E+02	1.55E+04
HTC	2.66E+04	3.08E+04	1.25E+04	9.80E+03	1.41E+03	2.71E+04
HTNC	1.84E+04	2.81E+04	3.27E+04	3.46E+04	7.93E+02	4.88E+04
ODA	1.95E+01	2.39E+01	6.77E+00	4.68E+00	4.15E-01	8.42E+00
RFF	3.62E+03	4.03E+03	2.99E+03	3.12E+03	2.76E+02	3.20E+03
SA	2.83E+03	4.88E+03	8.36E+03	9.19E+03	1.89E+02	1.23E+04
Types	**Gravity concentration**	**Second sulfur flotation**	**Second iron flotation**	**Niobium flotation**	**Scandium magnetic separation**	**Total value**
AD	1.22E+02	3.29E+01	8.12E+03	2.39E+03	6.11E+01	5.89E+04
ET	7.81E+01	3.12E+02	1.20E+04	7.51E+03	2.70E+01	1.45E+05
EP	2.04E+02	1.21E+02	2.64E+03	1.66E+03	4.69E+01	3.43E+04
GWA	2.62E+02	4.62E+01	7.17E+03	1.90E+03	8.74E+01	5.40E+04
HHPA	8.20E+02	5.66E+01	7.91E+03	2.16E+03	3.86E+02	6.13E+04
HTC	3.97E+02	3.11E+02	1.06E+04	6.64E+03	9.01E+01	1.26E+05
HTNC	3.97E+02	1.38E+02	2.42E+04	6.97E+03	1.84E+02	1.95E+05
ODA	1.26E-04	1.01E-01	3.11E+00	2.24E+00	2.65E-05	6.92E+01
RFF	3.26E+02	2.85E+01	1.62E+03	7.16E+02	7.36E+01	2.00E+04
SA	1.26E+02	2.65E+01	6.25E+03	1.68E+03	5.63E+01	4.59E+04

Weighting

Through characterization and normalization, summary value of specific assessment subject on each environmental impact type can be gotten. Each environmental impact type is assigned specific weighting factor, on the basis, normalization values were weighted. Then the comprehensive impact values get. Through this procedure, the total environmental impact of study object can be assessed. Meanwhile, the proportions of different environmental impact types in the overall environmental impact can be obtained. Through this result, the impact degree of different environmental impact types to the total environment can be analyzed and quantitative data can be calculated.

TRACI 2.1 environmental impact weight-values were used to get quantitative results of the total environmental impact. Calculation results are shown in *Table 4*.

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Table 4. *Weighting results of each environmental impact type*

Types	Iron separation	Rare-earth flotation	Bulk flotation	Fluorite flotation	First sulfur flotation	First iron flotation
AD	2.14E+04	3.67E+04	5.95E+04	6.56E+04	1.25E+03	8.26E+04
ET	2.08E+05	2.48E+05	9.54E+04	7.26E+04	8.63E+03	6.46E+04
EP	4.28E+04	4.59E+04	2.38E+04	3.75E+04	3.72E+03	1.82E+04
GWA	3.42E+04	5.39E+04	8.89E+04	9.36E+04	2.93E+03	1.19E+05
HHPA	3.15E+04	4.24E+04	7.09E+04	7.88E+04	4.06E+03	9.47E+04
HTC	2.08E+05	2.41E+05	9.76E+04	7.65E+04	1.10E+04	6.41E+04
HTNC	1.43E+05	2.19E+05	2.55E+05	2.70E+05	6.18E+03	3.25E+05
ODA	1.02E+02	1.24E+02	3.52E+01	2.43E+01	2.16E+00	1.08E+01
RFF	2.25E+04	2.50E+04	1.85E+04	1.93E+04	1.71E+03	1.72E+04
SA	1.95E+04	3.37E+04	5.77E+04	6.33E+04	1.31E+03	7.91E+04
Types	**Gravity concentration**	**Second sulfur flotation**	**Second iron flotation**	**Niobium flotation**	**Scandium magnetic separation**	**Total value**
AD	7.10E+02	1.91E+02	4.28E+04	1.09E+04	3.54E+02	3.22E+05
ET	5.23E+02	2.09E+03	3.12E+04	1.67E+04	1.81E+02	7.48E+05
EP	1.35E+03	7.97E+02	9.36E+03	5.44E+03	3.10E+02	1.89E+05
GWA	2.33E+03	4.12E+02	6.23E+04	1.59E+04	7.78E+02	4.74E+05
HHPA	5.41E+03	3.73E+02	4.95E+04	1.24E+04	2.54E+03	3.93E+05
HTC	3.10E+03	2.43E+03	3.17E+04	1.70E+04	7.03E+02	7.52E+05
HTNC	3.10E+03	1.08E+03	1.69E+05	4.13E+04	1.43E+03	1.43E+06
ODA	6.54E-04	5.23E-01	4.76E+00	3.87E+00	1.38E-04	3.08E+02
RFF	2.02E+03	1.76E+02	9.16E+03	3.82E+03	4.57E+02	1.20E+05
SA	8.66E+02	1.83E+02	4.12E+04	1.03E+04	3.89E+02	3.08E+05

Results and interpretation

According to the weighting results, environmental impact type values of integrated exploitation technology for tailings in Bayan Obo mine have been calculated (*Fig. 4*).

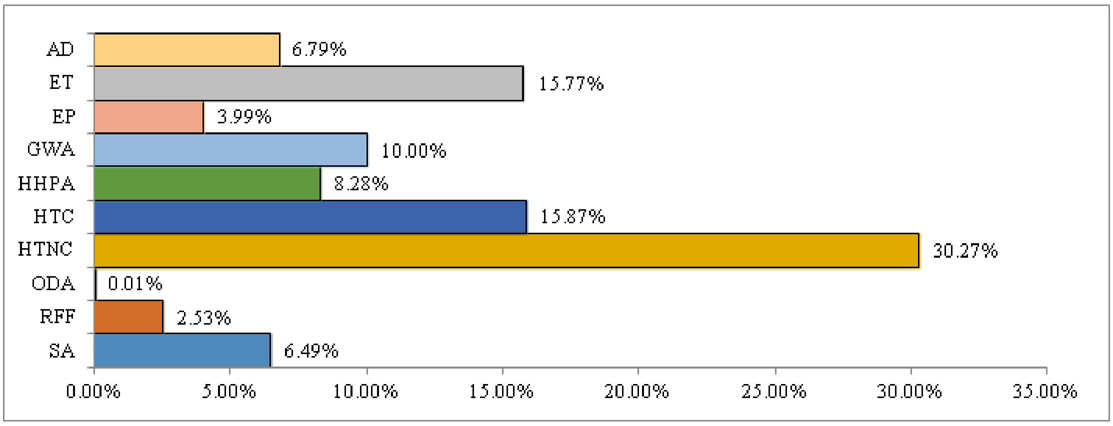

Figure 4. *Proportion of each environmental impact type in the total environmental impact*

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From *Figure 4,* the potential environmental impacts of integrated exploitation technology for tailings in Bayan Obo mine was HTNC, HTC, ET, GWA, HHPA, AD, SA, EP, RFF and ODA sorted by numerical value. Among these types, HTNC was the main environmental impact type, accounting for 30.27% of the total environmental impact. HTC was the next, accounting for 15.87% of the total environmental impact. ET, GWA and HHPA accounted for 15.77%, 10.00% and 8.28%, respectively.

The most serious impact process in HTNC was iron flotation, whose contribution rate was 22.64% (*Fig. 5*). The contribution rate of fluorite flotation, bulk flotation and rare-earth flotation were 18.82%, 17.80% and 15.29%, respectively. Steam was the main factor influencing HTNC in the stage of iron flotation, accounting for 96.33% (*Fig. 6*), which was mainly caused by organic matters discharge such as trichlopropane, trichloro ethylene etc.

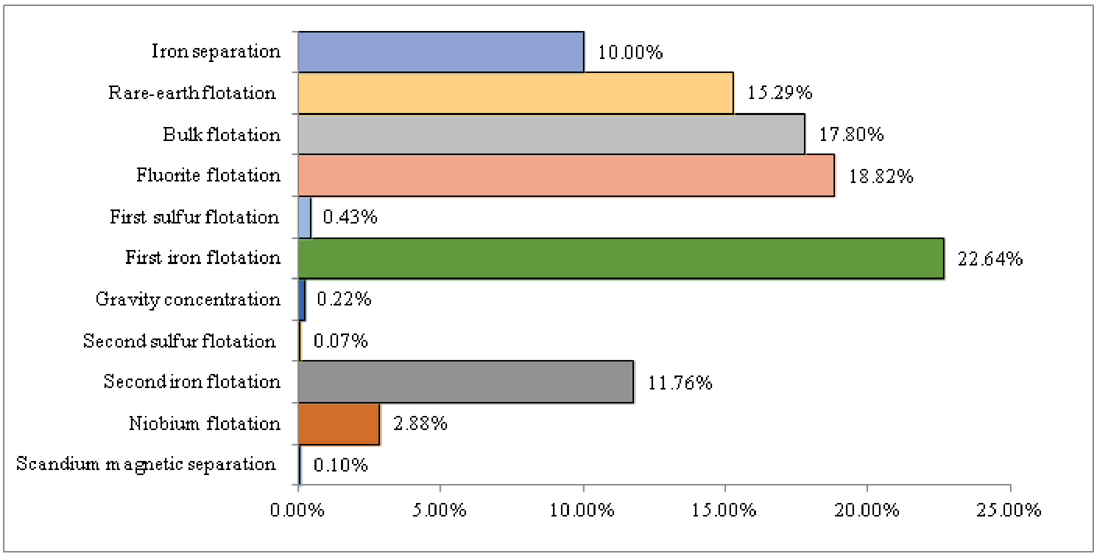

***Figure 5.** Proportion of HTNC in each process*

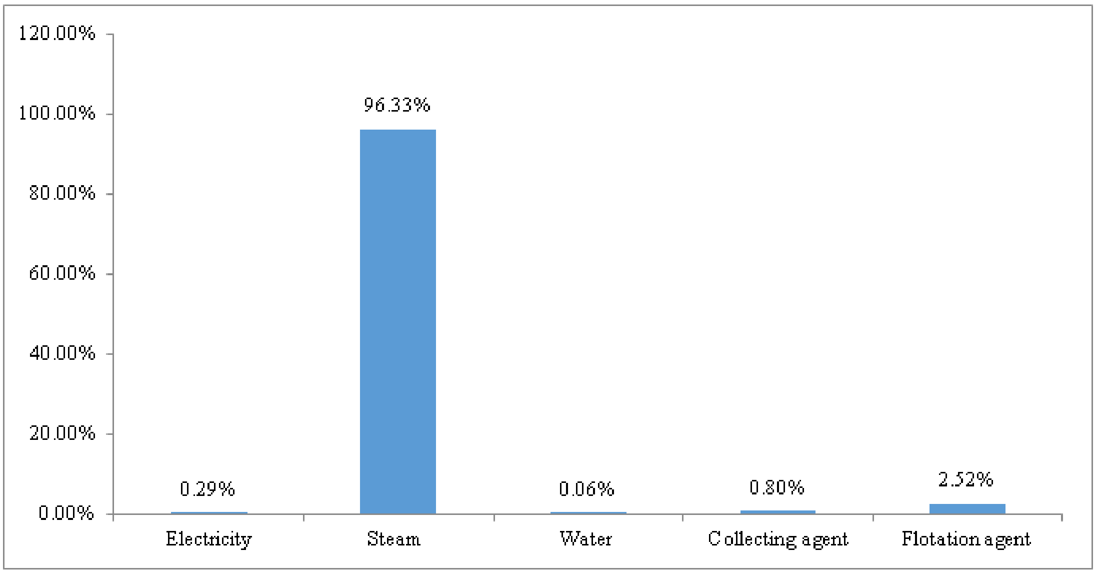

***Figure 6.** Proportion of environmental impact of each input in iron flotation in HTNC*

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From the LCA results of integrated exploitation technology for tailings in Bayan Obo mine, the second environmental impact type is HTC, in which, the most serious impact process was rare-earth flotation (*Fig. 7*), accounting for 31.97% of the total environmental impact of HTC. The next was iron separation process, accounting for 27.62%. Water glass was the main factor influencing HTC in the stage of rare-earth flotation, accounting for 83.16% (*Fig. 8*). The next was collecting agent, accounting for 11.87%. The main reason was discharge of arsenite, cadmium and benzene etc. in the manufacturing process of water glass and collecting agent.

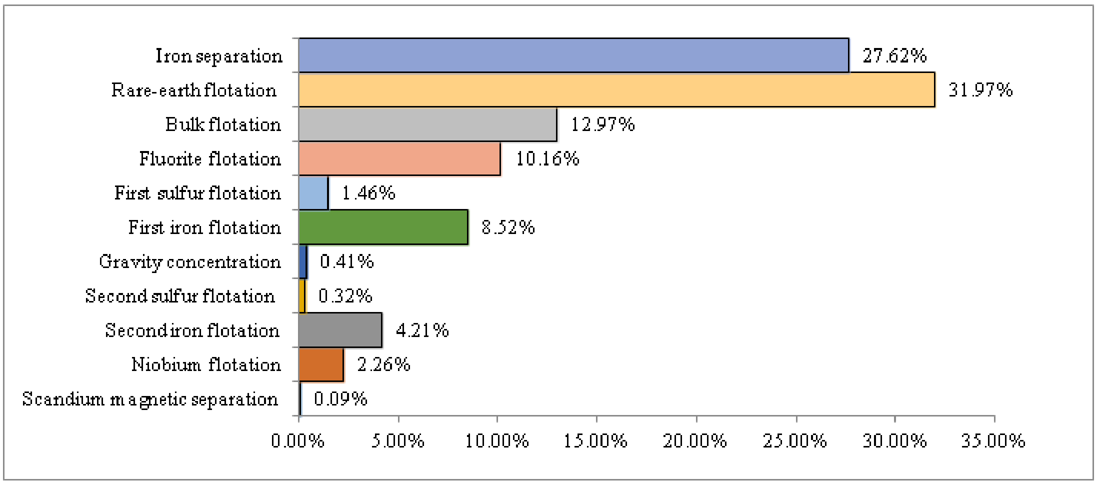

Figure 7. *Proportion of HTC in each process*

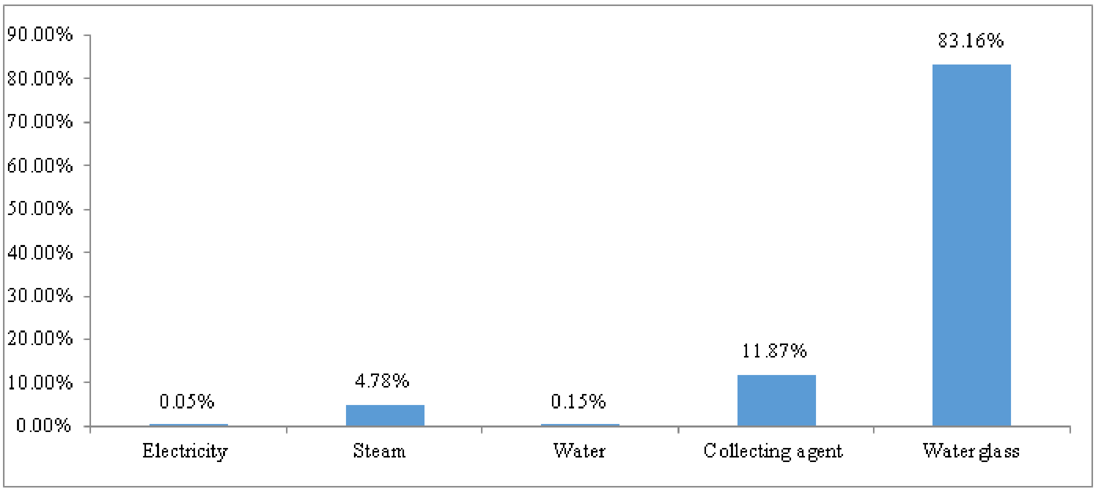

Figure 8. *Proportion of environmental impact of each input in rare-earth flotation in HTC*

The first environmental impact type, namely HTNC was mainly generated from iron flotation and the next was fluorite flotation. The second environmental impact type, namely HTC was mainly generated from rare-earth flotation and iron separation. The most serious impact in ET was rare-earth flotation and the next is iron separation. GWA was mainly generated from iron flotation and fluorite flotation. ODA was the least serious environmental impact among the total environmental impact types, which

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mainly generated from rare-earth flotation and iron separation (*Fig. 9*). From the above, the pollution caused by iron flotation was much higher than other processes.

The integrated exploitation technology for tailings in Bayan Obo mine covers 11 processes including iron separation, rare-earth flotation, bulk flotation, fluorite flotation, first sulfur flotation, first iron flotation, gravity concentration, second sulfur flotation, second iron flotation, niobium flotation, and scandium separation magnetic. The proportion of each environmental impact type in these processes is different (*Fig. 10*).

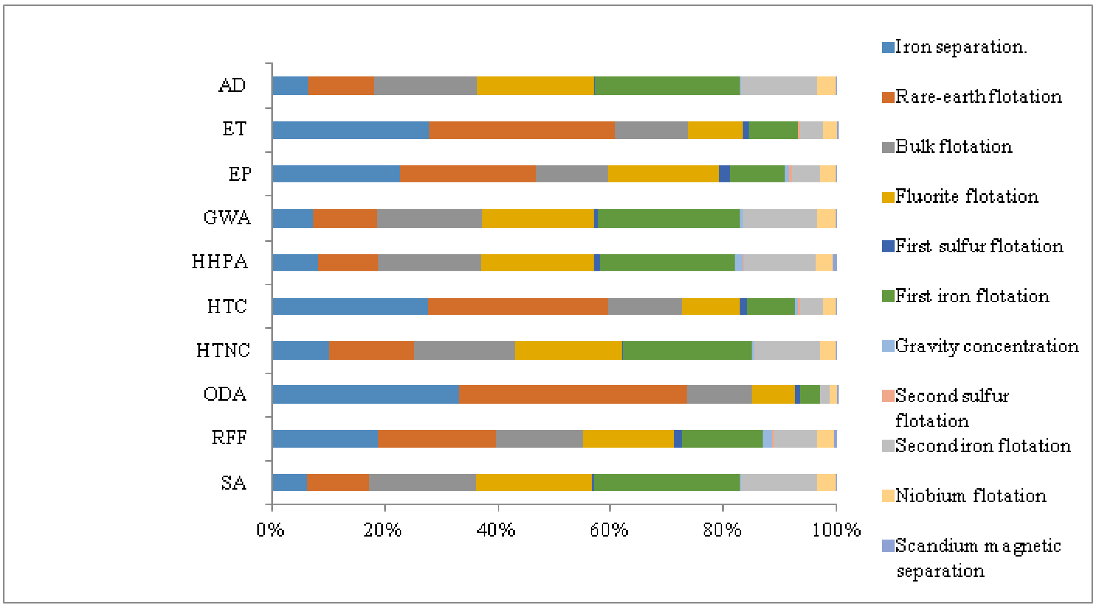

Figure 9. *Proportion of environmental impact of each process in each environmental impact type*

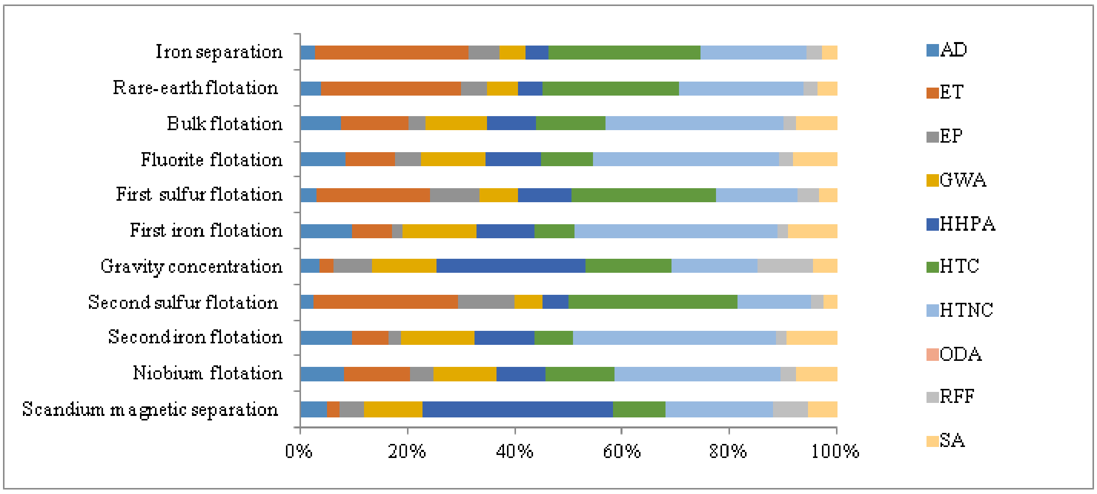

Figure 10. *Proportion of each environmental impact type in each process*

From the figure, in the processes of rare-earth flotation and iron separation, ET was the most important environmental impact type. HTC took second place. In the processes of bulk flotation and niobium flotation, HTNC was the major environmental impact

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type. HTC was the secondary. In the processes of first sulfur flotation and second sulfur flotation, HTC and ET were the major environmental impact types. In the processes of fluorite flotation, first iron flotation and second iron flotation, the most important environmental impact type was HTNC, and the next was GWA. However, in the process of gravity concentration, the main environmental impact type was HHPA, accounting for 27.88%. The next was HTC, accounting for 15.97%. In addition, in the process of scandium magnetic separation, HHPA and HTNC were the major environmental impact types, accounting for 35.59% and 20.06%, respectively.

In LCA of integrated exploitation technology for tailings in Bayan Obo mine, proportion of each environmental impact type in the total environmental impact is different (*Fig. 11*). The environmental impact order of these processes was rare-earth flotation, iron flotation, fluorite flotation, bulk flotation and iron separation etc. Environmental impact of rare-earth flotation was the most serious, accounting for 19.94% in the total environmental impact. Iron flotation was the next, accounting for 18.23% in the total environmental impact. Scandium magnetic separation had the minimal impact on environment, accounting for 0.15%. In the process of rare-earth flotation, the major environmental impact type was ET.

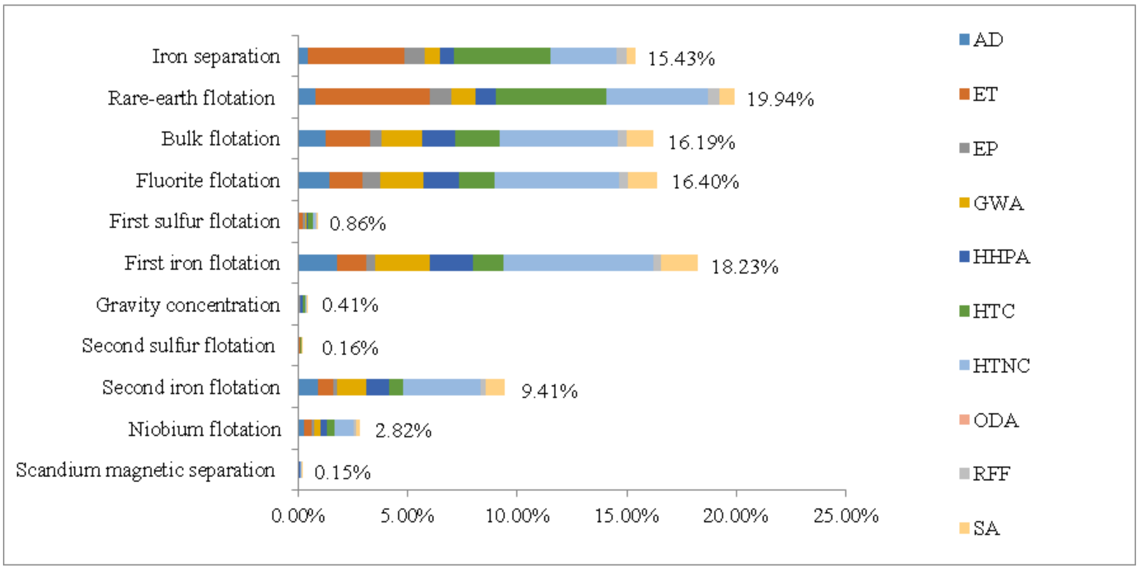

Figure 11. *Proportion of each environmental impact in the total environmental impact*

Conclusions and suggestions

Conclusions

(1) In LCA environmental impact types of integrated exploitation technology for tailings in Bayan Obo mine, HTNC is the main impact type and HTC is the next. All of these indicate that the major environmental problem in the processes of this technology is human health damage. The most serious process in HTNC is iron flotation. Steam is the major impact factor in HTNC, accounting for 96.33%. The most serious process in HTC is rare-earth flotation. Water glass is the major impact factor in HTC, accounting for 83.16%. From the above, it shows that environmental impact brought by raw material actually is much higher than that caused by pollution discharge in these actual processes.

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(2) In the total environmental impacts, flotation makes the heaviest pollution. Environmental pollution of some flotation processes is due to the production of flotation agents, such as rare-earth flotation. Environmental pollution of the other flotation processes is due to the pollution brought by energy production which meets the need of processes, such as iron flotation. Environmental impacts of rare-earth flotation and iron flotation account for 40%, which is the largest proportion of the total environmental impacts.

Suggestions

(1) Based on this study, future studies about LCA of integrated exploitation technology for tailings in Bayan Obo mine may do some research into the different minerals such as rare-earth, iron, niobium, scandium et al. Meanwhile, the researchers should pay more attention to collecting or measuring the emission of wastewater and waste-gas in order to conduct a more accurate and objective assessment. Based on this study, the ecological design of products can be carried out, which could solve the problem of "end-of-pipe treatment" for comprehensively utilizing tailings.

(2) Because of the environmental impact brought by raw material is actually much higher than that caused by pollution discharge in the processes of tailings integrated exploitation of Bayan Obo mine, the rare-earth industry needs investigation and management in the selection and purchase of raw material. Therefore, green purchasing for flotation agent, inhibitor and collecting agent etc. is needed.

(3) If the producing technology and purchasing channel of raw material cannot be changed, the enterprises can conduct some contrast tests to find environmental friendly material for replacing previous material in technology. For the heaviest pollution of rare-earth flotation and iron flotation, technology itself should be deeply studies. The rare-earth industry not only keeps seeking green environmental substitute, but also needs to improve technology itself, reduce intermediate links as much as possible and improve the utilization rate of equipments.

Acknowledgements. This study was supported by Inner Mongolia University of Science and Technology Innovation Fund (grant number 2016QDL-B28); Inner Mongolia Autonomous Region Scientific Research Project of Higher Education Institution (grant number NJZY18152); Inner Mongolia Science & Technology Plan (grant number 0901051701). We are thankful to Chen Shuilong from Gabi China for their kind help and guidance. We also thank Baotou Steel Group for their data support. Any opinions, findings, conclusions, or recommendations expressed in this study are those of the authors and do not necessarily reflect the views of Baotou Steel Group and related organizations.

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DOI: http://dx.doi.org/10.15666/aeer/1702_43434359

ENHANCEMENT OF GROWTH IN ORNAMENTAL PEPPER (*CAPSICUM ANNUUM* L.) PLANTS WITH APPLICATION OF A COMMERCIAL SEAWEED PRODUCT, STIMPLEX®

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(Received 8th Jan 2019; accepted 22nd Feb 2019)

Abstract. Seaweed extracts have been used as biostimulant or biofertilizers in agriculture to improve growth and development in plants, and enhance yield. There are a wide variety of commercial seaweed products for use on plant growth promotion. In this study, a greenhouse experiment was carried out to evaluate the effect of Stimplex®, a commercial liquid seaweed extract derived from *Ascophyllum nodosum*, on growth of ornamental pepper (*Capsicum annuum* L.) plants. Containing a complex array of bioactive compounds, Stimplex® is a premium organic biostimulant that promotes the growth, development, and yield in a wide range of conventional and organic grown horticultural and ornamental crops. Ornamental pepper plants were treated with Stimplex® as either a soil drench or foliar spray at 0, 0.25, 0.50, 0.75, and 1.0 ml·L^{-1} concentrations. The treatments were repeated at 10-day intervals until the end of the experiment. Application of Stimplex® to ornamental pepper plants improved stem diameter, plant height, number of leaves and leaf area, leaf chlorophyll content, shoot fresh weight, shoot dry weight, root fresh weight and dry weight compared to the control plants. The results of this study indicate that Stimplex® may be a viable tool for improving growth of ornamental pepper plants.
Keywords: *Ascophyllum nodosum, sustainable, biostimulant, relative chlorophyll content, biofertilizer*

Introduction

It is necessary to develop sustainable alternatives for agricultural production because of the growing consumer demand for agricultural products, increased costs of chemical fertilizers, and more importantly the detrimental effects of the residuals from chemical fertilizers and pesticides. In recent years, the use of biostimulants to promote plant growth in sustainable agriculture has gained increasing attention worldwide (Nardi et al., 2016). The use of biostimulants in agriculture has been estimated to increase at an annual rate of 13.58% during the forecast period, 2017-2022 and it is projected that the global market for biostimulants will reach to $3.68 billion in annual revenue by 2022 (Anonymous, 2017). Kauffman et al. (2007) defined the word biostimulant as "biostimulants are materials, other than fertilizers, that promote plant growth when applied in low quantities." Companies in the biostimulant sector have created an association called 'European Biostimulants Industry Council' (EBIC) in Europe. The EBIC proposes the following definition for the biostimulant: Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality (EBIC, 2018). A wide range of compounds, including humic and fulvic acids, seaweed extracts, protein hydrolysates and other N-containing compounds, chitosan and other biopolymers, beneficial

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43614375

microorganisms, and other potentially bioactive agents act as a biostimulant (Calvo et al., 2014; du Jardin, 2015). There are broad variety of commercial biostimulant products are available for use on plant growth promotion. One of these commercial products is Stimplex®. It is a concentrated liquid crop biostimulant extracted from seaweed (*Ascophyllum nodosum*) which stimulates plant growth and development, promotes yield, earlier maturity, improves resistance to some environmental stresses, improves fruit quality, increases fruit set in a wide range of conventional and organic grown horticultural and ornamental crops (Crouch et al., 1992; Zhang and Ervin, 2004; Spann and Little, 2010; Sidhu and Nanswani, 2016). Stimplex® contains trace amounts of some elements, vitamins, amino acids, auxins, cytokinins and natural chelating agents such as mannitol, fucoidins and alginic acids. It also induces the plant's own production of natural growth substances such as auxins and cytokinins to stimulate cell division and cell elongation initiating the development of larger root systems and promoting growth and yield (Crouch et al., 1992; Crouch and van Staden, 1993; Reitz and Trumble, 1996; Stirk et al., 2003; Spann and Little, 2010; du Jardin, 2015; Anonymous, 2018). Numerous studies have revealed that seaweed extracts promoted growth, increased yield and quality of horticultural crops such as grapes (Norrie and Keathley, 2006), apple (Basak, 2008), olives (Chouliaras et al., 2009), watermelon (Adbel-Mawgoud et al., 2010), cucumber (Sarhan et al., 2011), broccoli (Mattner et al., 2013), spinach (Fan et al., 2013), eggplant (Nandwani et al., 2015), strawberry (Nika et al., 2018), maize (Zermeno-Gonzalez et al., 2015), and tomato (Hernández-Herrera et al., 2014; Ali et al., 2016; Sidhu and Nandwani, 2017). To our knowledge no information on the application of Stimplex® to ornamental pepper plants has been published. The objective of the present research was to determine effective doses and application methods of Stimplex®, a commercial liquid extract of *Ascophyllum nodosum*, on growth of the ornamental pepper (*Capsicum annuum* L.) plants.

Materials and methods

Location and characteristics of the study area

The greenhouse experiment was conducted during the spring-summer 2016 at the Agricultural Sciences Research Center of Bingol University, in Bingol, Turkey (38°53'N, 40°29'E, at 1139 m altitude). The experiment was carried out in heated polycarbonate-covered greenhouse with natural daylight conditions at average day/night temperature of 29/18 °C and relative humidity of 60%.

Plant material

Seeds of ornamental pepper (cv. 'Thai Hot') were used as plant material. The 'Thai Hot' ornamental pepper plants are compact, and grow up 30 cm tall. The fruits on them grow up to 2.5 cm long, point upwards from the plant. It matures abut 80-85 days. This variety produces large numbers of fruits (averages 200 fruits per plant). The fruits range from green to yellow, orange, and deep red. The fruits are edible, although they are extremely hot. Thai ornamental hot peppers have a Scoville rating of 50,000 to 100,000.

The ornamental pepper seeds were surface sterilized with 1% (w/v) sodium hypochlorite for 15 min and rinsed thoroughly three times with distilled water. The sterilized seeds were sown into 70-cell plastic trays filled with a media consisting of peat and perlite in the ratio of 4:1 and grown on a greenhouse bench. The trays were

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irrigated as needed. Uniform 4-week-old pepper seedlings were transplanted into plastic pots (12 cm in diameter and 11 cm in deep) filled peat/perlite substrate in the ratio of 4:1. The pots were irrigated regularly and fertilized with 20-20-20 N-P-K soluble fertilizer to supply 200 mg.L^{-1} N weekly.

Biostimulant treatment

Stimplex® liquid commercial concentrate of *Ascophyllum nodosum* (Acadian Seaplants Limited, Dartmouth, NS, Canada) was used as biostimulant in this experiment. Stimplex® contains 99% *Ascophyllum nodosum* extract, 0.01% kinetin and other ingredients determined to be non-hazardous. Three days after transplanting, ornamental pepper plants were treated with Stimplex® crop biostimulant as either soil drench or foliar spray at 0, 0.25, 0.50, 0.75, and 1.0 ml.L^{-1} concentrations containing of 0.1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) as a wetting agent. Plants sprayed or drenched with water containing 0.1% Tween 20 served as the control. Foliar applications were applied with an atomizer to run-off, ensuring that upper and lower leaf surfaces were fully wetted, and the soil surface was covered during application to prevent soil contamination. Soil drench treatments were applied using 100 mL of biostimulant solution per pot. Treatments were repeated at 10-day intervals until week 10 (i.e., the end of the experiment).

Plant growth components evaluated

At the end of 10-week growth period, all replicates per treatment sampled for measurement of number of true leaves, leaf area using LI-3100C portable area meter (LI-COR Biosciences, Lincoln, Nebraska, USA)., open flowers (at anthesis), and leaf relative chlorophyll content of three recently mature leaves per plant using Minolta SPAD-502 Plus chlorophyll meter; Minolta Camera Co., Osaka, Japan), crown diameter, plant shoot fresh and dry weight, root fresh and dry weight, plant height. For dry weights, plant shoots and roots were dried separately in a forced air oven at 70 ^{o}C until dry weights remained constant.

Experimental design and statistical analysis

The treatments were arranged in a complete randomized design. One pot represented the experimental unit and each treatment contained five replicate pots. All data were subjected to analysis of variance using the general linear model program of SAS (version 9.1.3; SAS Institute, Cary, NC). Means comparison was performed using the Tukey's honestly significant difference test at $P \leq 0.05$.

Results

Stem diameter

The effect of Stimplex® treatments on basal stem diameter of ornamental pepper is depicted in *Figure 1*. There was a significant difference ($P \leq 0.01$ between Stimplex® levels in the increment of stem diameter at the end of 10-week growth period. The stem diameter ranged from 3.49 to 4.40 mm and the average stem diameter was 3.98 mm. Ornamental pepper plants treated with 0.75 and 1.00 ml.L^{-1} Stimplex® exhibited the highest increment of stem diameter (4.33 and 4.40 mm, respectively). The plants treated

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with 0 ml.L^{-1} Stimplex® (control) had the lowest increment of stem diameter (3.49 mm), which was closely followed by plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments (*Fig. 1*). Both spray and drench treatments significantly increased stem diameter, but variation in method of application was not significant. Interaction between Stimplex® levels and application methods were also found non-significant for stem diameter.

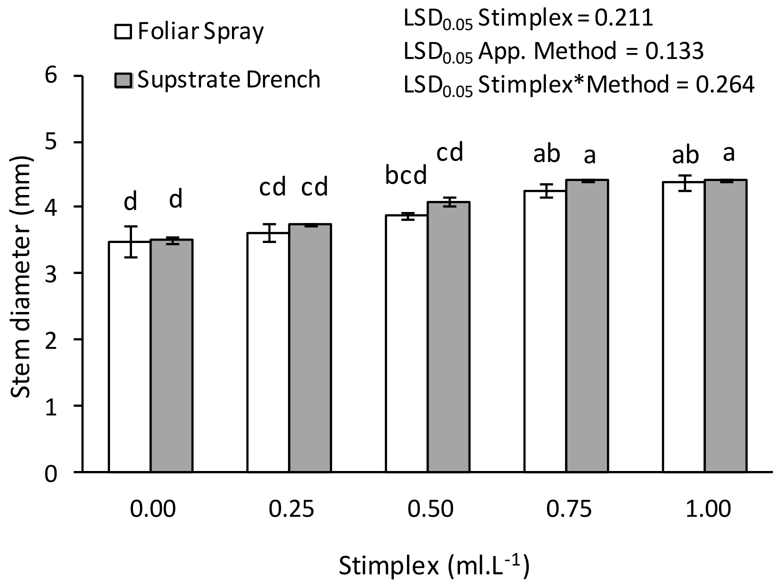

Figure 1. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on stem diameter of ornamental pepper. Levels not connected by same letter are significantly different at $P \leq 0.01$ by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

Plant height

Application of Stimplex® significantly increased plant height ($P \leq 0.01$). As shown in *Figure 2,* plant height ranged from 19.18 to 26.43 cm and the average plant height was 23.01 cm at the end of 10-week growth period. Ornamental pepper plants treated with 0.75 and 1.00 ml.L^{-1} Stimplex® treatments exhibited the greatest increment of plant height (26.16 and 26.43 cm, respectively). The plants treated with 0 ml.L^{-1} Stimplex® (control) had the lowest plant height value (19.18 cm), which was closely followed by plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments (*Fig. 2*). Variation in method of application was significant ($P \leq 0.01$) and the substrate drench method was found to be significantly effective than the foliar spray application in terms of plant height (*Fig. 2*). No significant interaction effect between Stimplex® levels and application methods were observed for the plant height.

Number of leaves

The effect of Stimplex® treatments on number of leaves per plant is presented in *Figure 3.* The number of leaves ranged from 41.00 to 46.00 leaves per plant and the average number of leaves was 43.63 at the end of 10-week growth period (*Fig. 3*). Application of Stimplex® significantly increased the number of leaves ($P \leq 0.01$). Ornamental pepper plants treated with 0.75 and 1.00 ml.L^{-1} Stimplex® treatments

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showed the highest number of leaves (45.62 and 46.00, respectively), whereas the values of plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments were not significantly different than the values of control plants treated with 0 ml.L^{-1} Stimplex® (41.00). Variation in method of application was significant ($P \leq 0.05$) and the substrate drench method was found to be significantly effective than the foliar spray application in terms of number of leaves (*Fig. 3*). No significant interaction effect between Stimplex® levels and application methods were observed for number of leaves.

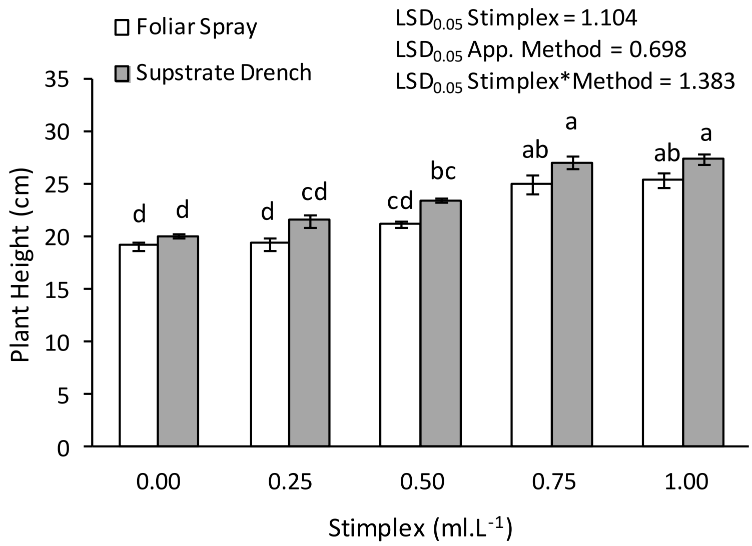

***Figure 2.** Effect of Stimplex® treatments applied as foliar spray and substrate drench on plant height of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

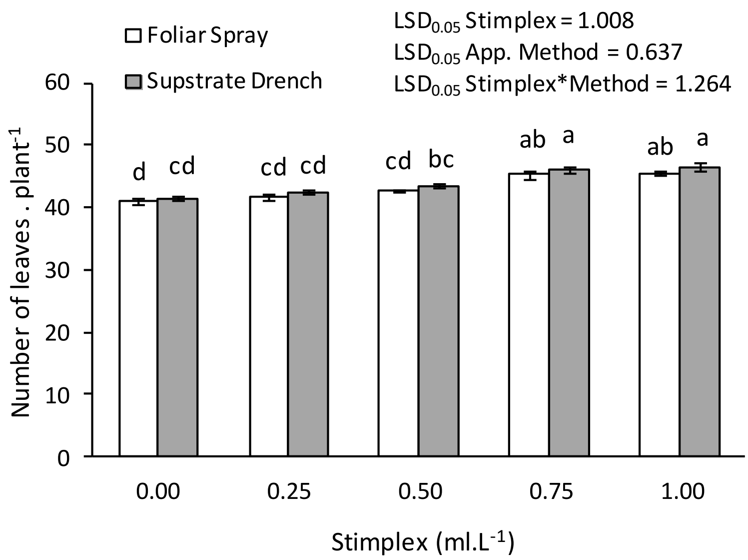

***Figure 3.** Effect of Stimplex® treatments applied as foliar spray and substrate drench on number of leaves of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

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DOI: http://dx.doi.org/10.15666/aeer/1702_43614375

Leaf area

The average leaf area per plant is illustrated in *Figure 4*. The leaf area per plant ranged from 235 to 353 cm^2 $plant^{-1}$ and the average leaf area was 294 cm^2 $plant^{-1}$ at the end of 10-week growth period. The plants treated with 0.75 and 1.00 $ml.L^{-1}$ Stimplex® treatments produced the highest leaf area (348 and 353 cm^2 $plant^{-1}$, respectively), whereas the leaf area values of plants treated with 0.25 and 0.50 $ml.L^{-1}$ Stimplex® treatments were not significantly different than the values of control plants treated with 0 $ml.L^{-1}$ Stimplex® (235 cm^2 $plant^{-1}$). Variation in method of application was significant ($P \leq 0.05$) and the substrate drench method was found to be significantly effective than the foliar spray application in terms of leaf area per plant. There was no significant interaction between Stimplex® levels and application methods for the leaf area.

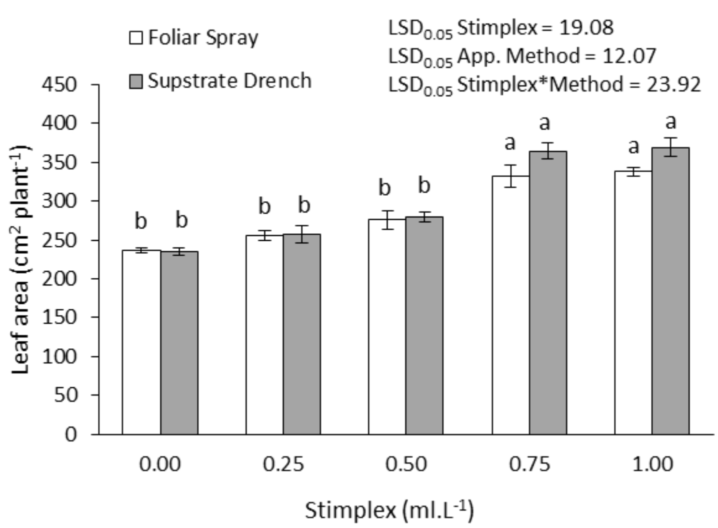

Figure 4. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on leaf area of ornamental pepper. Levels not connected by same letter are significantly different at $P \leq 0.01$ by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

Number of flowers

Application of Stimplex® either as foliar spray or substrate drench significantly increased the number of flowers ($P \leq 0.01$). The 0.75 and 1.00 $ml.L^{-1}$ Stimplex® treatments resulted in the highest number of flowers (23.62 flowers), whereas the values of plants treated with 0.25 and 0.50 $ml.L^{-1}$ Stimplex® treatments were not significantly different than the values of control (0 $ml.L^{-1}$ Stimplex®) plants (20.87 flowers). There were no significant differences between the two application methods of Stimplex® in the number of flowers. No significant interaction effect between Stimplex® levels and application methods were also detected for the number of flowers (*Fig. 5*).

Chlorophyll content

The effect of Stimplex® treatments on leaf chlorophyll content (SPAD) presented in *Figure 6*. Results revealed that application of Stimplex® either as foliar spray or substrate drench significantly increased leaf chlorophyll content of ornamental pepper ($P \leq 0.01$). The plants treated with 0.75 and 1.00 $ml.L^{-1}$ Stimplex® treatments showed

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the highest number of flowers (57.86 and 58.70 SPAD, respectively). The plants treated with 0 ml.L^{-1} Stimplex® (control) had the lowest chlorophyll content (50.71 SPAD), which was closely followed by plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments (*Fig. 6*). There were significant differences between the two application methods of Stimplex® in the chlorophyll content. Beneficial effect of Stimplex® in enhancing chlorophyll content was more apparent in substrate drenched plants than foliar sprayed plants. There were also significant interaction effects between Stimplex® levels and application methods on chlorophyll content. The highest chlorophyll content was obtained from interactions of substrate drench × 0.75 or 1.00 lg.L^{-1} Stimplex® levels.

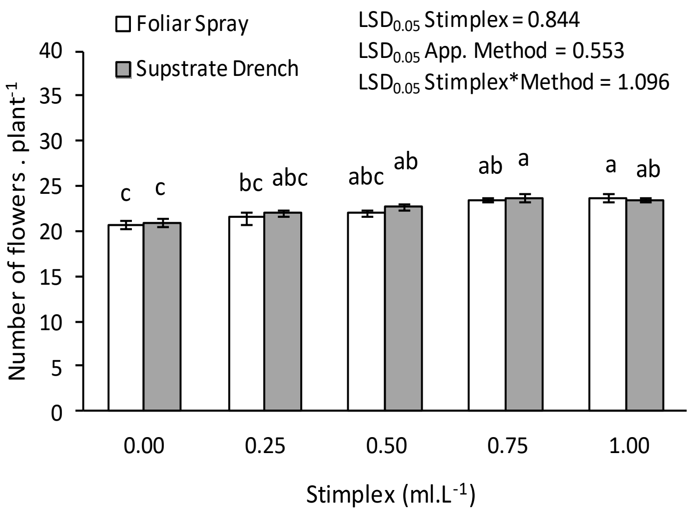

***Figure 5.** Effect of Stimplex® treatments applied as foliar spray and substrate drench on number of flowers of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

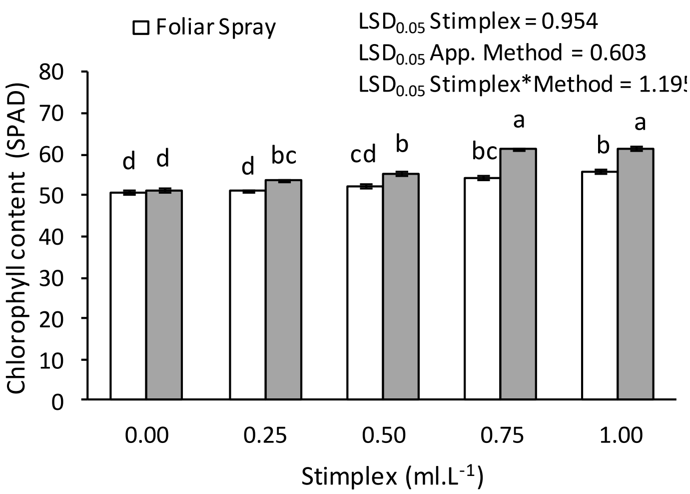

***Figure 6.** Effect of Stimplex® treatments applied as foliar spray and substrate drench on chlorophyll content (SPAD) of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

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Shoot fresh weight

Results indicated that application of Stimplex® either as foliar spray or substrate drench significantly increased shoot fresh weight of ornamental pepper plants ($P \leq 0.01$). As presented in *Figure 7,* the shoot fresh weight ranged from 15.67 to 20.30 g and the average shoot fresh weight was 22.45 g at the end of 10-week growth period. Ornamental pepper plants applied with 0.75 and 1.00 ml.L^{-1} Stimplex® treatments as foliar spray or substrate drench showed the highest shoot fresh weight values (20.30 and 20.22 g, respectively). The control plants (0 ml.L^{-1} Stimplex®) had the lowest shoot fresh weight value (15.67 g), which was closely followed by plants treated with 0.25 mg.L^{-1} Stimplex® (*Fig. 7*). There were no significant differences between the two application methods of Stimplex® in the shoot fresh weight. No significant interaction effect between Stimplex® levels and application methods were also detected for the shoot fresh weight.

Shoot dry weight

As shown in *Figure 8,* the shoot dry weights ranged from 2.11 to 2.46 g and the average shoot dry weight was 2.29 g at the end of 10-week growth period. Ornamental pepper plants treated with 0.75 and 1.00 ml.L^{-1} Stimplex® treatments produced the greatest shoot dry weight values (2.45 and 2.46 g, respectively), whereas the values of shoot dry weights of plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® were not significantly different than the values of control plants (2.11 g). Similarly to shoot fresh weight, there were no significant differences between the two application methods of Stimplex® in the shoot dry weight. No significant interaction effects between Stimplex® levels and application methods were also observed for the shoot dry weight.

Root fresh weight

Application of Stimplex® either as foliar spray or substrate drench significantly increased root fresh weight of ornamental pepper plants ($P \leq 0.01$). The 0.75 and 1.00 ml.L^{-1} Stimplex® treatments resulted in the greatest root fresh weight values (7.31 and 7.49 g, respectively). The plants treated with 0 ml.L^{-1} Stimplex® (control) had the lowest root fresh weight (5.69 g), which was closely followed by plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments (*Fig. 9*). There were significant differences between the two application methods of Stimplex® in the root fresh weight ($P \leq 0.01$). Beneficial effect of Stimplex® in enhancing root fresh weight was more evident in substrate drenched plants than foliar sprayed plants. No significant interaction effect between Stimplex® levels and application methods were found for the root fresh weight.

Root dry weight

The root dry weights ranged from 0.73 to 0.90 g and the average root dry weight was 0.81 g at the end of 10-week growth period (*Fig. 10*). Results indicated that application of Stimplex® either as foliar spray or substrate drench significantly increased root dry weight of ornamental pepper ($P \leq 0.01$). The plants treated with 0.75 and 1.00 ml.L^{-1} Stimplex® treatments showed the greatest root dry weight values (0.87 and 0.90 g, respectively). The plants treated with 0 ml.L^{-1} Stimplex® (control)

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DOI: http://dx.doi.org/10.15666/aeer/1702_43614375

had the lowest root dry weight value (0.73 g), which was closely followed by plants treated with 0.25 and 0.50 ml.L^{-1} Stimplex® treatments (*Fig. 10*). There were significant differences between the two application methods of Stimplex® in the root dry weight ($P \leq 0.05$). Substrate drench applications gave better response and increased the number of flowers more than foliar applications. No interaction effect was detected between Stimplex® levels and application methods on the root dry weight of ornamental pepper plants.

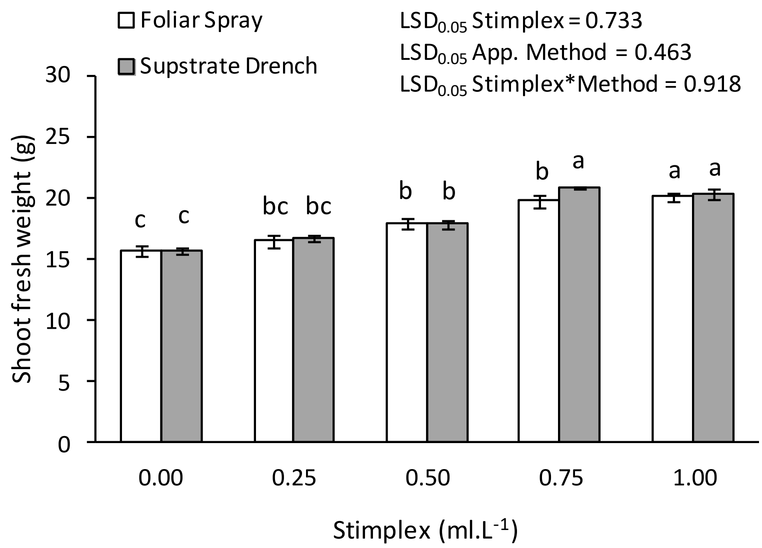

Figure 7. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on shoot fresh weight of ornamental pepper. Levels not connected by same letter are significantly different at $P \leq 0.01$ by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

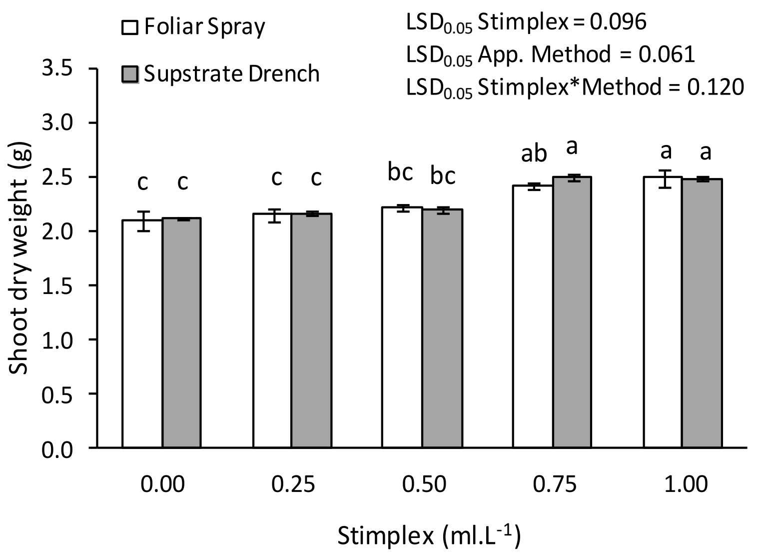

Figure 8. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on shoot dry weight of ornamental pepper. Levels not connected by same letter are significantly different at $P \leq 0.01$ by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

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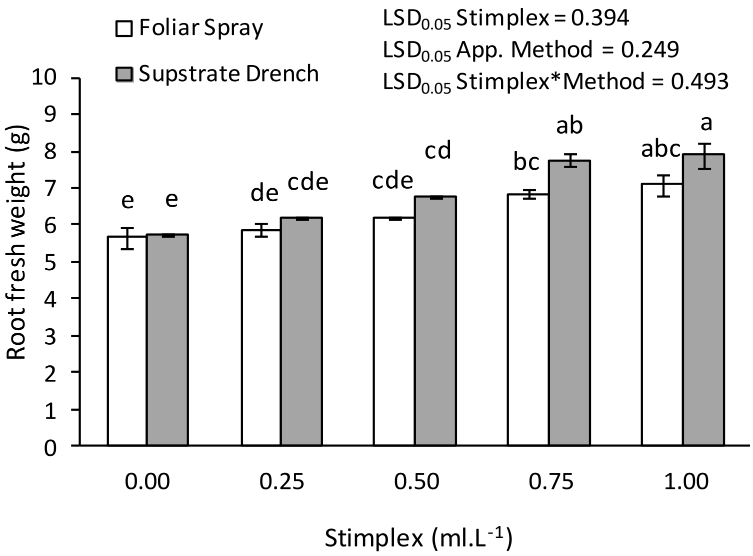

Figure 9. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on root fresh weight of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

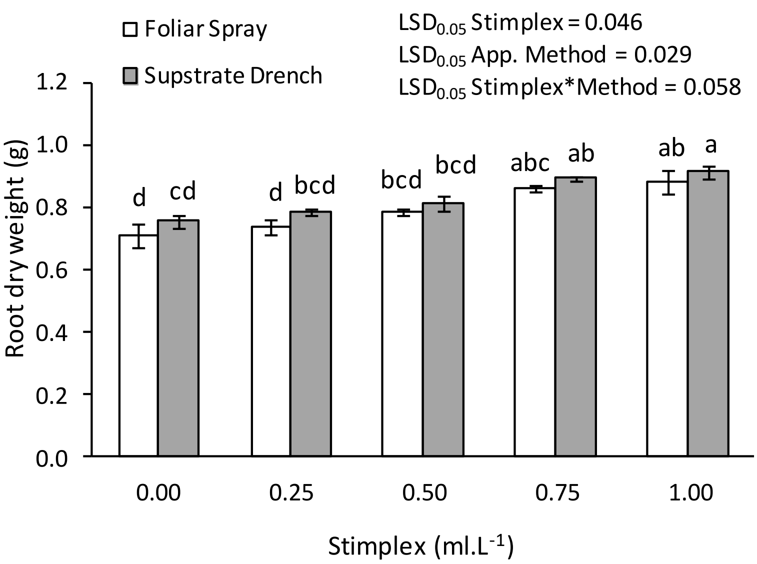

Figure 10. *Effect of Stimplex® treatments applied as foliar spray and substrate drench on shoot dry weight of ornamental pepper. Levels not connected by same letter are significantly different at P ≤ 0.01 by Tukey's honestly significant difference test. Values represent average (n = 5 plants); bars represent standard error*

Discussion

Seaweed concentrates have been reported to trigger early flowering and to increase number of flowers in crop plants. In this study, Stimplex® treatments increased the flower number in ornamental pepper plants. The promotion of flowering following application of Stimplex® was observed in some other plant species For example, Li and

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Mattson (2015) reported that substrate drench at 5–10 ml.L^{-1} significantly increased flower number of petunia and tomato. They used higher concentrations than ours. This could be explained by the fact that different plant species can have different responses to the application methods and rates of seaweed concentrates. Sarhan and Ismael (2014) also verified positive effects of seaweed extract on flower number by reporting that treatment with seaweed extracts significantly enhanced number of flowers and fruit setting percentage in cucumber. This can be attributed to the fact that the seaweed extracts have been reported to contain significant amounts of auxins, cytokinins, and betaines, which affect cell division during the early stages of growth along with the induction of flower formation and number (Roussos et al., 2009).

In the current study, the Stimplex® treated ornamental pepper plants had higher leaf chlorophyll content (SPAD) than those in the control plants. Leaf chlorophyll content was enhanced following seaweed application in a number of previous studies (Eris et al., 1995; Blunden et al., 1997; Spinelli et al., 2010; Fan et al., 2013; Jannin et al., 2013). It was also previously shown that cytokinin-like effects and betaines in seaweed (*A. nodosum*) extracts might be responsible for this effect (Whapham et al., 1993). Blunden et al. (1997) reported that application of an extract of *A. nodosum* resulted in higher concentrations of chlorophyll in the leaves of tomato, bean, wheat, barley, and maize plants in comparison to control plants. They also suggested that the enhanced leaf chlorophyll content of plants treated with seaweed extract was dependent on the betaines present. Betaines, while not traditionally included among the known classical plant hormones, are found in seaweeds and their extracts (MacKinnon et al., 2010). Recently, Fan et al. (2013) observed that the transcription of betaine aldehyde dehydrogenase and choline monooxygenase were greater in seaweed (*A. nodosum*) treated leaves compared to the control. Contrary to some earlier research findings, no beneficial effects were detected for seaweed (*A. nodosum*) applications on chlorophyll content of spinach grown under both normal and stress situations (Xu and Leskovar, 2015).

Numerous studies have revealed seaweed extracts promoted vegetative growth of horticultural crops (Arthur et al., 2003; Morales Payan, 2004; Spinelli et al., 2010; Kumari et al., 2011; Zodape et al., 2011; Zulaikha, 2013; Hernández-Herrera et al., 2014; Selvakumari and Venkatesan, 2017; Yildiztekin et al., 2018). In accordance with these earlier studies, application of Stimplex® to ornamental pepper plants improved stem diameter, plant height, number of leaves and leaf area, shoot fresh weight, shoot dry weight, root fresh weight and dry weight compared to the control plants in the present study. In a previous study, application of seaweed (*A. nodosum*) extract solution at 1% concentration to roots produced transplants with increased root length and shoot length compared to control in tomato (Poincelot, 1993). Spinelli et al. (2010) reported that seaweed extract derived by the algae *A. nodosum* increased the vegetative growth of strawberry up to 10%. They also reported that the most significant result was the increment of the plant biomass: the shoot dry matter was increased up to 27% and root dry matter up to 76%. In another study, Kumari et al. (2011) reported an increase in root length, shoot length, and fresh weight in tomato treated with drench and foliar applications of liquid seaweed extracts. Similarly, at 21 days following application, addition of 0.1 g L^{-1} of seaweed extract to the growth medium significantly increased spinach fresh weight and dry-matter content by 58% and 23%, respectively (Fan et al., 2013). In broccoli grown in greenhouse, drench application of seaweed extracts increased leaf area, stem diameter, and both shoot and root biomass (Mattner et al.,

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DOI: http://dx.doi.org/10.15666/aeer/1702_43614375

2013). Recently, Zermeno-Gonzalez et al. (2015) reported that the application of biological fertilizers derived from seaweed extracts to the soil and foliage on forage maize resulted in an increase in plant height (17.3%), stem diameter (10.5%) and plant dry weight (14.8%) compared to the control plants. In the study conducted by Xu and Leskovar (2015), *A. nodosum* seaweed extract treatments increased leaf fresh weight and dry weight.

It is possible that the beneficial effects of Stimplex® on growth parameters could be ascribed to the presence of macro and micro nutrients as well as growth promoting substances like cytokinins other constituents in the seaweed extract. Seaweed ingredients contains some macro and microelement nutrients, vitamins, amino acids, auxins, and cytokinins that affect cellular metabolism in treated plants, leading to enhanced growth (Crouch and van Staden, 1993; Khan et al., 2009; Craigie, 2011; Spann and Little, 2010; du Jardin, 2015). Zodape et al. (2011) have indicated that increased plant height of tomato treated with seaweed sap might be due the macro and micro nutrients as well as growth promoting substances like cytokinin promoting growth. Ramamoorthy et al. (2007) indicates that application of seaweed extract as foliar spray enhances the growth of the plants by making the growth promoting substances available to the plants through absorption and translocation. Rayorath et al. (2008) provided evidence that components of the commercial seaweed (*A. nodosum)* extracts regulate the concentration and translocation of auxins which could account for the enhanced plant growth. Khan et al. (2011) reported that that Stimplex® treatment resulted in increased cytokinin-like responses in *Arabidopsis thaliana* plants, which suggests that the seaweed extracts have compounds that may have contributed to cytokinin-like activity. Cytokinins induce cell division and proliferation, increasing the sink activity of roots, resulting in growth stimulation (Nelson and Van Staden, 1984). Increases in lateral root formation, total root volume, and root length have been observed and attributed to the presence of auxins and cytokinins in seaweed extracts (Crouch and Staden, 1993; Stirk and van Staden, 1997; Arthur et al., 2003; Khan et al., 2011; Sarhan and Ismael, 2014; Vijayanand et al., 2014).

Conclusion

Broadly, application of seaweed extracts as organic biostimulant has received a greater acceptance in agriculture because of their potent plant growth-enhancing properties. The interest in seaweed extracts to enhance agricultural productivity continues to grow globally. A number of commercial seaweed extract products are available for use in agricultural and horticultural crops. The application of Stimplex® seaweed extract (0.75 and 1.00 ml.L^{-1}) to the growing substrate and foliage caused improvement in plant growth parameters of ornamental pepper. The growth parameters increased with increasing concentrations of Stimplex®, up to 0.75 ml.L^{-1}, but increasing concentration beyond 0.75 ml.L–1 did not have any further effect on plant growth. The results of this study indicate that Stimplex® may be a viable tool for improving growth of ornamental pepper plants. Furthermore, the preparation of the product is handy and it has no hazardous side effects and offers ecofriendly production of the agricultural produce. However, further study is needed before adopting this practice.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43614375

EVALUATION OF THE TECHNOLOGICAL QUALITY OF GRAIN AND FLOUR OF TWO SPELT WHEAT (*TRITICUM AESTIVUM* SSP. *SPELTA* L.) CULTIVARS GROWN UNDER DIFFERENT CONDITIONS OF CROP PROTECTION AND SEEDING RATE

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(Received 9th Jan 2019; accepted 28th Feb 2019)

Abstract. A field study was conducted during the period 2014–2015 at the Czesławice Experimental Farm in Poland. The aim of this study was to determine the technological characteristics of the grain and flour of winter spelt (*Triticum aestivum* ssp. *spelta*) two cultivars ('Rokosz' and 'Schwabenspelz') depending on the density of sowing and crop protection methods. The present study showed that crop protection method and seeding rate did not cause most of the quality characteristics of spelt grain and flour to significantly vary. Both the Polish cultivar 'Rokosz' and the German cultivar 'Schwabenspelz' exhibited similar technological quality of grain and flour under organic farming and chemical crop protection conditions. Therefore, both these cultivars can be recommended for cultivation in organic farms. In 'Rokosz', gluten content, Zeleny sedimentation value, and dough development time increased with increasing protein content, whereas gluten weakening decreased, which is the evidence of a higher quality and baking value of this cultivar relative to 'Schwabenspelz'.
Keywords: *chemical and pro-ecological protection, sowing rate, farinographic features of flour and dough, quality characteristic of grain, winter spelt cultivar*

Introduction

Spelt wheat (*Triticum aestivum* ssp. *spelta*) is one of the oldest wheat subspecies (Szumiło and Rachoń, 2015; Stankowski et al., 2016). Numerous archaeological studies show that it originated from the south-western region of Asia (Krawczyk et al., 2008a; Cacak-Pietrzak et al., 2013), but for a long time it has also been grown in Germany, Switzerland, Belgium, Slovakia, the Czech Republic, Italy, Canada, and the USA (Stankowski et al., 2016). Despite the fact that in large-area cultivation it was replaced by the more threshable and productive common wheat (Podolska et al., 2015; Ugrenović et al., 2018), in recent years the beneficial properties of spelt wheat are being discovered anew. This species owes its renewal predominantly to the growing interest in healthy food and organic farms, where spelt is frequently grown due to its high resistance to adverse environmental conditions, diseases, and pests (Kraska et al., 2013; Callejo et al., 2015; Rachoń et al., 2016; Babenko et al., 2018). Moreover, spelt wheat has a long stem and high tillering ability, and hence it exhibits high weed competitiveness (Rachoń et al., 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43774395

According to 2015 data of the Central Statistical Office, in 2015 nearly 270,000 organic farms with an area of more than 11.1 million hectares were recorded in the EU, which accounts for 6.2% of the total agricultural land area. In the same year, in Poland there were more than 22,000 organic farms with an area of over 580,000 ha, which accounts for 3.8% of Poland's total agricultural land area (Statistical Yearbook of Agriculture, 2017).

Consumers seeking food of high nutritional value readily choose spelt products, driven by the nutrient richness and health-enhancing properties of spelt wheat (Callejo et al., 2015; Rachoń et al., 2016; Boukid et al., 2018). Spelt grain is most often used to make whole grain food products, e.g. bakery products, pasta, muesli, or cereals (Benincasa et al., 2015; Boukid et al., 2018). Compared to common wheat, eating spelt grain products prevents hypertension, reduces blood cholesterol level, improves the functioning of the digestive system, and aids the nervous system (Solarska et al., 2012; Spychaj-Fabisiak et al., 2014). The most important technological parameters of spelt flour are similar to those of common wheat flour, but the observed differences weigh in favour of spelt flour (Rachoń et al., 2011). It contains more protein, including gluten, vitamins A, E, D, B, PP, micro- and macronutrients, fats, and necessary amino acids (Lacko-Bartošová, 2010; Escarnot et al., 2012; Biel et al., 2016a; Zorovski et al., 2018). Its total protein content ranges from 13 to 19% DM (Biel et al., 2016c). Gluten obtained from spelt flour added to low protein flour increases the amount of protein and improves the technological properties of such flour (Rachoń et al., 2011). Due to the poorer milling properties of spelt grain, lower milling yields are obtained from it compared to common wheat grain (Krawczyk et al., 2008b). Flour extracted from spelt grain is a good raw material to produce high quality bakery products (Pruska-Kędzior et al., 2008). Spelt bread loaves bake well, do not crumble when cut, and smells and tastes slightly of walnut (Callejo et al., 2015). It should be noted that spelt dough can have a looser texture than dough made of common wheat flour (Biel et al., 2016b). This is due to the spelt gluten which is characterized by high weakening, while the dough by excessive extensibility (Krawczyk et al., 2008a). The rheological properties of spelt flour and dough primarily depend on the gluten content and a proper gliadin-to-glutenin ratio (Callejo et al., 2015; Biel et al., 2016b).

The study hypothesized that spelt grain and flour obtained from organically grown spelt (no crop protection, mechanical weed control) would have similar or more favourable technological properties than grain and flour obtained from a chemically protected crop (herbicide protection, complete chemical crop protection). An assumption was also made that an increased seeding rate (500 grains per m^2) of spelt wheat (which is the common practice in organic farms) will not result in a deterioration of the technological quality of this plant. The aim of this study was to compare the technological quality of grain and flour of two winter spelt cultivars grown under different agronomic conditions.

Materials and methods

A field study was carried out for two growing seasons 2013/2014 and 2014/2015 in the village of Czesławice where the Experimental Farm (51°18′23″ N, 22°16′2″ E) belonging to the Lublin University of Life Sciences, Poland is located (*Fig. 1*). The experiment on growing spelt wheat was established on a loess-derived *Luvisol*, classified as good wheat soil complex and soil class II. The arable layer of the soil was characterized by high availability of phosphorus (P_2O_5 – 175.1-178.2 mg kg^{-1} soil) and potassium (K_2O – 141.9 -159.4 mg kg^{-1} soil) as well as medium availability of magnesium (Mg – 79-85 mg kg^{-1} soil), slightly acidic pH (in 1 M KCl – 6.1-6.4), and a humus content of 1.59-1.63%. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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experiment was conducted in three replicates. Two winter spelt wheat cultivars, the German cultivar 'Schwabenspelz' (*Fig. 2*) and the Polish cultivar 'Rokosz', were the object of this research.

The experiment included the following factors: I – seeding rate: 1) optimum – 350 plants per m^2; 2) increased – 500 plants per m^2; II – crop protection: 1) pro-ecological: A – treatment without protection against pathogens (control treatment), B – harrowing in early spring at the beginning of the growing season; 2) chemical: C – application of the herbicides Sekator 125 OD (a.i. amidosulfuron, iodosulfuron, mefenpyr-diethyl) at a rate of 150 ml ha^{-1} and Attribut 70 WG (a.i. propoxycarbazone-sodium) at a rate of 60 g ha^{-1} at BBCH 22-24 stages of spelt wheat; D – application of complete chemical protection – the herbicides Sekator 125 OD (a.i. amidosulfuron, iodosulfuron, mefenpyr-diethyl) at a rate of 150 ml ha^{-1} and Attribut 70 WG (a.i. propoxycarbazone-sodium) at a rate of 60 g ha^{-1} at BBCH 22-24 stages of spelt wheat; the growth retardant Cerone 480 SL (a.i. ethephon) at BBCH 30-31 stages at a rate of 0.75 l ha^{-1}, the fungicide Wirtuoz 520 EC (a.i. prochloraz, tebuconazole, proquinazid) at BBCH 24-25 and 32-33 stages at a rate of 1.0 l ha^{-1} as well as the insecticide Decis 2,5 EC (a.i. deltamethrin) at BBCH 37-39 stages at an amount of 0.25 l ha^{-1}.

Figure 1. *Localization of the study site*

Figure 2. *'Schwabenspeltz' cultivar of spelt wheat*

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Mineral fertilization was applied at the following rates: N – 50 kg ha^{-1}, P_2O_5 – 60 kg ha^{-1} and K_2O – 70 kg ha^{-1}. All phosphorus and potassium fertilizers and part of nitrogen fertilizers (20 kg N) were applied before sowing the spelt wheat. The remaining portion of nitrogen fertilizers was applied right after the beginning of the growing season. The sown and harvested plot area was 13.5 m^2 (5.0 × 2.7 m). Winter wheat was the previous crop for spelt wheat. Tillage was typical for common wheat cultivation. The spelt wheat was sown on September 23, 2013 and September, 25, 2014 and it was harvested on August 12, 2014 and August 11, 2015.

Temperature range in both vegetation seasons of wheat spelt were similar. Mean temperature in season 2013/2014 was 8.7 and in season 2014/2015 was 0.1 higher (*Table 1*). Season 2013/2014 could be described as very wet as rainfall was by 281 mm higher than in season 2014/2015. In year 2014 the highest rainfall was in May, June and August. In year 2015 high rainfall was in May but August was very dry with only 5.9 mm of rain.

Table 1. *Rainfall and air temperature in the growing season of spelt wheat, according to the Meteorological Station in Czesławice*

Years	Months												Sum/Mean
	IX	X	XI	XII	I	II	III	IV	V	VI	VII	VIII	
Rainfalls (mm)													
2013/2014	49.5	7.3	60.6	13.7	54.5	5.8	49.1	63.9	230.2	110.2	61.4	102.0	808.2
2014/2015	21.8	27.5	24.1	57.8	50.9	15.8	48.6	39.1	169.6	13.5	52.6	5.9	527.2
Temperature (°C)													
2013/2014	11.3	9.5	4.9	1.7	-2.9	0.3	4.9	8.9	13.0	15.2	19.6	18.3	8.7
2014/2015	14.0	9.7	4.6	-0.1	1.0	-1.1	2.8	6.5	11.5	16.1	19.0	21.9	8.8

The following parameters were determined: 1000 grain weight (TGW) was determined according to PN-EN ISO 520:2011E. Clean material was placed in an automatic seed counter and 4 × 250 seeds were counted and weighed with an accuracy of 0.1 g. Test weight was determined according to PN-EN ISO 7971-1:2010P. To analyse α-amylase activity, SWD-SŻ type instrument (Sadkiewicz® Instruments, Bydgoszcz, Poland), was used, in which falling number was measured using the Hagberg-Perten method, according to PN-EN ISO 3093:2010E. The grain was milled in a laboratory 6-roller mill manufactured by the Research Institute of the Bakery Industry in Bydgoszcz. The milled flour was sieved in a laboratory sifter to obtain appropriate fractions for the individual assays, on sieves with proper mesh sizes (265 µm – for farinographic evaluation, 230 µm – for determination of gluten quantity and quality, 150 µm – for determination of sedimentation value). Determination of gluten content and weakening was performed according to PN-EN ISO 21415-2:2015-12E using the Gluten index system (Perten Instruments). Sedimentation value was determined according to PN-EN ISO 5529:2010E. This assay was performed on an apparatus consisting of a measurement panel and the Sadkiewicz vortex mixer (Sadkiewicz® Instruments, Bydgoszcz, Poland), SWD-89 model. Analysis of the farinographic properties of the dough was carried out on a farinograph (Brabender) using a 50 type head according to PN-EN ISO 5530-1:2015-01E. The following parameters were determined – flour water absorption, dough development time, dough stability, and degree of softening after 10 min and after 12 min.

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The obtained results were statistically analysed by two-way analysis of variance using a completely randomized design. Statistical software FR-ANALWAR 5.2. was used for calculations. Confidence half-intervals were calculated by Tukey's test at a significance level of 0.05. Significant differences between these two groups of treatments: organic protection (treatments A and B) and chemical protection (treatments C and D), were evaluated based on the Scheffe test (α = 0.05).

Results

Conducted research showed that in 2014 and 2015 experiment factors had significant influence on weight of 1000 grains of spelt wheat 'Rokosz' cultivar (*Tables 2 and 3*). In the first year of research the greatest TGW was described in cultivar with complete chemical protection (treatment D) but in 2015 in harrowing treatment (B) (*Table 2*). In both years increase in seeding rate had untoward influence on grain size 'Rokosz' cultivar (*Table 3*).

Table 2. *The impact of crop protection on quality characteristic of the spelt wheat grain cultivar 'Rokosz' (A^1 – control treatment, B^2 – harrowing in early spring at the beginning of the growing season, C^3 – application of the herbicides, D^4 – application of complete chemical protection, ns* – not significant at $p \le 0.05$)*

Years	Experimental factors	1000 grains weight (g)	Test weight (kg hl^{-1})	Falling number (s)	Gluten content (%)	Gluten weakening (mm)	Gluten index (%)	Zeleny sedimentation value (ml)
2014	A^1	28.41	72.20	304.00	31.93	0.75	51.00	17.65
	B^2	27.90	71.80	325.75	31.57	0.50	51.25	17.67
	C^3	28.41	70.50	248.00	33.37	0.25	41.25	17.43
	D^4	30.69	74.60	324.75	28.60	0.50	59.00	17.20
$LSD_{0.05}$ (Tukey test)		0.55	0.57	20.33	1.28	ns*	9.02	ns
2015	A^1	34.91	80.45	365.25	18.47	2.62	46.50	15.37
	B^2	37.77	80.55	372.25	19.13	4.88	49.00	15.60
	C^3	37.28	80.60	386.50	17.97	3.25	56.25	15.35
	D^4	36.79	79.10	359.25	17.98	3.50	54.50	15.35
$LSD_{0.05}$ (Tukey test)		0.36	0.24	13.84	1.12	0.61	5.73	ns
Mean for 2014-2015	A^1	31.66	76.33	334.63	25.20	1.69	48.75	16.51
	B^2	32.84	76.18	349.00	25.35	2.69	50.13	16.64
	C^3	32.85	75.55	317.25	25.67	1.75	48.75	16.39
	D^4	33.74	76.85	342.00	23.29	2.00	56.75	16.28
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	2.07	ns	ns	ns
Comparison of the pro-ecological and chemical crop protection (mean for 2014-2015)								
pro-ecological (A, B)		32.25	76.25	341.81	25.28	2.19	49.44	16.58
chemical (C, D)		33.29	76.20	329.63	24.48	1.88	52.75	16.34
Scheffe's test $LSD_{0.05}$		ns	ns	ns	1.82	ns	ns	ns

In 'Schwabenspelz' cultivar, during the experiment (2014-2015), crop protection had significant influence on this parameter (*Table 4*). In 2014 the greatest TGW was described in treatment B but in 2015 in the treatment D. Increase in seeding rate in 2015 significantly decreased of TGW of 'Schwabenspelz' spelt wheat (*Table 5*). Mean results from 2014-2015 did not confirm that crop protection and seeding rate had significant influence on grain size of both studied cultivars and there was also no difference between pro-ecological and chemical crop protection (*Tables 2* and *3,4,5*).

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Table 3. *The impact of seeding rate on quality characteristic of the spelt wheat grain cultivar 'Rokosz' (ns* – not significant at p≤0.05)*

Experimental factors	1000 grains weight (g)	Test weight (kg hl^{-1})	Falling number (s)	Gluten content (%)	Gluten weakening (mm)	Gluten index (%)	Zeleny sedimentation value (ml)
2014							
optimum	29.55	73.30	332.13	32.16	0.31	53.00	16.72
increased	28.15	71.25	269.11	30.58	0.69	48.25	18.25
$LSD_{0.05}$ (Tukey test)	0.65	1.27	ns*	ns	ns	ns	ns
2015							
optimum	37.62	80.25	378.25	18.79	3.50	50.12	15.48
increased	35.75	80.10	363.38	17.99	3.62	53.00	15.36
$LSD_{0.05}$ (Tukey test)	1.57	ns	ns	ns	ns	ns	ns
Mean for 2014-2015							
optimum	33.59	76.78	355.19	25.48	1.91	51.56	16.10
increased	31.95	75.68	316.25	24.28	2.16	50.63	16.81
$LSD_{0.05}$ (Tukey test)	ns	ns	ns	ns	ns	ns	ns

Table 4. *The impact of crop protection on quality characteristic of the spelt wheat grain cultivar 'Schwabenspelz' (A^1 – control treatment, B^2 – harrowing in early spring at the beginning of the growing season, C^3 – application of the herbicides, D^4 – application of complete chemical protection, ns* – not significant at p≤0.05)*

Years	Experimental factors	1000 grains weight (g)	Test weight (kg hl^{-1})	Falling number (s)	Gluten content (%)	Gluten weakening (mm)	Gluten index (%)	Zeleny sedimentation value (ml)
2014	A^1	36.87	66.77	73.50	43.55	4.13	38.00	17.20
	B^2	43.01	69.85	90.75	40.08	5.00	30.25	16.72
	C^3	36.84	68.45	98.25	43.30	5.50	41.50	17.20
	D^4	36.20	70.15	126.75	39.47	3.75	31.75	13.90
$LSD_{0.05}$ (Tukey test)		0.61	0.56	6.77	ns*	1.40	ns	1.66
2015	A^1	37.77	71.40	330.25	31.18	9.25	36.75	16.32
	B^2	35.30	70.05	341.00	36.22	11.12	38.00	15.83
	C^3	37.71	71.25	306.00	31.20	10.88	40.00	17.28
	D^4	38.54	72.05	311.00	30.05	9.12	37.00	16.77
$LSD_{0.05}$ (Tukey test)		0.79	0.27	21.57	0.85	2.31	2.09	1.10
Mean for 2014-2015	A^1	37.32	69.09	201.88	37.37	6.69	37.38	16.76
	B^2	39.16	69.95	215.88	38.15	8.06	34.13	16.28
	C^3	37.28	69.85	202.13	37.25	8.19	40.75	17.24
	D^4	37.37	71.10	218.88	34.76	6.44	34.38	15.34
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	ns	ns	1.68	ns
Comparison of the pro-ecological and chemical crop protection (mean for 204-2015)								
pro-ecological (A, B)		38.24	69.52	208.88	37.76	7.38	35.76	16.52
chemical (C, D)		37.33	70.48	210.51	36.01	7.32	37.57	16.29
Scheffe's test $LSD_{0.05}$		ns	ns	ns	ns	ns	ns	ns

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***Table 5.** The impact of seeding rate on quality characteristic of the spelt wheat grain cultivar 'Schwabenspelz' (ns* – not significant at p≤0.05)*

Experimental factors	1000 grains weight (g)	Test weight (kg hl^{-1})	Falling number (s)	Gluten content (%)	Gluten weakening (mm)	Gluten index (%)	Zeleny sedimentation value (ml)
2014							
optimum	38.86	68.69	84.88	38.42	4,25	35.00	16.50
increased	37.60	68.93	109.75	44.78	4.94	35.75	16.01
$LSD_{0.05}$ (Tukey test)	ns*	ns	11.12	ns	ns	ns	0.16
2015							
optimum	39.52	70.90	306.50	31.76	10.50	37.75	16.78
increased	35.14	71.48	337.62	32.56	9.69	38.12	16.32
$LSD_{0.05}$ (Tukey test)	0.79	0.32	30.18	ns	ns	ns	ns
Mean for 2014-2015							
optimum	39.19	69.80	195.69	35.09	7.38	36.38	16.64
increased	36.37	70.21	223.69	38.67	7.32	36.94	16.17
$LSD_{0.05}$ (Tukey test)	ns	ns	ns	ns	ns	ns	ns

In the research years methods of crop protection had significant influence on test weight 'Rokosz' and 'Schwabenspelz' cultivars (*Tables 2* and *4*). In 2014 and 2015, in both cultivars, the greatest test weight was amongst grains from treatment with complete chemical crop protection, furthermore in 'Schwabenspelz' cultivar test weight in 2014 did not vary much between treatments B and D. In the first year of research increase in seeding rate, from 350 to 500 grains per m^2, significantly decreased test weight of 'Rokosz' and in 2015 increased test weight of 'Schwabenspelz' cultivar (*Tables 3* and *5*). Mean result from research years did not confirm statistically significant relation between experiment factors and test weight.

In both research years crop protection had significant influence on falling number (*Tables 2* and *3,4,5*). In 2014 'Rokosz' cultivar shown the greatest falling number in treatment B and D (*Table 2*). In 2015 the greatest falling number was amongst treatment where herbicide was used (treatment C). In the first year of the study, which was characterized by o lot of rainfall (*Table 1*), in 'Schwabenspelz' cultivar, the falling number was very low. The greatest falling number (126 s) was found in the treatment D (*Table 4*). In 2015, positive influence on falling number of grain had pro-ecological crop protection (treatments A and B) of 'Schwabenspelz' spelt wheat (*Table 4*). Seeding rate significantly modified the falling number in the 'Schwabenspelz' cultivar only. In each year of the study the increase in sowing rate adversely affected this feature (*Table 5*). Mean result from research years, as in the case of the feature discussed earlier, showed no significant influence of experiment factors on falling number of spelt wheat grains.

Crop protection methods caused significant differences in the amount of gluten in 'Rokosz' grain (*Table 2*). In the first year of research, the greatest gluten content in grains from treatment B was found, while in 2015 from control (A) and mechanical weed management (B) treatments. Mean from 2014-2015, the gluten content in the treatment with complete chemical protection was significantly lower than in the treatment where only the herbicide was applied (C) and in the treatment with harrowing

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(B), whereas it did not differ significantly with the control treatment. It was statistically proven that pro-ecological crop management affected more favourably the trait described compared to chemical crop protection (*Table 2*). In the second year of study, in ‘Schwabenspelz’ cultivar significantly influence of crop protection on this feature was observed (*Table 4*). The highest content of gluten was in grain from the harrowing treatment (B). With regards to this cultivar, despite no significant differences, pro-ecological technologies also positively influenced grain gluten content (*Table 4*). In the case of the studied cultivars, the seedling rate did not significantly modify this feature (*Tables 3* and *5*).

In the years of research, the crop protection methods significantly differentiated the gluten weakening in grain of both spelt wheat cultivars (*Tables 2* and *4*). In 2015, in ‘Rokosz’ cultivar the highest gluten weakening was observed in the conditions where only herbicide was used (C), and the smallest in the control treatment (A) (*Table 2*). In both years of research in the grain of ‘Schwabenspelz’ cultivar harvested from the complete chemical protection treatment (D) was found the smallest gluten weakening (*Table 4*). In 2014, the highest value of discussed parameter in grain was recorded in the treatment C, while in the second year of research (2015) in variant B (*Table 4*). Mean result from research years did not confirm statistically significant influence of crop protection on gluten weakening in the grain of both spelt wheat cultivars.

In the 2014 and 2015, gluten index of grain of studied cultivars varied significantly (*Tables 2* and *4*). In 2014, in the ‘Rokosz’ cultivar, the gluten index in the variant with complete chemical protection (treatment D) was significantly higher than in treatment C, where only the herbicide was used (*Table 2*). In 2015, the value of the discussed parameter was different, as it was the highest in the treatments C and D, while the lowest gluten index was shown in the grain from the control treatment (A). In ‘Schwabenspelz’ cultivar, in the second year of research (2015) the value of the gluten index varied significantly (*Table 4*). The highest value of this indicator was obtained in the treatment C, where only the herbicide was used, and significantly lower in the treatment A and D. Mean results from 2014-2015 showed that the use of only herbicide (treatment C) in the crop protection of spelt wheat of the ‘Schwabenspelz’ cultivar significantly affects increase in the gluten index in comparison to other crop protection methods. In both spelt wheat cultivars the statistical analysis did not confirm the effect of seeding rate on gluten weakening and the gluten index (*Tables 3* and *5*). In the case of both cultivars, the values of gluten index were below normal in all treatments of experiment.

In 2014, in ‘Schwabenspelz’ cultivar significant influence of the crop protection on the value of the Zeleny sedimentation value was demonstrated (*Table 4*). In the treatment with complete chemical crop protection (D) a significantly lower Zeleny sedimentation value was obtained compared to other treatments. In 2014, in ‘Schwabenspelz’ cultivar proved the effect of seeding rate on Zeleny sedimentation value. A negative effect on the value of this indicator had increased seeding rate (*Table 5*).

All the grain quality characteristics were statistically proven to significantly vary between the cultivars studied, except for the sedimentation value (*Table 6*). ‘Schwabenspelz’, compared to ‘Rokosz’, showed better parameters with regard to TGW, gluten content, and falling number. On the other hand, ‘Rokosz’ was characterized by a significantly higher test weight and more favourable parameters of gluten weakening and index than ‘Schwabenspelz’.

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Table 6. *Quality characteristic comparison of the spelt wheat grain cultivars 'Rokosz' and 'Schwabenspelz' (mean for 2014-2015) (ns* – not significant at p≤0.05)*

Cultivar	1000 grains weight (g)	Test weight (kg hl^{-1})	Falling number (s)	Gluten content (%)	Gluten weakening (mm)	Gluten index (%)	Zeleny sedimentation value (ml)
Rokosz	32.77	76.23	335.72	24.76	2.03	51.09	16.45
Schwabenspelz	37.78	70.00	209.69	36.88	7.34	36.66	16.40
Mean	35.28	73.11	272.70	30.82	4.69	43.88	16.43
$LSD_{0.05}$ (Tukey test)	1.76	2.40	30.13	1.77	0.62	3.38	ns*

All studied parameters of both cultivars except for the gluten index and Zeleny sedimentation value differ significantly depending on the growing season (*Figure 3*). 'Rokosz' cultivar was characterized by a significantly higher TGW (A), test weight (B), falling number (C) and gluten weakening (E) in 2015 compared to 2014. Only gluten content (D) was significantly higher in the first year of experiment. In 2014 'Schwabenspelz' cultivar was characterized by significantly higher 1000 grains weight (A) and gluten content (D) than in the next year. In 2015 test weight (B), falling number (C) and gluten weakening (E) were characterized by significantly higher values.

When analysing the quality characteristics of spelt flour obtained from 'Rokosz', it was revealed that crop protection method caused significant differences in flour water absorption (*Table 7*). In all experimental treatments, the evaluated cultivars exhibited flour water absorption below 53% (*Tables 7* and *8*). For mean result from research years significantly the highest value of this trait was found in the mechanical weed management treatment (B), while it was significantly lower when complete chemical protection was applied (treatment D). In the second year of the experiment, a significant influence of the method of crop protectiom methods on degree of dough softening after 12 min was observed. Mechanical weed management (B) caused a significant increase in this parameter (121.75 min) compared to the control (A) and herbicide treatment (C). Crop protection methods did not significantly differentiate other analysed parameters of flour and dough. In both years of the experiment, a significant effect of the seeding rate of the Rokosz cultivar on the parameters of flour and dough was observed (*Table 8*). In 2014, this factor significantly differentiated only the dough development time, which was significantly longer when the optimal seeding rate was used (2.18 min). In 2015, the optimum seeding rate had a significant impact on the dough development time and dough stability, resulting in an increase of these parameters to respectively 2.18 min and 2.64 min. The degree of dough softening after 10 and after 12 min significantly increased in the variant of increased seeding rate.

During the experiment, in 'Schwabenspelz' cultivar, a significant influence of crop protection methods on flour water absorption and dough stability was observed (*Table 9*). In the first year of research, the flour obtained from the grain from the control treatment (A - 55.78%) was characterized by the highest water absorption, while in the second year of the experiment the flour obtained from harrowing treatment (B - 50.73%). In 2014, the dough made from grain harvested from the complete chemical protection treatment (D) was characterized by the highest stability, whereas in 2015 the highest value of this feature was found in the herbicide treatment (C) and in the complete chemical protection treatment (D), which did not significantly differ.

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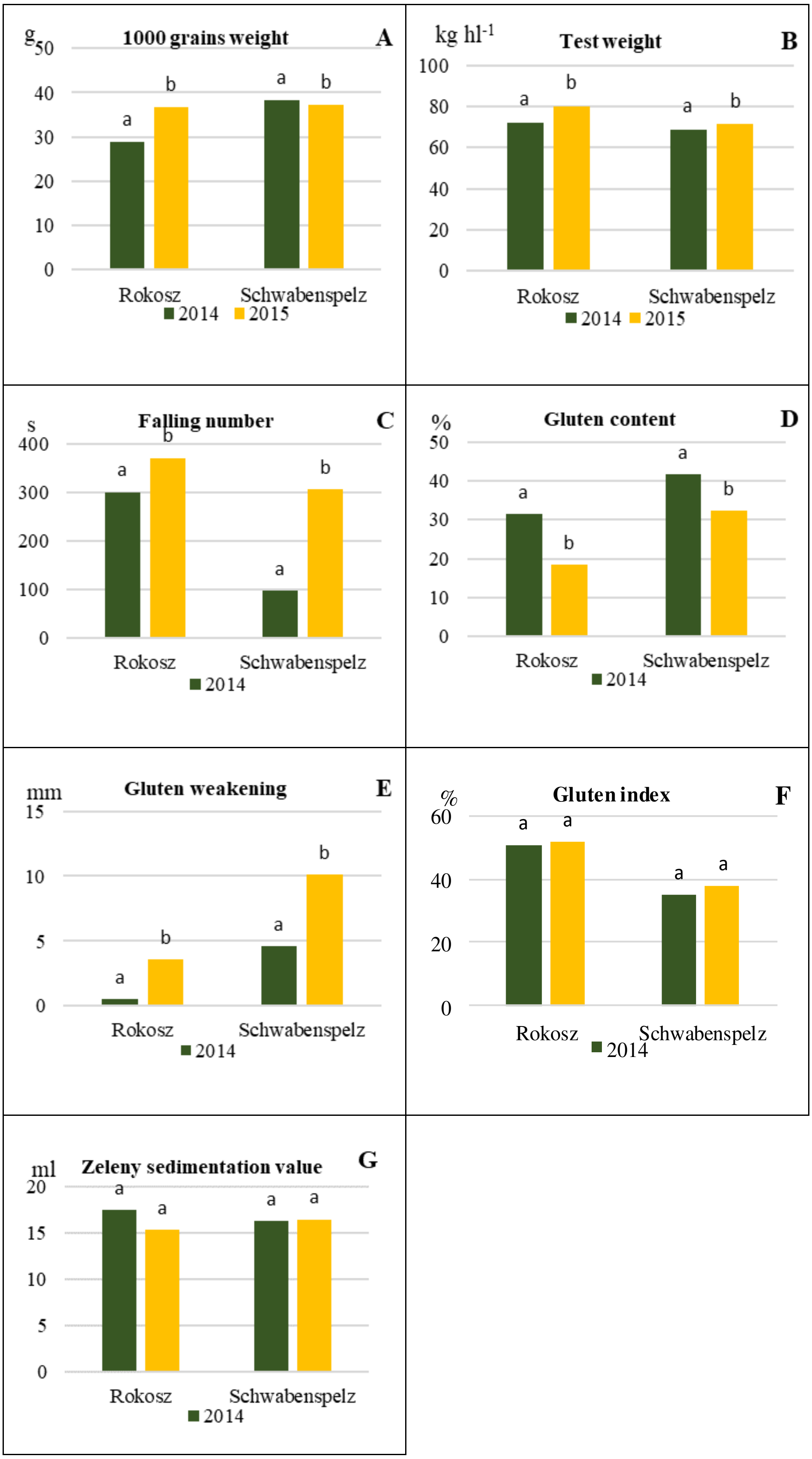

Figure 3. *(A-G) Grain quality of the spelt wheat cultivars 'Rokosz' and 'Schwabenspelz' during the study years (independent on experimental factors) (different letters indicate significant difference at $p \leq 0.05$)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_43774395

Table 7. *The impact of crop protection on farinographic features of flour and dough of the spelt wheat cultivar 'Rokosz' (A[1] – control treatment, B[2] – harrowing in early spring at the beginning of the growing season, C[3] – application of the herbicides, D[4] – application of complete chemical protection, FU[5] – farinographic unit, ns[*] – not significant at p≤0.05)*

Years	Experimental factors	Water absorption of flour (%)	Dough development time (min)	Dough stability (min)	Degree of dough softening after 10 min (FU[5])	Degree of dough softening after 12 min (FU)
2014	A[1]	55.03	1.90	2.80	113.50	139.50
	B[2]	55.55	2.00	3.12	99.25	117.75
	C[3]	56.10	2.18	2.50	108.00	133.25
	D[4]	54.00	1.97	2.75	92.00	123.25
$LSD_{0.05}$ (Tukey test)		0.59	ns[*]	ns	ns	ns
2015	A[1]	48.07	1.45	2.63	99.00	112.00
	B[2]	48.85	1.32	2.70	106.00	121.75
	C[3]	48.25	1.35	2.55	99.25	114.00
	D[4]	48.35	1.30	2.77	103.75	118.50
$LSD_{0.05}$ (Tukey test)		0.64	ns	ns	ns	4.76
Mean for 2014-2015	A[1]	51.55	1.68	2.72	106.25	125.75
	B[2]	52.20	1.66	2.91	102.63	119.75
	C[3]	52.18	1.77	2.53	103.63	123.63
	D[4]	51.18	1.64	2.76	97.88	120.88
$LSD_{0.05}$ (Tukey test)		0.98	ns	ns	ns	ns
Comparison of the pro-ecological and chemical crop protection (mean 2014-2015)						
pro-ecological (A,B)		51.88	1.67	2.82	104.44	122.75
chemical (C,D)		51.68	1.71	2.64	100.76	122.26
Scheffe's LSD (p = 0,05)		ns	ns	ns	ns	ns

Table 8. *The impact of seeding rate on on farinographic features of flour and dough of the spelt wheat cultivar 'Rokosz' (FU[1] – farinographic unit, ns[*] – not significant at p≤0.05)*

Years	Experimental factors	Water absorption of flour (%)	Dough development time (min)	Dough stability (min)	Degree of dough softening after 10 min (FU[1])	Degree of dough softening after 12 min (FU)
2014						
	optimum	55.61	2.18	2.64	105.13	129.00
	increased	54.72	1.85	2.95	101.25	127.87
$LSD_{0.05}$ (Tukey test)		ns[*]	0.32	ns	ns	ns
2015						
	optimum	48.79	1.46	3.21	93.12	109.50
	increased	47.98	1.25	2.11	110.88	123.6
$LSD_{0.05}$ (Tukey test)		ns	0.16	0.63	3.18	4.76
Mean for 2014-2015						
	optimum	52.20	1.82	2.93	99.13	119.25
	increased	51.35	1.55	2.53	106.07	125.74
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	ns	ns

Mean result from research years showed a significant effect of the studied factors only on dough stability. The highest value of this parameter was obtained in the complete crop protection (treatment D), which was significantly higher than in the pro-

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DOI: http://dx.doi.org/10.15666/aeer/1702_43774395

ecological crop protection variants (treatments A and B), and statistically the same as in the treatment C. Seeding rate did not significantly affect the tested parameters of flour and dough obtained from the grain of 'Schwabenspelz' cultivar (*Table 10*). In 2015, only significant differences in water absorption of flour were demonstrated. The flour from grain obtained from the increased seeding rate treatment was characterized by significantly higher water absorption compared to the optimum seeding rate treatment.

Table 9. *The impact of crop protection on farinographic features of flour and dough of the spelt wheat cultivar 'Schwabenspelz' (A[1] – control treatment, B[2] – harrowing in early spring at the beginning of the growing season, C[3] – application of the herbicides, D[4] – application of complete chemical protection, FU[5] – farinographic unit, ns[*] – not significant at $p \leq 0.05$)*

Years	Experimental factors	Water absorption of flour (%)	Dough development time (min)	Dough stability (min)	Degree of dough softening after 10 min (FU[5])	Degree of dough softening after 12 min (FU)
2014	A[1]	55.78	1.92	1.35	153.50	182.50
	B[2]	55.20	1.95	1.40	137.25	162.00
	C[3]	54.90	1.95	1.32	149.50	173.50
	D[4]	54.92	2.10	1.70	123.75	152.25
$LSD_{0.05}$ (Tukey test)		0.39	ns[*]	0.30	ns	ns
2015	A[1]	49.45	1.85	1.83	132.25	149.00
	B[2]	50.73	1.80	1.77	125.25	149.75
	C[3]	49.57	1.85	2.08	110.75	126.25
	D[4]	48.95	1.70	2.22	105.00	120.75
$LSD_{0.05}$ (Tukey test)		0.97	ns	0.23	ns	ns
Mean for 2014-2015	A[1]	52.62	1.89	1.59	142.88	165.75
	B[2]	52.97	1.88	1.59	131.25	155.88
	C[3]	52.24	1.90	1.70	130.13	149.88
	D[4]	51.94	1.90	1.96	114.38	136.50
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	0.28	ns
Comparison of the pro-ecological and chemical crop protection						
pro-ecological (A,B)		52.80	1.89	1.59	137.07	160.82
chemical (C,D)		52.09	1.90	1.83	122.26	143.19
Scheffe's LSD ($p = 0.05$)		ns	ns	ns	ns	ns

Table 10. *The impact of seeding rate on on farinographic features of flour and dough of the spelt wheat cultivar 'Schwabenspelz' (FU[1] – farinographic unit, ns[*] – not significant at $p \leq 0.05$)*

Years	Experimental factors	Water absorption of flour (%)	Dough development time (min)	Dough stability (min)	Degree of dough softening after 10 min (FU[1])	Degree of dough softening after 12 min (FU)
2014						
	optimum	55.00	1.99	1.46	142.25	164.00
	increased	55.40	1.97	1.42	139.75	171.12
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	ns	ns
2015						
	optimum	49.50	1.78	1.95	120.37	139.75
	increased	49.85	1.82	2.00	116.25	133.12
$LSD_{0.05}$ (Tukey test)		0.32	ns	ns	ns	ns
Mean for 2014-2015						
	optimum	52.25	1.89	1.71	131.31	151.88
	increased	52.63	1.90	1.71	128.00	152.12
$LSD_{0.05}$ (Tukey test)		ns	ns	ns	ns	ns

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The performed variance analysis proved significant differences in dough stability and degree of softening between the cultivars evaluated (*Table 11*). 'Rokosz' was characterized by higher dough stability and a lower degree of softening after 10 and 12 min. Despite that no significant differences were revealed, this cultivar showed lower flour water absorption and shorter dough development time compared to 'Schwabenspelz'.

Table 11. *Comparison of the farinographic features of flour and dough of two cultivar of spelt wheat (mean for 2014-2015) (FU[1] – farinographic unit, ns[*] – not significant at p≤0.05)*

Cultivar	Water absorption of flour (%)	Dough development time (min)	Dough stability (min)	Degree of dough softening after 10 min (FU[1])	Degree of dough softening after 12 min (FU)
Rokosz	51.78	1.68	2.73	102.59	122.50
Schwabenspelz	52.44	1.89	1.71	129.66	152.00
Mean	52.11	1.79	2.22	116.13	137.25
$LSD_{0.05}$ (Tukey test)	ns[*]	ns	0.31	10.84	12.85

Water absorption of flour and dough development time of 'Rokosz' cultivar were significantly higher in 2014 compared to 2015 (*Figure 4*) (A-B). The other parameters of this cultivar were statistically the same (C-D). In the first year of experiment 'Schwabenspelz' cultivar was characterized significantly higher water absorption of flour and dough development time (A-B). Dough stability of this cultivar was significantly higher in 2015 compared to 2014 (C). Degree of dough softening after 10 min and after 12 min similarly to the 'Rokosz' cultivar, did not differ statistically.

The relationship between the quality characteristics and grain protein content (*Table 12*) differed for both cultivars studied.

Table 12. *Straight correlation coefficients and simple regressions for the relationship between protein content (x) and qualitative characteristics (y) of grain, flour and dough of two cultivars of winter spelt (n=16) (r_{emp}[1] – Pearson correlation coefficient, R^2 – coefficient of determination, FU[3] – farinographic unit, * – significance level 0.05, ** – 0.01, *** – 0.001)*

Quality parametr	r_{emp}[1]	Significance	R^2	Regression equation
Rokosz				
Gluten content (%)	+0.81	***	0.76	$y = -19.8 + 0.431x$
Gluten weakening (mm)	−0.61	*	0.66	$y = 10.4 - 0.0813x$
Zeleny sedimentation value (ml)	+0.53	*	0.34	$y = 10.8 + 0.0514x$
Dough development time (min)	+0.71	**	0.82	$y = -1.06 + 0.0268x$
Schwabenspelz				
Gluten content (%)	+0.76	***	0.56	$y = 8.18 + 0.223x$
Dough stability (min)	−0.65	**	0.38	$y = 3.15 - 0.0112x$
Degree of dough softening after 12 min (FU[3])	+0.52	*	0.42	$y = 61.8 + 0.707x$

In 'Rokosz', with increasing protein content the amount of gluten, flour sedimentation, and dough development time increased, whereas gluten weakening decreased – which is evidence of its better quality. The values of determination

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coefficients (R^2) indicate that from 34% to 82% of the variability of these features is explained by the presented regression equations. 'Schwabenpeltz' was characterized by a positive relationship between protein content and gluten content – 56% of the variability was explained by the regression equation. As regards the other characteristics for which a statistically proven relationship was found (dough stability and weakening), gluten quality deteriorated with increasing protein content.

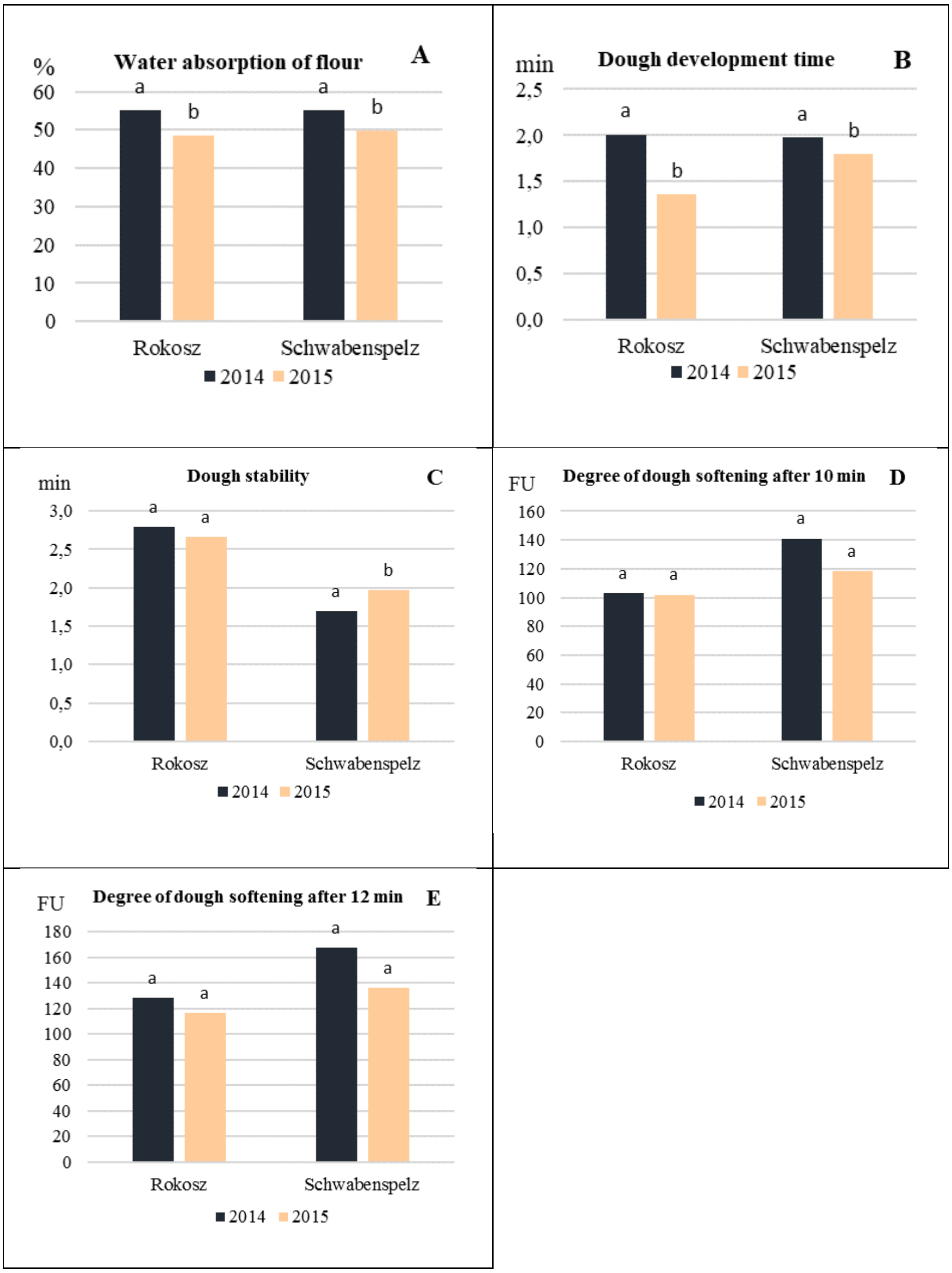

Figure 4. *(A-E) Farinographic features of flour and dough of the spelt wheat cultivars 'Rokosz' and 'Schwabenspelz' during the study years (independent on experimental factors) (different letters indicate significant difference at* $p \leq 0.05$*)*

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Discussion

Grain size is characterized by TGW. In the present study, TGW of cultivar 'Rokosz' was 32.77 g, while for 'Schwabenspelz' it was 37.78 g. The values of this trait were lower than those obtained by Capouchová (2001) (TGW = 52-54 g), Krawczyk et al. (2008b) (TGW = 38.7-52.7 g), Biel et al. (2016b) (TGW = 38.5-46.2 g), Andruszczak (2017) (39.3-50.7 g) and Petrenko et. al. (2018) (TGW = 56.5–56.7 g). As regards the test weight of 'Rokosz' grain, other authors reported similar values of the trait in question for different spelt wheat cultivars (Bastard, Oberlander, Burgdorf, Weisser, Schweizer, and Schwabenkorn) and lines (Makowska et al., 2008; Krawczyk et al., 2008b; Podolska et al., 2015; Biel et al., 2016b; Stankowski et al., 2016; Petrenko et al., 2018). Podolska et al. (2015) and Dorval et al. (2015) did not show seeding rate to have a significant effect on 1000 grain weight.

Falling number is a measure of α-amylase activity (the higher the falling number, the lower the activity) (Rachoń et al., 2011). The processing suitability of flour can be determined on this basis. In this study, the falling number for 'Rokosz' was 335.72 s, whereas for 'Schwabenspelz' 209.69 s, which is evidence of the latter cultivar's higher amylolytic activity. Podolska et al. (2015) obtained slightly lower values for 'Rokosz', whereas the grain falling number of other cultivars and lines studied by Stankowski et al. (2016) ranged from 188 to 308 s, Biel et al. (2016b) – 134-295 s, Rachoń et al. (2016) – 334 s, Krawczyk et al. (2008b) – 259-287 s, and Petrenko et al. (2018) – 287-303 s. Dorval et al. (2015) did not show seeding rate to have a significant effect on this parameter.

Gluten content and quality are an important indicator of baking quality of flour because they give proper elasticity to dough, and a spongy structure and springiness to bread. The average gluten content in 'Rokosz' spelt grain tested was 24.76% and weakening was 2.03 mm, whereas in 'Schwabenspelz' grain these values were 36.88% and 7.34 mm, respectively. Rachoń et al. (2013) found a significantly higher gluten content in spelt grain as affected by complete chemical protection (42.5%) compared to the herbicide treatment (37.2%). In other study, Rachoń et al. (2016) showed an increased content of gluten (44.9%) in wheat grain spelt under the influence of increased chemical protection intensity. In the study by Podolska et al. (2015), an increasing trend in the amount of gluten in 'Rokosz' grain was observed with a simultaneous increase in the seeding rate from 400 to 500 seeds per 1 m^2. It was only when 600 seeds were sown that a significant decrease in this parameter occurred. These authors found much higher gluten weakening (6.3-6.5 mm) than in the present study, but seeding rate did not cause significant differences in this trait. Andruszczak (2018) did not find a significant effect of seeding rate on the content of gluten in the spring spelt grain.

Gluten index shows the quality of gluten and what grain and flour can be used for. Wheat flour used to make bakery products should be characterized by the gluten index at a level of 60-90%. Determinations above and below this range indicate low baking quality (Stępniewska, 2015). This study showed the gluten index of 'Rokosz' (51.09%) to be significantly higher than in 'Schwabenspelz' (36.66%), but these values do not qualify the cultivars in question for material of high baking value. Podolska et al. (2015) found similar values of this indicator and seeding density did not result in significant differences in it.

The Zeleny sedimentation value was almost identical in both cultivars, averaging 16.43 ml, and the experimental factors did not cause variations in it. In the study by

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Stankowski et al. (2016), lower values of the investigated trait were observed (14.5 ml) compared to the present study. On the other hand, Podolska et al. (2004), who proved a significant increase in values of the parameter studied with increasing intensity of chemical crop protection, found a higher sedimentation value. Likewise, Andruszczak (2017) demonstrated that under complete chemical crop protection spelt wheat was characterized by a significantly higher sedimentation index than under extensive cropping conditions. Krawczyk et al. (2008a) proved that the Zeleny sedimentation value of the studied cultivars and lines ranged 30-40 ml. In the study on the cultivar 'Rokosz' conducted by Podolska et al. (2015), this value was at a level of 29-29.5 ml and it was not dependent on seeding rate. Andruszczak (2018) also did not find a significant effect of seeding rate on this parameter. Rachoń et al. (2011) obtained similar values for line STH 3 (28.2 ml), whereas for line 715 they were significantly lower (20.7 ml).

According to Mikos and Podolska (2012), water absorption of spelt flour may reach even 65%. 'Schwabenpeltz' was characterized by slightly higher flour water absorption compared to 'Rokosz', which is evidence of higher dough yield (*Table 6*). In the study by Podolska et al. (2015), in which seeding rate did not affect significantly this parameter, similar results were obtained (53-53.3%). The spelt wheat lines evaluated by Rachoń et al. (2011) exhibited slightly higher water absorption (56.7-59%). Cultivar 'Blauer Samtiger'evaluated in other study by Rachoń et al. (2016) are also characterized by a higher water absorption (65.2%) than in the present study.

Dough stability is one of dough quality indicators and characterizes the resistance of flour to mixing (Rachoń et al., 2011). In 'Rokosz', this parameter was on average 2.73 min and significantly higher than in 'Schwabenspelz' (1.71 min) (*Table 6*). Information on higher values of the trait in question can be found in available scientific literature. The studies by Biel et al. (2016b), Rachoń et al. (2016) and Stanowski et al. (2016) demonstrated dough stability values at a level of 3.5, 5.8 and 4.48 min, respectively. Podolska et al. (2015) did not show seeding rate to have a significant effect on this parameter, but a decreasing trend in these values could be observed with increasing plant density – from 3.2 min at a seeding rate of 400 seeds per 1 m^2 up to 3.0 at a rate of 600 seeds. The higher the sum of the dough development time and dough stability, the longer the dough should be mixed. Therefore, spelt doughs require longer mixing than doughs made of common wheat flour (Krawczyk et al., 2008a).

The variance analysis proved significant differences in the degree of softening between the cultivars evaluated (*Table 6*). 'Schwabenspelz', compared to 'Rokosz', showed longer development time and a higher degree of softening. A higher value of the trait in question was obtained in the research conducted by Makowska et al. (2008) and Stankowski et al. (2016), which indicates that dough is characterized by a high degree of softening. Evaluating 5 spelt wheat cultivars, Makowska et al. (2008) demonstrated that their degree of softening was at a level of 107-142 FU, whereas in the study by Stankowski et al. (2016) it was on average 95.9 FU after 10 min and 122 FU after 12 min. According to Wiwart et al. (2017) and Geisslitz et al. (2018), only some cultivars of spelt wheat have high breadmaking potential.

Similarly as in the present study, spelt wheat cultivars are characterized by high variation in chemical composition and grain technological quality also in the evaluation of other authors (Capouchová, 2001; Lacko-Bartošová et al., 2010; Świeca et al., 2014; Andruszczak, 2017). Weather conditions also have a great impact on grain quality of this species since they additionally increase intervarietal variation (Lacko-Bartošová et

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al., 2010; Wojtkowiak and Stępień, 2015). According to Pruska-Kędzior et al. (2008), in technological terms spelt wheat can be considered among the most valuable cereals. Most authors stress that it is a species that tolerates well extensive cropping without plant protection chemicals and is characterized by favourable grain and flour characteristics under organic farming conditions (Capouchová, 2001; Kwiatkowski et al., 2015). Nevertheless, Andruszczak (2017) demonstrated that application of complete chemical crop protection contributed to an improvement in some grain quality parameters.

Conclusions

The present study showed that crop protection method and seeding rate did not cause most of the quality characteristics of spelt grain and flour to significantly vary. Both the Polish cultivar 'Rokosz' and the German cultivar 'Schwabenspelz' exhibited similar technological quality of grain and flour under organic farming and chemical crop protection conditions. Therefore, both these cultivars can be recommended for cultivation in organic farms. In 'Rokosz', gluten content, Zeleny sedimentation value, and dough development time increased with increasing protein content, whereas gluten weakening decreased, which is evidence of better quality and baking value of this cultivar relative to 'Schwabenspelz'.

Currently, there is a continuous necessity to look for effective agrotechnical solutions that can be introduced to the organic farming. Due to the high nutritional value of spelt wheat and its usefulness for tillage under organic farming conditions, it is necessary to select cultivars of this species that best tolerate pro-ecological cultivation and which are characterized by the high technological values. It seems be necessary to undertake research into non-chemical methods of limiting the presence of pests, such as: sowing rate, mechanical crop protection, use of biostimulators, etc. in this crop.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43774395

EFFECTS OF *TRICHODERMA HARZIANUM* AND BORON ON SPRING BROCCOLI

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(Received 9th Jan 2019; accepted 26th Feb 2019)

Abstract. This paper deals with the effects of *Trichoderma harzianum* application and boron (Nitrabor) topdressing on the growth, yield, and quality of spring broccoli. A field experiment was carried out in central-eastern Poland, in 2014-2015.The experiment was established as a split-block design with three replicates. There were the following combinations of *Trichoderma harzianum* treatment: added to the nursery substrate; added to the nursery substrate and as topdressing in a form of spray after seedlings were planted out; as pre-planting treatment just before seedlings were planted out, followed by topdressing in a form of spray; control without *Trichoderma harzianum*. Two types of late topdressing for broccoli were used: nitrogen topdressing or Nitrabor to the soil. The treatment significantly increased the marketable yield of broccoli curds. This increase relative to control ranged from 17 to 49%, depending on the *Trichoderma harzianum* treatment. The best results were obtained when boron topdressing was combined with *Trichoderma harzianum* added to the nursery substrate and then the fungus was applied in a form of spray after seedlings were planted out. Broccoli inoculation with *Trichoderma harzianum* also increased L-Ascorbic acid content in the plants. Topdressing with boron significantly increased the marketable yield of curds, their weight, the diameter of their circumference, the diameter of the stem, the leaf greenness index value (SPAD), as well as the content of dry matter, total sugars, and L-Ascorbic acid.
Keywords: *bio-stimulators, Brassica oleracea L. var. italica, mineral fertilization, nutritional value, yield*

Introduction

Broccoli (*Brassica oleracea* var. *italica*) is an important vegetable grown worldwide. The plant originates from the Mediterranean region and belongs to the *Brassicaceae* family. It is tasty and more nutritious than any other vegetables of the same kind, considered a valuable source of vitamins, antioxidants, glucosinolates, and other compounds of proven anticancer activity (Parente et al., 2013). Broccoli is an important component of human healthy diet, and demand for this vegetable on Polish and international markets is constantly growing. Its producers use new growing methods enhancing its growth and improving its quality. Hence, there is a need for research to improve the yield of broccoli and to increase its production; some biological agents should be implemented either on their own, or in combination with mineral fertilisers to increase its cultivation and production.

Among various biological methods currently used, microbial inoculants applied at the seedling stage could prove promising. Several symbionts like arbuscular mycorrhizal fungi and *Trichoderma* ssp. can be implemented for broccoli cultivation.

The efficacy of *Trichoderma* as a biostimulator agent has been proven when fungal species have been applied to soil, seeds, or plants. Their benefits are related to increased

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

nutrient solubility, improved nutrient uptake by roots, and to the mode of action of plant growth promoting bacteria (Molla et al., 2012; Martínez et al., 2015). *Trichoderma harzianum* releases volatile organic antibiotic compounds against pathogenic fungi, and it also stimulates plant growth (Vinale et al., 2008). *Trichoderma* species improve the growth and development of plants grown in vitro, in greenhouses, and in the field. Some studies have demonstrated beneficial effects of *Trichoderma* spp. on vegetable plants, like cabbage, cucumber, tomato, and lettuce, stimulating seed germination, or vegetative and generative growth (Bal and Altintas, 2006; Studholme et al., 2013).

Boron represents one of the essential micronutrients necessary for proper plant growth, the latter becoming limited both when it is deficient or at elevated level (Davies et al., 2011). It increases the growth and yield of plants because it stimulates division and elongation of the cell and development of its walls. Boron plays an important role in the metabolism of carbohydrates and proteins (Goldbach and Wimmer, 2007; Miwa et al., 2007). It is also crucial for the development of nitrogen-fixing cyanobacteria (Bolanos et al., 1996; Bonilla et al., 1997). Boron deficiency causes many anatomical, physiological, and biological disorders (Brown et al., 2002; Xu et al., 2007). Vegetable plants with the highest demand for boron include, among others, broccoli.

The natural content of boron in the soil depends mainly on the type of material from which it has developed. Clay soils are generally rich in boron, in contrast to sandy ones, in which this chemical element may be present in small amounts. Boron concentration in soils varies from 2 to 200 mg B kg^{-1}, but generally less than 5-10% of its content is in a form available to plants (Diana, 2006). In Poland sandy soils dominate, and with the content of this chemical element being too small it is necessary to apply boron with mineral fertilizers to the soil or as a foliar spray (Szulc and Rutkowska, 2013).

The purpose of this research was to determine the effect of *Trichoderma harzianum*, applied as a biostimulator to nursery substrate and after seedlings were planted out, and of boron topdressing on the size and quality of the broccoli yield.

Material and methods

Experimental site

The experiment was carried out between 2014 and 2015 at the Experimental Station of the Siedlce University of Natural Sciences and Humanities, located in central-eastern Poland (52°03'N, 22°33'E). The soil was classified as luvisol (IUSS, 2015) with the average organic carbon content of 0.97%, the humus layer reaching the depth of 30-40 cm, and pH_{KCl} of 6.0. The content of available forms of nutrients ($mg \cdot kg^{-1}$) was as follows: 4.7 NO_3-N, 2.5 NH_4-N, 12 P, 27 K, 10.8 Mg, 87.5 Ca, 0.11 B.

Experimental design

The experiment was established as a split-block design with three replicates, and it included two factors: factor I – mineral fertiliser, factor II – different methods of using *Trichoderma harzianum* (*Table 1*). The studies used Trianum-G and Trianum-P produced by the Koppert B.V. company (Netherlands). Trianum-G is microbiological granular containing spores of *Trichoderma harzianum (T. harzianum)* Strain T-22 Rifai (1.5×10^8 spores per 1 g). It is insoluble in water and used as pre-planting treatment, mixing granules with the soil. Trianum-P is a microbiological product in a form of

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DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

granules to prepare aqueous suspension. It contains 1.5 × 10^9 spores of *T. harzianum* Strain T-22 Rifai per 1 g. The combinations of Trianum-G and Trianum-P application are presented in *Table 1*. In the experiment both Trianum-G and Trianum-P were used in doses recommended by the manufacturer. Nitrabor (Yara Poland) contains calcium nitrate with 15.4% N (including 14.1% NO_3-N and 1.3% NH_4-N), 25.6% CaO, and 0.3% B. It is recommended for use on vegetable plants, especially on species that require increased doses of boron.

***Table 1.** Factors of the experiment*

	Two mineral fertiliser combinations	
Factor I	NPK	Basic pre-planting treatment NPK (206 kg N, 146 kg P_2O_5, 273 kg K_2O per 1 ha – in the form of ammonium nitrate, granular superphosphate and 60% potassium chloride, respectively) + nitrogen topdressing to the soil (62 kg·ha^{-1} – in the form of ammonium nitrate)
	NPK+B	Basic pre-planting treatment NPK (206 kg N, 146 kg P_2O_5, 273 kg K_2O per 1 ha – in the form of ammonium nitrate, granular superphosphate and 60% potassium chloride, respectively) + Nitrabor topdressing to the soil (400 kg·ha^{-1})
	Five combinations with *Trichoderma harzianum*	
Factor II	Th_0	control – without *Trichoderma harzianum*
	Th_1	*T. harzianum* added to the substrate before planting seeds (Trianum-G 750 g per 1 m^3 of the substrate)
	Th_2	*T. harzianum* added to the substrate + spraying plants in the field with *Trichoderma harzianum* (Trianum-G 750 g per 1 m^3 of the substrate + Trianum-P 40 g·100 m^{-2} dissolved in 15 dm^3 H_2O, plants sprayed 21 days after transplanting seedlings to the field)
	Th_3	*T. harzianum* added to the soil before transplanting seedlings (Trianum-G 1 g per 1 plant)
	Th_4	*T. harzianum* added to the soil before transplanting seedlings + spraying plants in the field with *Trichoderma harzianum* (Trianum-G 1 g per 1 plant + Trianum-P 40 g·100 m^{-2} dissolved in 15 dm^3 H_2O, plants sprayed 21 days after transplanting seedlings to the field)

Seedling preparation

Broccoli seedlings 'Wiarus' (PNOS, Poland) were grown in a non-heated greenhouse. Seeds were sown in the successive growing seasons on 17 and 20 March to multi-trays with the size of 400 × 600 mm and 54 cells with the diameter of 54 mm. 60% of the seedlings were produced on substrate without *Trichoderma harzianum*, and 40% on substrate with *Trichoderma harzianum* (Trianum-G). The Aura substrate produced by Hollas - Greenyard Horticulture Poland Ltd. was used for the production of seedlings. It was made of de-acidified 'highmoor' peat with 5.5-6.5 pH and salinity not greater than 2 g NaCl per litre. The substrate was enriched with mineral fertiliser containing NPK (14-16-18%) and Mg (5%). On average nutrient content in the substrate was as follows (in mg·dm^{-3}): 238 NO_3-N, 18 NH_4-N, 70 P, 207 K, 1016 Ca, and 158 Mg.

Field work

The crop preceding broccoli was triticale. In the autumn the field was ploughed, and in the spring, two weeks before seedlings were planted, disc harrowing was used. After that, mineral fertilizers were applied up to the optimal level for broccoli: 206 kg N, 146 kg P_2O_5, 273 kg K_2O per 1 ha. Seedlings were planted on 18 and 22 April, at a spacing of 50 × 50 cm. The area of a plot (unit) was 9 m^2 (3 m × 3 m), with 36 plants in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

each of them. The area of the whole field together with paths between experimental combinations and replicates was 650 m^2. Then *Trichoderma harzianum* was added to adequate plots, in accordance with the combinations set out in *Table 1*. For three weeks the plants were covered with Pegas Agro 17UV polypropylene fibre (Rybnik, Poland). After removing the fibre the plants were topdressed with mineral fertilizers. To plants with the NPK treatment 62 $kg \cdot ha^{-1}$ of nitrogen was applied (the same amount of nitrogen as the NPK applied, together with B, in a form of Nitrabor. On the NPK+B combination a Nitrabor dose of 400 $kg \cdot ha^{-1}$ was used, which made it 1.2 $kg \cdot ha^{-1}$ of Boron. The Nitabor dose to be applied in the experiment had been determined by taking into account boron content in the soil and in the fertilizer, as well as broccoli requirements for this chemical element. 21 days after planting seedlings *Trichoderma harzianum* was applied as a spray to Th_2 and Th_4 combinations (*Table 1*).

Sample collection and laboratory analysis

Broccoli was harvested by hand on 12 June in 2014, and on 11 June in 2015. The area of each plot to be harvested was 3 m^2. Marketable yield, weight of marketable curd, length of curd circumference, and stalk diameter were determined during the harvest. From each plot a broccoli sample was also taken (four randomly selected curds) for chemical analysis to determine: dry matter content by drying to the constant weight at 105°C; L-ascorbic acid content with the Tillmans method (PN-A-04019, 1998); monosaccharide content with the Luff-Schoorl method (EU, 2009); protein content with the Kjeldahl method (using a factor of 6.25). Seven days before the plants were harvested the leaf greenness index (SPAD) of broccoli curds was measured with SPAD-502 Plus Chlorophyll Meter (Konica Minolta Inc., Tokyo, Japan).

Statistical analysis

The results were statistically processed with ANOVA for the split-block design. The significance of differences was determined with Tukey's test at the significance level of $P \leq 0.05$. All the calculations were performed with Statistica software (version 10, Statsoft, USA).

Weather conditions

The basic weather conditions of the experimental area in individual experimental years are presented in *Table 2*. To assess pluvio-thermal conditions during the growing season Selyaninov's hydrothermal coefficient was calculated (Eq. 1):

$$K = \frac{M_O \times 10}{D_t \times days} \qquad \text{(Eq.1)}$$

where: K – hydrothermal coefficient for individual months; M_o – total monthly precipitation; D_t – mean daily temperatures in a particular month (Szymańska et al., 2017).

During the broccoli growing season from April to June 2014 the air temperatures and rainfall were higher than in 2015. However, the second year, 2015, turned out to be more favourable for broccoli growth. High air temperatures during the final ten days of April and in May 2014 negatively affected survival and growth of seedlings despite the large amount of rainfall. This translated into the smaller yield of curds.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

Table 2. *Weather condition in the experiment area in 2014 and 2015 (Zawady Meteorological Station, Poland)*

Month	2014			2015		
	T (°C)	P (mm)	*K*	T (°C)	P (mm)	*K*
April	9.8	44.7	1.53 o	8.2	30.0	1.22 qd
May	13.5	92.7	2.29 h	12.3	100.4	2.71 vh
June	15.3	55.4	1.20 qd	16.5	43.3	0.87 d
Mean	12.9	-	-	12.3	-	-
Total	-	192.8	-	-	173.7	-

T – temperature; P – precipitation; K – Sielyaninov's hydrothermal index: d – dry, qd – quite dry, o – optimum, h – humid, vh – very humid

Results and discussion

For two years of the research the marketable yield of broccoli curds was on average 13.1 $t{\cdot}ha^{-1}$, and the mass of the marketable curd was 352.5 g (*Table 3*). In 2015, when weather conditions for broccoli growth were more favourable, the yield was 20.2% higher than in 2014.

Table 3. *The effect of different treatment combinations on the yield of broccoli and the weight of curds*

Treatment	Marketable yield ($t{\cdot}ha^{-1}$)					Weight of marketable curd (g)				
	Year		Mineral fertilization		Mean	Year		Mineral fertilization		Mean
	2014	2015	NPK	NPK+B		2014	2015	NPK	NPK+B	
Th_0	10.46^{a}	9.89^{a}	9.20^{aA}	11.15^{aB}	10.18^{a}	320.7	336.5^{a}	314.8	342.3	328.6
Th_1	10.41^{a}	13.51^{b}	11.27^{bA}	12.64^{bB}	11.96^{b}	278.0	390.8^{ab}	350.7	318.2	334.4
Th_2	13.23^{b}	17.12^{d}	14.12^{cA}	16.23^{dB}	15.17^{d}	318.2	420.2^{b}	353.2	385.2	369.2
Th_3	11.65^{ab}	15.14^{cd}	12.01^{bA}	14.78^{cB}	13.39^{bc}	292.0	423.3^{b}	344.8	370.5	357.7
Th_4	13.73^{b}	15.87^{d}	14.01^{cA}	15.60^{dB}	14.81^{cd}	304.2	441.3^{b}	359.8	385.7	372.8
Mean	11.90^{A}	14.30^{B}	12.12^{A}	14.08^{B}	13.10	302.6^{A}	402.4^{B}	344.7	360.4	352.5

Source of variation	F-value	*P*	$HSD_{0.05}$	F-value	*P*	$HSD_{0.05}$
Th	35.01	<0.001	1.469	2.38	>0.05	NS
MF	299.59	<0.001	0.278	2.93	>0.05	NS
Y	59.11	<0.001	0.650	73.57	<0.001	24.21
Th × MF	3.03	0.03	0.750	1.23	>0.05	NS
MF × Th	3.03	0.03	0.650	1.23	>0.05	NS
Y × Th	6.51	0.003	2.088	3.55	0.02	77.56
Y × MF	5.91	>0.05	NS	1.35	>0.05	NS

Means followed by different lowercase letters in columns and different uppercase letters in rows differ significantly at $p \leq 0.05$; NS – not significant; Th – *Trichoderma harzianum*; MF – mineral fertilization; Y – years; Th_0, Th_1, …, NPK, NPK+B – combinations with *Trichoderma harzianum* and mineral fertilization, see *Table 1*

Similarly, the weight of the marketable curd was significantly higher. Inoculation of broccoli with *Trichoderma harzianum* contributed to the growth of the marketable yield of curds relative to uninoculated control. The effect was especially visible in 2015, when a significant increase in the yield compared to control was observed on all combinations of *T. harzianum*, regardless of the manner of application. The average increase in the curd yield for both years ranged from 17.5 to 49%, with the highest for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

the Th_2 combination (*T. harzianum* added to the nursery substrate and as topdressing in a form of spray after seedlings were planted out). The beneficial effect of broccoli inoculation with *T. harzianum* on the curd marketable yield was recorded only in 2015. Tanwar et al. (2013) also noted a significant increase in the weight and size of broccoli curds as a response to inoculation with *Trichoderma viride*. Abd Alla and El-Shoraky (2017) found a significant increase in the yields of white cabbage and cauliflower after *Trichoderma harzianum* inoculation. Altintas and Bal (2008) noted an upward trend in the yield of onion after applying *T. harzianum*, but the increase, when compared with control, was not statistically significant.

Regardless of the manner of *Trichoderma harzianum* application there was a beneficial effects of boron treatment on broccoli yields. The marketable curd yield on plots with Nitrabor (NPK+B) increased on average by 2.68 $t \cdot ha^{-1}$ in relation to the combinations without boron (NPK). The largest marketable yield was reported after combined application of *Trichoderma harzianum* (Th_2) and NPK+B topdressing. An increase in the yield of broccoli curd as a response to boron application was also noted by Moniruzzaman et al. (2007), Hussain et al. (2012), Singh et al. (2015), Islam et al. (2015) and Farooq et al. (2018). Additionally, Moniruzzaman et al. (2007), Hussain et al. (2012) and Singh et al. (2015) observed a statistically significant increase in the mass of curds. Optimum levels of NPK and boron applied to broccoli positively affect photosynthetic efficiency and improve other processes like enzyme activation, protein and carbohydrate accumulation, and translocation of sugar and starch; because of all that the yield also increases (Shahah et al., 2010; Singh et al., 2015).

There was no effect of *Trichoderma harzianum* on the curd circumference length and stem diameter. It was noted, however, that the SPAD value evolved under the influence of the inoculation, depending on how it was applied (*Table 4*).

Table 4. *The effect of different treatment combinations on broccoli curd biological parameters and SPAD*

Treatment	Curd circumference length (mm)	Stalk diameter (mm)	SPAD
Trichoderma harzianum			
Th_0	226	31.6	80.53^a
Th_1	225	28.8	81.83^{ab}
Th_2	221	32.3	86.94^c
Th_3	232	29.3	80.54^a
Th_4	224	32.1	84.46^{bc}
Mineral fertilization			
NPK	220^a	29.8^a	82.05^a
NPK+B	232^b	31.8^b	83.67^b
Years			
2014	239^b	31.9^b	82.99
2015	212^a	29.8^a	82.05
Mean	226	30.8	82.86
Source of variation	$HSD_{0.05}$		
T. harzianum	NS	NS	2.565
Mineral fertilization	5.2	1.71	0.710
Years	7.8	1.85	NS
Th × MF	NS	NS	NS
MF × Th	NS	NS	NS
Y × Th	NS	NS	NS
Y × MF	NS	NS	NS

Means followed by different letters in columns differ significantly at $p \leq 0.05$; NS – not significant; Th – *Trichoderma harzianum*; MF – mineral fertilization; Y – years; Th_0, Th_1, …, NPK, NPK+B – combinations with *Trichoderma harzianum* and mineral fertilization, see *Table 1*

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DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

Compared to control a significant SPAD increase was reported on Th_2 and Th_4 combinations, where *T. harzianum* was applied additionally as a spray. The results indicated that this kind of treatment increased the amount of nitrogen taken up by plants. A significant increase in chlorophyll content in broccoli leaves after vaccination of the substrate with *Trichoderma viride* was noted, among others, by Tanwar et al. (2013). Harman et al. (2012) and Hermosa et al. (2012) report that *Trichoderma* strains colonise plant roots, establishing chemical communication and systemically altering the expression of numerous plant genes that alter plant physiology and may result in the improvement of abiotic stress resistance, nitrogen fertiliser uptake, and photosynthetic efficiency.

Both NPK+B topdressing significantly increased curd circumference length, the diameter of the stem, and the SPAD value compared with the basic fertilization (*Table 4*). Similar results were obtained by Husain et al. (2012) and Singh et al. (2015). Regardless of the dose size, they recorded a significant increase in the diameter of broccoli curds and stems after boron treatment. Similarly, Chatterjee and Bandyopadhyay (2017) recorded a significant SPAD increase in cowpea after boron application. The increase was directly proportional to the applied doses. In the present experiment, there was no significant effect of the interaction of *T. harzianum* and boron on the circumference length, the diameter of the stem, and the SPAD value.

The average content of dry matter in broccoli curds was 11.61%, with protein constituting 4.04% in fresh matter (FM), total sugars 2.66 $g{\cdot}100g^{-1}$ FM, and monosaccharides 1.68 $g{\cdot}100g^{-1}$ FM (*Table 5*).

Table 5. *The effect of different treatment combinations on dry matter, protein, and sugar content in broccoli*

Treatment	Dry matter (%)	Protein (% FM)	Total sugars ($g{\cdot}100g^{-1}$ FM)	Monosaccharides ($g{\cdot}100g^{-1}$ FM)
Trichoderma harzianum				
Th_0	11.51	4.05	2.68	1.64
Th_1	11.67	4.03	2.61	1.65
Th_2	11.52	4.04	2.66	1.62
Th_3	11.62	4.04	2.71	1.63
Th_4	11.74	4.06	2.66	1.86
Mineral fertilization				
NPK	11.17^a	4.04	2.71^b	1.68
NPK+B	12.05^b	4.06	2.61^a	1.68
Years				
2014	11.40^a	4.02	2.64	1.76^b
2015	11.82^b	4.07	2.69	1.60^a
Mean	11.61	4.04	2.66	1.68
Source of variation	$HSD_{0.05}$			
T. harzianum	NS	NS	NS	NS
Mineral fertilization	0.435	NS	0.039	NS
Years	0.231	NS	NS	0.136
Th × MF	NS	NS	NS	NS
MF × Th	NS	NS	NS	NS
Y × Th	NS	NS	NS	NS
Y × MF	NS	NS	NS	NS

Means followed by different letters in columns differ significantly at $p \leq 0.05$; NS – not significant; Th – *Trichoderma harzianum*; MF – mineral fertilization; Y – years; Th_0, Th_1, …, NPK, NPK+B – combinations with *Trichoderma harzianum* and mineral fertilization, see *Table 1*

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DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

There was no significant effect of *Trichoderma harzianum* inoculation on the above content. However, the amount of dry matter and total sugars in plants was dependent on fertilizer treatment. Broccoli plants treated with boron contained significantly more dry matter and less total sugars than those grown without the Nitrabor fertilizer. A significant increase in dry matter content in broccoli curds as a response to boron was also noted by Islam et al. (2015). Ningawale et al. (2016) recorded an increase in dry matter content in cauliflower heads treated with Borax, but the increase was statistically insignificant. In the studies of Patel et al. (2017) broccoli treated with boron and without it had similar content of sugars, but Meena et al. (2015) found a significant increase in the content of total sugars and monosachcarides in the fruits of tomato treated with this chemical element. In the present experiment there was no significant effect of the interaction between *T. harzianum* and boron on the content of dry matter, protein, and sugars in broccoli. It was found, however, that weather conditions throughout the research differentiated the content of dry matter and monosaccharides in broccoli curds. In 2014 dry matter content was lower and monosaccharide content was greater than in 2015.

Ascorbic acid (AA) content in broccoli curds was on average 67.20 mg per 100 g FM (*Table 6*). With 2.95 mg·100g^{-1} FM in 2014 it was higher than in 2015, and this difference was statistically significant. There was a significant effect of broccoli inoculation with *Trichoderma harzianum* on AA content. On average, for both growing seasons, broccoli grown on plots with *T. harzianum* contained more AA than on the control plot. In the case of Th_2 and Th_3 combinations these differences were statistically significant. The beneficial effect of *T. harzianum* on AA content in broccoli was especially visible in 2015. Similarly, in the studies of Hale et al. (2012) it was found that *T. harzianum,* added to compost and applied in a form of solution in the cultivation of tomato, significantly influenced the growth of the content of AA, protein, and total sugars in fruits.

Table 6. *The effect of different treatment combinations on ascorbic acid content in broccoli (mg 100·g^{-1} FM)*

Treatment	Year		Mineral fertilization		Mean
	2014	2015	NPK	NPK+B	
Th_0	69.01	62.84^{a}	65.87	65.99^{a}	65.93^{a}
Th_1	69.78	64.13^{ab}	67.63	66.27^{ab}	66.95^{ab}
Th_2	70.06	66.43^{bc}	66.98^{A}	69.52^{cB}	68.25^{b}
Th_3	68.27	67.29^{c}	67.39	68.17^{bc}	67.78^{b}
Th_4	68.76	65.48^{bc}	65.86^{A}	68.38^{cB}	67.12^{ab}
Mean	68.18^{B}	65.23^{A}	66.75^{A}	67.67^{B}	67.20

Source of variation	*F-value*	*P*	$HSD_{0.05}$
T. harzianum	5.04	0.005	1.659
Mineral fertilization	6.64	0.04	0.888
Years	125.21	<0.001	0.733
Th × MF	3.79	0.02	1.778
MF × Th	3.79	0.02	2.051
Y × Th	6.94	0.003	2.347
Y × MF	5.84	>0.05	NS

Means followed by different lowercase letters in columns and different uppercase letters in rows differ significantly at $p \leq 0.05$; NS – not significant; Th – *Trichoderma harzianum*; MF – mineral fertilization; Y – years; Th_0, Th_1, …, NPK, NPK+B – combinations with *Trichoderma harzianum* and mineral fertilization, see *Table 1*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_43974407

A significant increase in AA content (on average by 1.35%) in broccoli was also noted after application of Nitrabor, a fertilizer containing boron. Beneficial effects of boron on the content of ascorbic acid in broccoli was also observed by Islam et al. (2015), and in the fruit of tomato by Lester (2006). However, in the studies of Patel et al. (2017) and Meena et al. (2015) there were no significant changes in the content of vitamin C in broccoli heads and tomato fruits after boron treatment, compared to plants treated with basic fertilizer.

Combined application of *T. harzianum* and boron had a significant impact on the amount of ascorbic acid. The highest amount of AA was recorded in plants topdressed with boron and with *T. harzianum* added to the substrate during seedling production and then sprayed on plants in the field (Th_2), and when *T. harzianum* were applied in the field first as pre-planting treatment and then as a spray (Th_4).

Conclusions

1. Trichoderma harzianum, irrespective of the application combination, had a significant impact on the growth of the marketable broccoli curd yield.
2. The highest yield increase was recorded after applying Trichoderma harzianum to seedling substrate, supplemented with spraying plants with Trichoderma harzianum during their growth. Favourable yield parameters resulted from greener leaves, with more efficient photosynthetic production.
3. Boron nutrition favourably affected yield parameters studied in the experiment. As a chemical element affecting synthesis and transport of carbohydrates boron contributed to a substantial increase in the curd marketable yield (on average by 16.2%), curd weight (on average by 4.6%) and an increase in the content of dry matter, total sugars, and L-Ascorbic acid in broccoli tissues in comparison to that after mineral fertilization without the addition of boron.
4. In the production of broccoli, the use of Trichoderma harzianum can be recommended, especially as a combined application, first to the seedling substrate, and then in a form of spray in the field, supplemented with boron.

Acknowledgements. The research carried out under the theme No 226/06/S was financed by the science grant of the Ministry of Science and Higher Education.

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GROWTH CURVE PREDICTION OF HOLSTEIN-FRESIAN BULLS USING DIFFERENT NON-LINEAR MODEL FUNCTIONS

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(Received 31st Jan 2019; accepted 28th Feb 2019)

Abstract. This study aimed to determine the best model to explain the variations in the live weight of Holstein bulls using non-linear function models such as Brody's, Gompertz's and Richards Logistic. For this purpose, live weight records of 51 Holstein Fresian male calves reared in Dicle University Cattle Research Farm were used. In order to estimate the best model, the coefficient of determination (R^2) and the residual mean squares (RMS) statistics were utilized. The coefficient of determination (R^2) for Gompertz's, Richards Logistic and von Bertalanffy models were found to be 0.999, 0.999, 0.998 and 0.999 respectively. Residual mean squared were found to be 21.41, 16.82, 50.94 and 22.21, respectively. As a result, the Richards model used in the study was found to be the best fitted model based on RMS and R^2 criteria. It is the more suitable model due to its accurate ability to predict mature weight, which is an important selection goal.
Keywords: *bodyweight, growth, Holstein bull, inflection point, Richards*

Introduction

The increase in size, number or mass with time is the primary definition of growth but does not include the phenomenology and etiology of growth, such as the energy transactions in the growing animal including metabolism, nutrition and genetics (Parks, 1982).

Growth is usually defined as an increase in tissue mass caused by hyperplasia early in life and hypertrophy later in life (Owens et al., 1995). The accurate knowledge of the growth curve is important due to its relation with the efficiency of production (Fitzhugh, 1976).

Prediction of growth in animals can be found by age-weight graphical plotting using mathematical models (Bathaei and Leory 1996).

Growth curve study in cattle has mainly used non-linear models that relate the animal weights to ages (Garnero et al., 2006; Forni et al., 2009; Souza et al., 2010).

In beef cattle, different equations for growth model have been used and compared in Hereford (Brown et al., 1976), Angus (Beltran et al., 1992), Retinta Cattle (López de Torre et al., 1992), Belgian Blue (Behr et al., 2001), Salers (Garcia et al., 2008) and Nelore (Forni et al., 2009).

The many equations that have been used to predict growth for cattle include Gompertz, Robertson's logistic, Brody, Bertalanfy, Feller, Weiss and Kavanau, Fitzhugh, Richards, Laird, and Parks equations. Detailed descriptions for the models were summarized by Parks in 1982. The most used models to describe growth patterns in beef cattle are: Brody, Bertalanfy, logistic, Gompertz and Richards (Brown et al., 1976, Fitzhugh Jr 1976). Difficulties in obtaining convergences within Richards and Janoschek functions have been reported by Sarmento et al. (2006).

The objective of the study was to assess and compare the non-linear models by Gompertz, Richards and Logistic for goodness of fit of the weight-age data for Holstein Frisan bulls.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44094416

Materials and methods

The experiment was conducted in Dicle University, Cattle Research Center in Diyarbakir Province, Turkey (37°57'41 N and 40°13'54 E, 650 m asl) (*Figure 1*).

Figure 1. *Study location map*

The material of this study consisted of monthly live weight records of 51 Holstein Fresian male calves from 4th. to16th. month reared in Dicle University Cattle Research Farm. The research did not require the Bioethical Committee's Certification.

The study material consisted of monthly live weight and body measurement records of 51 male Holstein-Friesian bulls collected between the years 2016 and 2018. The study was carried out in two different locations. First stage was between the days born-120 d. in private farm and second was in Cattle Research Farm of Dicle University. Calves born in private dairy farm received milk as much as 10% of their body weight with starter feed after 2 weeks. Calves were weaned at the 9th of weeks. Different amounts of concentrate and roughage were provided ad libitum to the bulls considering the average live weights obtained from the monthly measurements.

All data of means and standard errors of weights during different age stages were analyzed using SPSS (Statistical Package for the Social Sciences, version 10). Non-linear models of Gompertz (1825), Richards (1959), von Bertalanffy (1957) and the Logistic model introduced by Verhulst (1838) were fitted to the weight-age data using non-linear regression procedure option.

Assessment of goodness of fit among three models was applied having the highest determination coefficient (R^2) percentage. Models parameters (A, b, k, and M) were iterated at a set of a maximum of 150 times the initial of their values using the Levenberg-Marquardt method option.

The used model functions (Gompertz, Richards, Logistic and von Bertalanffy) to fit Holstein male growth curve present in *Table 1*.

Table 1. *Non-linear functions used for modeling the growth curves [Yt= weight (kg) at time (month). A= asymptotic weight. b= scale parameter. k= maturing index. e= logarithm base. m= inflection point. ε = random error]*

Model	Function
Gompertz	$Y_t = Ae^{-b\exp(-kt)} + \varepsilon$
Richards	$Y_t = A(1 - be^{-kt})^m + \varepsilon$
Logistic	$Y_t = A(1 + e^{(-kt)})^{-1} + \varepsilon$
Von Bertalanffy	$Y_t = A*(1-B*\exp(-k*t))^3$

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DOI: http://dx.doi.org/10.15666/aeer/1702_44094416

In the mathematical expressions, yt represents the weight of the animal at a given age (t); parameter A is the asymptotic weight, if $t \rightarrow \infty$; when the adult weight of the animal is not reached, this reflects in an estimate of the weight of the last weighings; b is a constant without biological interpretation, but it is important to model the sigmoidal format of the growth curve from birth (t = 0) until the adult age of the animal ($t \rightarrow \infty$); K is the maturity index, which expresses the ratio of the maximum growth rate in relation to the adult size, where lower k values indicate delayed maturities and higher k values indicate accelerated maturity; M is the parameter that shapes the curve; e is the natural base logarithm; the L parameter has no biological meaning, but together with K constitutes b, which has the function of modeling the sigmoidal curve; and ε represents the random error associated with each weighing (Marinho et al., 2013)

The statistical criteria and biological interpretation of the parameters were used to compare the non-linear models for goodness of fit. The statistical criterias were mean square error (MSE) and coefficient of determination (R^2).

Weight and age at the point of inflection were calculated as *Eq.1* for logistic; *Eq.2* for Gompertz; and *Eq.3* for Richards.

$$Y_i = A/2 \; and \; t_i = ln(B)/k \qquad \text{(E.q.1)}$$

$$Y_i = A/e \; and \; t_i = ln(B)/k \qquad \text{(Eq.2)}$$

$$Y_i = A/(d + 1)^{1/d} \; and \; t_i = -1/k * ln|d/B| \qquad \text{(Eq.3)}$$

By using the three obtained equations in terms of the formula, then the predicted growth curves were plotted and presented with observed live weight data.

Results

The observed live weight of Holstein bulls and standard error was shown below. According to the data obtained an average birth weight and final live weight was found as 33.41±0.84 kg and 553.66±7.41 respectively during the 16 month of age (*Table 2*).

Growth curve parameters (A, b and K) from tree non-linear (Gompertz, Richards and Logistic) models with their relative determination coefficient (R^2) and residual mean squared values was shown in *Table 3*.

The models explaining the growth of Holstein bulls according to R^2 and RMS values were arranged in terms of efficiency as Gompertz, Richards and Logistic. Comparison of the models based on the coefficient of determination (R^2) showed that Richards model led to an improved fit of data compared to Gompertz Logistic and von Bertalanffy equations (0.999, 0.998 and 0.997 and 0.999 respectively). The Richards and von Bertalanffy model having the highest coefficient of determination (0.999) and the lowest residual mean squared (16.82and 22.21) value was the best model explaining the growth of the Holstein bulls (*Table 3*). Considering that lowest RMS value determines a greater reliability of the model, so Richards function have the best fit with 16.82 RMS value. The highest value for the coefficient A was obtained with von Bertalanffy model. The value of the coefficient K was found similar in all models.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44094416

The results indicate that all the growth functions were easily fitted to the observed data by nonlinear regression. The estimated growth curve patterns compared with the observed live weights for Holstein bulls was shown in *Figure 2*.

Table 2. *Observed means and standard error of Holstein bulls weights by age*

			95% Confidence Interval	
Age(Month)	**Weight (Kg)**	**SE**	**Lower Bound**	**Upper Bound**
Birth	33.41	0.84	24	42
1	48.38	1.05	32	59
2	68.34	1.36	42	83
3	86.21	1.57	53	101
4	99.91	2.98	65	126
5	135.01	3.25	100	168
6	170.99	3.51	132	205
7	207.52	3.72	162	247
8	244.42	4.07	193	282
9	281.38	4.33	230	320
10	317.16	4.56	260	358
11	355.52	5.51	298	408
12	394.24	5.85	335	455
13	432.86	6.15	372	497
14	471.72	6.67	409	542
15	516.38	7.25	446	580
16	553.66	7.41	486	601

Table 3. *Means and standard error of Holstein-Friesan bulls growth curve parameters, coefficients of determination and residual mean square by different non-linear models*

Model	A	b	K	M	R^2	RMS
Gompertz	986.440 ± 3.403	3.354 ± 0.033	0.004 ± 0.00	-	0.998	21.41
Richards	1110.243 ± 51.881	0.299 ± 0.02	0.003 ± 0.00	10.632 ± 17.4	0.999	16.82
Logistic	672.940 ± 25.817	13.760 ± 0.696	0.008 ± 0.00	-	0.997	50.94
Bertalanffy	1565.604±134.683	0.737±0.005	0.002 ± 0.00	-	0.999	22.21

A: asymptotic weight. b: scale parameter(constant of integration) K: maturity rate. R2: determination coefficient. RMS: residual mean squared

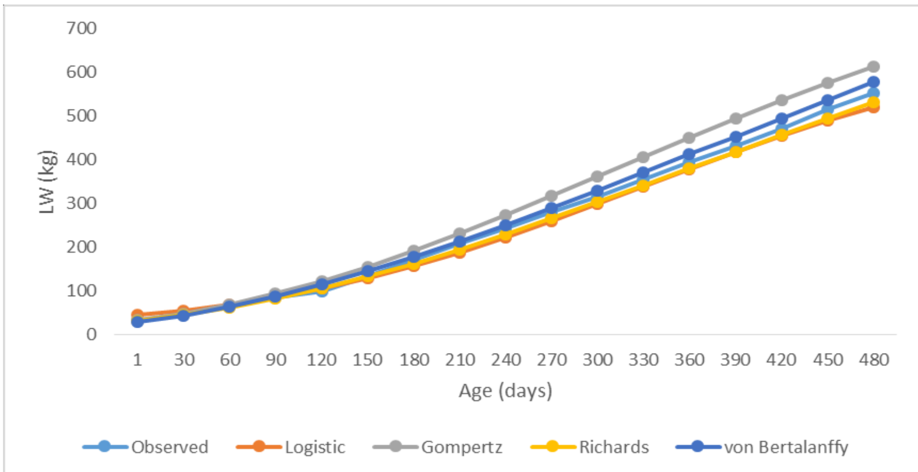

Figure 2. *Growth curve obtained by the avarage of weight observed and weight stated by Gompertz, Richards, Logisticand von Bertalanffy models in different ages.*

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DOI: http://dx.doi.org/10.15666/aeer/1702_44094416

Inflection point traits

The estimates of weight and age at the inflection point was reported in *Table 4*. Age at the inflection point (t_i) is the maximum growth rate longitudinal time frame of live weight. Different from the values of t_i estimated by using Richards model (400.14 days) and von Bertalanffy (396.72 days) were found similar to each other, those calculated using Gompertz and Logistic were lower and similar to each other (327.72 and 306.75 days, respectively). Moreover, the highest value of ti was obtained using the Richards model (400.14 days) with a Wi of 545.01 kg. By using Gompertz, Logistic and von Bertalanffy equations the values of Yi obtained as 362.89, 463.88 and 336.47 kg, respectively (*Figure 3*).

Table 4. *The inflection point traits by different non-linear models*

	Gompertz	**Richards**	**Logistic**	**von Bertalanffy**
Weight at the inflection point (Y_i), kg	362.89	545.01	336.47	463.88
Age at the inflection point (t_i), day	306.75	400.14	327.72	396.72
Shape parameter (d)	-	10.63	-	-

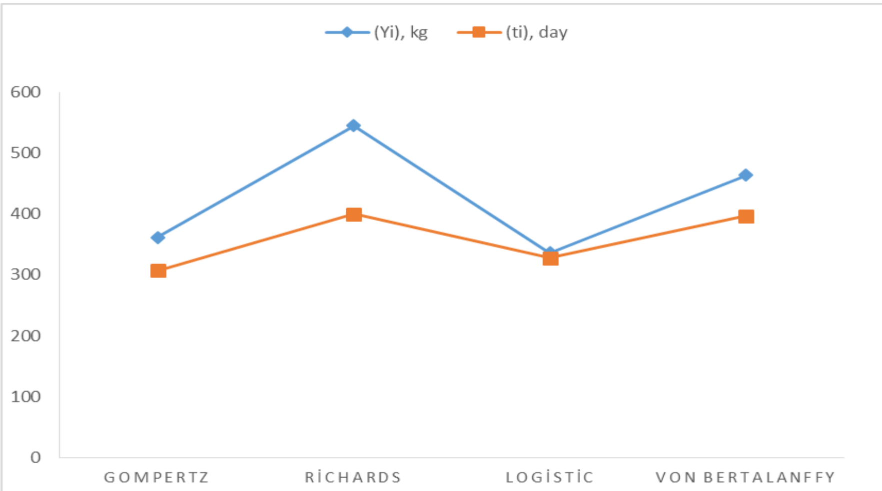

Figure 3. *The inflection point traits (Y_i and w_i) curve by different non-linear models*

The Logistic and Gompertz models overestimate the initial weights as 38% and 4 % respectively. The Richards and Von Bertalanffy underestimate as 2.39% and 12.27% respectively. Overestimation of the final weights by Gompertz and von Bertalanffy was 11% and 4.47, underestimation of those was 5.92% and 3.90 % respectively (*Table 5*).

Table 5. *Overestimation and underestimation of initial and final live-weights by different non-linear models (OE: Overestimation; UE: Underestimation)*

	Initial live weights		**Final live weights**	
Models	OE%	UE%	OE%	UE%
Logistic	38	-	-	5.92
Gompertz	4	-	11	-
Richards	-	2.39	-	3.90
von Bertalanffy	-	12.27	12.27	-

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According to different non-linear models, over-underestimate curve of initial and final live weights plot was shown in *Figure 4*.

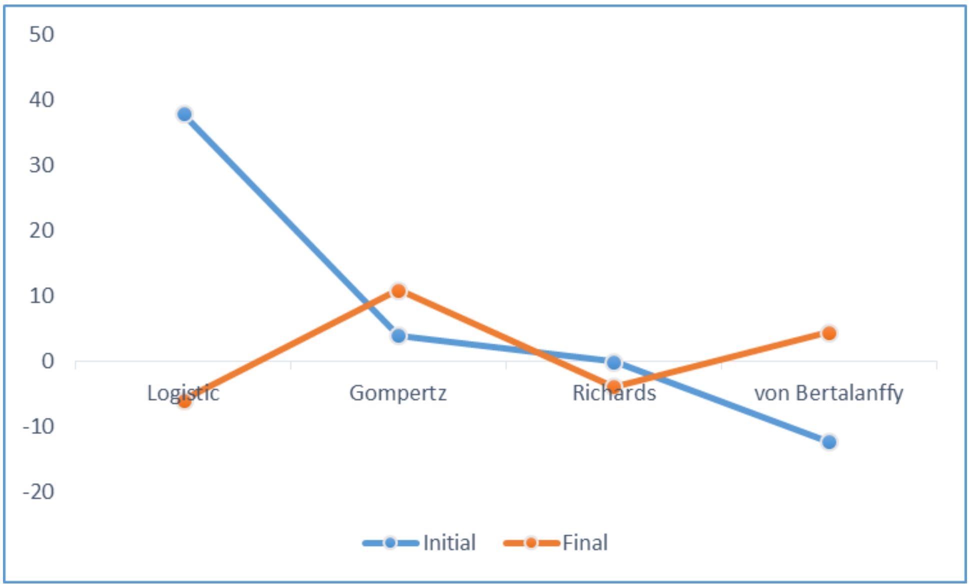

Figure 4. *Over-underestimate curve of initial and final live weights plot*

Discussion

According to findings Richards and Gompertz model was found the best overall fit for the growth of Holstein bulls.

Selvaggi et al. (2017) reported that the Logistic and Richards models provided the best fit, being useful to study the growth of Padolica bulls. Various studies reported that different growth functions gave a best data fit in cattle. In particular, DeNise and Brinks (1985) and Beltran et al. (1992) reported the Richards function as the best for cattle. Conversely, Mazzini et al. (2003) stated that Brody and Richards functions were inadequate to describe the growth pattern of Hereford bulls. Mgberel and Olutogun (2002) stated that Richards model with least residual mean squares was the best fit to the observed growth pattern of male and female N'Dama cattle.

Koşkan and Özkaya (2014) obtained the highest R^2 values of 0.992 and 0.991 respectively by using the Logistic and Gompertz model.

In a recent study conducted by Gano et al. (2015) on Parda de Montaña breed, the Richards function provided the best goodness of fit as also suggested by DeNise and Brinks (1985) and Doren et al. (1989). Furthermore, Gano et al. (2015) reported that the von Bertalanffy function also described the dataset satisfactorily, whereas the logistic equation had the worst fit. Goldberg and Ravagnolo (2015) analyzed the growth curves for Angus cows using different nonlinear models and the results showed that the Richards model was the best to fit the data

The best estimations according to the birth weights were obtained from the Gompertz and Richards models, even if the estimations were lower by R and higher by G than the actual weights. On the other hand, in their study Perotto et al. (1992) stated that the G and L models estimate birth weights higher than the actual values and the R model would be more convenient to estimate birth weights.

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Conclusions

On the basis of our results, Richards model is the best fitted model based on RMS and R^2 criterias which is adequate to establish mean growth pattern of Holstein bulls. Although Richards is a four-parameter model having more computational difficulty, it is the more suitable one due to its accurate ability in predicting mature weight, which is an important selection goal. The growth curve parameters will provide an opportunity to design selection strategies since they may be included in genetic improvement programs as successfully done in other species as well. Richards growth curve model should be used for the other cattle genotypes because there are differences in their growth patterns.

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DOI: http://dx.doi.org/10.15666/aeer/1702_44094416

PHYTOCHEMICAL, ANTIBACTERIAL, ANTIOXIDANT AND PHYTOTOXICITY SCREENING OF THE EXTRACTS COLLECTED FROM THE FRUIT AND ROOT OF WILD MT. ATLAS MASTIC TREE (*PISTACIA ATLANTICA* SUBSP. *KURDICA*)

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(Received 31st Jan 2019; accepted 27th Feb 2019)

Abstract. The goals of this research were to analyze the phytochemical, antioxidant, antibacterial, and allelopathic activities of extracts collected from *P. atlantica* subsp. *Kurdica* fruit and root. The fruit extract showed the highest phenolic (5.20 mg GAE/g dry extract) and flavonoid contents (1.01 mg QR/g dry extract). Qualitative and quantitative analysis of the diverse, biologically active elements from crude extracts displayed different compounds with varying molecular weights using gas chromatography–mass spectrometry. Likewise, both extracts possess unique phytochemical constituents. Additionally, the fruit extract has strong antioxidant and antibacterial properties compared to the root extract. The results of the allelopathic assay revealed strong and significant effects of both extracts on germination, seedling growth and root length for the test crops and weeds. According to the bioactivity analysis, the result of the variable importance in the projection (VIP) revealed that fruit extracts possessed a stronger biological activity than root extracts. The principal component analysis (PCA) plot detected a relationship between different constituents and biological activeness of both extracts and divided them into four clades. The results of this study showed that *P. atlantica* subsp. *Kurdica* may serve as a powerful natural antioxidant, antibacterial and herbicide source.
Keywords: *Mt. Atlas mastic tree, GC/MS, total phenolic and flavonoid contents, bioactivity analysis, radical scavenging, allelopathy*

Introduction

Isolation and recognition of phytochemical constituents from several innate sources can be interesting to scientists (Wheelwright, 1974). The World Health Organization (WHO) focuses on the advancement of native medicaments and ethnomedicines, conducting many investigations on natural therapies in order to search alternative medicines and innovative phytochemical mediators to control eczema, paralysis, diarrhoea, throat infections, jaundice, asthma and stomach pain and kidney stones (Borrelli and Izzo, 2000). North of Iraq, an area with high plant diversity, appears to be excellent for these studies. One of the plants with several industrial and medicinal properties, is the mastic. The *Pistacia* genus is held by the family Anacardiaceae; this genus comprises 11 shrub species belonging to the order Sapindales. *P. atlantica* is a

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long-lived and dioecious plant, with different sorts of blooms. The trees rise up to 7 m in height, with branches spreading and growing erect to form a thick crown. It originates from Asia and the Mediterranean (Rougemont, 1989). In the north of Iraq, *P. atlantica* grows in natural forests, where it is used for several traditional meals and remedies, typically utilizing its gum and aromatic fruits.

Different phytochemical compounds, such as essential oils, phenolic, flavonoid compounds and several fatty acids like eicosanoic, linolenic, palmitic, lignoceric, palmitoleic, pentadecanoic, hexadecanoic and octadecanoic have been recognized in *P. atlantica* (Adams et al., 2009; Trabelsi et al., 2012). Due to their abundances in these bioactive compounds, *P. atlantica owns* some pharmacological characteristics and beneficial usages. Likewise, microorganism resistance is a contest for health and nourishment manufacture, and the search for substitute natural antimicrobial mediators endures (Soković et al., 2010). Contamination and illness as a result of multidrug-resistant (MDR) bacteria in both community and hospital situations have been difficult for many decades, for example, methicillin-resistant *Staphylococcus aureus* (MRSA) is presently unaffected by several antibiotic remedies (Dubey et al., 2013). Different organs like stem, leave and bark, as well as components from *P. atlantica*, demonstrated significant allelopathy and antimicrobial (Tahir et al., 2019). Nevertheless, there has been no information of extracts obtained from the *P. atlantica* subsp. *Kurdica* root, as alternative spontaneous products to reduce the free radical and to control the weeds and bacteria. Established along the request for natural products as an antiradical, and to control microorganisms and weeds, the current investigation was conducted to screen the ethanolic extract composition, antioxidant, antibacterial, and allelopathic activities in fruit and root of *Pistacia atlantica* subsp. *Kurdica.*

Materials and methods

Sampling site

The plant materials were collected in the Sarga mountain in the municipality of Sharbazher, Sulaimani, Iraq (Altitude: 35.85759, Longitude: 45.59086 and level sea: 1352.1 masl). Fruits and roots of *P. atlantica* subsp. *Kurdica* were acquired at 11:00 A.M. in August 2018. The plant was characterized and authenticated at the College of Agricultural Sciences of the University of Sulaimani by the taxonomist Assistant Professor Dr Rupak Abdulrazaq. A voucher specimen (NRT 03) was prepared. The plant materials were dried at 41 °C for eight days.

Chemicals agent

Ethanol and methanol, potassium acetate, Folin–Ciocalteau reagent, gallic acid and quercetin, aluminium chloride hexahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical (ABTS) were purchased from Sigma-Aldrich (Munich, Germany).

Extract isolation

The phytochemical of fruit and root were obtained by the Soxhlet method by placing 85 grams of dried-ground tissues of *P. atlantica* subsp. *Kurdica* in a cellulose thimble. An amount of 220 mL of ethanol (Pure ethanol) was practiced for the extraction using a standard Soxhlet method for 150 min in a Soxhlet extraction system (BÜCHI Extraction

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System Model B-811). The solvent from extracts was then expelled utilizing a vacuum a vacuum rotary evaporator.

Determination of total phenolic compounds

The Folin-Ciocalteau method was applied to determine the amount of total compounds of phenolic acids as described by Singleton et al. (1999) with little modifications. The sample test contained 0.03 mL of each extract (root or fruit), which mixed with 1.7 ml Folin-Ciocalteau (diluted ten times). After 4 min of incubation, 1.3 mL of sodium carbonate solution (10%) was complemented. The calibration curve was determined by mixing aliquots of 5, 10, 15, 20, and 25 µg/mL gallic acid solutions with the same reagents, as the sample tests. The blank contained all solutions with 0.03 mL of distilled water in the state of plant extract. All test tubes were kept for 48 min in the dark at 39 °C. The absorptions of the samples were determined with a spectrophotometer at 750 nm. Each sample was repeated three times. The quantity of phenolic compounds in samples was determined by the calibration curve ($y = 0.0267x - 0.0269$, $R^2 = 0.99$) and expressed in mg gallic acid equivalent/ g dry extract using this formula:

Total phenolic content (mg gallic acid equivalent/g dry extract) = sample concentration calculated from the calibration curve of gallic acid in mg/mL × [extract volume (mL)/weight of the dry extract (g)].

Determination of total flavonoid compounds

The $AlCl_3$ method was used by three triplicates to determine the total flavonoid content of ethanolic fruit and root extracts (Madaan et al., 2011). A stock solution of quercetin was composed by dissolving 10.0 mg quercetin in 10 mL pure methanol, then the series of standard solutions of quercetin in concentration 0.005, 0.010, 0.020, 0.040 and 0.080 mg/mL were prepared by using pure methanol. Three mL of a solution composing of 1 mL of methanol 80%, 0.33 mL of 2% $AlCl_3$, 0.07 mL of 1 M potassium acetate and 1.60 mL of autoclaved distilled water was supplemented to 40 µl of the sample extract or 1 mL of the standard solutions. The mixture was hardly agitated and incubated for 32 min at room temperature. The absorption of the reaction compound was estimated at 415 nm against the blank (contains all above reagents and 40 µL of distilled water in the state of plant extract). The equation of linear regression of the standard curve ($y = 0.0243x + 0.0005$, $R^2 = 0.99$) of the series of quercetin dilutions, was plotted by MS Excel software and exploited to calculate the total flavonoid content by using this formula:

Total flavonoid content (mg quercetin equivalent/g dry extract) = concentration of samples derived from the quercetin calibration curve in mg/mL × [volume of the extract (mL) / dry extract weight (g)].

Qualitative and quantitative screening of ethanolic extracts by GC/MS

The qualitative and quantitative screening of constituents was accomplished using GC/MS. The GC/MS analysis was completed by using Shimadzu GC-QP 2010 Ultra gas chromatograph, The GC oven temperature was stated at 40 °C and then progressively upturned to 280 °C at a rate of 15 °C min^{-1}. Helium was exploited as a healer gas; inlet pressure was 96.1 kPa; linear velocity was 47.2 cm sec^{-1}. Column flux was 1.71 mL min^{-1}; injector temperature 280 °C; injection mode: split. MS scan

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conditions: source temperature 200 °C; interface temperature 280 °C; detector gain 0.69 kV + 0.10 kV and mass range of m/z 50-800. The constituents of the extracts in different parts were set by matching the retention indices with those of recognized constituents stored in the NIST library (2005) or either those of the literature. Peak area concentrations of the constituents were calculated to based on GC peak areas.

Antibacterial test

The antibacterial activity was measured by the agar-well diffusion method (Mounyr et al., 2016). The bacteria employed in this exploration, were *Staphylococcus aureus (*ATCC® 6538P™), methicillin-resistant *Staphylococcus aureus* (MRSA: Clinical isolates), *Bacillus subtilis* (ATCC® 6633™) and *Bacillus cereus* (Clinical isolates). Test microorganisms were initially cultivated in nutrient broth at 37 °C. The antibacterial potential of *P. atlantica* subsp. *Kurdica* was studied using the ethanolic extracts dissolved in Tween-80 (1%). For each pathogenic bacterium, a suspension (10^7 CFU/mL) was first dispersed generally on the solid agar surface. At that point, a hole with a diameter of 0.60 cm has penetrated with an autoclaved cork borer, and a volume (80 µL) of the extract solution (255 mg/mL) was inserted into the well. For 15 h, the plates were incubated at 37 °C and the diameter of the suppression areas was calculated in millimeters.

Ability of radical scavenging extracts by DPPH assay

The antioxidant ability of both extracts was determined using the method, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging as defined by Shimada et al. (1992) with some amendments. Two mL (6×10^{-5} M) of DPPH solution was mixed with 20 µL sample. A control with methanol and a DPPH solution have also been implemented. The samples and control were incubated in the dark at 23 °C for 31 min and absorbed against a blank containing only methanol at 517 nm. The triple experiment was conducted. The inhibition percentage of samples were calculated from the data of absorbances by the formula:
% inhibition = [(Absorbance of control - Absorbance of the sample) / Absorbance of control] × 100.

Radical scavenging ability of extracts by ABTS radical cation

The free radical scavenging activity of root and fruit extract was set by 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid radical (ABTS) radical cation decolorization assay (Re et al., 1999). ABTS radical cation was made by the mixing of ABTS (7 mM) in water and 2.45 mM of potassium persulfate (1:1). The mixed solution was hoarded in the dark at 21 °C for 15 h before use. For obtaining an absorbance of 0.700 ± 0.002 at 734 nm, the ABTS radical cation solution was made weaker with methanol in ratio 1:37.50. Seven µL of root or fruit extract was added to 2.993 mL of diluted ABTS radical cation solution. A control solution contained 7 µL of methanol and 2.993 mL of diluted ABTS radical cation solution. After 8 min of incubation, the absorbance was read against the blank (methanol) at 734 nm. All tests have been repeated three times. Percent of inhibition was calculated using the formula:
ABTS cation scavenging (%) = [(Absorbance of control-Absorbance of the sample)/Absorbance of control] × 100.

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Allelopathy assay

The allelopathic power of the root and fruit extracts was performed on the seed germination parameters, of four plant species: wheat (*Triticum aestivum* L. variety Aras), barley (*Hordeum vulgare* L. variety Alkher), wild mustard (*Sinapis arvensis* L.), and narbon vetch (*Vicia narbonensis* L.*)*. Plant seed was sterilized with NaClO (0.90%) for 12 min and washed with autoclaved distilled water six times. Five replicates, each comprising of 15 seeds, were exercised for each of the control, 1.10 and 2.20 mg/mL plant extracts, using sterile Petri dishes (0.90 cm diameter) padded with three-sterile filter papers (Whatman). The plant extracts were mixed in a distilled water-acetone mixture (98:2) (Tahir et al., 2018). Nine mL of distilled water/acetone (control) or 9 mL different doses of the two plant extracts (1.10 or 2.20 mg/mL) were added to each Petri dishes. In order to prevent loss of moisture and microbial contamination, the dishes were covered with parafilm and deposited in a dark incubator at 19 °C for 7 days. In the eighth day, the germination percentage [%G = (Total germinated seeds/15) × 100] and seedling growth were measured. A seed was considered as a germinated seed when the appearance of the radicle become obvious (*Figure 1*).

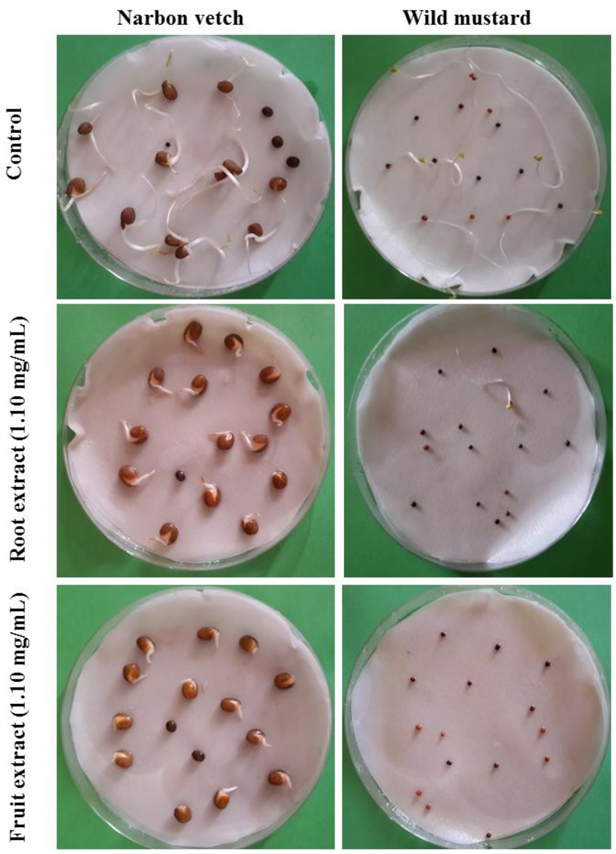

Figure 1. *Allelopathy effects of P. atlantica subsp. Kurdica fruit and root extracts on the germination parameters of two weeds: narbon vetch and wild mustard*

Statistical data analysis

Data on bio-activities were submitted to the analysis of variance (One way-ANOVA) using XLSTAT 2016 software. Means were matched by Duncan's new multiple range

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test at the 5% significance level. The data was exposed to a PCA analysis using the software XLSTAT 2016 to define the relation between chemical components and inhibitory effects.

Results and discussion

Screening of the polyphenolic constitutions

The results of the phytochemical contents of the fruit and root ethanolic extracts are detailed in *Figure 2*. The polyphenolic constitutions were considered by the Folin–Ciocalteu method and there was not significant variance ($p < 0.05$) among all tested samples for total phenolic content (TPC), while significant differences were noticed between the extracts collected from fruit and root for the flavonoid content (TFC). The maximum values of phenol and flavonoid compounds with 5.20 mg gallic acid equivalents/g and 1.01 mg quercetin equivalents per gram of dry extract, were registered by fruit extract. The outcome showed that fruit extract was rich in polyphenolic compounds. The TPC amount in both extracts was varied from the earlier assessments in methanolic, water, butanol and ethyle acetate extracts of the same species and another plant part (Ben Ahmed et al., 2017; Benamar et al., 2018; Tahir et al., 2019). Likewise, the variation between our results and other researchers in the quantity of polyphenolic compounds in *P. atlantica* may be contributed to a growing region, growing season, growth stage. solvent, a method of extraction and plant organ.

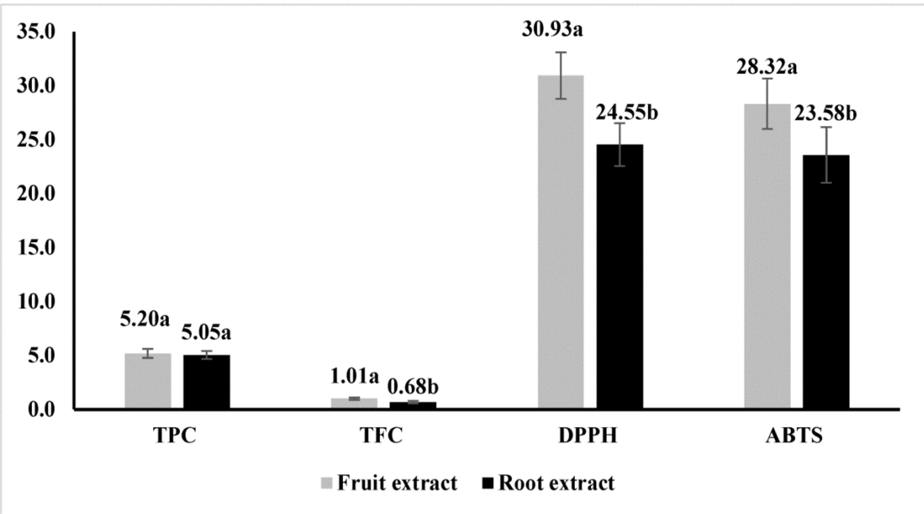

Figure 2. *Total phenolics (TPC in mg gallic acid equivalent/g dry extract), total flavonoids (TFC in mg quercetin equivalent/g dry extract) contents and antioxidant potentials (%) in P. atlantica subsp. Kurdica fruit and root extracts. The letters attached to each bar by the different letters mark substantial differences according to Duncan's multiple-range test. Each value is an average of three replicates*

GC/MS analysis of the extracts

Chromatographies analysis of the extracts allowed 26 compounds to be detected (*Table 1*). Several classes of compounds, including essential oil, polyphenol, and aliphatic compounds, were noticed in these extracts. Among its 18 components detected

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in the fruit extracts, 6 constituents dominated the composition of this extract, with a content of 72.83%. Constituents: 2-isoamyl-6-methylpyrazine (38.57%), oxalic acid, hexadecyl 2-methyl phenyl (9.26%), (NB)-O-[(diethyl boryl oxy)(ethyl) boryl]-caprolactone oxime (7.19%), pyrogallol (6.78%), 3-cyclopentylpropionic acid, 3-methyl phenyl ester (5.62%), and benzoic acid, 3-hydroxy-benzoic acid (5.41%) were the predominated components. Among the detected fractions, seventeen compounds were limited to fruit extracts. As displayed in the GC/MS analysis of the root extracts, 9 compounds were determined. Among them, five constituent concentrations were ranged from 5 to 37%. The major ones were 2-fromyl-3-benzyl-3-cholestanol (36.97%), l-(+)-ascorbic acid 2,6-dihexadecanoate (15.98%), cis-9-hexadecenal (9.83%), alpha-amyrenone (6.34%) and 1-heptacosanol (5.62%). Eight of ten compounds were constricted to this organ. Variations in the extract composition among the different tissues can be expounded by the volatility and biotransformation of monoterpenes, which often occur in several metabolic pathways leading to a mixture of products and, which may undergo spontaneous auto-oxidation (McGarvey and Croteau, 1995). To our awareness, there are no works on the chemical composition, antioxidant, antibacterial and allelopathic activities of the ethanolic extracts from *P. atlantica* subsp. *Kurdica* root. Hence, our findings are of attentiveness. In the previous works of *P. atlantica*, various compounds of several phytochemical clades have been known. Most of the predominated components found from different parts of *P. atlantica* subsp. *Kurdica*, have made it as a fountain of pharmaceutical and industrial usages. For instance, 2-isoamyl-6-methylpyrazine is used as foodstuffs (Xu-Yan et al., 2012). Studies revealed that pyrogallol had antibacterial properties averse to food-borne gram-positive (Han and Wang, 2017). Compound l-(+)-ascorbic acid 2,6-dihexadecanoate recognized as an antioxidant food additive (Cort, 1974). Previous investigations using α-amyrenone have proved its pharmacological potential as a new healing tool for the controlling of painful and inflammatory sicknesses (Quintão et al., 2014).

Antioxidant spectrophotometric assay

The effectiveness of the radical scavenging associated with the site and the number of compound hydroxyl groups. Most components with the OH group in *ortho* position of the ring, showed the greatest powerful scavengers of DPPH radical while the constituents with more of OH in the structure were the most powerful ABTS radical scavengers (Baba and Malik, 2014). The antioxidant ability of different extracts of fruit and root was conducted using DPPH and ABTS assays as shown in *Figure 2*. Then, a lower percentage value designates a lower antioxidant activity. As reported in the DPPH method, the antioxidant activity was ranged significantly from 24.55 to 30.93%. Fruit extract had the maximum DPPH scavenging activity (30.93%), while the lowest action (24.55%) was stated by root extract. In the ABTS radical, the scavenging activities in the various extracts were difference significantly and ranged from 23.58 to 28.32%. The fruit extract exhibited the maximum antiradical ability by ABTS. Interestingly, the consequences of antioxidant activities revealed that fruit extract appears to heavily affect the radical scavenging ability. These findings suggested that the antioxidant powers of fruit extracts can be attributed to their high flavonoid quantity. Our results of antiradical ability are lower than the output (87.00-97.50%) obtained by Hatamnia et al. (2014) and Hatamnia and Malekzadeh (2015). Several factors could be clarified this variation like the genetic variability of the plant, harvesting time of the samples, a method of extraction and the effect of the climate.

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Table 1. *GC/MS analysis of different extracts acquired from fruit and root of P. atlantica subsp. Kurdica*

No.	Compounds name	Fruit	Root
1	Tetradecane	0.86	—
2	Pentadecanoic acid	4.53	—
3	9,12-octadecadienoic acid	1.64	6.53
4	Cis-9-hexadecenal	—	9.83
5	1-heptacosanol	—	5.62
6	Tetrahydrocyclopenta[1,3]dioxin-4-one	3.26	—
7	Pyrogallol	6.78	—
8	Benzoic acid, 3-hydroxy-benzoic acid	5.41	—
9	Elemol	0.98	—
10	6-deoxy-D-galactose	4.02	—
11	Beta-eudesmol	1.47	—
12	Dichloroacetic acid, tridec-2-ynyl ester	3.57	—
13	2-isoamyl-6-methylpyrazine	38.57	—
14	3-cyclopentylpropionic acid, 3-methylphenyl ester	5.62	—
15	3-pentadecyl-phenol	2.85	—
16	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	1.73	—
17	2,2-dimethyl-3-vinyl-bicyclo[2.2.1]heptane	0.71	—
18	Oxalic acid, hexadecyl 2-methylphenyl	9.26	—
19	(NB)-O-[(diethylboryloxy)(ethyl)boryl]-caprolactone oxime	7.19	—
20	Alpha-tocopherol-beta-D-mannoside	1.55	—
21	l-(+)-ascorbic acid 2,6-dihexadecanoate	—	15.98
22	2-fromyl-3-benzyl-3-cholestanol	—	36.97
23	Cis-13-octadecenoic acid	—	4.39
24	Dibenzo[a,h]cyclotetradecene, 2,3,11,12-tetraethyl-1,2,3,4,5,6,7,8,9,10,11,12,13,14,15	—	4.78
25	22-alpha-hydroxy-3,4-secostict-4(23)-en-3-oic acid	—	4.56
26	Alpha-amyrenone	—	6.34

Antibacterial activity of different extracts

The tested extracts of *P. atlantica* subsp. *Kurdica* fruits and roots held the antibacterial potent versus Gram-positive than Gram-negative bacteria. The cause of the increased sensitivity of Gram-positive bacteria than Gram-negative bacteria could be related to their differences in cell membrane structures. The antimicrobial potentials of *P. atlantica* subsp. *Kurdica* fruit and root extracts were determined primarily by the disc diffusion technique against different Gram-positive bacteria strains, which commonly encountered in infectious diseases. As stated in *Figure 3*. It can be noted that both plant extracts, revealed varying grades of antibacterial potential against the four bacterial strains tested. The effects of both extracts varied considerably from all bacteria tests except methicillin-resistant *S. aureus*. The zones of fruit extract inhibition against four types of bacteria were ranged from 3.38 to 7.35 mm. The ethanol fruit extracts exhibited the strongest anti-*S. aureus* activity with an inhibition zone diameter of 7.35 mm and a feeble activity against methicillin-resistant *S. aureus* (6.40 mm). The extracts of root presented a relatively moderate activity principally against all bacterial species with the inhibition diameters ranged from 3.38 to 7.13 mm. This extract of plant parts showed a small activity mainly against *B. subtilis* (3.38 mm) and the maximal inhibition zones against *B. cereus*. It has been described in this section that the zone of inhibition could

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DOI: http://dx.doi.org/10.15666/aeer/1702_44174429

be importantly affected by the structure of crude extracts that are a combination of phyto-components which may impact the diffusion power of the active elements. Similar results have been obtained from an investigation carried on *P. atlantica* subsp. *Kurdica* leaf, stem and bark by Tahir et al. (2019).

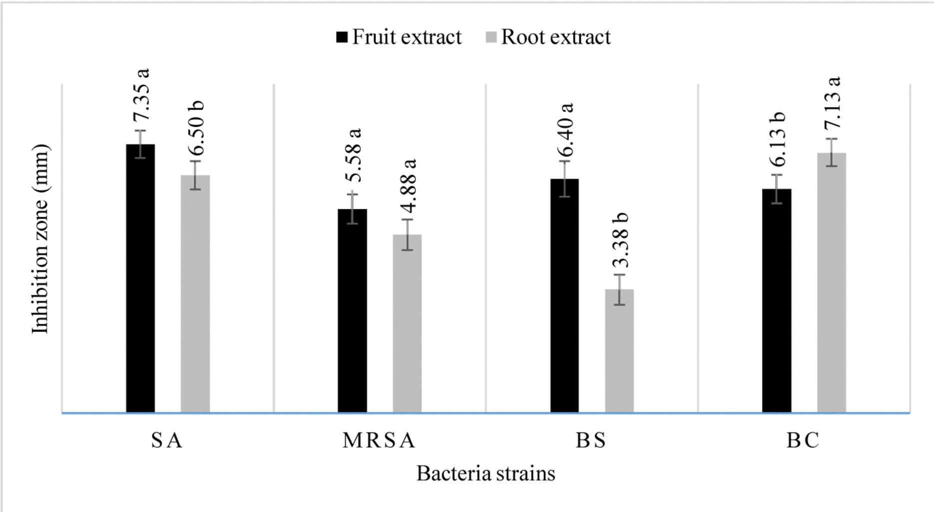

Figure 3. *Inhibition zones (mm) of the fruit and root extracts against four different species of bacteria. SA: S. aureus, MRSA: methicillin-resistant S. aureus, BS: B. subtilis, BC: B. cereus. The letters attached to the numbers of each bar by the different letters refer to substantial differences based on Duncan's multiple-range test. Each value is an average of three replicates*

Responses of crop and weed species to different extracts

All the applied concentrations of fruit extracts significantly blocked the germination and growth of the test plant species (*Table 2*). Fruit extracts at 2.20 mg/mL were more effective than fruit extracts at 1.10 mg/mL. The highest preventing of germination percentage (81.36% on wild mustard), root length (95.06% on narbon vetch) and shoot growth (88.34% on barley) were recorded by fruit extracts at a concentration of 2.2 mg/mL, while the lowest values of blocking of germination percentage (3.41% on bread wheat), root elongation (59.67% on wild mustard) and shoot length (56.20% on wild mustard) were reported by fruit extracts at 1.1 mg/mL concentration. All the doses of root extracts significantly inhibited the germination and growth of test crops and weeds. Root extracts at 2.20 mg/mL concentration were comparatively more toxic. The results presented that fruit and root extracts seemed more active in preventing bread wheat and narbon vetch germination. In addition, both extracts had more phytotoxic power against root elongation of all test species because of its directness contact with the extracts. Overall, the reduction of germination parameters was attributed to the presence of some allelochemical compounds in both extracts such as alpha-amyrenone, benzoic acid, 3-hydroxy-benzoic acid, pentadecanoic acid and -(+)-ascorbic acid 2,6-dihexadecanoate (Javaid et al., 2006; Ferreira et al., 2017). In addition, these allelochemical compounds modify the central vascular cylinder, layers of cortex cells and the mitochondrial metabolism, thereby altering some physiological and metabolic reactions related with growth of the plants (Gatti et al., 2010). Similarly, Tahir et al. (2019) found that the bark, stem and leaf extracts of *P. atlantica* subsp. *Kurdica* significantly decreased the growth of some crops and weeds.

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DOI: http://dx.doi.org/10.15666/aeer/1702_44174429

Relationship between chemical components and bioactivity parameters

The objective of variable importance in projection (VIP) was to identify and select the most important predictor variables. In a given model, a variable with a VIP score near or above 1 can be considered important. As displayed in *Figure 4,* the fruit extract had a high impact (VIP > 1) and it was considered as variable importance in the bioactivity assays. For reducing the multidimensional structure of the data and providing a two-dimensional map to explain the relationship and variance between the data obtained from phytochemical and bioactivity analysis of both extracts, a PCA plot was performed and two main PCAs for the analyzed two extracts accounted for 100% of the total variation, PC1 for 88.32% and PC2 for 11.68% (presented in *Figure 5*). The plot obtained from PCA using the linkage method among classes indicated the presence of four clusters:

a. Group 1 with higher concentrations of pyrogallol (C4), benzoic acid, 3-hydroxy-benzoic acid (C5), 3-cyclopentylpropionic acid, 3-methylphenyl ester (C7), Oxalic acid, hexadecyl 2-methylphenyl (C8), and (NB)-O-[(diethylboryloxy)(ethyl)boryl]-caprolactone oxime (C9). Moreover, a positive correlation with total phenolics content, total flavonoids content, inhibition growth of *S. aureus* (SA), methicillin-resistant *S. aureus* (MRSA), and *B. subtilis* (BS).
b. Group 2 with high amount of 9,12-octadecadienoic acid (C1), Cis-9-hexadecenal (C2), 1-heptacosanol (C3), l-(+)-ascorbic acid 2,6-dihexadecanoate (C10), Alpha-amyrenone (C12), high inhibition growth of *B. cereus* (BC), high germination inhibition of wheat (INW) and narbon vetch (INNV).
c. Group 3 with a high content of 2-isoamyl-6-methylpyrazine (C6), high inhibition of germination of barley (INB) and high radical scavenging activity (DPPH and ABTS).
d. Group 4 with a high concentration of 2-fromyl-3-benzyl-3-cholestanol (C11) and high blocking of germination in wild mustard (INM)

Table 2. *Inhibition percentages of various concentrations of fruit and root extracts of P. atlantica subsp. Kurdica on the germination and growth of four plant species*

		Inhibition (%)				
Plant species	**Parameters**	**Control**	**Fruit (1.10 mg/mL)**	**Fruit (2.20 mg/mL)**	**Root (1.10 mg/mL)**	**Root (2.20 mg/mL)**
T. aestivum	Germination	0.00b	3.41b	13.57ab	5.09b	22.04a
	Root length	0.00b	81.42a	88.50a	84.96a	88.50a
	Shoot length	0.00c	58.86b	64.88b	55.18b	83.61a
H. vulgare	Germination	0.00d	40.00c	79.38a	41.88c	62.81b
	Root length	0.00d	65.08c	88.83a	75.42b	91.62a
	Shoot length	0.00d	50.44c	88.34a	70.85b	89.21a
V. narbonensis	Germination	0.00b	8.18b	8.48b	8.18b	22.56a
	Root length	0.00b	93.83a	95.06a	93.83a	93.83a
	Shoot length	0.00c	63.16ab	82.30a	55.50b	79.90a
S. arvensis	Germination	0.00d	68.38c	81.36ab	75.20bc	87.68a
	Root length	0.00c	59.67b	80.66a	80.25a	85.60a
	Shoot length	0.00b	56.20a	58.21a	67.44a	74.06a

The different letters connected to each row mark substantial differences according to Duncan's multiple-range test. Each value is an average of five replicates

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DOI: http://dx.doi.org/10.15666/aeer/1702_44174429

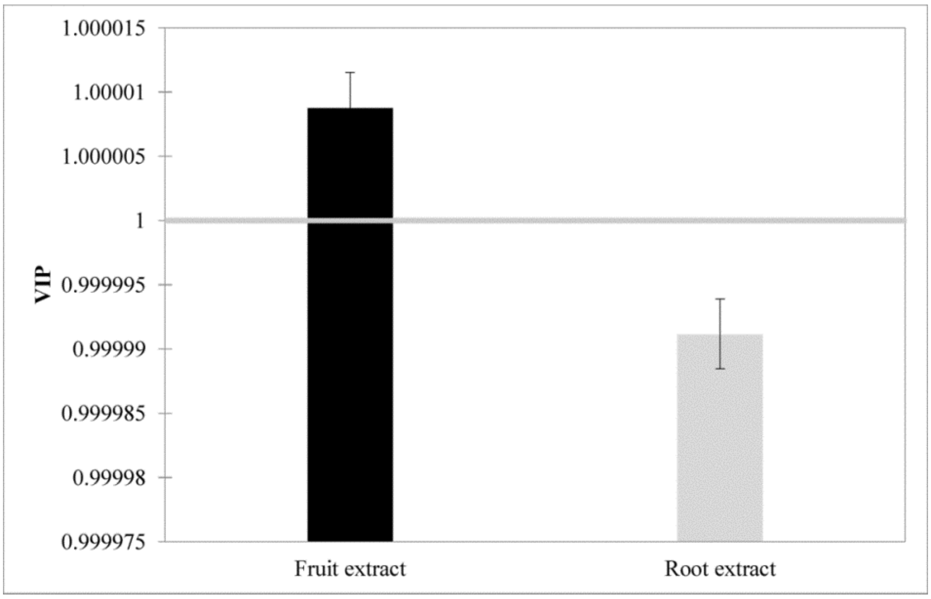

Figure 4. *Variable importance in the projection (VIP) showing the importance of fruit extract*

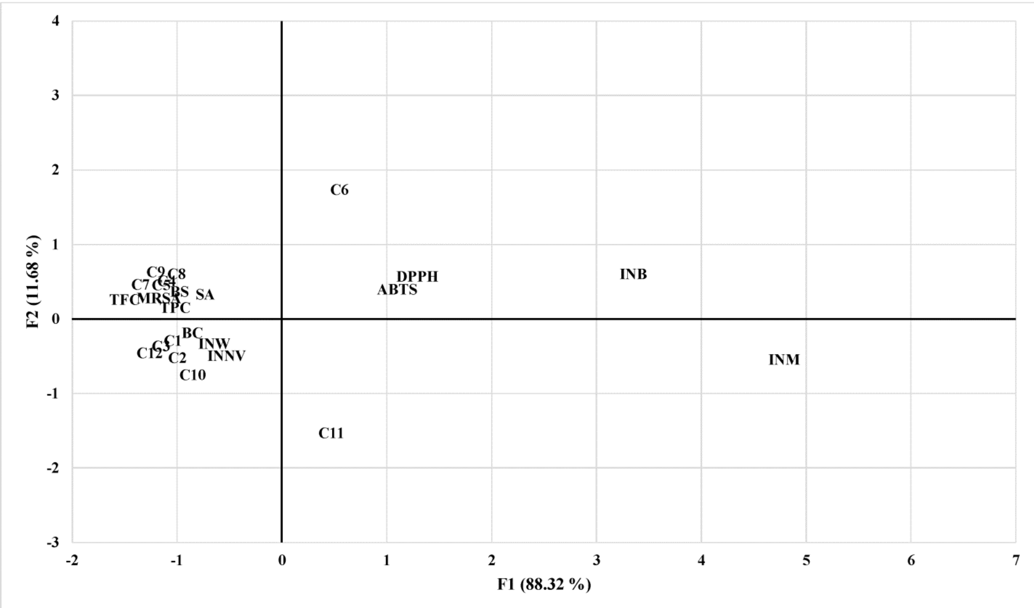

Figure 5. *PCA biplot showing the distributions of chemical compounds (12 fundamental compounds having an average concentration of at least 5.00% of the total extract composition in at least one plant tissue) and biological activity parameters. C1: 9,12-octadecadienoic acid, C2: Cis-9-hexadecenal, C3: 1-heptacosanol, C4: Pyrogallol, C5: Benzoic acid, 3-hydroxy-benzoic acid, C6: 2-isoamyl-6-methylpyrazine, C7: 3-cyclopentylpropionic acid, 3-methylphenyl ester, C8: Oxalic acid, hexadecyl 2-methylphenyl, C9: (NB)-O-[(diethylboryloxy)(ethyl)boryl]-caprolactone oxime, C10: l-(+)-ascorbic acid 2,6-dihexadecanoate, C11: 2-fromyl-3-benzyl-3-cholestanol, C12: Alpha-amyrenone, SA: S. aureus, MRSA: methicillin-resistant S. aureus, BS: B. subtilis, BC: B. cereus, TPC: Total phenolics content, TFC: Total flavonoids content, INB: Inhibition of germination in barley (average of both concentrations), INM: Inhibition of germination in mustard (average of both concentrations), INW: Inhibition of germination in wheat (average of both concentrations), INNV: Inhibition of germination in narbon vetch (average of both concentrations)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_44174429

Conclusions

Qualitative and quantitative analysis of the diverse biologically active elements from crude extracts of *P. atlantica* subsp. *Kurdica* displayed different types of molecular weight compounds in each extract. The high radical scavenging and antibacterial capacity are detected in the ethanol fruit extract compared to the root extract. The current work determines that fruit and root extracts have significant herbicide effects on the germination and growth of seedlings in four plants. Fruit and root residues of *P. atlantica* could be disseminated on wilderness, causing to the leaching of allelochemical compounds that would prevent or decrease the seed germination of weeds. Therefore, both extracts are considered to be new natural sources of antioxidants, antibacterial and weedicides. The prospect investigates on *P. atlantica* may be conducted on the following characteristic:

a. More resources are needed to verify the primary mechanisms of *P. atlantica* extracts as inhibitory agents.
b. For making the bio-weedicide to control the weeds. It is important to purify individually the allelopathic components, which have the inhibitory properties, presenting in the *P. atlantica* extracts.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44174429

ECONOMIC EVALUATION OF A PASSIVE SOLAR GREENHOUSE HEATING SYSTEM IN CRETE, GREECE

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(Received 26th Jan 2019; accepted 28th Feb 2019)

Abstract. The objective of this study is to evaluate economically the passive solar heating system. Data collected from tomato greenhouses in the Agriculture Research Center of Northern Greece was analyzed. For Comparison, two other conventional heating systems were used: the traditional anti-frost system and the diesel-fired furnace system. Models have been constructed to assess each heating method and several approaches of appraisal such as net present value, internal rate of return, benefit/cost ratio and payback period were measured in order to fulfill the assessment. The importance of the passive solar system was revealed not only as an energy-saving method but also as a significant contributor to farmer's income and an appropriate system avoiding the risk of high initial investment, which is generally associated with unconventional systems. Meanwhile, it was indicated that the diesel-fired furnace system is ineffective compared to the passive solar system, this ineffectiveness is particularly due to its high fuel consumption and relatively high initial investment. Finally, sensitivity analysis highlighted the importance of the product price in affecting benefits and indicating that income will increase dramatically with earlier production.
Keywords: *cost-benefit analysis, energy conservation, farm income, renewable energy, stochastic efficiency*

Introduction

Out-of-season production of good quality vegetables, ornamentals, and fruit in greenhouses in the Mediterranean is greatly influenced by energy costs. Tataraki et al. (2018) have stated earlier that the greenhouse heating costs have increased dramatically and the use of other sources of energy in response to escalating costs and occasional scarcities of petroleum shall be investigated (Bustos et al., 2016).

Several heating systems are used in the Mediterranean, it can be generally classified into conventional and non-conventional sources. Conventional sources include: Solid fossil fuels, Oil (fossils), Gas (natural) and Hydraulics. Non-conventional sources include: Solar, Wind, Biomass, Geothermal and Waste and reject energy (Balana et al., 2015).

Methods of investment appraisal which takes into account the time value of money are based on compound interest principles. The Net Present Value (NPV) method involves calculating the present value of a project's cash flows, both positive (inflows) and negative (outflows). Buchholz et al. (2017) defined the NPV of a project as the difference between the present values of its future cash inflows and outflows: all annual cash flows should be discounted to the zero points in time (the start of implementation) at a predetermined discount rate (Gautam et al., 2017).

Another way of using discounted cash flows to measure the overall of a project is to find the discount rate which makes the NPV of the cash flow equal to zero. This

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

discount rate is termed the Internal Rate of Return (IRR) and represents the average earning power of the money used in a project over its life (Boardman et al., 2017). Larson and Gray (2013) defined the IRR as the maximum rate of interest that can be paid to finance a project without causing harm to the shareholders.

Besides NPV and IRR, a number of other measures of investment performance are in use, the most common of which is the pay-back period criterion, defined as the period of time by which the investments will be covered by the returns (Boardman et al., 2017). The pay-back period is defined as the length of time required for the stream of cash proceeds produced by an investment to equal the original cash outlay required by the investment (Nowotny et al., 2016).

Dixon et al. (2013) has defined the benefit/cost ratio as the present value of benefits over the present value of the costs. The corresponding decision rule requires that the project is accepted if the benefit/cost ratio is greater than one to compute the benefit/cost ratio, the discount rate must be determined. Dixon et al. (2013) has stated probably the best discount rate is the opportunity cost of capital. Another which is often chosen for the benefit/cost ratio calculation is the borrowing rate for the project. A third rate sometimes suggested is the social rate of return, which may more adequately reflect the time preference of society than does the opportunity cost of capital (Benli, 2013).

Ranking projects or alternatives is an important step in evaluation. Differences occur when two or more appraisal methods may yield differing orderings when applied to the same project. Brigham and Ehrhardt (2013), Boardman et al. (2017) and Johnson and Pfeiffer (2016) have suggested several methods of solving the problem.

According to Dogbe et al. (2013), Break-Even Analysis (BEA) establishes the lowest production and/or sales levels at which a project can operate without endangering its financial viability. In the manual for evaluation of industrial projects (Song et al., 2013), the term Break-Even Point (BEP) is used to indicate a level of operation at which a project yields neither profit nor loss, expressed as a volume of sales revenue.

Dogbe et al. (2013) and Elhag (2016) state that sensitivity analysis may be used in the early stages of project preparation to identify the variables in the estimation for which special care should be taken. According to Elhag (2014) and Boardman et al. (2017), sensitivity analysis consists of varying key parameter values, usually one at a time but sometimes in combination, and assessing the effect of such changes on the central tendency estimate of profitability (or the optimal solution).

In this study, three methods of greenhouse heating, two conventional methods (the Anti-Frost System (AFS) and the Diesel-Fired Furnace System (DFS)) and an unconventional method (the Passive Solar System (PSS)), all of which are used in greenhouses producing off-season tomatoes, were evaluated for production and gross returns, and the magnitude of the benefits provided by such technology, using different approaches of appraisal (net present value, internal rate of return, benefit/cost ratio and pay-back period).

Materials and methods

Data collection

Data are obtained from experiments conducted at the National Agricultural Research Foundation (NAGREF) in the region of Thessaloniki for 3 years started from 2012 and lasted until 2014. The designated study area is a semi-arid Mediterranean environment with a cold variant: the summer is hot and dry; the winter is cold and rainy.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

Production and gross returns

A comparison of production and gross returns were made between the three greenhouses. The output of each greenhouse was the statistical mean of the production of tomatoes for three years (2012-2014). Production and gross returns were estimated for the total area of each greenhouse.

Fuel consumption

Annual fuel consumption was estimated as the average fuel consumption for the three years for each greenhouse for tomatoes and the average of two years for melons for each greenhouse.

Labor costs

Labor costs intervene in this analysis, particularly in the installation cost of the three systems in consideration, and especially for the PSS, which is more time-consuming. It intervenes also in the harvest costs.

Maintenance costs

Maintenance costs apply, especially to the PSS. At the beginning of each season, farmers need to fill the plastic tubes and position them, and later empty them and prepare them for the next season. AFS and DFS also have maintenance costs, but these were considered negligible in the calculations.

Models

The problems were formulated to allow the determination of the NPV as a measure of investment worth and to permit comparisons with investment alternatives. Partial budgeting procedures were used in which values with and without the PSS in the first case and with and without the DFS in the second case were used to calculate the NPV and other economic parameters: IRR, benefit/cost ratio and pay-back period (Udayakumara and Gunawardena, 2018).

Model for evaluating the PSS

Simulation methodology was used to evaluate the worth of the investment under various influences occurring in the Thessaloniki region. Three factors as having a major impact on the production benefits (Levidow et al., 2014) were incorporated into the analysis: (1) recoverable yield, which increases with suitable temperature; (2) fuel consumption, which depends on the type of heating system; (3) price of the crop, which varies over the harvest period, with generally higher prices earlier in the season. Following Boardman et al. (2017), the effect of inflation was ignored by assuming that it acts in the same way on revenues and costs and the effects cancel each other. The specific expression was extracted from that utilized by Elhag (2017) and modified to the needs of the problem:

$$NPV = -\sum_{t=1}^{8} \frac{\frac{[1-(-1)^t]IS_t}{2}}{(1+R)^t} + \sum_{t=1}^{8} AR_t + SF_t - AE_t - MN_t + \frac{IC_1}{(1+R)} + \frac{IC_5}{(1+R)^5} \quad \text{(Eq.1)}$$

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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where:
IS_t = purchase price, including the cost of installing the PSS in year t for a 0.1 ha greenhouse;
IC_i and IC_S = purchase price, including the cost of installing the AFS in years one and five for a 0.1 ha greenhouse;
ARt = additional annual returns;
SFt = annual fuel costs saved;
AEt = additional annual harvest costs;
MNt = additional annual maintenance costs;
R = interest rate;
t = year of operation;
where:
$AR = \sum_i^n P_i\,(Q_{is} - Q_{ic})$;
$SF = H(F_c - F_s)$;
$AF = K(Q_s - Q_c)$;
and where:
Pi = price of the crop on Day i (dr/kg);
Qis = production of greenhouse S on Day i (kg);
Qic = production of greenhouse C on Day i (kg);
H = fuel price (dr/1);
Fc = annual quantity of fuel used in greenhouse C (1);
Fa = annual quantity of fuel used in greenhouse S (1);
K = harvest costs (dr/kg);
Qa = annual production of greenhouse S (kg);
Qc = annual production of greenhouse C (kg);
n = number of harvest days.

Model for evaluating the DFS

This model was similar to that used for the PSS, with certain parameters altered, such as the life of the system and the electricity needed to operate it. The assumptions used for the evaluation of the PSS were also made.

The NPV was determined by considering the monetary flows of the two greenhouses F and C, using the following expression which also extracted from that utilized by Agil and Hosseinian (2014) and modified to the needs of the problem:

$$NPV = \frac{-IF + IC_1}{(1+R)} + \frac{IC_5}{(1+R)^5} + \sum_{t=1}^{8} \frac{AR_t - CF_t - AE_t - EC_t}{(1+R)^t} \quad \text{(Eq.2)}$$

where:
IF = purchase price, including the cost of installing the DFS, for a 0.1 ha greenhouse;
ICi and IC5 = purchase price, including the cost of installing the AFS in years one and five for a 0.1 ha greenhouse;
ARt = additional annual returns;
CFt = additional annual fuel expenses;
AEt = additional annual harvest costs;
ECt = annual electricity consumption by the furnace;
R = interest rate;
t = year of operation;

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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where:
$AR = \sum_i^n P_i \left(Q_{iF} - Q_{iC}\right)$;
$CF = H(F_F - F_c)$;
$AF = K(Q_F - Q_C)$;
and where:
Pi = price of the crop on Day i (dr/kg);
QiK = production of greenhouse F on Day i (kg)
Qic = production of greenhouse C on Day i (kg)
H = fuel price (dr/1);
Ff = annual quantity of fuel used in greenhouse F (1);
Fc = annual quantity of fuel used in greenhouse C (1);
K = harvest costs (dr/kg);
Qf = annual production of greenhouse F (Kg);
Qc = annual production of greenhouse C (kg);
n = number of harvest days.

Results

Evaluation of the PSS

Variables used in the evaluation of the PSS with no distinction between varieties are depicted in *Table 1*. This evaluation is based on the application of the model which gives the NPV. Interest is high in Greece: an annual rate of 20% was assumed ($R = 0.2$). An assisted computer program was used for financial analysis; the application of the discount cash flow approach is illustrated in *Table 2*.

NPV

The formal selection criterion for NPV is to accept all investments with a positive NPV. The use of the PSS, over an 8-year period, produced an NPV of almost 5,500.0 euro, indicating a significant increase in earnings of greenhouse farmers and a contribution to energy saving.

Table 1. *Base case values for variables included in the model evaluating the PSS.*

Variable	Symbol	Unit	Value
The total cost of the PSS	IS	Euro	0.3
The total cost of the AFS	IC	Euro	505.2
Discount rate	R	%	275.5
Additional annual returns	AR	Euro	20
Fuel price	H	Euro/L	1562.6
Fuel consumption in greenhouse C	F_C	1	0.2
Fuel consumption in greenhouse S	F_S	1	1112
Annual fuel expenses saved	SF	Euro	240
Harvest cost	K	Euro/kg	164.4
Quantity harvested from greenhouse S	Q_S	Kg	0.0
Quantity harvested from greenhouse C	Q_C	Kg	20900
Additional annual harvest costs	AE	Euro	18078
Maintenance cost of the PSS	MN	Euro	40.9

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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***Table 2.** Financial analysis of the PSS (0.l ha tomato greenhouse)*

Year	C.O.F	D.F (20%)	D.C.O.F	C.I.F	D.C.I.F	D.C.F
1	633.10	0.83	527.37	2002.48	1668.07	1140.69
2	127.92	0.69	88.77	1726.98	1198.52	1109.75
3	633.10	0.58	366.56	1726.98	999.92	633.36
4	127.92	0.48	61.66	1726.98	832.40	770.75
5	633.10	0.40	254.50	2002.48	805.00	550.49
6	127.92	0.34	42.85	1726.98	578.54	535.69
7	633.10	0.28	176.63	1726.98	481.83	305.19
8	127.92	0.23	29.81	1726.98	402.39	372.58
Total	**3044.07**	**3.84**	**1548.16**	**14366.83**	**6966.66**	**5418.50**

NPV at 20% = 6966.66 - 1548.16 = 5418.50. Benefit/cost ratio at 20% = 4.5
C.O.F = cash outflows; D.F = discount factor; D.C.O.F = discounted cash outflows; C.I.F = cash inflows; D.C.I.F = discounted cash inflows; D.C.F = discounted cash flows

IRR

The IRR was not taken into consideration because all the terms of the discounted cash flows are positive (*Table 2*). In this case, the NPV is positive at each value of interest rate, so that the NPV does not cross the interest rate axis and the determination of the IRR is impossible.

Benefit/cost ratio

The evaluation of a project by the benefit/cost ratio method requires acceptance of the project if the ratio is greater than one. The ratio here was 4.5, confirming the NPV method and indicating the profitability of the PSS when it replaces an AFS.

Evaluation of the PSS by variety (Arietta)

The evaluation of the PSS for each tomato variety is similar to that above, which did not distinguish between varieties; the only change concerns the additional annual returns (AR) and the additional annual harvest costs (AE). Financial analysis for each variety is presented in *Table 3* and the financial analysis of Arietta variety is presented in *Table 4*. The determination of the IRR is impossible for all varieties since the terms of the discounted cash flows columns are positive so that the NPV will never be zero.

Evaluation of the DFS

Several parameters were determined before calculation, summarizing the variables included in the case of no distinction between varieties (*Table 5*). As in the case of the evaluation of the PSS, the discount rate was assumed to be 20% and an assisted computer program was used to determine the parameters (NPV, IRR, and benefit/cost ratio). *Table 6* summarizes the discounted cash flow approach.

***Table 3.** Financial analysis of the PSS for the four tomato varieties*

Parameter	Carmello	Arietta	Dombo	Ramy
NPV (Euro)	6675.86	6736.97	3359.73	4901.28
Benefit/cost ratio	5.2	5.3	3.3	4.2

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

Table 4. *Financial analysis of the PSS (0.1 ha tomato var. arietta greenhouse)*

Year	C.O.F	D.F (20%)	D.C.O.F	C.I.F	D.C.I.F	D.C.F
1	640.90	0.83	533.87	2353.90	1960.80	1426.93
2	135.72	0.69	94.19	178.84	1442.41	1348.22
3	640.90	0.58	371.08	2078.40	1203.40	832.31
4	135.72	0.48	65.42	2078.40	1001.79	936.37
5	640.90	0.40	257.64	2353.90	946.27	688.63
6	135.72	0.34	45.47	2078.40	696.26	650.80
7	640.90	0.28	178.81	2078.40	579.88	401.06
8	135.72	0.23	31.62	2078.40	484.27	452.64
Total	**3106.50**	**3.84**	**1578.11**	**15278.67**	**8315.08**	**6736.97**

NPV at 20% = 8315.08 - 1578.11 = 6736.97. Benefit/cost ratio at 20% = 5.3

Table 5. *Base case values for variables included in the model evaluating the PSS*

Variable	Symbol	Unit	Value
The total cost of the DFS	IS	Euro	2914.50
The total cost of the AFS	IC	Euro	362.50
Discount rate	R	%	0.06
Additional annual returns	AR	Euro	1324.29
Fuel price	H	Euro/L	0.19
Fuel consumption in greenhouse C	F_C	1	3.22
Fuel consumption in greenhouse F	F_F	1	15.10
Annual fuel expenses saved	SF	Euro	772.10
Harvest cost	K	Euro/kg	0.01
Quantity harvested from greenhouse F	Q_F	Kg	57.75
Quantity harvested from greenhouse C	Q_C	Kg	52.43
Additional annual harvest costs	AE	Euro	26.64
Annual electricity consumption	EC	Euro	14.27

Table 6. *Financial analysis of the DFS (0.1 ha tomato greenhouse)*

Year	C.O.F	D.F (20%)	D.C.O.F	C.I.F	D.C.I.F	D.C.F
1	3727.50	0.83	3105.01	1686.79	1405.09	-1699.91
2	813.00	0.69	564.22	1324.29	919.09	354.84
3	813.00	0.58	470.73	1324.29	766.76	296.03
4	813.00	0.48	391.87	1324.29	638.31	246.44
5	813.00	0.40	326.83	1686.79	678.09	351.26
6	813.00	0.34	272.36	1324.29	443.64	171.28
7	813.00	0.28	226.83	1324.29	369.48	142.65
8	813.00	0.23	189.43	1324.29	308.56	119.13
Total	**9418.50**	**3.84**	**5547.26**	**11319.30**	**5528.98**	**-18.28**

NPV at 20% = 5528.98- 5547.26= -18.28. IRP = 19.6%. Benefit/cost ratio at 20% = 0.997. Pay-back period = 5 years

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

NPV

The application of the model leads to an NPV of -18.28 euro. Thus, there is no need, in terms of profitability, to replace the AFS by the DFS despite its effectiveness in increasing production and gross returns.

IRR

Here, the conditions of Vafaeipour et al. (2014) for the existence of the IRR are satisfied; the IRR was 0.196, slightly lower than the assumed discount rate.

Benefit/cost ratio

Using the benefit/cost ratio to evaluate the DFS, a ratio of 0.997 was found. This value is less than one, so this system did not add anything when it replaces the AFS. This confirms the results for NPV and IRR, which also showed the ineffectiveness of the DFS compared with the AFS.

Pay-back period

The stream of expected proceeds was not constant from year to year. The pay-back period was determined by adding up the proceeds expected in successive years until the total was equal to the original outlay. Subtracting cash inflows from cash outflows (*Table 6*) showed that investment will be covered by the returns after 5 years, which is the pay-back period.

Evaluation of the DFS by a variety

The only changes concerned the AR and the AE. Financial analysis for each variety is presented in *Table 7* and the financial analysis of Arietta variety is presented in *Table 8* in which NPV, IRR, benefit/cost ratio and pay-back period are illustrated. Thus, it is profitable to replace the AFS by the DFS only for arietta; NPVs for the other varieties were negative, varying between -1162.29 euro (dombo) and -223.85 euro (ramy), the IRRs were lower than the assumed discount rate (20%), and the benefit/cost ratios were less than one. The implementation of the DFS was also characterized by a long pay-back period, more than 8 years for dombo.

Risk and uncertainty

Because the PSS appeared to be an efficient method for greenhouse heating, NPVs were estimated by assuming changes in certain critical factors expected to affect the results such as discount rate, fuel price, and product price. The analysis did not distinguish between varieties.

Table 7. *Financial analysis of the DFS for the four tomato varieties*

Parameter	Carmello	Arietta	Dombo	Ramy
NPV (Euro)	-473.70	1960.10	-1162.29	-223.85
IRR %	10	62.5	-5.5	15.3
Benefit/cost ratio	0.91	1.35	0.79	0.96
Pay-back period (year)	6	3	>8	5

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

Table 8. *Financial analysis of the DFS (0.1 ha tomato var. arietta greenhouse)*

Year	C.O.F	D.F (20%)	D.C.O.F	C.I.F	D.C.I.F	D.C.F
1	3739.78	0.83	3115.26	2169.48	1807.17	-1308.09
2	825.31	0.69	572.77	1806.98	1254.04	681.27
3	825.31	0.58	477.86	1806.98	1046.24	568.38
4	825.31	0.48	397.80	1806.98	870.96	473.16
5	825.31	0.40	331.78	2169.48	1045.69	713.91
6	825.31	0.34	276.48	1806.98	605.34	328.86
7	825.31	0.28	230.26	1806.98	504.15	273.88
8	825.31	0.23	192.30	1806.98	421.02	228.73
Total	**9517.01**	**3.84**	**5594.51**	**15180.80**	**7554.61**	**1960.10**

NPV at 20% = 7554.61 - 5594.51 = 1960.10. IRR = 62.5%. Benefit/cost ratio at 20% = 1.35. Pay-back period = 3 years

Effect of the discount rate

To determine the effect of the discount rate on the NPV, decreases, and increases in this discount rate were examined. Thus, the NPV is related to the discount rate: when this rate decreased by 50%, the NPV increased by 39%; the NPV increased by 6% when the discount rate decreased by 10%. However, when the rate increased by 10%, the NPV decreased by 5.5% and a 50% increase in the discount rate caused a 24% decrease in the NPV. Such changes in the interest rate are more significant when the percentages are lower (*Fig. 1*).

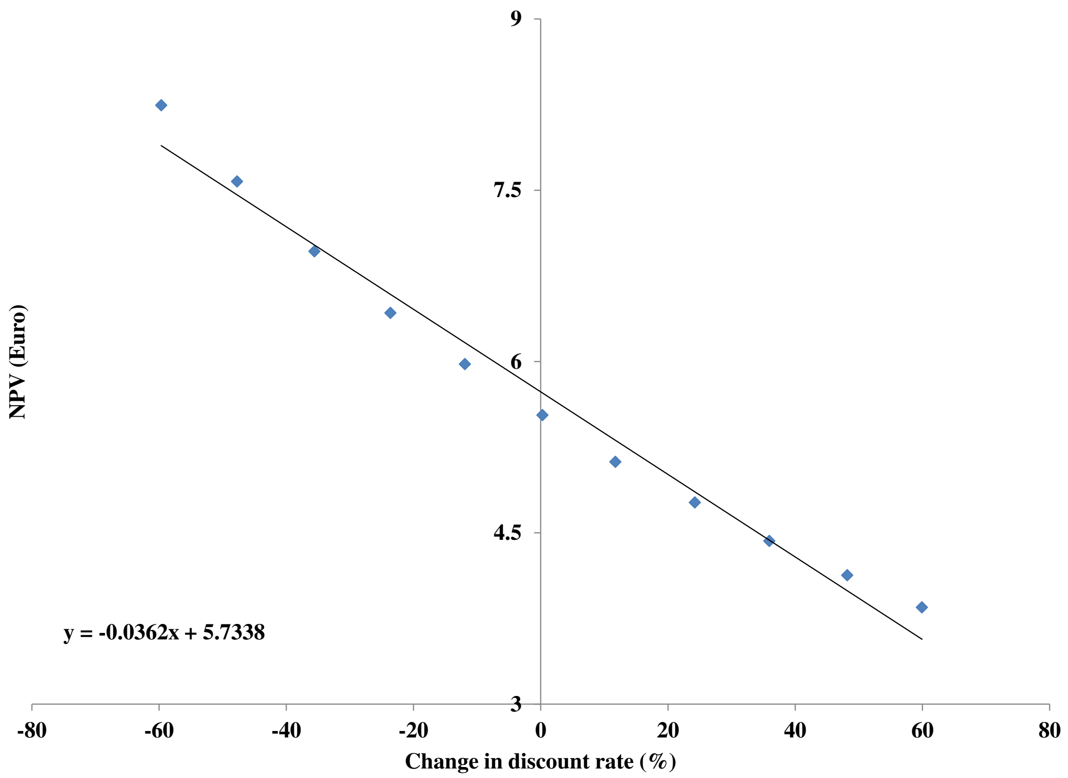

Figure 1. *The effect of a change in discount rate on the NPV for the PSS*

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

Effect of the fuel price

The effect of the fuel price on farmer income with the PSS instead of the AFS is summarized in *Figure 2*. The effect of a change in the price of fuel on NPV is not important: an increase of 10% caused an increase of 1%, and a decrease of 10% caused a decrease of 1%. The relationship between fuel price and NPV is linear so that the NPV increased by 5% when the fuel price increased by 50%. The effect of fuel price on NPV is considerable when a DFS is used. In this case, an increase in fuel price affected dramatically and negatively the benefits obtained by farmers utilizing the DFS: an increase of 10% caused a decrease of more than 1500% in the NPV.

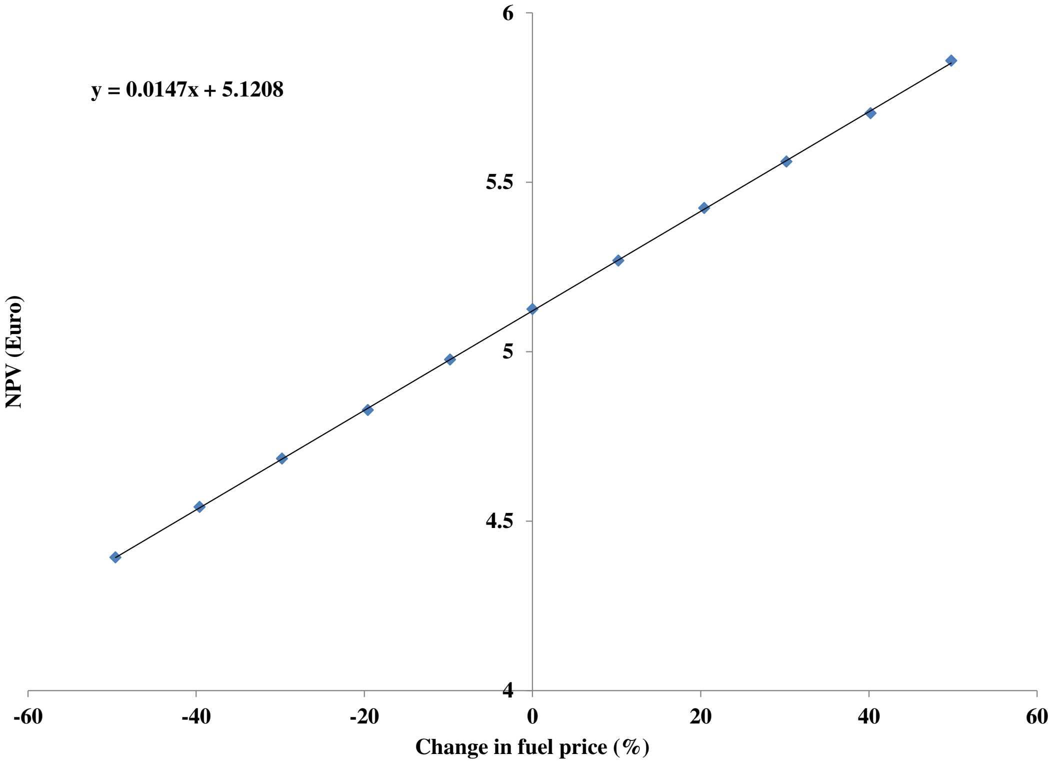

Figure 2. *The effect of a change in fuel price on the NPV for the PSS*

Effect of the product price

The product price is an important factor which affects the NPV. To demonstrate this effect, the NPV was calculated for decreases and increases in price (*Fig. 3*). Product price has a considerable effect on NPV since an increase of 10% caused an increase of 11% in the NPV. There is a linear relationship between product price and NPV, so that if the price increases by 50%, the NPV increases by 55%.

Generally, renewable energy sources require a large initial investment, but they have relatively low operating costs. The PSS significantly reduced energy consumption. The reduction in fossil fuel needs was from 80% compared with AFS to more than 2000% compared with the DFS. For an initial investment, the PSS is only 17% of the cost of the DFS, and slightly higher than the AFS. Besides its low initial investment, the PSS also has the advantage of low annual costs (13% that of the DFS and 63% that of the AFS).

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

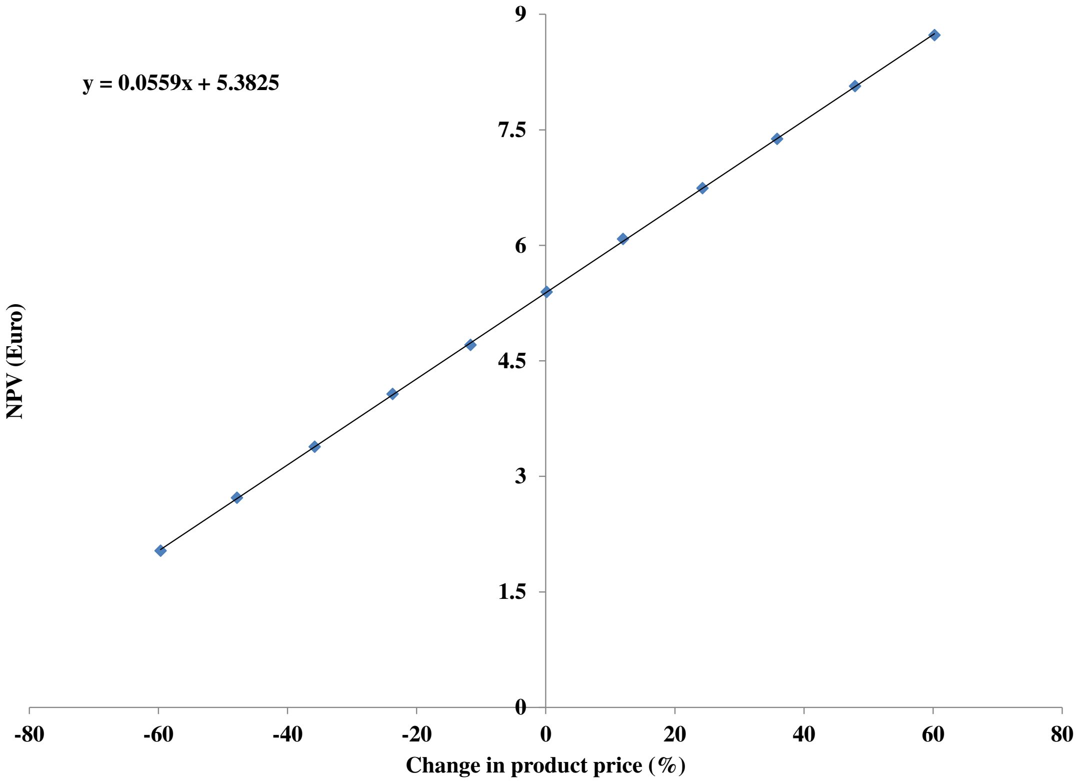

Figure 3. *The effect of a change in product price on the NPV for the PSS*

Discussion

The effect of heating systems on production and gross returns

The effect of the heating methods was that greenhouse C (AFS) had the lowest annual average production for tomato crop, greenhouse F (DFS) was 10% for tomatoes, greenhouse S (PSS) had the largest annual average production for both crops. The low production of greenhouse C was due to inefficient heating which did not maintain a uniform temperature and which may have damaged the plants (Wright, 2012).

The heating systems permitted early production which led to higher market prices. The average market price for crops grown in greenhouse C was the lowest particularly because of the competition from other field crops; since the harvest in this greenhouse started at the end of May for tomatoes (Dannehl et al., 2012).

Furthermore, gross return of tomatoes in greenhouse S was 23% greater than that in C, in agreement with Esen and Yuksel (2013), who stated that gross return of tomatoes in greenhouse with the PSS exceeds that in the control by an average of 22%, gross return in greenhouse F exceeded that of C by 19%. However, in term of production, those percentages were only 16% and 10% respectively. Gross return of melons in greenhouse F was 50% greater than that of C, with 44% more production; greenhouse S was 66% and 60% more in gross return and production, respectively. These results show how early production allows farmers to exploit the higher market prices; the earlier the production, the higher the market price achieved, this accords with Gandhi and Zhou (2014), who stated that early production permits to increase dramatically gross return.

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

Evaluation of the considered heating systems

The analyses of production and gross returns show that the PSS and the DFS should be accepted over the AFS for greenhouse heating. This acceptance should be affirmed by a financial analysis which permits the adoption or the rejection of technological innovation, which is determined not by its contribution to the increase of production or to the increase of output value, but by the magnitude of the net benefits that can be provided (Cerón-Palma et al., 2012). It was clearly seen that it is on the profit of the farmers to replace the AFS by the PSS, since the NPV is positive and equal to 5500 euro in the case of tomatoes over an 8-year period (2012-2014), with 2012 as the base year.

Presented results indeed manifest PSS's importance as a significant contributor to farmer's income, thus a farmer with a 0.1 ha greenhouse cultivated with early spring tomatoes, will add annually about 750 euro just by utilizing the PSS and the greater the area cultivated, the greater the income added, thus a farmer who has five 0.1 ha tomato greenhouse will add approximately 3650 euro/year to his net benefits just by introducing this renewable energy-saving method (Jamel et al., 2013; Nabat et al., 2015).

As well as the NPV method, the benefit/cost ratio which was greater than 1 for the tomato crop indicates the importance of this system, the pay-back period is so short (less than one year) avoiding the problem of risk and confirming the decision provided by the previous discounted techniques (NPV and benefit/cost ratio) this is especially due to the low initial investment and fuel consumption characterizing PSS. The IRR method was not taken into consideration because this investment project does not meet the conditions imposed by Vafaeipour et al. (2014).

Besides its effectiveness in term of income and net benefits, the PSS presented the advantage of an energy-saving method which is very important particularly for a country like Greece, which is characterized by its heavy dependence (more than 70%) on primary energy from imported oil (Mourmouris and Potolias, 2013) and (Markaki et al., 2013), thus the reduction in fuel consumption was more than 20 times when compared with the DFS in the case of tomatoes.

On the other hand, the implementation of a DFS for heating tomato greenhouses against an AFS is not profitable: NPV was negative, IRR was lower than the assumed discount rate, benefit/cost ratio was less than one and pay-back period of 5 years is relatively long compared to the project life, at a much higher risk characterizing this system (Mehrpooya et al., 2015). The exception of arietta variety which had a positive NPV and an IRR greater than 20% and a relatively short payback period is especially due to its considerable difference in term of production between greenhouses F and C, which permitted to produce higher additional annual returns (AR) (Mekhilef et al., 2013).

It is important to note that despite its effectiveness in term of production and gross returns, the DFS did not provide any benefits for farmers when it replaces the AFS, this is particularly due to its relatively high initial investment and its large fuel consumption (Sueyoshi and Goto, 2015).

Sensitivity analysis

Sensitivity analyses were performed by varying certain critical factors such as discount rate, fuel price and product price over the range established, keeping the other

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DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

variables at base case levels, this showed how the value of the NPV changes with variations in the value of any variable. Product price was the most important factor affecting the NPV: a price increase of 10% caused an increase of 11% in the NPV for tomatoes, and there was a linear relationship between product price and NPV, in an agreement with Vafaeipour et al. (2014), who stated that product price appears to cause the greatest effect on estimated income benefits. Thus farmers should choose the most appropriate heating system to have early production and therefore higher prices and benefits (Orgerie et al., 2014).

However, an increase in fuel price decreases income significantly when a DFS is used against an AFS; an increase of 10% caused a decrease of more than 1500% in the NPV of tomatoes, increasing risk associated with the implementation of the DFS because fuel prices generally increase with time, especially for oil-importing countries such as Greece (Markaki et al., 2013).

The NPV is also related to the discount rate: an increase of 10% caused a decrease of 6% in the NPV's of tomatoes. It is also important to note that a change in the discount rate is more significant in the case of negative change: for example, when R increased by 50% (R = 30%) NPV decreases by 24%, but a decrease of 50% (R = 10%) caused an increase of 39% in the NPV, giving an advantage to farmers from countries with low-interest rates (Vadiee and Martin, 2014).

Risk and uncertainty associated with renewable energy

In contrast to the use of fossil fuel energy sources, renewable energy sources generally require a large initial investment because of the large initial costs. This is especially important in countries which have high-interest rates (Mohammed et al., 2014). The relatively large initial investment required means that such projects will have relatively long pay-back periods and therefore, they involve much higher risk than that associated with fossil fuels (Castilla, 2013).

Renewable energy projects also face uncertainty regarding possible changes in economic conditions between the time the initial investment takes place and the length of the project's useful life. Of course, there is a wide range of risk associated with different renewable energy sources. Economic uncertainty is mainly due to possible changes in future energy prices. For example, few who anticipated the decrease in crude oil prices in 1985 and 1986, and many analysts were surprised to discover that 1986 real oil prices were at the pre-1973 level (Xu et al., 2014).

PSS differs from other renewable energy systems in that it has a low initial investment (17% of that of conventional DFS), thus indicating its advantage in avoiding the risk of high initial investment which is generally associated with renewable energy (Mourmouris and Potolias, 2013). In term of operating costs, the PSS has the advantage of renewable energy sources, which are characterized by the low operating annual costs. Thus by the low initial investment and the low annual costs, it may be useful to encourage the use of the PSS for greenhouse heating (Foteinis and Tsoutsos, 2017).

Conclusions

The evaluation of the current system in comparison with the most common non-renewable greenhouse heating system revealed its importance not only as an energy-saving technology, with considerable reductions in fossil fuel consumption, but also as a significant contributor to farm income. The implementation of the passive solar system

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

instead of the traditional anti-frost one has dramatically increased production and gross returns particularly through its promotion of early production. The financial analysis which is based on different methods used as a basis on which to assess the investment profitability of a project (NPV, IRR, benefit/cost ratio and pay-back period) demonstrated that the adoption of the passive solar system significantly increases the earnings of greenhouse farmers. This study also revealed the advantage of this system in avoiding the risk of high initial investment which generally associated with renewable energy systems, and sensitivity analysis indicated the importance of product price in affecting benefits, thus early production allows farmers to exploit the higher market prices. This system is appropriate for any region whose climatic conditions are sufficiently similar to those of northern Greece. Financial analysis also revealed that investment in a diesel-fired furnace system did not provide any considerable benefits when it replaces an anti-frost system; its inefficiency is particularly due to its relatively high initial investment and its large fuel consumption.

For further consideration where the costs are not the only issue, the major obstacle to the wide application of renewable energy sources is higher risk and, uncertainty and not simply the cost of the energy utilized. Therefore, the main role of the public in such projects should be directed toward sharing some of the risk involved in construction and the operation.

Acknowledgements. This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. D-021-155-1440. The authors, therefore, acknowledge with thanks, DSR technical and financial support.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44314446

EFFECTS OF DROUGHT STRESS ON ENDOGENOUS HORMONES AND OSMOTIC REGULATORY SUBSTANCES OF COMMON BEAN (*PHASEOLUS VULGARIS* L.) AT SEEDLING STAGE

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(Received 21st Jan 2019; accepted 20th Feb 2019)

Abstract. Drought is an important factor that restricts the growth of common bean. In the present study, Longyundou 10 (non-drought-resistant variety) and Longyundou 17 (drought-resistant variety) were selected to analyze the effects of drought stress on endogenous hormones and osmotic regulatory substances of common bean at seedling stage. Under mild drought stress, the ABA accumulation and response of drought-resistant Longyundou 17 were stronger than that of no drought-resistant Longyundou 10. The effects of CTK and IAA on drought resistance of common bean at seedling stage are complicated. Under normal water condition, the soluble protein content of Longyundou 10 was slightly higher than that of Longyundou 17. However, there was no significant difference in the degree of inhibition between Longyundou 10 and 17. Therefore, we believe that the soluble protein of common bean has little effect on resisting the threat of drought stress. The content of proline and soluble sugar in common bean responded strongly to drought stress, these two osmotic regulatory substances in drought-resistant varieties were higher than those in drought-resistant varieties. Therefore, ABA, proline and soluble sugar content can be used as important indicators for breeding of drought-resistant Varieties of common bean at seedling stage.
Keywords: *common bean, drought stress, ABA, proline, soluble sugar*

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most popular edible bean widely cultivated in more than 90 countries and regions around the world (Acostagallegos et al., 2007). Common bean is rich in protein, unsaturated fatty acid, dietary fiber and other nutrients (Kuto et al., 2003; Yang et al., 2016). Drought stress is not only a hot research topic, but also a common adversity for crop growth. It is also a decisively restrictive factor for sustainable agricultural development which always threatens food security (Schindler et al., 2007). With the change of global climate, the drought situation in common bean producing areas is getting worse and worse. According to statistics, 60% of common bean producing areas in the world suffer from drought, and the yield reduction in Kenya and South Africa is as high as 80% (Beebe et al., 2008; Franca et al., 2000).

Under drought conditions, it is a complex physiological change for plants to respond to adversity. Many researches reported that drought causes responses of endogenous

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DOI: http://dx.doi.org/10.15666/aeer1702_44474457

hormones in plants, which regulate and control plant physiological activities to adapt to drought stress (Yordanov et al., 2000; Sauter et al., 2001; Farooq et al., 2009). Abscisic acid (ABA), indole acetic acid (IAA) and cytokinin (CTK) are drought stress response hormones (Bartels et al., 2005; Acharyaet a., 2009; Tang et al., 2005), which can regulate plant physiological and biochemical processes and enhance drought resistance of crops. ABA exists in almost all higher plants and participates in many physiological processes during plant growth and development, such as stomatal movement, fruit maturation, seed dormancy and germination (Cutler et al., 2010). Under drought stress, ABA concentration in plants increased, which promote stoma to close to reduce transpiration loss, thereby to enhance plant drought resistance (Schroeder et al., 2001; Verslues et al., 2005).

IAA is a kind of plant endogenous hormones containing an unsaturated aromatic ring and an acetic acid side chain. Most IAA is concentrated in tissues and organs with strong division and growth metabolism, such as in root tips, young leaves, developing seeds and fruits. Drought stress can affect the change of IAA concentration. The mechanism of IAA drought regulation is complex. CTK is a kind of adenine derivative. Under drought stress, the regulation mechanism of CTK varies in drought degrees and crops. Osmotic regulation is the physiological mechanism which plants are adapted to water stress. Under water stress, plant cells can actively change the content of osmotic regulatory substances of small molecules or their existing state in plants to regulate osmotic potential in vacuoles and ensure normal growth and development of plants. Soluble sugars, proline and soluble proteins are considered to be important osmotic regulatory substances in plants, which can respond positively to drought stress. It has been reported that under drought stress, the proline content of wheat leaves increased and the soluble protein content decreased (Bai et al., 2007). Proline and soluble sugar are the main osmotic regulatory substances of common bean, which can maintain the leaf water balance (Li et al., 2014). Under drought stress, the content of osmotic regulatory substances in common bean was different among different drought-resistant varieties (Rosales et al., 2012; Lizana et al., 2006).

Drought is the second major factor restricting the production of common bean besides diseases (Schneider et al., 1997). Therefore, drought resistance of common bean has become one of the important indexes in breeding and production (Li et al., 2014; Cortés et al., 2012). In the present study, the changes of endogenous hormones and osmotic regulatory substances in common bean were studied in order to provide technical guidance and theoretical support for efficient production and breeding of drought-resistant of common bean varieties.

Materials and methods

Both Longyundou 10 (non-drought-resistant variety) and Longyundou 17 (drought-resistant variety) were provided by Crop Breeding Research Institute of Heilongjiang Academy of Agricultural Sciences, Heilongjiang province, China.

The experiment was carried out in an artificial climate chamber with humidity of 60%+10%, 27 °C under 16-h light conditions, 16 °C under 8-h dark conditions. The experimental plots were completely randomized with three replications and three water treatments at each site. Mild drought stress (LS): Relative water content of soil (RWC) = 60% + 5%; severe drought stress (SS): RWC = 45% + 5%; control: RWC = 75%+5% under normal water condition. Seeds were planted in plastic pots

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filled with a potting mixture of 3 perlite : 3 vermiculite : 4 nursery soil. Each plastic pot contained 2 kg potting mixture and 3 seeds. From sowing to the appearance of the second true leaf, the soil was controlled under normal water condition (RWC = 75% + 5%). When the second trifoliate leaves are fully developed and mature, the soil moisture content of each treatment was monitored and controlled to reach the set target by weighing method every morning and evening.

On the 2nd, 4th, 6th, 8th, 10th, 12nd, 14th and 16th days after drought stress treatment, the parietal lobe of the second mature trifoliate were taken from 9:00 to 9:30 a.m. The contents of ABA, CTK, IAA, free proline, soluble sugar and soluble protein were determined by ELISA. All the kits are provided by Shanghai Enzyme-linked Biotechnology Co., Ltd. Microsoft Excel 2010 and SPSS 18.0 software are employed for data collation and analysis.

Results

Effects of drought stress on ABA content of common bean

ABA is a stress response hormone, which can accumulate rapidly when stress occurs and play a significant role in plant response to stress. As shown in *Figure 1,* at the beginning of severe drought stress, ABA content of Longyundou 17 increased abnormally. In addition, the accumulation of ABA content in common bean leaves showed a single-peak curve at seedling stage. The peaks of ABA content of Longyundou 17 in mild drought stress, severe drought stress and control treatments were 941.81 ng g^{-1}, 829.97 ng g^{-1} and 747.16 ng g^{-1}, respectively. The peaks of ABA content of Longyundou 10 in mild drought stress, severe drought stress and control treatments were 849.33 ng g^{-1}, 731.04 ng g^{-1} and 724.58 ng g^{-1}, respectively.

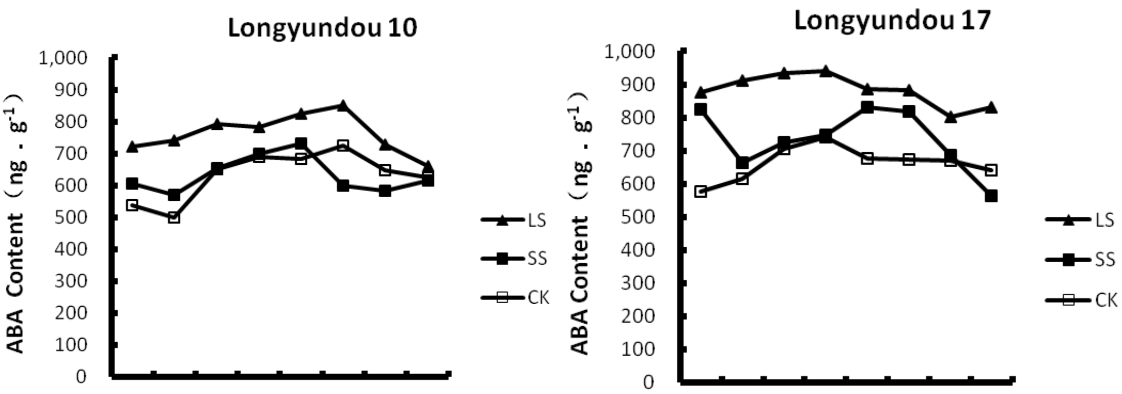

Figure 1. *Changes of ABA content in common bean leaves under drought stress*

Under mild drought stress, the accumulation of ABA in leaves increased significantly, but with the increase of water stress time, the content of ABA in severe drought stress began to decrease and accumulated slowly, which indicated that mild drought stress stimulated the ABA response. Common bean could alleviate the effect of water stress through the accumulation of ABA in a certain period of time, but severe drought stress can destroy the formation and accumulation of ABA.

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ABA content in leaves of drought-resistant variety Longyundou 17 was slightly higher than that of Longyundou 10. Under drought stress, the increase of ABA content was also greater than that of common bean varieties with weak drought resistance. On the 12th day after severe drought treatment, ABA content of Longyundou 10 was lower than that of normal water treatment, while Longyundou 17 decreased on the 16th day after severe drought treatment, indicating that the accumulation and response of ABA under drought stress were stronger than those of non-drought resistant varieties.

Effect of drought stress on CTK content of common bean

As shown in *Figure 2,* under normal water condition, CTK content fluctuated in the leaves of common bean at seedling stage, but there is no distinct tendency. Under drought stress, CTK content increased first, then decreased, and then increased again. The performance of CTK content was different among different varieties, which indicated that the role of CTK in drought resistance of common bean at seedling stage is complex.

There were no significant differences in CTK content of Longyundou 10 among drought stress treatments, but the change tendency was slightly different. When Longyundou 10 was treated with severe drought stress for 2-4 days, the content of CTK content increased significantly, with an average increase of 50.6% compared with the control. After 6 days of treatment, CTK content declined sharply, slightly lower than the control treatment on the 8th day, and increased again after 12 days of treatment. Under mild drought stress, the CTK content in Longyundou 10 was significantly higher than that under normal water condition after 2nd-6th and 16th days of treatment, but increased slightly after other treatments, even decreased slightly on the 8th day of treatment.

Except for the 4th day after treatment, the CTK content of Longyundou 17 under severe stress treatment was higher than that in mild stress treatment. The CTK content under drought stress was higher than that under no stress treatment, indicating that drought stress stimulated the CTK production in Longyundou 17.

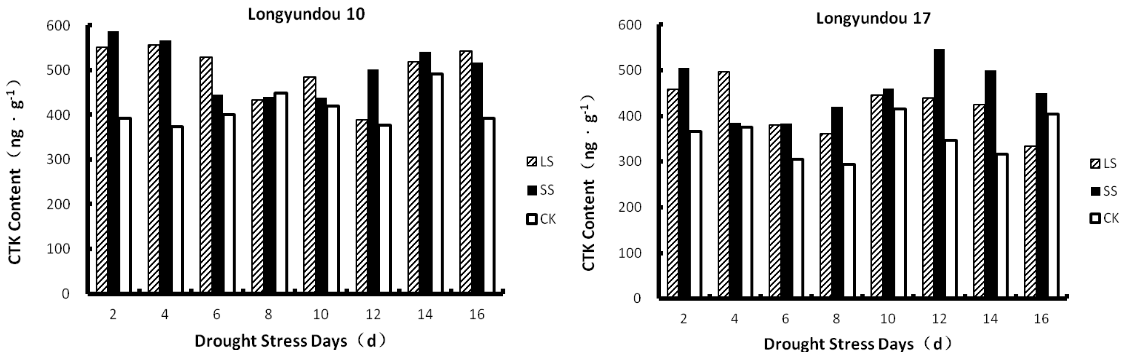

Figure 2. *Changes of CTK content in common bean leaves under drought stress*

Effect of drought stress on IAA content of common bean

As shown in *Figure 3,* IAA content of common bean showed a downward trend of fluctuations under drought stress. The change of IAA content under drought stress was more complex. The changes of IAA content under both severe and mild drought stress were alternate, and no drought stress showed greater impact on IAA content. At the initial stage

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of drought stress, IAA content was higher than that under no stress. However, with the prolongation of drought stress time, IAA accumulation was inhibited and content began to decline. Especially from the 8th day after drought treatment, IAA content of Longyundou 10 was lower than that of control. The IAA content of Longyundou 17 was slightly higher than Longyundou 10 in all treatments.

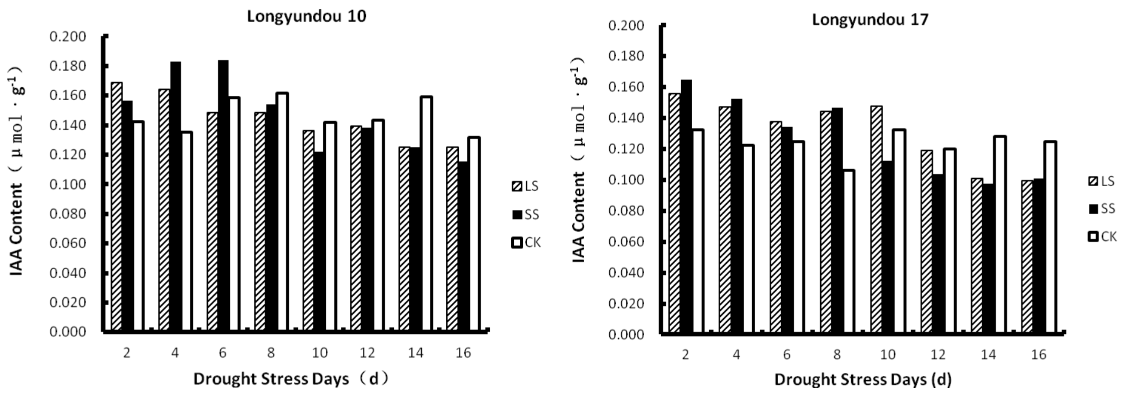

Figure 3. *Changes of IAA content in common bean leaves under drought stress*

Effects of drought stress on free proline content of common bean

In *Figure 4,* under different degrees of drought stress, the free proline content in common bean leaves increased significantly with the maximum increase more than 1.6 times than that under non-drought stress, while free proline accumulation fluctuated under mild drought stress.

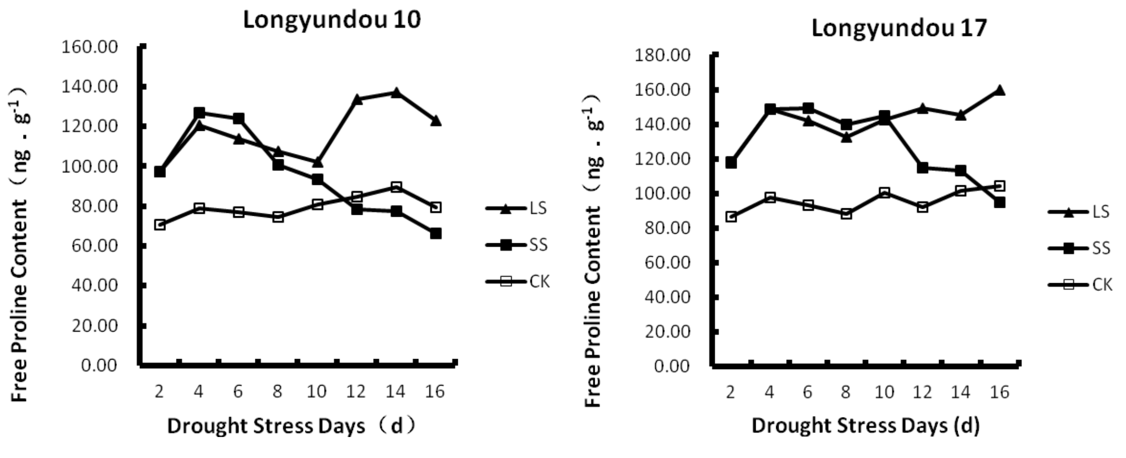

Figure 4. *Changes of free proline content in common bean leaves under drought stress*

Under severe drought stress, free proline content tends to increase at first and then decrease. At the beginning of drought treatment, the content of proline under severe drought stress was higher than that under other water treatments. With the prolongation of drought stress time, the accumulation of proline under severe drought stress was inhibited and the content of proline decreased. Longyundou 17 maintained high free proline accumulation within 10 days under severe drought stress, but began to decrease 12 days after treatment, which was lower than that under mild drought stress. The free proline content of Longyundou 10 under

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mild stress was higher than that under severe stress after 8 days of drought treatment, which indicated that Longyundou 17 could maintain higher free proline accumulation for a long time under severe stress than Longyundou 10, and its osmotic regulation ability was also higher than Longyundou 10. The free proline content of Longyundou 17 was higher than Longyundou 10 in all treatments, and the increase of free proline under drought stress was also higher than Longyundou 10, indicated that free proline was an important osmotic regulatory substance of drought resistance of common bean.

Effect of drought stress on soluble sugar content of common bean

Figure 5 shows that the accumulation of soluble sugar content in common bean leaves showed a single peak curve, and the peak appeared at different times according to different varieties under drought stress. The peaks of soluble sugar content of Longyundou 17 appeared on the 10th and 8th day after severe drought stress, and the peaks were 6.52 mg g^{-1} and 5.61 mg g^{-1} after mild drought stress, respectively, which were 2.2 times and 1.84 times higher than those under normal water condition. Under severe and mild drought stress, the peaks of soluble sugar content of Longyundou 10 appeared on the 8th day after stress, and the peak values were 4.63 mg g^{-1} and 3.78 mg g^{-1}, respectively, which increased 1.6 times and 1.3 times as much as that under normal water condition. The soluble sugar content of Longyundou 17 was higher than that of Longyundou 10 at all stages and treatments. In the early stage of drought stress, the soluble sugar content of common bean increased significantly. With the prolongation of drought time, the accumulation of soluble sugar was seriously inhibited, especially under severe drought stress, the content of soluble sugar decreased sharply. The decrease of soluble sugar content in Longyundou 17 occurred 14 days after drought treatment with the soluble sugar content decreases by 16.9% and 22.5% under severe and mild drought stress, respectively, compared with those in normal water. Under drought stress, the content of soluble sugar in Longyundou 10 decreased earlier than that in Longyundou 17. After 10 days of severe drought stress, the content of soluble sugar in Longyundou 10 was significantly lower than that in normal water.

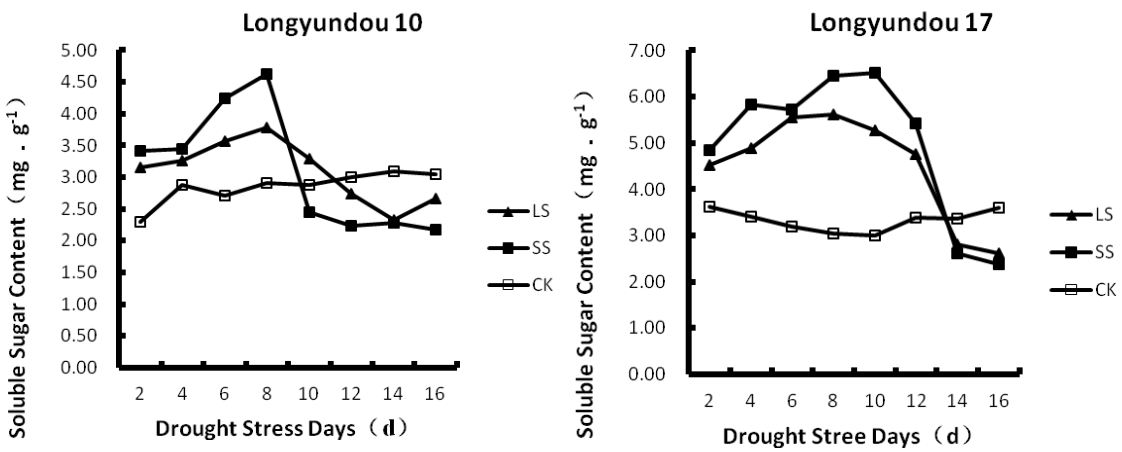

Figure 5. *Changes of soluble sugar content in common bean leaves under drought stress*

Effect of drought stress on soluble protein content of common bean

In *Figure 6,* the accumulation of soluble protein in common bean leaves at seedling stage showed a fluctuating upward trend under normal water conditions. The

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accumulation dynamics of soluble protein showed irregular changes under drought stress. At the initial stage of drought stress, soluble protein content increased, especially under severe drought stress. However, with the increase of drought stress time, the accumulation of soluble protein in common bean was inhibited, and the content was lower than that in normal water condition. Under normal water condition, the soluble protein content of Longyundou 10 was slightly higher than that of Longyundou 17.

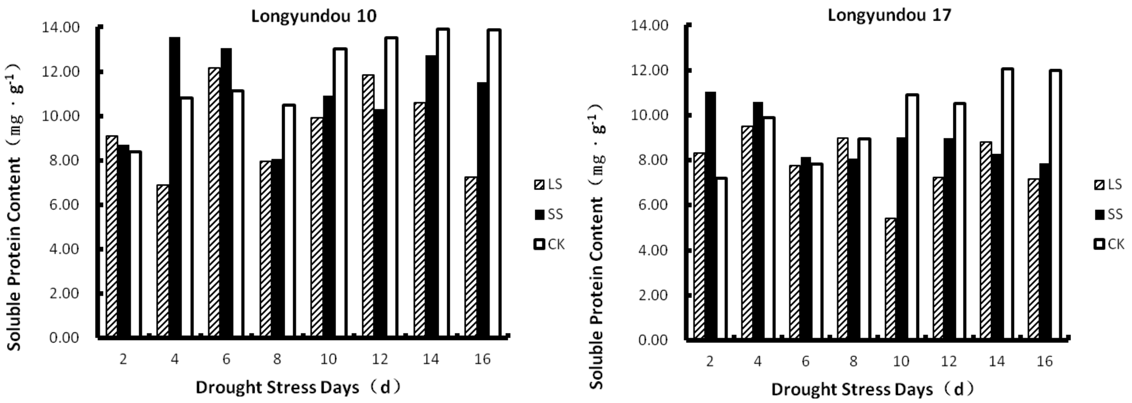

Figure 6. *Changes of soluble protein content in common bean leaves under drought stress*

Discussion

Changes of endogenous hormones in common bean under drought stress

Many researches have proved that plant endogenous hormones play an important regulatory role under drought stress (De Ollas et al., 2016; Sharp et al., 2004; Kurepin et al., 2015; Forner-Giner et al., 2011). Different crops and their ABA, IAA and CTK contents have different response mechanisms. Plant hormone ABA plays an important regulatory role in plant growth, development, stress resistance, stomatal movement and gene expression (Neill et al., 1999).

In the present study, ABA accumulation in common bean leaves increased significantly under mild drought stress, indicating that mild drought stress stimulated the ABA response, but severe drought stress would destroy the formation and accumulation of ABA. The ABA content in drought resistance variety Longyundou 17 was significantly higher than that in non drought-resistant variety Longyundou 10. Therefore, we believe that ABA can be used as important drought resistance indexes of common bean at Seedling Stage.

CTK is formed and accumulated in the root system, which is transported to the the aboveground part. At the same time, CTK is formed in large quantities in the root system under drought stress. In our study, CTK increased in the leaves of common bean seedlings under drought stress, especially in common bean varieties with stronger drought resistance. Drought stress stimulated the CTK production in Longyudou 17, suggested that moderate drought stress exercise is helpful to the stress-resistant growth of common bean.

Endogenous IAA is synthesized by plant apical tissues and growing leaves as a growth hormone. Under drought stress, the change of IAA content increases in some plants (Zhao et al., 2012; Mei et al., 2017), while decreases in others (Zhang et al., 2018; Chang et al., 2012). In the present study, IAA content in common bean leaves

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DOI: http://dx.doi.org/10.15666/aeer1702_44474457

decreased in a fluctuation way under drought stress. IAA content alternate change under severe stress and mild stress. No change was observed in IAA content under two drought stresses, indicating that the mechanism of IAA response to drought stress was complex, which was consistent with previous studies.

Changes of osmotic regulatory substances in common bean under drought stress

Osmotic regulation is not only an important way for plants to resist drought adversity, but also an important physiological mechanism for plants to induce protective response under drought stress (Cabuslay et al., 2002; Alla et al., 2012; Moustakas et al., 2011). Under drought stress, osmotic regulatory substances such as proline and soluble sugar accumulate constantly in plant cells, reduce cell osmotic potential and improve water retention capacity through response, thus enhance drought resistance of crops. However, there are some differences in the accumulation of osmotic regulatory substances among different crops (Osmotic et al., 2010; Trung et al., 2018). In the present study, two common bean cultivars with obvious difference in drought resistance were chose to analyze osmotic regulatory substance changes under drought stress. These results showed that different osmotic regulatory substances had different responses. Among them, the contents of soluble sugar and proline were correlated with the drought resistance of cultivars. The drought-resistant varieties had higher expression level under drought stress compared with non drought-resistant varieties, but the soluble protein did not show the same conclusion.

Conclusion

Endogenous hormones and osmotic regulatory substances showed differences in different degrees of drought stress and among common bean varieties. When common bean was subjected to mild drought stress, the endogenous hormones and osmotic regulatory substances of common bean showed a positive response. The contents of ABA, proline and soluble sugar in drought-resistance common bean varieties were higher than those in varieties with weak drought resistance. Therefore, these substances could be considered as important indicators for drought resistance breeding.

The accumulation of endogenous hormones and osmotic regulatory substances in common bean was severely affected when drought stress lasted for a long time. Although common bean could respond to drought stress, the accumulation of related regulatory substances would be inhibited with the persistent threat of drought stress. This also indicated that the tolerance of common bean to drought stress was weak at seedling stage. Therefore, it should pay special attention to water management in seedling stage in the production of common bean.

In the present study, we discussed the response mechanism of endogenous hormones and osmotic regulatory substances of common bean under drought stress, and found the response law. We will further study the response mechanism at molecular level, especially for ABA, proline and soluble sugar, which can be used as physiological indicators of drought resistance of common bean, and explore and utilize related genes to change the physiological and biochemical characteristics of common bean for the improvement of drought-resistance of common bean.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_44474457

Acknowledgements. This research was supported by China Agriculture Research System (CARS-09); National Infrastructure for Crop Germplasm Resources (NICGR2018-024) and Protection and Utilization of Crop Germplasm Resources of Ministry of Agriculture (2130135).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer1702_44474457

DETERMINING THE INTERACTIONS OF BLACK PINE NET PRIMARY PRODUCTIVITY AND FOREST STAND PARAMETERS IN NORTHERN TURKEY

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(Received 22nd Jan 2019; accepted 22nd Feb 2019)

Abstract. Net primary productivity (NPP) is a vital dataset to assess carbon cycling, carbon budget and interpreting global warming. There are many approaches to calculate NPP, and Carnegie-Ames-Stanford approach (CASA) is one of the most popular approaches that was applied in this study. Black pine forest NPP was calculated with the CASA model in a transection zone between humid black sea and dry middle Anatolia region of Turkey for the year of 2016. Model parameters and homogeneity were tested with one-way ANOVA. Results was showed that annual NPP values were varied from 194 to 1213 (g C m^{-2} $year^{-1}$) for pure black pine stands. Model validation was made with stand increment, growing stock, and stand carbon values. Correlation co-efficiencies were obtained to be 0.92 and 0.85 respectively. It was found that NPP was higher in young stands where the mass accumulation potential was higher than areas, where crown closure was between 11% and 70%. According to this study, young stands should be established in the forests that were operated with the highest NPP objective. NPP models that can be used on a global scale is required intense data and time consuming. In addition, it has been determined that mechanical models which are allowed more practical calculation and can be used with the stand parameters easily.
Keywords: *black pine ecosystem, CASA model, forest productivity, stand attributes, forest characterization*

Introduction

Net primary productivity (NPP) is an important indicator on terrestrial carbon cycle from local to global scales. It is the net amount of carbon the plant cover receives after the photosynthetic activity. The sum of autotrophic respiration and NPP is gross primary productivity (GPP). GPP is a parameter that is not directly measured and also measurement of autotrophic respiration is laborious (Gower et al., 1999; Berberoğlu et al., 2007; Ardö, 2015; Chen et al., 2016; Wang et al., 2018).

NPP is an important key variable in terms of carbon trading, which was established by 180 countries in 1997 Kyoto Protocol to reduce carbon emissions and global warming. Countries that has high carbon emissions, have been allowed to purchase more carbon dioxide emissions to the atmosphere from the countries with lower carbon emissions thanks to this agreement (Dong and Whalley, 2010; Klein et al., 2016). Forests are our one of the most efficient weapon in the battle with global warming and climate change. NPP can be used as indicator and control variable on this issue. It will be right strategy to cultivating forests with maximum NPP in this struggle in appropriate regions according to the ecological requirements.

There are many models that can be used to calculate NPP. These models are divided into three categories (Cramer et al., 1999; Schloss et al., 1999; Ruimy et al., 1999). First group is based on satellite data such as CASA (Potter et al., 1993), GLO-PEM (Prince, 1991),

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SDBM (Knorr and Heimann, 1995), TURC (Ruimy et al., 1996) and SIB2 (Sellers et al., 1996). Second group is based on seasonal biogeochemical fluxes such as HRBM3.0 (Esser et al., 1994), CENTURY4.0 (Parton et al., 1993), TEM4.0 (McGuire et al., 1995) and SILVAN2.2 (Kaduk and Heimann, 1996). Third group is based on seasonal biogeochemical fluxes and vegetation structure such as BIOME3 (Haxeltine and Prentice, 1996), DOLY (Woodward et al., 1995) and HYBRID3.0 (Friend, 1995).

Spatial NPP models particularly CASA model are widely applied from regional to global scale accurately using remotely sensed datasets (Turner et al., 2006; Wang et al., 2013). Liang et al. (2015) and Chen et al. (2016) used the CASA model to research the temporal and spatial changes in NPP of different vegetation types, from 1982 and 2010-from 1984 to 2014, respectively. Liu et al. (2018) estimated aboveground NPP using CASA model for forest ecosystems. Tripathi et al. (2018) selected CASA model to explore the spatio-temporal patterns of NPP for 2009 and 2010 years in forest plantations. Li and Zhou (2015) predicted NPP using CASA model for forest types and reported that how NPP changed by forest stand age.

In our study, we focused on areas where covered by black pine. Black pine trees have been started to use main afforestation tree against soil erosion and to mitigate the some land degradation effects in Turkey during the more than ten years. So this study also important to understand the black pine tree contribution to carbon budget of the country. The objectives of this study were; (i) to calculate the NPP with CASA model, (ii) to validate the CASA model system for pure black pine stands under continental climate conditions, (iii) to research model possibilities of NPP with stand parameters and (iv) to determine the optimal stand criteria for maximum NPP in pure black pine areas.

Materials and methods

Study area

The study area is located on the Black Sea backward region of Turkey (*Fig. 1*). The coordinates of the study area are between 33°21'56" - 33°25'27" north latitude and 33°34'22" - 33°20'01" east longitude. The study area is about 18488.30 ha and pure Anatolian black pine (*Pinus nigra subsp. Pallasiana var. Pallasiana (Arnold)*) stands are covered totally 5517.44 ha areas. There are also Scots pine (*Pinus sylvestris*), Poplar (*Populus sp.*) and Oak (*Quercus sp.*) in the region. The elevation has been varied between 1000 and 1600 m.

Climate type of the study area is defined as semi-arid, mesothermal with excessive wetness during winters. Mean, maximum and minimum annual temperature are 11, 18 and 5 °C, respectively. This area is one of the transection zones between Euro-Siberien and Irano-Turanian phytogeographical regions. Mean annual precipitation is 412 mm. Daily total highest precipitation in the region is 74 mm.

Materials

Three main materials were used in the study to be forest inventory, satellite dataset and climate dataset (*Table 1*).

Forest inventory

Data from national forest management inventories were used. Sample plots were taken systematically at intervals of 300 × 300 m. According to the crown closure of the sample plots, sample point size was chosen from low (11-40% = 800 m^2), medium (41-70% = 600 m^2) and full crown closure (71-100% = 400 m^2). Then, diameter at breast height (DBH, 1.30 m), age and height were measured in all trees with a diameter greater than 7.9 cm in each sample plot (Anonymous, 2008). Stand map was prepared using combine inventory method through satellite image or aerial photograph and field measurements and 2015-2016 plot measurements were used in this study. Detailed stand area, stand increment and growing stock tables were added to forest management plan.

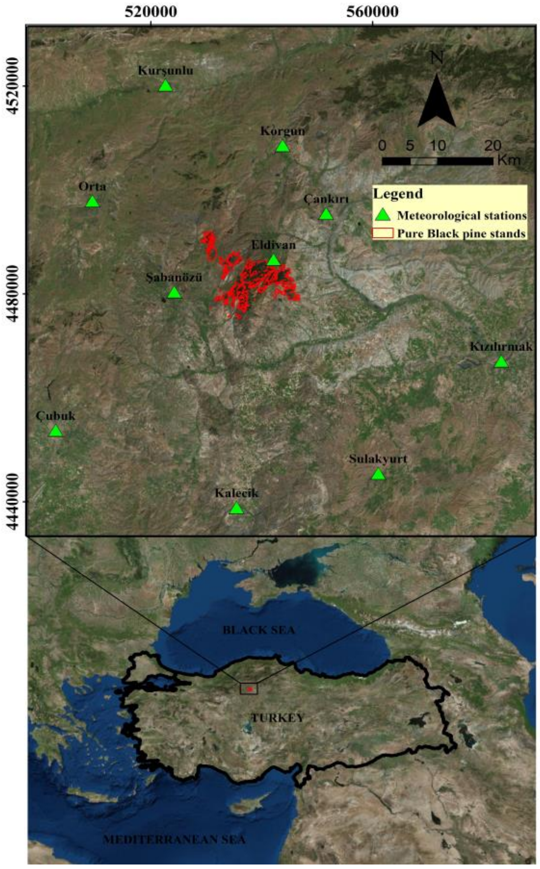

Figure 1. *Study area*

Table 1. *Characterizations of the study materials*

Dataset	Usage purpose	Source
Forest inventory	Ground truth of NPP, comparison analyses	Republic of Turkey, General Directorate of Forestry
Satellite dataset	NPP calculations	USGS Landsat database
Climate dataset	NPP calculations	State meteorological works of Turkey

Satellite data

Landsat 8 satellite images were acquired for each month of 2016 from the United States Geological Survey Earth Explorer data portal (USGS, 2000). Band 4 (Red), Band 5 (Near Infrared) and Band 7 (Short-wave Infrared) were used to run the CASA model for twelve months of a year.

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Meteorological data

Climate data were included daily mean, maximum, minimum temperature, precipitation and solar radiation with the same periods of Landsat image's records (2015-2016) and obtained from 10 Turkish State Meteorological Service climate stations. Monthly mean, maximum, minimum temperature and solar radiation were interpolated with appropriate interpolation techniques such as ordinary kriging and radial basis function. Climate data maps prepared for the study area were produced with 30 m spatial resolutions. Data interpolation and analysis were performed using.

Methodology

The CASA model

Carnegie, Ames, Stanford Approach (CASA) model was used to predict Black pine NPP in local scale (Potter et al., 2003, 2004). This model is run based on photosynthesis progress of a plant. It was designed for global studies, however; it is contained plant specific variables and it may be used for the local studies for modifying the variables such as maximum light use efficiency and photosynthesis temperature range.

The CASA model was used for predicting NPP in the study area. It is defined as follows (*Eq. 1*):

$$NPP = \varepsilon_n x \, APAR \quad \text{(Eq.1)}$$

where APAR is absorbed photosynthetically active radiation (MJ/m^2 month) and ε_n is light use efficiency (g C/MJ).

$$\varepsilon_n = \varepsilon_{max} \, x \, f(T) x \, f(W) \quad \text{(Eq.2)}$$

Maximum light use efficiency (ε_{max}) for evergreen needleleaf stands was separated into three age classes. Mean ε_{max} of young, middle age and mature stands are 0.72, 0.57 and 0.52 (g C/MJ), respectively (Li and Zhou, 2015).

Temperature on the maximum light use efficiency of vegetation (T) is defined as follows (*Eq. 3*):

$$f(T) = (T - T_{min}) x (T - T_{max}) / ((T - T_{min}) x (T - T_{max})) - \left(T - T_{opt}\right)^2 \quad \text{(Eq.3)}$$

where T is the atmospheric temperature (°C); and T_{min}, T_{opt}, and T_{max} are the minimum, optimal, and maximum temperatures (°C) for photosynthetic activities, respectively (Huang et al., 2010).

Water on the maximum light use efficiency of vegetation (W) is defined as follows (*Eqs. 4* and *5*):

$$W = (1 + LSWI) / (1 + LSWI_{max}) \quad \text{(Eq.4)}$$

$$LSWI = (p_{nir} - p_{swir}) \, / \, (p_{nir} + p_{swir}) \quad \text{(Eq.5)}$$

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where LSWI is the land surface water index and $LSWI_{max}$ is the maximum LSWI. The variables p_{nir} and p_{swir} represent the surface reflectance of the NIR and MIR bands in Landsat 8 images, respectively (Huang et al., 2010).

$$APAR = FPAR \ x \ PAR \quad \text{(Eq.6)}$$

Fraction of photosynthetically active radiation (FPAR) is defined as follows (*Eq. 7*):

$$FPAR = \frac{(NDVI - NDVI_{min}) \ X \ 0.95}{(NDVI_{max} - NDVI_{min})} + 0.05 \quad \text{(Eq.7)}$$

where NDVI is Normalized Difference Vegetation Index, $NDVI_{max}$ and $NDVI_{min}$ are the maximum and minimum Normalized Difference Vegetation Index, respectively (Los et al., 2000; Zhu et al., 2006; Huang et al., 2010; Chen et al., 2016).

Photosynthetically active radiation (PAR) is defined as follows (*Eq. 8*):

$$PAR = Sr \ x \ 0.50 \quad \text{(Eq.8)}$$

where Sr is solar radiation (MJ/m^{-2} day^{-1}) (Potter, 1993, 1998; Huang et al., 2010).

Tree cover classification

Stand map was acquired from Turkey General Directorate of Forestry to determine the pure black pine cover. The stand map is contained the classification of the area according to the tree type, development age and crown closure. Within these areas, there may be areas that are not covered by trees because it was obtained as polygons and caver degree is variable inside the polygons. Therefore, only forest covered areas were extracted from the polygons applying a supervised classification approach. NPP will be calculated for pure black pine areas, so the opening areas had to be removed. We used maximum likelihood classification method (MLC) that is one of the most effective parametric classifier when there are enough training points for forest cover classification (Şatır and Berberoğlu, 2012). Almost 270 training points were used to be forest and non-forest areas, and 100 points were used for accuracy assessment. This area is not too complex, also it is included only black pine forest formation so point samples were enough for the classification.

Calculating carbon stock

Initial parameter in calculating the carbon stock was total stand growing stock volume (V), and it was obtained from stand map and forest management plan. Other parameters were above-ground biomass (AGB), below-ground biomass (BGB), above-ground carbon (AGC), below-ground carbon (BGC), dead wood biomass (DWB), dead wood carbon (DWC), litter carbon (LC) and forest soil carbon (FSC). The carbon stocks in the biomass were calculated using AGC, BGC, DWC, LC and FSC (*Eq. 9;* Tolunay, 2011; Değirmenci and Zengin, 2016). The equations of these parameters were presented in *Table 2*.

$$Carbon \ stock = AGC + BGC + DWC + LC + FSC \quad \text{(Eq.9)}$$

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Table 2. *Carbon stock coefficients*

Cover type	Parameter	Equation
Coniferous	AGB	V x 0.446 x 1.212
	BGB	AGB x 0.29
	AGC	AGB x 0.51
	BGC	BGB x 0.51
	DWB	AGB x 0.01
	DWC	DWB x 0.47
	LC	Area (ha) x 7.46
	FSC	Area (ha) x 76.56

Variance analysis

Digital Elevation Model (DEM), forest management plan and stand map were used for calculating testing parameters to be age, site index, crown closure and elevation. Homogeneity between NPP and these parameters was tested using one-way ANOVA by followed Duncan procedure. Classes were created for elevation, age, crown closure and site index (*Table 3*). Elevation was between 1000 and 1600 m in the study area. It was separated into six classes. Age was created as a seven classes with a period of 20 years. There are no stands of the sixth age class in the study area. Crown closure was divided into three classes by coverage of ground cover. Site index was determine according to dominant height at standard age (100) and separated into three classes.

Table 3. *Classes for variance analysis*

Criteria	Value	Class	Criteria	Value	Class
Age (year)	0-20	1	Elevation (m)	1000-1100	1
	21-40	2		1101-1200	2
	41-60	3		1201-1300	3
	61-80	4		1301-1400	4
	81-100	5		1401-1500	5
	121-140	6		1501-1600	6
Site index (m)	20-24	1	Crown closure (%)	11-40	1
	15-19	2		41-70	2
	10-14	3		71-100	3

The validation of CASA model

NPP was predicted using the CASA model and we did not have actual values to validation of the model. So, stand increment, growing stock and stand carbon were used for the CASA model validation. The relationships between these parameters and NPP were compared. Relationship levels were determined by calculating correlation (r) and coefficient of determination (R^2). In addition, CASA model was compared and validated in many studies (Cramer et al., 1999; Potter et al., 2012), and these were showed that this model was provided significant results when it was applied correctly.

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Results

Study results were presented in three stages to be; mapping the tree cover, mapping of the NPP, and defining the relationship between NPP and some forest stand variables.

Mapping the tree cover in black pine stands

The CASA model used for NPP computation was created for tree covered fields. It was necessary to remove the opening areas. So the NPP was more consistently calculated in terms of spatial position. For the operation of this process, Landsat 8 satellite image was used for supervised classification (kappa = 0.92, overall accuracy = 96.4%). The opening and tree cover areas were determined and NPP was calculated to tree cover areas (*Fig. 2*).

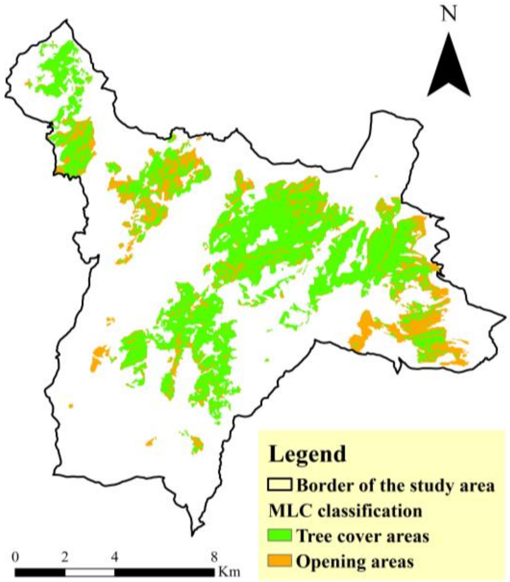

Figure 2. *Tree cover map of the black pine stands*

Mapping the NPP using CASA model

The CASA model was used to predict NPP values of the pure black pine forests in 2016. NPP values were integrated with forest stand map and calculated variables such as stand increment, growing stock and stand carbon in 676 plots. Descriptive statistics for NPP, stand increment, growing stock and stand carbon were presented in *Table 4*. The NPP values ranged between 15.16 and 1893.93 (ton C year^{-1}) with a mean value of 382.82 (ton C year^{-1}). Mean values of stand increment, growing stock and stand carbon were defined to be 20.90, 548.79 and 677.57, respectively.

Table 4. *Descriptive statistics of the NPP and forest attributes*

Variable	N	Min	Max	Mean	SD	Skew	Kurt
NPP (ton C year^{-1})	676	15.16	1893.93	382.82	385.26	1.651	2.306
Stand increment (m^3)	676	0.60	175.39	20.90	27.07	2.468	6.756
Growing stock (m^3)	676	7.32	4010.47	548.79	746.09	2.024	3.813
Stand carbon (ton C year^{-1})	676	45.30	4221.33	677.57	716.82	1.831	3.193

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Monthly mean NPP values were calculated for all months of 2016 (*Fig. 3*). The highest mean NPP was calculated in May to be 120.83 (g C m^{-2} $month^{-1}$) and the lowest mean NPP was calculated in February as 5.89 (g C m^{-2} $month^{-1}$). According to the monthly analyses, there was a significant improvement on NPP in March, April and May. However, NPP was decreased fast after the September. The highest NPP value was predicted to be 1213.40 (g C m^{-2} $year^{-1}$) and the lowest NPP value was 193.70 (g C m^{-2} $year^{-1}$) per unit area over the year (*Fig. 4*).

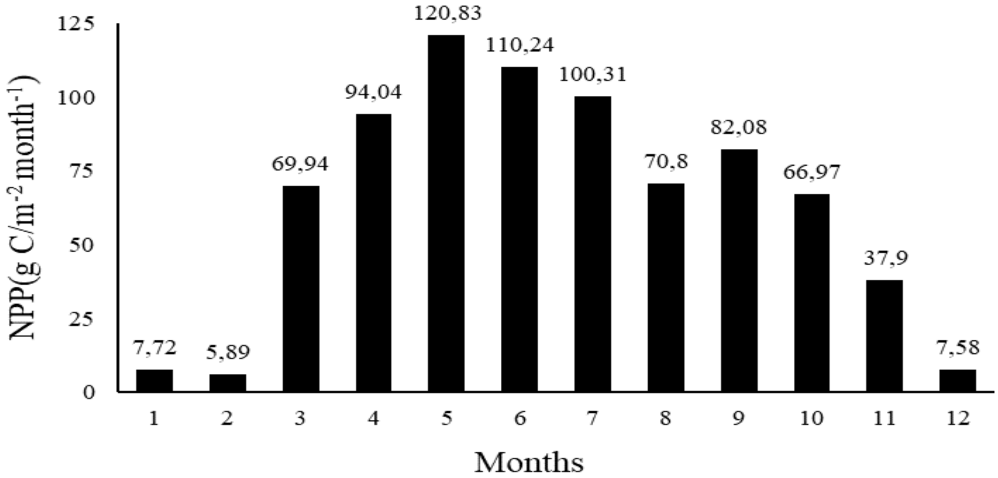

***Figure 3.** NPP distribution in the pure black pine stands for months of 2016*

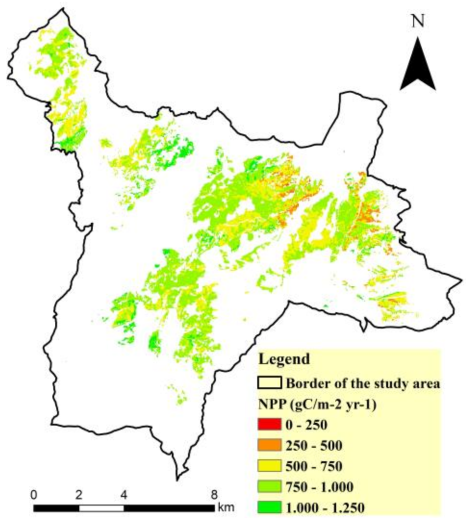

***Figure 4.** Spatial distribution of NPP derived from the CASA model in the black pine stands for the year 2016*

Defining the relationship between NPP and some forest attributes

Relationship levels between the calculated NPP data and stand increment, growing stock and stand carbon were showed in *Table 5* and *Figure 5*. The highest correlation was obtained with stand carbon (r = 0.92), and the lowest correlation was defined with stand increment (r = 0.87). Linear models and relationships were created between NPP

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DOI: http://dx.doi.org/10.15666/aeer/1702_44594473

and these variables. Linear model results were showed that predicted NPP and calculated stand variables related with NPP were matched, and NPP prediction was significant for this area.

Table 5. *NPP models and performance criteria*

Variable	N	Model	r	R^2	p
Stand increment (m^3)	676	NPP = (12.351 × Stand increment) + 124.69	0.87	0.75	0.000
Growing stock (m^3)	676	NPP = (0.4552 × Growing stock) + 133.04	0.88	0.78	0.000
Stand carbon (ton C $year^{-1}$)	676	NPP = (0.4954 × Stand carbon) + 47.155	0.92	0.85	0.000

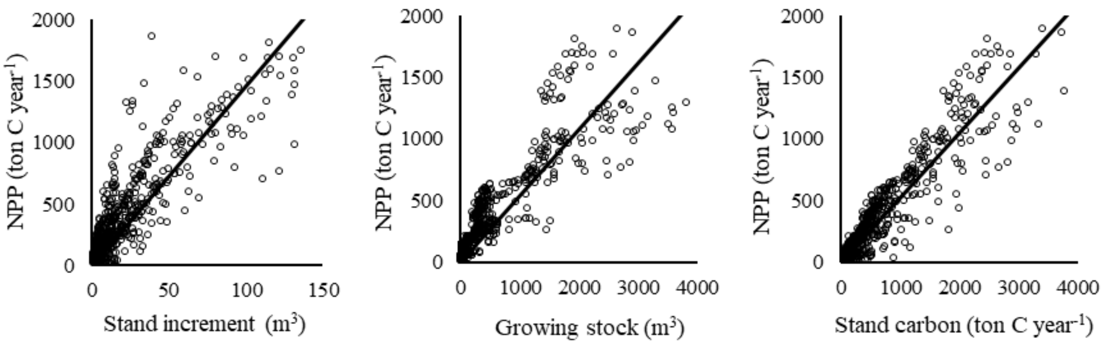

Figure 5. *Comparison between NPP and validation parameters*

Results of the one - way ANOVA test were summarized in *Table 6*. Duncan test showed that NPP values were statistically different by age (F = 88.465, p < 0.05), elevation (F = 39.354, p < 0.05), site index (F = 63.118, p < 0.05) and crown closure (F = 23.114, p < 0.05). According to the results, age classes were divided into 5 groups (a < b < c < d < e), elevation classes were divided into 3 groups (a < b < c), site index and crown closure classes were divided into 2 groups (a < b). Results showed that NPP was higher in young and low-medium closed stands than old stands in an altitude of 1300-1600 m with an average dominant height of 12-17 m.

Table 6. *Comparison of stand parameters in terms of NPP according to one-way ANOVA by followed Duncan*

Criteria	Class	N	Mean	Criteria	Class	N	Mean
Age (year)	1	87	946.30[e]	Elevation (m)	1	53	670.13[a]
	2	510	849.91[d]		2	278	686.12[a]
	3	935	743.97[b]		3	470	766.84[b]
	4	245	778.67[c]		4	714	799.54[c]
	5	827	750.33[b]		5	723	793.62[bc]
	6	100	710.30[a]		6	466	777.65[bc]
Site index (m)	1	828	730.81[a]	Crown closure (%)	1	469	782.86[b]
	2	1727	792.21[b]		2	905	795.78[b]
	3	149	808.51[b]		3	1330	756.68[a]

a, b, c, d and e letters show groups that are statistically different each other at 95% significance level (a < b < c < d < e)

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Discussion

NPP was quite low in the first two months of the year. This situation was caused by the seasonal low temperature, solar radiation and leave chlorophyll activities (NDVI). NPP was increased rapidly in March and reached the highest value in May because of ideal weather conditions (precipitation, temperature and solar radiation) for black pine photosynthesis process. Summer temperatures were between 19–22 °C, and the highest temperature were recorded in August based on the nearest climate station. Therefore there was a little increase in September due to lower temperature than August. According to the NPP and temperature relationships, the ideal temperature for the black pine vegetation was defined to be 14.5–15 °C. NPP of the black pine vegetation was impacted from the temperature rise in June, July and August, negatively. NDVI in the last three months of the year was decreased regularly based on the weather conditions in these months. Particularly, temperature, precipitation and solar radiation were indicative on anomalies in seasonal transition (*Fig. 3; Table 7*).

Table 7. *The monthly mean parameter values used in the CASA model*

Month	Mean temperature (°C)	Mean precipitation (mm)	Mean solar radiation (MJ/m^{-2})	NDVI	NPP ($g\ C\ m^{-2}$)
January	-1.30	57.66	5.542	0.255	7.72
February	0.27	39.15	8.809	0.186	5.89
March	5.04	49.29	13.677	0.343	69.94
April	9.96	41.10	16.776	0.367	94.04
May	14.69	73.47	20.521	0.455	120.83
June	19.31	54.60	22.474	0.442	110.24
July	22.79	20.77	23.479	0.429	100.31
August	22.89	29.76	20.964	0.401	70.80
September	17.83	17.40	16.739	0.376	82.08
October	12.18	42.47	11.364	0.356	66.97
November	5.38	24.00	7.934	0.305	37.90
December	1.46	48.36	5.972	0.229	7.58

There are many different models that can be used to calculate NPP. Schloss et al. (1999), Cramer et al. (1999) and Ruimy et al. (1999) compared the different global NPP models. Ruimy et al. (1999) evaluated twelve global NPP models and assessed the performance of the models. LUE was derived from models and obtained the linear correlation coefficients with NPP. The highest correlation between NPP and LUE was obtained with SIB model ($r = 0.71$). APAR was also used in evaluation of the models. The highest accuracy was obtained by CASA model based on APAR ($r = 0.98$).

The CASA model is widely used in both locally and globally scales for NPP calculation. Taskınsu Meydan and Berberoglu (2008) performed NPP calculations for black pine stands in Mediterranean part of Turkey. Productivity evaluation can be made for northern and southern regions between continental and sub-Mediterranean climate zones. In this study, the lowest NPP value of the year was obtained in February (5.89 g C m^{-2} $month^{-1}$), and the highest NPP value was obtained in May (120.83 g C m^{-2} $month^{-1}$). In the study conducted at the Mediterranean region, the lowest and highest values were detected in March and June (0.28-52.25 g C m^{-2} $month^{-1}$), respectively (*Fig. 6*). NPP

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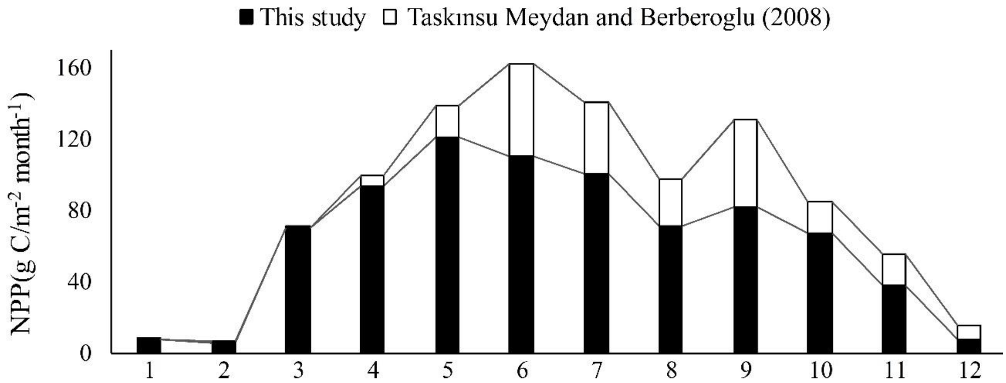
obtained for the northern region was obviously higher than southern region of Turkey. The average for all months was 64.53 g C m^{-2} year^{-1} for the northern region and 19.79 g C m^{-2} year^{-1} for the southern region. Evrendilek et al. (2006) was estimated the NPP of conifer forests in the eastern Mediterranean region of Turkey. They presented that the annual total NPP of black pine forests was 1302.00 g C m^{-2} year^{-1}. It was 774.30 g C m^{-2} year^{-1} in our study. Because, studies were applied in different regions that has been various climatic conditions, and difference of the NPP values were significant even if they have similar land cover.



	1	2	3	4	5	6	7	8	9	10	11	12
□	0,37	0,34	0,28	5,58	18,41	52,25	40,24	27,1	48,93	18,37	18,08	7,48
■	7,72	5,89	69,94	94,04	120,83	110,24	100,31	70,8	82,08	66,97	37,9	7,58

Figure 6. *Comparison of monthly NPP values for pure black pine stands between southern and northern Turkey*

Besides the climatic features, forest forms and land cover types were also affected to the NPP. Ma et al. (2008) were estimated the annual average NPP for *Pinus elliottii* plantation. They focused 20 years period from 1885 to 2005. The average yearly GPP was predicted 630.88 g C m^{-2} year^{-1} by their study. In our study, the yearly NPP value was around 775 g C m^{-2} year^{-1}. In our work was done in natural pine forest, and their work was conducted in pine plantation areas that was included different pine species from our study. However, it can be seen clearly in Ma et al. (2008) that NPP values have been variable based on climatic effects.

Pei et al. (2018), Zhang and Zhang (2017) and Chen et al. (2016) were calculated the annual mean NPP using CASA model for different land cover types in China. Pei et al. (2018) obtained the NPP for evergreen broadleaf forest (EBF, 774 g C m^{-2} year^{-1}), deciduous broadleaf forest (DBF, 471 g C m^{-2} year^{-1}), evergreen needleleaf forest (ENF, 308 g C m^{-2} year^{-1}), deciduous needleleaf forest (DNF, 444 g C m^{-2} year^{-1}) and mixed forest (MIF, 460 g C m^{-2} year^{-1}). Zhang and Zhang (2017) reported that the NPP in ENF, DNF, DBF and MIF were 489.73, 415.71, 954.19 and 650.94 g C m^{-2} year^{-1}. Chen et al. (2016) calculated the NPP for EBF (771 g C m^{-2} year^{-1}) and DBF (734 g C m^{-2} year^{-1}). These differences in the NPP of land cover types were particularly affected by climate and topographic factors (Zhang and Zhang, 2017). Donmez et al. (2016) calculated the NPP using CASA model for 2000-2010 and estimated for 2070-2100 period using RCP climate scenarios in the eastern Mediterranean region of Turkey. The annual mean NPP was calculated 1042 g C m-2 year-1 for ENF between 2000 and 2010. The highest and lowest NPP values were obtained for DBF (1529 g C m^{-2} year^{-1})

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and shrubland (452 g C m^{-2} $year^{-1}$) cover types, respectively. The highest and lowest grand mean change according to RCP climate scenarios was obtained for the DBF (-3.2%) and shrubland (0.7%) cover type between 1476-1483 g C m^{-2} $year^{-1}$ and 454-457 g C m^{-2} $year^{-1}$, respectively.

NPP is generally higher at young stands that are accumulating biomass in forest ecosystems (Field et al., 1995; Li and Zhou, 2015; Wang et al., 2018). As a result of the one-way ANOVA test applied to age classes ($6 < 3 = 5 < 4 < 2 < 1$), NPP was higher in young stands that were 1st and 2nd age classes. The areas with an average dominant height of 12-17 m were more favorable than other areas for NPP ($1 < 2 = 3$). Black pine covered areas in the study area were 5517 ha. Nearly half of the black pine areas (2303.19 ha) was located to 2nd-3rd site index and 1st-2nd-3rd age classes. Thus, the NPP in areas with lower dominant height was found to be higher than other places because, these areas were covered by young stands with a high NPP. Wang et al. (2018) researched the relationship between age and NPP for broadleaved and conifer forests under the various site conditions. They reported that NPP of young forests increases rapidly, reaches the highest value in mature forests and decreases in old forests. In addition to the forest age, site conditions also affect the NPP. Chen et al. (2002) indicated that NPP was increasing faster in area of the high site index. That is, site index is critical in identifying the relationship between NPP and age.

When the results were examined in terms of the elevation that a linear relationship was appeared between NPP and elevation in negative way. Donmez et al. (2015) reported that there were an inverse relationship between NPP and elevation ($R^2 = 0.8129$). Both studies were similar results in same points of view. This study was carried out for an area of 1000000 ha and an elevation range of 0-2300 m. These results for site index and elevation have been influenced by the local and managed forest area.

NPP was higher in stands with low-medium crown closure (11-70%) than high crown closure areas because of the efficient sun light availability. NPP may be high because the rate of fall to the surface of rain and the rate of water utilization of plants are high (especially in arid or semi-arid climates). These effects may have an enhancing effect on photosynthesis. So it may be caused NPP to increase.

Conclusions

In this study, NPP was predicted using CASA model for pure black pine stands in the Black Sea backward region of Turkey. The CASA model can be used efficiently that the region where this study was conducted. The satellite and climate data were required to run the CASA model and it was effort and time consumed. In addition, there was also the possibility of modeling with the stand increment ($r = 0.87$), growing stock ($r = 0.88$) and stand carbon ($r = 0.92$) parameters that were correlated positively with the NPP in the study. Results of the variance analysis showed that optimal stand criteria for maximum NPP were detected in 0–40 years of age and 12-17 m mean dominant height, 11-70% of level of crown closure and 1300-1600 m of elevation. It was believed that these results can be helpful to create forest management plans considering maximum NPP operating objective studies in forest ecosystems or afforestation studies. Recently, black pine trees were used in afforestation in all around the Turkey to be coniferous, and this study can be a good guide to use the black pine for carbon absorption more efficiently. It is also very important to create a sustainable carbon budget strategy in country scale.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Acknowledgements. We would like to thank to Turkish State Meteorological Service and General Directorate of Forestry for providing data.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44594473

THRESHOLD RANGELAND CONDITION FOR RANGELAND RESTORATION INVESTMENTS AND THE FINANCIAL EQUIVALENT OF LIVEWEIGHT LOSSES DUE TO RANGELAND DEGRADATION

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(Received 23rd Jan 2019; accepted 6th Mar 2019)

Abstract. In this paper, the relations between live weight (LW) gain of the grazing cows and rangeland condition (RC), LW gain and concentrate supplement and LW gain and genetics of the grazing cows were investigated to quantify LW and body condition score (BCS) losses driven by rangeland degradation to estimate the threshold RC over which rangelands can compensate these losses. This study was conducted in Erzurum Province, Turkey. LW gains were estimated using farmer- animal- and rangeland-related variables during June-August and June-October periods. RC was calculated employing the classical condition assessment method. Ordinary least squares (OLS) were used in data analyses. The results show that a 10% enhancement or setback in RC can result in about 10 kg LW gain or loss per head. The financial equivalent of these LW gain or losses was 314.6 Turkish lira (TRY) or 59.0 USD (I TRY = 0.1875 USD) per farm, which accounts 15.5 TRY or 2.9 USD per hectare of rangeland. It is concluded that rangeland with an RC value below 4.3 requires restoration and that cows of higher genetic merit more than crossbred genotypes are not suitable for extensive production in the study area and areas sharing similar agroecological conditions.
Keywords: *threshold rangeland condition, rangeland restoration investments, grazing cows, live weight gain, body condition score, genetic merit, concentrate supplement, Erzurum, Turkey*

Introduction

As a natural resource, rangeland is the common name for extensive natural landscapes, such as grasslands, shrub lands, woodlands, wetlands and deserts. It is the most common land type that about 50% of the total land area of the world is covered with rangelands (Holechek et al., 2004). Beside the immeasurable outputs of ecosystem services such as biodiversity, soil fertility, water quality, pollination, they ensure the sustainability of extensive livestock production by providing free of charge forage for the farm animals (Williams et al., 1968; Altın et al., 2005). For that reason, extensively managed livestock production is the most sustainable and common form of agriculture. Although they produce relatively agriculturally low value, rangelands have significant overall, economic and social value due to their geographic magnitude (Schacht and Reece, 2009). For that reason, rangelands with low-input, extensive livestock production have been a way of life and important in sustaining livelihoods for centuries in many areas worldwide (Williams et al., 1968; Gintzburger et al., 2006; Pardini, 2009).

However, heavy grazing is reported to be the most important factor causing the deterioration on rangelands. through replacement of species of higher nutritive value with those of lower nutritive value over time. Especially in public rangelands, continuous heavy grazing, irregular and misuse, accelerated with other type of pressures, such as increasing land demand for industry, urbanization and crop cultivation, results in irreversible changes in botanical composition and undesired

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species become dominant (Gökkuş and Koç, 2001; Holechek et al., 2004; Pardini, 2009). Thus, these valuable ecosystems have been degraded over time and they lost their hay production potential. Because, rangeland degradation means losses of livestock revenues for the national economy, rehabilitation and restoration of the degraded natural rangeland sites is of great importance to increase and maintain productivity.

Rangeland condition is defined as an evaluation of the existing state of health of the vegetation cover in a definite site relative to an expected norm with a given set of prevailing environmental and managerial factors, which measures range deterioration and improvement (Heady, 1975; Koç et al., 2003; Ludwig and Bastin, 2008).

As in intensive livestock production systems, extensive, rangeland dependent dairy cattle production is profitable only with high milk yields and the birth of a calf each year per head of dairy cows, both of which require optimal care and feeding conditions. Dairy cow energy reserves are important indicators of these optimal care and feeding conditions, reflecting the animals' underlying physiological state. Because rangelands have been the main feeding sources especially during the grazing seasons (Kara et al., 2009; Ünal et al., 2010; Sayar et al., 2015), energy reserves of the grazing animals are, of course, closely linked to rangeland condition in extensive production.

One method for determining an animal’s energy reserves is the body condition score (BCS). The BCS rates farm animals according to visual and/or tactile appraisals of specific body regions made by trained staff to assess body energy reserves (Aktaş et al., 2011; Berry et al., 2011; Anonymous, 2012). In their review, Bewley and Shulz (2008) concluded that changes in BCS throughout lactation can have an impact on milk yield, herd health, reproductive performance, and animal well-being. Therefore, management of BCS must play a key role in achieving profitable animal production by maximising animal potential.

Modified versions of the BCS have been used in many countries for different kinds of farm animals since it was first developed for sheep by Jefferies (1961). Different countries employ different BCS scoring systems (e.g. 1–4, 1–5, 1–8, 1–9, and 1–10 scales). In all systems, thin animals receive lower scores than fat animals (Bewley and Schulz, 2008).

In addition to BCS, live body weight (LW) is routinely used to estimate the body energy reserves of farm animals. Although changes in LW are influenced by factors other than fat content (e.g. endogenous water and protein content, changing organ weights, gastrointestinal contents, and foetal development) (Schroder and Staufenbiel, 2006), thereby rendering interpretation of LW changes difficult (Morris et al., 2002), it is accepted that LW and LW changes have effects similar to those of BCS (Roche et al., 2007) and that monitoring LW after parturition can be used as a management tool to prevent reproductive problems (Řehák et al., 2012).

Consequently, the relation between BCS and LW has been the subject of numerous studies. Otto et al. (1991) calculated an r^2 of 0.62 for the relation between BCS and LW in US Holstein cows. Enevoldsen and Kristensen (1997) reported correlations between BCS and LW of 0.53, 0.34, and 0.57 for Danish Friesian, Danish Jersey, and crossbred Jersey × Red Danish cows, respectively. Yıldız et al. (2011) reported a correlation of 0.4 between BCS (1–5 scale) and LW for Brown Swiss × Simmental crosses. Berry et al. (2011) reported a moderate correlation (r = 0.49) between BCS (1–5 scale) and LW for Irish Holstein-Friesian dairy cows in pasture-based dairy cattle production.

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Although LW explains only 25–62% of the variation in BCS changes, it is still used as an indicator of BCS losses or gains in cows (Anonymous, 2012). In fact, these values underestimate the accuracy of LW as an indicator of BCS since BCS is affected by body shape, and more fat is needed for each additional BCS point on a large-framed than on a small-framed cow, because visual changes in BCS take longer to become apparent (minimum 4 weeks) than do changes in LW (1 or 2 weeks) (Moran, 2005).

Morris et al. (2002) reported that 15-kg and 30-kg LW increments were necessary to increase BCS by one unit in 1–10 and 1–5 systems, respectively. Berry et al. (2006) reported that, on average, 1 BCS unit equalled 31 kg in LW. In a later study, Berry et al. (2011) reported a 50-kg LW change per unit change in BCS, varying from 39–66 kg, depending on the parity and stage of the inter-calving interval.

The BCS of farm animals fluctuates throughout the year, and different BCSs have been suggested for certain stages of cow production cycles to maximize economic returns through optimizing milk production while minimizing health and reproductive disorders (DEFRA, 2001). During the parturition and dry-off periods, optimum BCS is expected to be 3 and 4, respectively, and to be between 2 and 3 during peak milk yield (Serin, 2004; DeLaval, 2006; Hulsen, 2007; OMAFRA, 2015). The reason for low BCS during peak milk yield is insufficient daily nutrient intake coupled with higher energy requirements, which lead to a negative net energy balance. To cover the energy gap, the energy reserves of the body are mobilised, which brings about BCS losses after calving. Gallo et al. (1996) pointed out that BCS is lowest in the third and fourth months of lactation in low- and high-yielding animals, respectively, and it is compensated for only in the middle of or at the end of lactation, although replacement of decreased body fat reserves starts in the seventh and twelfth weeks after calving.

With one third of total rangeland asset, Eastern Anatolia ranks first among the geographical regions of Turkey. Not only is arable land limited due to varied topography but also crop pattern because of short vegetation period and low mean temperature. Consequently, rangeland dependent extensive animal production has been an important component for livelihoods of the rural people from past to present. In this region and especially in the study area, calving occurs in February and March, and rangeland grazing starts by early May (Kara et al., 2009). The beginning of the grazing season coincides with the 60^{th}–90^{th} day of lactation, when BCS is lowest. In the study area, cow BCS is likely < 2.5, and even drops as low as 2.1 (Aktaş et al., 2011).

Given that the only nutrient source for grazing cows is rangeland herbage (except in the early and late grazing season; Kara et al., 2009), high-quality rangeland is necessary to compensate for BCS and LW losses and achieve profitable production in extensive farming. However, the decades-long use of rangeland above its carrying capacity has resulted in deteriorating rangeland quality and losses in herbage production potentials. Therefore, rehabilitation of degraded rangeland can lead to LW and BCS gains, ultimately leading to an increase in beef and milk production for the local farmers whose livelihoods depend on animal production.

Although an increasing awareness has been developed on the importance of rangelands worldwide and the governments allocate considerable funds to restore degraded rangelands it is necessary to keep the rangelands on the top of the list in the agenda in facilitating sustainable fund allocation to rangeland restoration investments providing cause-effect results with some concrete data. This article covers an on-farm research study of 11 carefully selected villages and their rangelands in a socioeconomic environment. By connecting rangeland and grazing animal attributes in extensive dairy

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cattle production at farm level, I aimed to quantify LW and BCS losses driven by rangeland degradation to estimate the threshold RC over which rangelands can compensate BCS and LW losses, and under which restoration is required.

I also aimed to grasp attention of the policy makers to the importance of rangeland restoration efforts and discuss the importance of sustainable rangeland use from a different view point by revealing the effect of rangeland degradation on LW and BCS losses and calculating the cost equivalent of the feeds required to compensate for the LW or BCS losses. Study results may also be important for researchers and the others having an interest on rangeland-animal relationships prevailing under on-farm conditions. Although the study presents results and arguments from Erzurum Province, Turkey, the findings are expected to be relevant to countries sharing similar agro–ecological conditions, production patterns, and cultural and historical backgrounds.

Materials and methods

Materials

The primary study materials were vegetation study results and LW records of lactating cows in farms selected from the study villages. Secondary materials included official records of the Eastern Anatolia Agricultural Research Institute and other relevant official institutions.

Methods

Study area

The study area covers Erzurum Province, which is representative of the entire region in terms of topography, altitude, climate, and production pattern (*Fig. 1*). It has 12% and 10% of the total meadow and rangeland pastures in Turkey, respectively (TURKSTAT, 2013). A continental climate prevails, with long harsh winters and short hot summers. The lowest and highest recorded temperatures between 1975 and 2006 were −37.2°C and 36.5°C, respectively, while average annual temperature and rainfall were 5.5°C and 453 mm, respectively. The number of frosty days was 154, and the number of days with snow cover was 113 (TÜMAS, 2013). The average annual rainfalls in 2006 and 2007 were 357.4 mm and 436.6 mm, respectively, of which 43.4 and 134.1 mm were recorded from June–August in the first and second grazing seasons, respectively, a difference of 90.7 mm. Despite the positive difference in annual precipitation between the first and second study years, there was considerably more rain in September–October in 2006 than in 2007 (*Table 1*).

Figure 1. *The study area*

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Table 1. *Precipitation in June–August and June–October periods of the grazing seasons (mm)*

Grazing Season	June–August	June–October	September–October	Annual Total
2006	43.4	162.7	119.3	357.4
2007	134.1	167.9	33.8	436.6
Difference	90.7	5.2	−85.5	79.2

Source: TÜMAS (2013)

Indigenous cattle breeds and their various crosses constitute the largest ruminant population, whereas Red Karaman sheep and Anatolian Black goats make up the small-ruminant assets in the region (Kara and Kızıloğlu, 2012). They are hardy and well adapted to natural rangeland. In *Figure 2* are presented some photos to illustrate the study area rangelands.

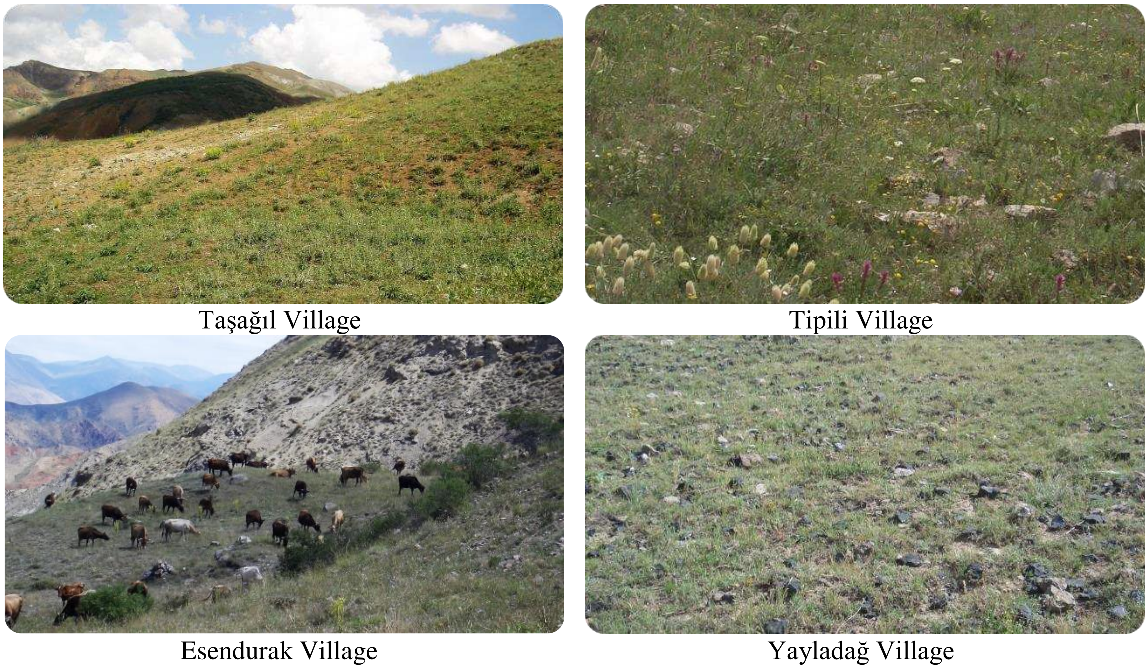

Figure 2. *The study area rangelands*

Selection of villages

Based on how well they represented the surrounding area and province as a whole, villages were selected from different districts, both east–west and north–south, that were free of nomadic movements and boundary problems and for which rangeland demarcation and allocation studies were completed. In total, 11 villages were selected from Aşkale, Yakutiye, Tortum, Narman, Pasinler, Köprüköy, and Horasan districts. Although the selected villages dispersed in different districts of Erzurum province, there was no significant differences among them regarding climatic conditions, production techniques, customs and the habits. At least, it was assumed that the only factors differentiate the villages from each other were the condition of their rangelands, the altitude and the stocking rate.

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Vegetation studies and calculation of the rangeland condition

Vegetation studies were carried out in 2006 to determine the botanical composition and conditions of the village rangeland. They were conducted in 12 representative sites in rangelands of each village with four replications along 100-m transects in easterly, westerly, southerly, and northerly directions, following Koç and Çakal (2004). The condition of the rangeland was determined following the methods of De Vries et al. (1951), cited in Koç et al. (2003), using the vegetation study data for each site in every village.

Rangeland condition was calculated according to the Rangeland Quality Degrees method (De Vries et al., 1951, cited in Koç et al., 2003), using the equation given below.

$$R_{Condition} = \sum P_i \times QS_i \qquad \text{(Eq.1)}$$

In *Equation 1*, P_i shows the relative abundance of each species, calculated as the proportion of individuals of i^{th} species to the total number of individuals encountered in the studied site, as QS_i represents the quality score of the i^{th} species. Quality score reflects the values given to each species according to the grazing and productivity traits of the encountered species such as productivity, post-grazing regeneration ability and palatability, varying between -1 to 10. Poisonous plants take -1 point as the scores 1 to 10 indicate the degree of other desirable traits (Koç and Gökkuş, 1996; Altın, 2001; Koç et al., 2003). In this method, vegetation cover is accepted as the product of climate and soil, as such that information on climax vegetation is not needed.

The arithmetic means of 12 sites in each village were used as a measure of the village's overall degree of rangeland quality. Rangeland condition was given a value between 0 and 10 (0–2.0: very poor, 2.1–4.0: poor, 4.1–6.0: moderate, 6.1–8.0: good, and 8.1–10.0: very good).

LW measures

The LWs of grazing lactating cows from the selected farms were measured and recorded by the study staff using digital scales three times per year during the study: at the beginning (early June), in the middle (late August), and at the end of the grazing season (late October). The scales were calibrated at the time of each weighing in every study village.

To reveal the effect of concentrated supplements on LW gain, each lactating grazing cow was regularly supplied with supplements on a daily basis (1 kg manufactured milking concentrate) during the grazing season. About half the study farms were provided with milking concentrate (16% crude protein and 2,400 Kcal kg^{-1} metabolic energy) in the first year, and the rest were provided with it in the following year. The amount of concentrate was standardised using a 1-kg capacity pan.

Data analysis

Forage allowance is high in June to August and thereafter continuously declines until the end of the season due to grazing and the maturation of the vegetation respectively. For that reason, the LW gain of the grazing cows in randomly selected farms in 11 studied villages was calculated for two periods (June–August and June–October) for each lactating cow by subtracting the initial LW from that measured in the middle and

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at the end of the grazing season. In the socioeconomic environment of extensive production systems, many factors interact and affect the LW gain of grazing animals. Some of these factors relate to farmers (age, education) and some relate to the attributes of the grazing animals (e.g., lactation order and LW at the beginning of the grazing period), whereas other factors are related to the rangeland itself (e.g., rangeland condition, bare ground, altitude, and stocking rate), which determine herbage production and quality.

Considering the fact that most of the variables under consideration are affected by more than one cause in the real world, it is important to isolate multiple independent variables affecting the dependent variable in the model of interest. This feature can be achieved at *ceteris paribus*, which means "holding other things constant". The assumption of *ceteris paribus* is important to determine causation, especially in economics. Linear regression, on the other hand, is the workhorse of the econometrics and ordinary least squares (OLS) method is the most popularly used method to estimate the regression coefficients and describes well the relationship between dependent and independent variables in the presence of *ceteris paribus* assumption (Gujarati, 2012).

Moreover, the linear estimators, i.e., the regression coefficients, are easy to understand and simple to deal with. It is easier to explain the effect of independent variables on a dependent variable (Gujarati, 1995). However, Baltagi (2005) warns that individuals, firms, states, countries, etc., are heterogenous units and if not controlled this heterogeneity, the studies considering other than the panel data will have the risk of obtaining biased results. The most prominent techniques used to analyze panel data are fixed effect and random effect models. If a specific set of N entities (e.g., firms, countries, cities or the villages as in the present study) is focused on and the inferences have to be restricted to the behavior of these entities or whenever the aim is to analyze the impact of variables that vary over time, the fixed effects model is appropriate and therefore should be used (Baltagi, 2005; Torres-Reyna, 2007).

In the present study, however, as stated earlier, the study villages share the same agro-ecological conditions and production pattern, habits and customs inherited from the past. I assumed that heterogeneity among the villages was solely due to rangeland condition, altitude, and stocking rate. Further, I also assumed that heterogeneity among the farmers was due to their age and education level. As observation units, the cows were differentiated in terms of their lactation orders, initial LWs and breeds, all of which were controlled with relevant variables in the models.

In this study, I would like to reveal the effect of rangeland condition on LW gain in a two-year study and rangeland condition is a long-time concept (Heady, 1975), i.e., it is time invariant and does not change for several years. For that reason, it had a fixed value for each village during the study years. Only the average rangeland condition and average altitude value were calculated and assigned to each selected village, which is a significant limitation and makes it impossible to run fixed effect panel data models for the study data. In case of setting or treating the village variable as a panel variable, the rangeland condition, stocking rate, and altitude variables are dropped out. Whereas the rangeland condition is the backbone of the research and dropping it out would render the whole study inconclusive.

On the other hand, unlike the fixed effects model, the variation across entities is assumed to be random and uncorrelated with the predictor or independent variables included in the model (Torres-Reyna, 2007). The random effects model is appropriate if a set of N entities are randomly selected from a large population. If there is a reason to

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believe that the differences across entities (the villages here) have some influence on the dependent variable (e.g., rangeland condition in the present study), then RE seems to be the most appropriate and so should be used (Baltagi, 2005; Torres-Reyna, 2007). Nevertheless, following the Breusch-Pagan Lagrange Multiplier test with a null hypothesis suggesting the use of simple OLS regression, we found it to be more appropriate (p>0.05) for the present study (Torres-Reyna, 2007; Gujarati, 2012).

OLS linear regression model can be written as in *Equation 2* given below (Gujarati, 1995);

$$Y_i = \beta_1 + \beta_2 X_{2i} + \beta_3 X_{3i} + \cdots + \beta_k X_{ki} + u_i \qquad \text{(Eq.2)}$$

where;

- Y represents dependent variable.
- X represents explanatory (independent variables).
- β_1 represents intercept.
- β_2 to β_k represent slope coefficients.
- k represents kth coefficient.
- i represents ith observation.
- u represents random or stochastic error term.

In a linear regression analysis following assumptions should be met (Gujarati, 1995);

- There is no multicollinearity (exact linear relationship) among the independent variables.
- Error term has a homoscedastic (constant) variance.
- Error terms are not correlated with each other, that is, there is no autocorrelation among error terms.
- Error terms are normally distributed.

In the regression models, categorical variables were represented by dummy variables whose number was less by one than that of the classification of the qualitative variables (Gujarati, 2012). In our study, the variable of the cow breed had three categories including local cattle breed. Thus, it was represented by two dummies of crossbred and purebred. Since the dummy variable for local breed was not included in the regression models, coefficients of other dummy variables should be interpreted in relation to reference category (local breed) as coefficients of other continuous independent variables represent the marginal change in dependent variable as a result of one-unit change in the continuous dependent variable of interest at *ceteris paribus.*

LW gain of the grazing cows was considered to be a function of the continuous and discrete variables given in *Table 2*.

The F-test and Ramsey RESET test were used to determine the significance and the omitted variables of the model, respectively. Multicollinearity was controlled with the variance inflation factor. Heteroskedasticity was tested with the Breusch-Pagan test, and the normality assumption was controlled with skewness and kurtosis tests, along with the Graph Method (Neter et al., 1989; Gujarati, 1995; Callaghan and Chen, 2008; Park, 2008; Cameron and Trivedi, 2010). Autocorrelation was not tested due to the fact that it is the problem of time series data and the data used in the present study is cross-sectional (Gujarati, 1995).

The skewness and kurtosis tests appeared to suggest that the assumption of the normality of the residual was violated. However, despite some small deviations that

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could be omitted, the normal probability and quantile-quantile plot distributions of the residuals indicated this was not the case (Park, 2008; Anonymous, 2013).

As the variable 'altitude' caused serious multicollinearity problem and inconsistent estimators for the models, it was omitted. Similarly, 'concentrate supplement × season' and 'concentrate supplement × animal breed' interactions were not included into the model because they also caused serious multicollinearity problems. According to the variance inflation factor values (range: 1.02–2.38, mean: 1.48), the regression models without altitude variable and above-mentioned interaction terms did not have a serious multicollinearity problem (Gujarati, 1995; Callaghan and Chen, 2008; Park, 2009). However, the Breusch-Pagan test revealed that the model had heteroskedasticity problem ($p < 0.01$) and to correct this problem Robust Standard Errors procedure was applied (Gujarati, 2012). Moreover, the Ramsey RESET test proved that the model had no omitted variable bias ($p=0.32$). Stata SE 14 software package was used for all analyses.

Table 2. *Explanations of the study variables*

Variables	Explanations
LW Gain-1	LW gain of grazing dairy cows during June-August period (kg.head^{-1})
LW Gain-2	LW gain of grazing dairy cows during June-October period (kg.head^{-1})
Distance	Approximate distance between village and rangeland site (m)
Farmer Age	Age of the farmer (year)
Schooling	Education level of the farmer (in schooling years)
Rangeland Condition	Rangeland condition (rangeland quality)
Altitude	Average altitude of the village rangelands (m)
Lactation Order	Lactation order (number of giving birth) of the cows
Initial LW	Live weights of the cows at the beginning of the grazing period (kg)
Stocking Rate	Rangeland stocking rate (animal unit (AU) per hectare)
Bare Ground	Bare ground percentage of the village rangeland (%)
Season	Grazing season (1 = Second year; 0 = First year)
Crossbred	Crossbred cows (If crossbred 1, otherwise 0)
Purebred	Purebred cows (If purebred 1, otherwise 0)
Concentrate Supplement	Supplement feed during grazing season (1 = Yes, 0 = No)
Season × Supplement	Season (Year) × Supplement feed interaction
Crossbred × Supplement	Crossbred × Supplement feed interaction
Purebred × Supplement	Purebred × Supplement feed interaction
U	The error term

The data for the variables of rangeland condition, altitude, distance, and bare ground were obtained from the vegetation studies and the live weight data were obtained from the live weight measures of the farm animals as stated earlier. The data used for stocking rate calculations were from the official records of the provincial and district directorates of agriculture.

Results

Vegetation survey results

The rangeland attributes of the study villages are presented in *Table 3*. As mentioned by Kara et al. (2015), 140 different species were found in the studied rangeland, of which 26, 24, and 90 were species of legumes, grasses, and other families respectively.

Medicago varia (5.0%), *Astragalus lineatus* (3.3%), *Astragalus microcephalus* (1.9%), *Trifolium hybridum* (1.7%), *Coronilla varia* (1.4%), and *Astragalus lagurus*

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(1.3%) were the most common legumes, whereas *Festuca ovina* (13.3%), *Stipa lagascae* (3.3%), *Agropyron intermedium* (3.1%), *Dactylis glomerata* (1.5%), *Bromus tectorum* (1.3%), and *Phleum montanum* (1.3%) were the most common grasses. The most widespread species of other families were *Thymus pubescens* (6.7%), *Artemisia spicigera* (3.4%), *Salvia candidissima* (3.0%), *Eryngium campestre* (2.8%), *Galium verum* (2.6%), *Achillea millefolium* (2.4%), *Verbascum cheiranthifolium* (2.1%), *Plantago atrata* (2.1%), *Helichrysum plicatum* (2.0%), *Tanacetum balsamita* (1.7%), *Alyssum pateri* (1.5%), *Acantholimon caryophyllaceus* (1.4%), *Artemisia austriaca* (1.4%), and *Euphorbia virgata* (1.3%).

Table 3. *Rangeland attributes of study villages*

Villages	Species Richness	Of Species Found in Rangeland Vegetation (%)			Rangeland Condition (in fragments of 10)	Bare Ground (%)	Altitude (m)
		Legumes	Grasses	Forbs			
1	64	17.9	30.4	51.7	3.70	29.3	2202.3
2	66	16.4	34.0	49.6	3.26	29.7	1921.4
3	56	22.4	28.7	48.9	3.46	21.4	2269.4
4	36	6.2	25.3	68.5	2.53	27.5	2192.8
5	58	17.5	29.7	52.8	3.19	28.7	1862.1
6	54	17.2	22.3	60.5	2.95	28.3	1723.9
7	62	15.3	23.4	61.3	2.79	15.9	2010.2
8	58	25.6	24.6	49.8	3.73	17.9	2221.2
9	57	12.0	35.0	53.0	2.77	30.3	1775.1
10	51	29.4	36.2	34.4	4.18	27.7	2515.2
11	62	29.2	21.8	49.0	3.60	25.0	2298.7
Average	56	20.0	27.6	52.4	3.28	24.1	2135.8

Descriptive statistics

Average number of lactating cows per farm was 8.5 head. They had an average LW of 287.1 ± 1.8 kg at the beginning of the grazing season and gained 16.1 ± 0.9 kg and 14.9 ± 1.0 kg per head in June–August and June–October, respectively. The lactation orders of the cows were between 1 and 13. The rangeland condition of the study village rangeland varied from 2.53 to 4.18, indicating poor to moderate condition. About 24% of the rangeland area was bare, without any plant covers. The average stocking rate in the studied villages' rangeland was 0.68 AU (1 AU equals to 500 kg LW) per hectare varying between 0.1 and 3.0.

Multiple regression analysis

The factors affecting LW gain per head of dairy cows in June–August and June-October periods in two grazing seasons were examined using OLS regression (*Tables 4 and 5*).

Season, farmer age, rangeland condition and breed of the cows had all positive and very significant effects ($p < 0.01$) as concentrate supplements had also significant impact ($p < 0.05$) on LW gain of the grazing cows (*Table 4*). Additionally, the effects of initial LW of the cows, stocking rate, and bare ground of the rangelands on LW gain were all negative and very significant ($p < 0.01$). Distance from the village to rangeland, schooling years of the farmers and lactation order of the grazing cows had not significant effect on LW gain ($p > 0.05$).

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***Table 4.** Multiple regression analysis results with robust standard errors procedure for June–August period*

LW Gain-1	Coefficient	Robust Std. Err.	t	P > I t I
Season	18.7042	1.6560	11.30	0.000
Farmer Age	0.3323	0.0702	4.73	0.000
Schooling	0.4733	0.3386	1.40	0.162
Distance	-0.0025	0.0016	-1.53	0.127
Rangeland Condition	12.2968	1.6597	7.41	0.000
Crossbred	9.0896	1.9258	4.72	0.000
Purebred	23.5637	4.3770	5.38	0.000
Concentrate Supplement	3.4844	1.4592	2.39	0.017
Lactation Order	-0.0598	0.3581	-0.17	0.867
Initial LW	-0.1481	0.0214	-6.93	0.000
Stocking Rate	-5.4770	1.2610	-4.34	0.000
Bare Ground	-0.4797	0.1484	-3.23	0.001
Constant	4.1739	8.9289	0.47	0.640

Number of observations= 1019; F=45.39; p= 0.000; R-squared= 0.3182

Similar results can be seen in *Table 5*, with the exceptions that contribution of age and schooling years of farmers were negative and insignificant, as effect of bare ground was positive but not meaningful. Again, the effect of concentrate supplement shifted from significant to very significant as the effect of distance was altered to marginally significant.

***Table 5.** Multiple regression analysis results with robust standard errors procedure for June–October period*

LW Gain-2	Coefficient	Robust Std. Err.	t	P > I t I
Season	21.1888	1.8120	11.69	0.000
Farmer Age	-0.0091	0.0765	-0.12	0.905
Schooling	-0.3132	0.3937	-0.80	0.426
Distance	-0.0031	0.0017	-1.86	0.063
Range Condition	13.4401	1.9577	6.87	0.000
Crossbred	10.5889	2.2512	4.70	0.000
Purebred	25.9715	4.3310	6.00	0.000
Concentrate Supplement	4.7480	1.6464	2.88	0.004
Lactation Order	-0.4559	0.4236	-1.08	0.282
Initial LW	-0.1756	0.0250	-7.03	0.000
Stocking Rate	-4.6123	1.4598	-3.16	0.002
Bare Ground	0.2049	0.1747	1.17	0.241
Constant	10.7721	10.3155	1.04	0.297

Number of observations= 1019; F=35.73; p= 0.000; R-squared= 0.2997

The OLS regression results revealed that season had a strongly significant effect on LW gain in both models. There was a positive LW gain difference between the first and second grazing seasons; specifically, there was a difference of about 16.1 and 14.9 kg per head in June–August and June–October, respectively (*Table 6*).

***Table 6.** LW gain of cows by grazing season*

Grazing Season	June–August Period			June–October Period		
	N	Mean	Se	N	Mean	Se
2006	492	4.71	1.199	492	2.16	1.224
2007	527	26.79	1.063	527	26.83	1.290
Total	1019	16.13	0.870	1019	14.92	0.971

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The effect of cow breeds or genetic on LW gain was also very significant ($p < 0.001$). According to *Table 4* and *Table 5* crossbred and purebred cows gained 9.1 and 23.6 kg per head more LW than local cows during June–August and 10.6 and 26.0 kg per head during June–October periods respectively at *ceteris paribus*.

However, when I consider the non-supplemented groups in both grazing seasons, it is obvious that performances of the different breeds or genotypes differed in June–August and June–October periods of the grazing seasons. Indigenous or local cows seemed to be hardier and more insensitive for environmental conditions whereas high genetic merit cows, crossbred and especially purebred cows, are more sensitive. For example, considering the June–August period high genetic cows acted in opposite directions. They lost much weight in draught season as gained much in humid season (*Fig. 3*).

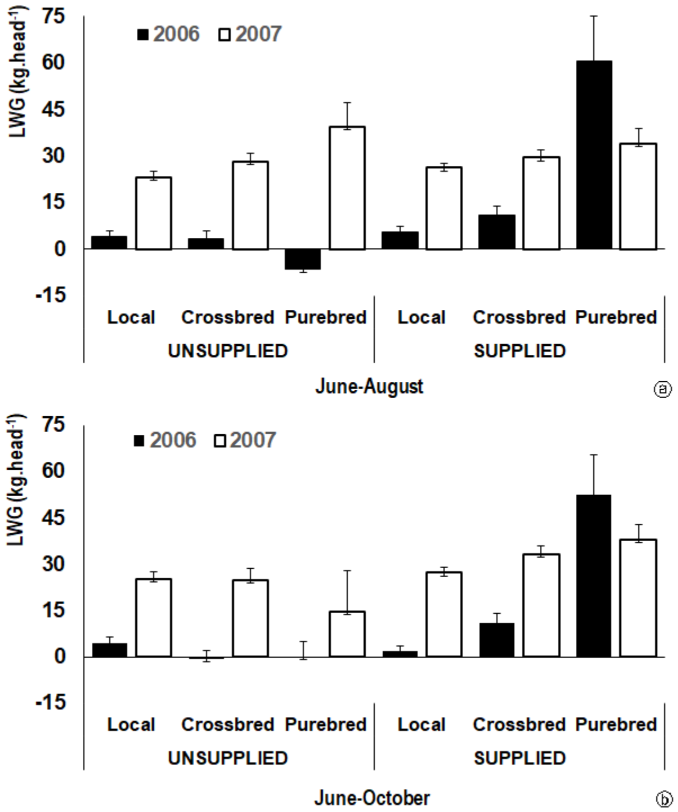

Figure 3. *Effect of concentrate supplement on LW gain by grazing seasons and cow breeds a) June–August period, b) June–October period*

Yet, as the season advanced, purebred cows did not maintain LW gain and lost much of what they gained during June–August period in humid season. It is also obvious that purebred cows lost LW during June–August period as local and crossbred cows gained some LW in draught season (2006). Again, another remarkable finding is that purebred cows balanced the LW losses at the end of June–October period through the late rainfalls in the draught season (*Fig. 3 and Table 1).*

The use of concentrate supplement during the grazing season improves LW gain because it enhances animal performance, thereby increasing LW. For that reason, the effect of concentrate supplement on LW gain was also significant ($p < 0.05$) and very significant ($p < 0.01$) in June–August and June–October periods, respectively.

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DOI: http://dx.doi.org/10.15666/aeer/1702_44754497

Concentrate supplementation increased LW 3.1 kg per head and 4.4 kg per head in these periods respectively (*Tables 4 and 5*).

More importantly, however, the LW gain due to supplement feed was well below that due to rangeland condition. *Tables 4 and 5* show that a 10% increase in rangeland condition was associated with 12.3±1.6 and 13.4±2.0 kg LW gains per head of cow in June–August and June–October, respectively. This is 3.5 and 2.8 times the LW gain (3.5±1.5 kg and 4.8±1.6 kg, respectively) provided by a daily supplement of 1 kg of concentrate in June–August and June–October, respectively.

LW losses as proxies for BCS losses due to rangeland degradation

RC was found to be one of the most important factors affecting LW gains in extensive dairy farming *(Tables 4 and 5*). LW gain as a result of the impact of RC can be converted to BCS following Berry et al. (2011).

Berry et al. (2011) quantified 39 kg LW per 1-unit BCS (1–5 scale) for Holstein-Friesian cows with a LW of 564 kg; this would be 6.9% of the total LW of the cow. The average amount of weight gain/loss for every unit of BCS change was found to be equivalent to 6.58% of a cow's total LW on the 1–10 scaling BCS system used in New Zealand (Anonymous, 2012). These two conversions of LW gain or loss for every unit of BCS are roughly the same despite the different scaling systems. However, this can be explained by the fact that the 10-point scale is essentially a 5-point scale, as, in practice, only 3–7 is generally used in New Zealand (Morris et al., 2002).

This study related BCS to changes in LW due to the impact of RC throughout the June–August grazing period. To this end, the findings reported by Berry et al. (2011) regarding the relation between the BCS and LW for the post-calving stage (101st to 200th day) were adapted for the present study.

As previously stated, in north-eastern Anatolia, calving occurs in February and March, and rangeland grazing starts by early May (Kara et al., 2009). Therefore, the beginning of the grazing season occurs between the 60th and 90th day of lactation, coinciding with the time of the lowest BCS (*Fig. 4*).

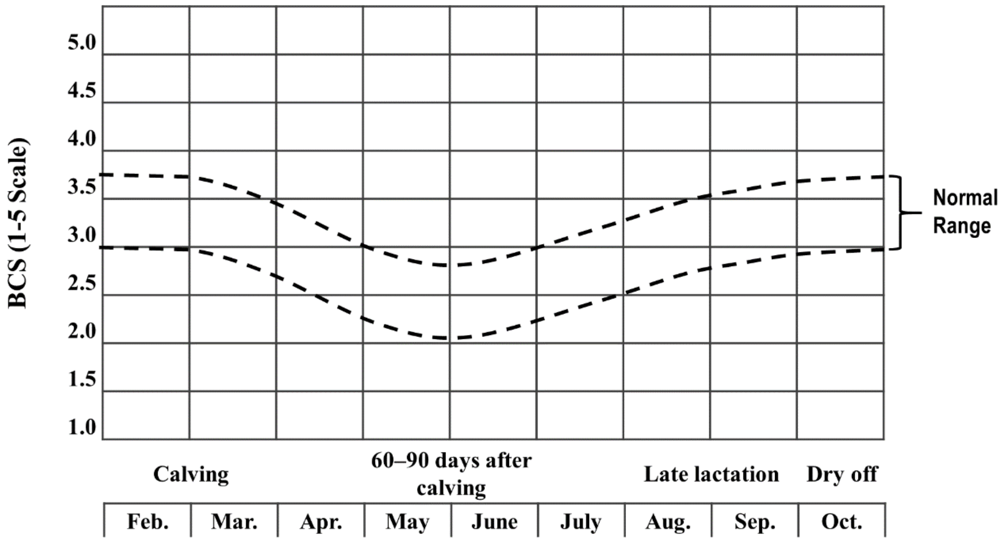

Figure 4. *Approximation of BCS changes in dairy cows after calving in the extensive dairy farming system in Erzurum province, Turkey, Source: Adapted from Hulsen (2007)*

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The grazing period considered in the present study roughly overlapped with the post-calving stage reported by Berry et al. (2011). The relation between BCS and LW for post-calving reported by Berry et al. (2011) was adapted for this study, although the breed and parity of the grazing cows in the study area were mixed. However, Nielsen et al. (2003) documented no significant effect of breed or parity on the relation between LW and BCS.

The average LW of the cows at the beginning of the grazing period was 287.1 ± 1.8 kg per head of cow. Using the rate of 6.9% LW gain per BCS point from Berry et al. (2011), I calculated 19.8 kg LW (287.1 kg × 6.9%) per BCS point at this stage. Because a 10% increase or decrease in RC would cause a 12.3±1.7 kg LW gain or loss (*Table 4*), it can be inferred that a 10% (one unit) degradation in RC will cause more or less a 0.5 point of BCS loss in the study area and in areas with similar conditions.

The average LW gain at the end of the June–October period was about 15 kg (302.0 kg−287.1 kg). That is, the BCS of grazing cows increased by only 0.75 points, not fully compensating for a 1-point BCS loss.

This study also revealed that, after August, grazing cows started to lose LW (*Table 6*), although expected to compensate fully for BCS losses before dry off. As calculated above about 20 kg of LW gain were required to increase BCS by one point.

Financial equivalent of LW and BCS losses due to rangeland degradation

The financial equivalent of LW and/or BCS losses due to the degradation of rangeland used by grazing dairy cows can be quantified by calculating the cost of feed required to compensate for BCS or LW losses. In calculation, barley, the most frequently and easily accessible feed on the farms, was considered. In general, one kg of barley contains 90% dry matter and has a metabolic energy value of about 13 mega joules per kg dry matter (Anonymous, 2015). Following this, financial equivalent of LW losses per farm due to 10 percent rangeland degradation (the energy cost to achieve the same amount of LW gain) are given in *Table 7.*

Table 7. *Cost equivalent of LW losses per farm due to 10 percent rangeland degradation*

Number of Lactating Cows per Farm (head)	(a)	8.5
LW Gain per Cow[1] (kg) in Grazing Period	(b)	12.3
Energy requirement for 1 kg LW gain (MJ)[2]	(c)	44.0
Total Energy requirement to re-gain LW loss in case of 10% degradation in RC (MJ)	(d=bc)	541.2
Energy Content of Barley DM[3] ($MJ.kg^{-1}$)	(e)	13.0
Total Barley Requirement per farm Containing 90% DM (kg)	$(f=ad(0.9e)^{-1})$	393.2
Average Barley Price[4] ($₺.kg^{-1}$)	(g)	0.8
Cost Equivalent of LW Loss per Farm Due to Rangeland Degradation (₺)	(h=fg)	314.6

Source: [1]from *Table 4*; [2]Anonymous (2012); [3]Anonymous (2015); [4]ETB (2018)

The financial equivalent of LW and/or BCS losses in the case of a 10% setback in RC due to rangeland degradation was 314.6 Turkish lira (TRY) per farm. Thus, it can be inferred that, *ceteris paribus*, farmers operating under 10% better rangeland conditions will likely save 314.6 TRY, or 59.0 USD (I TRY = 0.1875 USD).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44754497

I further estimated that the LW or BCS gain or loss per unit of rangeland area in response to a 10% enhancement or deterioration of RC was 15.5 TRY or 2.9 USD per hectare of rangeland (*Table 8*). Thus, *ceteris paribus*, farms operating with 10% poorer rangeland condition are likely to pay an extra 15.5 TRY per hectare of rangeland to compensate for the LW or BCS loss compared with their counterparts operating with rangeland in better condition.

Table 8. *Estimated total LW or BCS gain or loss per unit rangeland area due to a 10 percent enhancement or setback in RC*

Total Number of Villages	(a)	11
Total Rangeland Area (ha)	(b)	15 556
Total Number of Farm Households	(c)	768
Energy Feed Cost Equivalent of LW Loss per Farm Due to Rangeland Degradation[1] (TRY)	(d)	314.6
Total Energy Feed Cost Equivalent of LW Loss (TRY)	(e=cd)	241 612.8
Estimated Cost Equivalent of LW Loss per Unit Rangeland Area (TRY.ha^{-1})	(f=eb^{-1})	15.5

Source: [1]from *Table 7*

Estimation of the threshold RC value for rangeland restoration

The total contribution to LW gain made by both rangeland and concentrate supplements at the end of June–October was about 15 kg (*Table 6*). The marginal contribution of RC to LW gain was about 10 kg (12.3±1.7 kg in *Table 4* and 13.4±2.0 kg in *Table 5*), and the gap was an extra 10 kg of LW gain for full compensation of a 1-point BCS loss (20 kg) after calving in the absence of supplement use during the grazing season. This is possible in the case of a 10% enhancement (one-point increment) in RC. Because the average RC was calculated to be 3.3 for the studied rangelands (*Table 3*), it can be inferred that rangeland with a 4.3 RC (= 3.3+1.0) is able to recover BCS losses in full. In another word, rangeland with an RC value below 4.3 is in need of restoration to recover its herbage production potential and fully compensate for the BCS losses of grazing cows.

Discussions

According to OLS regression results, farmer age had significant ($p < 0.01$) positive effect on LW gain (*Table 4*). Contrary to expectations (Rogers, 1983; Lionberger, 1960) but being in tune with Schmit et al. (2007), an explanation for the positive sign and the significance of the variable “farmer age” might be that older farmers are more experienced, and they are more successful in animal keeping. They might have possibly engaged in training activities previously and been able to take timely measures reducing the possibility of unwanted outcomes. On the other hand, although not significant, a positive sign for the schooling variable suggests that the more the schooling years of farmer, the more LW gain per head cow during June–August period. However, it turns to negative for the June–October period probably due to outshining effect of forage scarcity towards the end of the grazing season.

The LW gain differences between the first and second grazing seasons can be explained by higher annual total precipitation in the second year of the study (357.4 mm

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in 2006 vs. 436.6 mm in 2007) and the difference in precipitation between 2006 and 2007 grazing seasons (90.7 mm in June–August vs. 5.2 mm in June–October periods) (*Table 1*). Higher levels of precipitation, especially in June–August period, result in greater herbage production (O'Connor and Rouxt, 1995; Khumalo and Holechek, 2005; Browning et al., 2012), which, in turn, result in higher LW gains.

Of particular note is that the LW gains were lower considering the whole season (June–October period) than that in June–August period in average and especially in draught season. This can be explained by the greater herbage mass during June–August and the decrease in rangeland herbage due to grazing coupled with the decreasing nutrient content of maturing plants over the course of the season (Cook and Harris, 1950, 1979; Cogswell and Kamstra, 1976; Mermer et al., 2011; Koç et al., 2014).

Although, concentrate supplements increased LW gain across all data-collection periods, LW gains driven by concentrates were more distinctive in draught season of the year 2006 than the humid season in the year 2007. Again, cows lost some what they gained during June–August period as the range forage decreases in amount and maturates due to grazing and lignination during the first grazing season of 2006 (Cook and Harris, 1950; Cogswell and Kamstra, 1976; Mermer et al., 2011; Koç et al., 2014).

The present study also revealed that re-scaling effect (Hammami et al., 2009) was of question regarding genetic merit x environment interaction in both years (i.e. genetic merit x humidity and genetic merit x concentrate supplement). That is, performances of the high and low genetic cows differed significantly under different environmental conditions. As seen in *Fig. 3*, the effect of concentrate supplementation was not the same across the all genotypes. Conforming to Ferris et al. (1999) and Kennedy et al. (2002), local cows responded the least of higher genetic merit cows to concentrate supplementation. In both grazing seasons, 2006 and 2007, purebred cows benefitted the most from the concentrates. However, in humid season responses to concentrate supplementation among the breeds were not as more distinctive as in draught season. This was more likely because of substitution effect since concentrate supplementation can increase total dry matter intake only in case of low herbage allowance (Stockdale, 2000; Delaby et al., 2001; Bargo et al., 2002; Vázquez et al., 2006; Sheahan et al., 2011; Ueda et al., 2016). Due to abundant rangeland forage allowance during humid season and especially in June–August period, concentrate supplementation did not increase total digestible dry matter intake. However, the positive effect of supplements was more obvious in June–October than in June–August, likely because of alleviated substitution effect (Stockdale, 2000) due to decreased forage till the end of the season (*Fig. 3*).

The OLS regression analysis also revealed that initial LW had a negative and significant effect on LW gain (*Tables 3 and 4*). This negative effect might be related to the maturity of the cows. Young animals have lower body weight and make higher body weight gains due to development, whereas heavier cows are likely to be mature animals that do not develop as much as young ones. Furthermore, large-frame animals have higher maintenance requirements even after dry-off due to the higher energy requirements of larger vital organs (DiCostanzo et al., 1990), which may cause cows to suffer longer periods of negative net energy balance (Řehák et al., 2012). It should also be remembered that cows usually mature until the age of 5 years (Tüzemen, 1990; Çakır et al., 1995). Therefore, less LW loss and a short period of recovery can be explained by the growth of young animals.

Similar to previous reports, I found that a high stocking rate had a negative and significant ($p < 0.05$) effect on LW gain (*Tables 4 and 5*). A low stocking rate may

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result in high output per animal due to selective grazing, or vice versa, because a high stocking rate reduces the degree of selectivity (Hull et al., 1965). Moreover, rangeland herbage production will decrease under heavy or above-carrying-capacity grazing conditions, which result in insufficient dry matter intake and poorer performance of grazing animals (Gökkuş and Koç, 2001).

The results also show that the bare ground rate had a very significant negative effect on LW gain (*Table 4*) for the period June–August because more bare ground indicates lower levels of herbage production in the rangeland. However, although not significant, the positive relationship between LW gain and the bare ground in the model for June-October period could be explained with the regrowth of grasses with late rainfalls in the season remembering the relatively high correlation (r=0.4778) between the percentage of grasses in botanical composition and high bare ground in the rangeland sites.

On the other hand, the village herds graze under the guidance of herders. In their excursions that start with sunrise and end at sunset (Kara et al., 2014), the total distance they walk every day is several times more than the actual distance between the village and the grazing site decided for the day. The actual distance varies between 360 m and 6800 m, making an average of 2350 m in the study area. This implies that the greater is the distance between the village and the target grazing site for the day, the more is the total walking distance for that day. In the present study, the effect of distance on LW gain was shifted from insignificant in June–August period (*Table 4*) to marginally significant in the June–October period (*Table 5*). This result suggests that energy cost of walking long distances becomes more apparent as the season proceeds most likely due to decrease in the forage and its lignination because the cost of walking could have only a minor effect on the energy requirement of the grazing cattle (Di Marco et al., 1998) and can be tolerated with abundant rangeland forage allowance. Accordingly, Pratumsuwan (1994) reported that dairy cows with a high productivity can walk horizontally at a comfortable walking speed up to 7.5 km per day with no significant effect on their milk production if the pasture allowance is not restricted. Similarly, D’Hour et al. (1994) pointed out that walking distances above 6.4 km only brought about modifications in the yield and composition of the milk.

Conclusion

The aim of this study was to estimate the threshold RC over which rangelands can compensate BCS and LW losses, and under which restoration is required. This was achieved by revealing the effects of RC on the LW and BCS gains or losses of grazing dairy cows in the presence of farmer, animal- and rangeland-related factors in a socioeconomic environment other than research station trials executed under controlled conditions. This makes the present study important not only for policy makers but also researchers and the others having an interest on rangeland-animal relationships prevailing under on-farm conditions.

Regression analysis showed several significant relations with LW gains in June-August and June–October. Therefore, the dataset and models can be used to draw useful inferences, because the signs of all variables in the models can be explained.

To achieve profitable dairy production by maximising animal potential, it is important to manage BCS, and BCS losses after calving should be compensated for. To this end, RC was shown to be one of the most important factors affecting LW gain or

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loss, and I used this as the proxy for BCS gain or loss during the grazing season. It is critically important to compensate for LW losses and maintain BCS in extensive dairy cattle production. Even a 10% reduction in RC will likely cause a 0.5-unit BCS loss in the grazing period when animals are not generally fed with concentrate supplements. The financial equivalent of this LW or BCS loss was calculated to be 314.6 TRY (59.0 USD; 1 TRY=0.1875 USD) per farm and 15.5 TRY (2.9 USD) per hectare of rangeland. However, it should be noted that economic losses may not be limited to energy feed cost equivalents and may include losses related to health disorders, low reproductive performance, and low milk yields. Similar to previous studies, this study showed that concentrate supplements are ineffective during the June–August period, especially in years of good herbage production. Therefore, supplemental feed may not be a good solution to compensate for the BCS or LW losses in June–August.

Nevertheless, this study made it obvious that, after August, grazing cows start to lose LW (*Table 5*), although they were expected to fully compensate for BCS losses before dry off. I calculated that a LW gain of about 20 kg was required to increase BCS by one unit. The LW gain at the end of the June–October period was about 15 kg, representing the total contribution of rangeland and concentrate supplement together. However, an additional 5-kg LW gain was required to compensate fully for the 1-unit BCS loss (*Table 6*). As a short-term solution, grazing cows in the study area and in areas with similar conditions should be given concentrates after August when rangeland herbage is scarce and fully maturated, which in turn, as stated earlier, brings about a more apparent effect of walking long distances on LW gain.

The present study also revealed that Local or indigenous cows exhibited more stable performance in all environmental conditions (*Fig. 3*). For that reason, I suggest that in extensive dairy production in the study area and the areas sharing similar agroecological and geographic conditions, high genetic merit purebred cows are not a good selection although they have rapid LW gain and so the cows to be used in extensive production should not be higher genetic merit than crossbred genotypes are.

Finally, I estimated that the threshold RC at which grazing cows fully compensated for BCS losses was 4.3. Therefore, rangeland with an RC value below 4.3 requires restoration to recover its herbage production potential and fully compensate for the BCS losses of grazing cows. It is evident that rangeland restoration investments are crucial, even considering only BCS losses and disregarding other benefits of rangeland. Yet, follow-up studies are needed to support the findings of this study.

Acknowledgements. This work is part of research project numbered TAGEM/HAYSÜD/05/01/01/01, and entitled “Analysis of the factors affecting milk production and live weight gain in cattle farms dependent on natural grazing lands in Erzurum”. The research is financially supported by the former Ministry of Agriculture and Rural Affairs, Turkey. The author extends his gratitude to the Rangeland and Animal Science Teams of Eastern Anatolia Agricultural Research Institute for their valuable work on rangeland vegetation surveys and farm animal live weight records in the study area.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44754497

EFFECTS OF POTASSIUM PHOSPHITE ON BIOCHEMICAL CONTENTS AND ENZYMATIC ACTIVITIES OF CHINESE POTATOES INOCULATED BY *PHYTOPHTHORA INFESTANS*

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(Received 30th Jan 2019; accepted 28th Feb 2019)

Abstract. Potato late blight caused by *Phytophthora infestans* dominates the entire world where potatoes and other Solanaceae crops are grown. In this study, the effects of potassium phosphite (KPhi) based fungicide on two potato varieties infected by two strains of the pathogen were studied. Tubers coming from foliar spray of potassium phosphite wounded and/or inoculated with pathogens were sampled at 0, 6, 12, 24, and 48 hours. Phytoalexins, phenols, β-1, 3-glucanase (PR-2), chitinase (PR-3), peroxidase (POD), polyphenol oxidase (PPO), superoxidase dismutase (SOD) and catalase (CAT), were analyzed. Results demonstrated that plants applied with KPhi produced tubers with enhanced resistance to the pathogen than their untreated plants. Moreover, tuber slices from KPhi applied plants following infection showed a significant increase in the contents of phytoalexins and phenols. PR-3 activities were induced by KPhi and wounding with the highest level at 48 hours. The activities of PR-2 were not significantly induced by KPhi or wounding, but its content was significantly increased by pathogen infection with the highest in untreated tubers after 48 hours. The KPhi treated tubers produced more enzymatic activities significantly after wounding and pathogen infection than those that were not treated. Our findings suggested that KPhi stimulates a quick and vigorous response in tubers against the pathogen infection via activation of defense responses, such as defense biochemical compounds, pathogenesis-related enzymes and antioxidant enzyme activities.
Keywords: *potassium phosphite, potato late blight, antioxidants enzymes, pathogenesis-related (PR) enzymes, Phytophthora infestans*

Abbreviation: KPhi - Potassium Phosphite, PR-2 - β-1, 3-glucanase, PR-3 - chitinase, POD - peroxidase, PPO - polyphenol oxidase, SOD - superoxidase dismutase, CAT - catalase, BABA - ß-aminobutyric acid, ROS - Reactive Oxygen Species, SAR - systemic acquired resistance, FW - fresh weight，GAE – gallic acid equivalent

Introduction

Solanaceae is an important plant family which has a diverse group of plants, ranging from wild species to several economically important cultivated crops. Potatoes (*Solanum tuberosum* L.) belongs to this family and it is the first non-grain food crop worldwide and third highly consumed food crop in the world, after wheat and rice (Norton and Swinton, 2018). Global potato production in 2017 was revealed as 376.8 million metric tons with

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44994514

major production from developing countries. Nowadays, China is leading potato producers throughout the world accounting for 99.1 million tons, followed by India and the Russian Federation (Norton and Swinton, 2018).

The causative agent of potato late blight is oomycete *Phytophthora infestans* (Mont.) De Bary. It is one of the most devastating plant diseases throughout the world. This disease causes leaf death, which leads to significant yield reductions. The pathogen can also infect tubers which cause storage reduction as well as reduced seed quality. Pathogenic infections trigger plant defense responses by changing their biochemical contents. Biochemicals can limit the multiplication of pathogens, making the host environment unsuitable for growth of pathogens or directly by targeting and eliminating the attack of the microorganism (Hammerschmidt, 1999).

Activating the plant's immune system by different chemical and symptomatic factors could be an alternative way to increase plant resistance to biotic stresses (Lim et al., 2013). It has been reported that various chemical and biological compounds can trigger plant defense reactions without a real attack of pathogens. These compounds are known as resistance inducers or plant enhancers (Silva et al., 2011). Phosphite ($H_2PO_3^{2-}$, Phi), an alkaline salt of phosphorous acid (H_3PO_3) and phosphonate [$HPO(OH)_2$], is commonly known for controlling plant diseases by enhancing plant defense responses (McDonald et al., 2001). Phi has direct effects on inhibiting oxidative phosphorylation in oomycete metabolism (Lobato et al., 2008), and indirect effects that stimulate host protection responses eventually inhibit the pathogen growth (Daniel and Guest, 2005). It plays an important role as a fungicide, fertilizer or biostimulator or can work with at least one of these properties in various research systems (Thao and Yamakawa, 2009). Also, Wang-Pruski et al. (2010) and Borza et al. (2017) also report a comprehensive protective effect of Phi, which increased when applied in combination with a chlorothalonil protective herbicide. Further, Borza et al. (2014) studied foliage and postharvest treatments of Phi and its uptake and translocation in leaves and tubers. Machinandiarena et al. (2012), Wiesel et al. (2014) showed that Phi triggers disease resistance through increased hydrogen peroxide (H_2O_2) production, gene expression of pathogenesis-related (PR) protein PR1, glucanase (PR 2) and phenylalanine ammonia lyase, and increased soluble protein accumulation in *Arabidopsis*, potato and tomato plants (Chandrasekaran et al., 2017; Silva et al., 2011).

Potassium phosphite (KH_2PO_3) has been applied on plants to induce resistance to various oomycete pathogens, such as *Phytophthora* species (Kim et al., 2010) and *Pseudoperonospora* species (Silva et al., 2011). KPhi has been studied to inhibit normal metabolism of oomycetes, limiting their growth and stimulating defense mechanisms of plants, as well as boosting the synthesis and transportation of secondary metabolites (Kuć, 2001). KPhi can entourage reposition of defense molecules (Dalio et al., 2014; Ramezani et al., 2017), such as phytoalexin and phenols that are defensive chemicals for resistance to diseases and to overcome pathogenic attacks. The accumulations of phytoalexins and phenols were recorded in potato tubers after plants were treated with ß-aminobutyric acid (BABA) (Olivieri et al., 2009). KPhi can induce plant resistance by initiating hypersensitive reactions, resulting in programmed cell death of infected cells, increasing activities of phenylpropanoid biosynthetic enzymes, resulting in increased acquired systemic immunity against pathogens (Eshraghi et al., 2011). PR proteins are vital factors for the interaction of plants with pathogens and many groups of PR proteins can be a part of

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first defense responses against pathogens (Kadota et al., 2014). Two PR proteins, chitinase and ß-1, 3-glucanase, were investigated in potato tubers in this study. Chitinase hydrolyzes chitin in cell wall of fungi (Dehestani et al., 2010), while the ß-1, 3-glucanase plays key role in plant defense responses to pathogen infections by catalyzing the cell wall cleavage of many pathogens (Adams, 2004). It has been shown that ß-1, 3-glucanase activity is correlated with systemic acquired resistance (SAR) that protects plants from various biotic stresses. Cellular defense function against oxidative stress can also be measured by increased POD, PPO, SOD, and CAT (Debnath et al., 2018b; Majer et al., 2014). Also, Lobato et al. (2011) studied that, after KPhi-treated potato leaves were mechanically wounded and/or infected by different pathogens, phytoalexins, antioxidant enzyme activities, and pathogenesis-related protein production were increased. In this study, we analyzed the protection function of KPhi against two pathogenic strains of *Phytophthora infestans*, in stored tubers obtained from the plants foliar treated with KPhi during the growing season. We also measured the contents of phytoalexins and phenols, and the enzymatic activities of chitinase, ß-1, 3-glucanase, POD, PPO, SOD and CAT, in two tubers of two potato varieties that vary in their resistant levels to Late blight.

Materials and Methods

Experiment location

The plants were grown in the College of Horticulture greenhouse at Fujian Agricultural and Forestry University, Fuzhou city (latitude 260, 5 '16 "N, longitude 190, 14 '6" E, altitude 42.09 m), in China between February, and May 2017 from October 2017 to January 2018. During the growing seasons, the temperatures in the greenhouse varied between 15-24°C and natural daylight cycle were 10-14 hours.

Biological samples

Two Chinese potatoes (*Solanum tubrusum* L.) varieties Xingjia No. 2 (moderately resistant to late blight) and Zhongshu No. 3 (moderate susceptible to late blight) were used in this study. Xingjia No. 2 was provided by Institute of Agricultural and Forestry Sciences in Daxinganling region, Heilongjiang Province; Zhongshu No. 3 was provided by Institute of Vegetables and Flowers, Chinese Academy of Agricultural Science. Pieces of seed potatoes (~ 50 g of weight with 2-3 eyes) were planted in 5-liter plastic pots containing a mixture of vermiculite, peat and perlite (1: 3: 1, v/v) in the greenhouse. Plants were watered by using a sprinkler irrigation system. The plants were grown between February 26, 2017 and May 25, 2017 and repeated once from October 15, 2017 to January 15, 2018. In each season, 50 plants were used for each variety. The 50 plants were divided into two groups of 25 each: Group 1 was sprayed with water; Group 2 was sprayed with 1% KPhi biweekly, total three times during the growing season. Plants from different treatment groups were randomized in the greenhouse. KPhi was first sprayed to the leaves 35 days after the emergence. The second spray was 15 days after the first spray and the third spray was 15 days after the second spray. The control plants applied with sterilized water. Two strains of *Phytophthora infestans* (Pi) (Mont.) ASO and 1-12-25, isolated from Yunnan province by personnel of College of Plant Protection at Fujian Agriculture and Forestry University, were used in this study. They were grown on Zhongshu No. 3 potato tuber

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slices and kept at 18°C and 90% RH. After seven days, mycelium was collected in sterile distilled water and stimulated to release zoospores by incubation at 4°C for 6 hours. After filtration through 5 layers of cheesecloth, the sporangial suspension was observed under a microscope for quantification before use as an inoculum. The rate of sporangia was adjusted to 4 x 10^{-4} sporangia/mL using a hematocytometer (Lobato et al., 2011).

KPhi stock solution, preparation and treatment

To prepare the KPhi stock solution (1%), the phosphorous acid crystals (Sigma-Aldrich) was neutralized with potassium hydroxide (KOH) by slowly mixing of phosphorous acid and potassium hydroxide solution until the pH was adjusted to 6.3. KPhi stock solution was diluted to 1% (1 g/100 mL) and sprayed to plants at the value of 30 mL per plant (4.5 L/ha) using a hand sprayer. Greenhouse plants were treated by KPhi three times during the growing season. KPhi was first sprayed to the leaves 35 days after the emergence. The second spray was 15 days after the first spray and the third spray was 15 days after the second spray. The control plants applied with sterilized water.

Wounding procedure and sample preparation

Tuber wounding procedure was performed according to the mechanism described by Kim et al. (2010) with a small modification. Collected tubers were first peeled, and internal flesh tissues were cross-sectioned by a mandolin cutter to pieces of about 10 mm thickness. The pith of the tubers was removed and tuber pieces were placed on a rack with a wet paper towel in five litter plastic boxes with lids at 18ºC in the dark up to 7 days. Water was added to the bottom of the boxes in order to keep the humidity. Periderm samples were collected at 0, 6, 12, 24, 48 hours and seven days after wounding. Samples were immediately frozen in liquid nitrogen and stored at -80ºC until use.

Late blight resistance evaluation

Tubers from plants of Xingjia No. 2 and Zhongshu No. 3 treated with KPhi and control were stored at 8°C and 55% RH for three months. The tubers were then washed in distilled water, sterilized by soaking in 2% sodium hypochlorite for 5 min and rinsed with distilled water and then used for phytopathological tests. Tubers slice (5-6 cm in diameter, 10 mm thickness) were infected with 50 µL of sporangia suspension (4 × 10^{-4} sporangia/mL) and incubated at 18°C in darkness. The disease severity symptom was evaluated on the upper surface of potato slices seven days after infection according to the method described by Lobato et al. (2008). The disease severity was recorded with the scale from 1 to 10, where 1 = no lesions, 2 = a few circles, 3 = up to 5%, 4 = 5–10%, 5 = 10–25%, 6 = 25–50%, 7 = 50–75%, 8 = 75–85%, 9 = 85–95% and 10 = 95–100% of leaf area with late blight symptoms (Lobato et al., 2008). Two strains of *Phytophthora infestans* (Pi) (Mont.) ASO and 1-12-25 were used in this study, and a total of 25 tubers in each variety and each treatment were used in three replications. The negative control has KPhi treated tuber slices but not infected by *P. infestans*.

Extraction and determination of phytoalexin and phenol contents

Phytoalexins was extracted and measured according to the method described by Lobato et al. (2008) Tubers (1 g) were mixed in 10 mL chloroform/methanol/acetic acid (50:5:45

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v/v/v) using a blinder. The homogenate was kept overnight at room temperature (15°C) and then filtered through cheesecloth. Chloroform and 0.2 M acetic acid were added in equal volumes to the filtered mix. The blends were shaken well and let stand to separate into two layers. The top chloroform layer containing phytoalexins was removed and evaporated in 60°C an electrothermal blast dryer (Shanghai Yiheng scientific instrument Co., Ltd.) until it is dry. The dried sample is dissolved into 1 mL of cyclohexane and 2 mL of sulfuric acid (H_2SO_4) were added to the solution. The mixture was stirred and centrifuged at 12,000 rpm for 30 min. Then the red color of the lower sulfuric acid layer was measured at 500 nm with a spectrophotometer (METASH UV5100H) to determine the concentration of phytoalexin as µg/g FW. Phenols were extracted according to the Folin-Ciocalteu colorimetric procedure with minor modifications (Škerget et al., 2005). Briefly, 0.5 g of the tuber sample was homogenized with 2.5 mL Folin-Ciocalteau 0.2 N (Solarbio Life Sciences) for 5 min and then 2.0 mL sodium carbonate (75 g/L) was added. The mixture was kept at room temperature for 2 hours before it was measured at 760 nm using a spectrophotometer (METASH UV5100H). The phenol concentration was represented as gallic acid equivalent (GAE) (mg) on fresh weight (FW) (mg GAE/g FW).

Measurement of antioxidant enzyme activities

The square cut (100 mg) in the central part of tuber disks was grounded in a mortar and pestle at 0, 6, 12, 24 and 48 hours after inoculation or wounding. The mixture was filtered through four layers of cheesecloth and centrifuged at 12,000 rpm for 15 min. The supernatant, which represented the soluble tuber extract, was immediately processed.

The activity of chitinase and ß-1, 3-glucanase, was measured spectrophotometrically using the [Commercial chitinase and ß-1, 3-glucanase Assay Kits (GA-1-Y and JDZM-2-G), Beijing Suolai Bao Technology Co., Ltd, China] following the procedures described by the manufacturer.

POD was measured using the method by Shannon et al. (1966). The assay mixture containing 2.5 mL of phosphate buffer, 0.2 mL of suitably diluted tuber extract, and 0.1 mL of o-dianisidine (50 mg of o-dianisidine was dissolved in 50 mL of methanol) was incubated at 28°C in a water bath for 2 min. The reaction commenced by adding 0.2 mL of H_2O_2 (0.6%) and the absorbance was recorded at 470 nm against reagent blank.

PPO it was measured in 1 ml reaction mixture consisting of 20 µl of raw extract, 35 mM sodium phosphate buffer pH 6 and 100 µl 0.2 M catechol. The reaction was initiated by the addition of catechol to the mixture and absorption 420 nm was measured in 1 minute. Total SOD activity was determined by it is the ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in a spectrophotometer at 560 nm as previously described (Cakmak and Marschner, 1992). The assay of CAT activity was based on its ability to decompose H_2O_2, with the absorbance of the supernatant at 240 nm (Choo et al., 2004).

Statistical analysis

All the experiments were organized in a complete random block design (CRBD) with three biological replicates for each treatment and repeated at least three times. The surface area showing damage symptoms data were analyzed by the *t-tes*t, phytoalexin and phenol contents, as well as enzymatic activities, were analyzed for significant variances and differences using one way ANOVA. SPSS (version 22, SPSS, Chicago) was used for

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means value analysis. Differences between means were detected, when using Tukey's test (P < 0.05) and graphs generated by Microsoft Office Excel 2016.

Results

Effect of KPhi on late blight suppression

The effects of leaf treatments of KPhi in greenhouse conditions have caused reduced sensitivity to *Phytophthora infestans* in the tubers. The comparison between inoculated tuber pieces of Xingjia No. 2 and Zhongshu No. 3 showed a decreased diameter development of pathogen growth from the plants treated with KPhi in both cultivars (*Figure 1A*). Data in *Figure 1B* showed a significant effect of the KPhi treatment, which caused reductions in lesion size caused by *P. infestans* in tubers of both cultivars. Also, it is confirmed that Xingjia No. 2 was less susceptible to both strains of *P. infestans*, and Zhongshu No. 3 showed higher susceptibility to *P. infestans* as seen in the control tuber slices (*Figure 1, A and B*). As well, the two strains showed a different range of aggressiveness, ASO is more aggressive that of 1-12-25 (*Figure 1, A and B*).

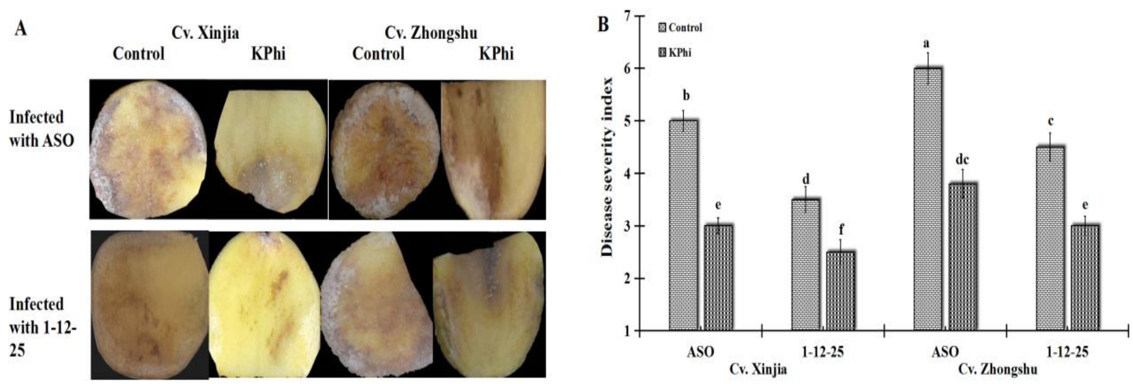

Figure 1. *Effect of foliar application of KPhi on tuber lesion diameter caused by two Phythophthora infestans strains (A). Effect of foliar application of KPhi on disease severity in tuber slices against Phytophthora infestans (B). Means that not sharing a common letter within a graph are significantly different at p-value < 0.05 according to the Tukey's test. The experiments included three biological replications and were performed two times*

Effect of KPhi on phytoalexin and phenol accumulations in tubers

The contents of phytoalexins and phenols were quantified in tuber pieces taken from plants treated by KPhi or non-treated and infected by ASO isolate of *P. infestans* (*Figure 2*). The highest amount of phytoalexins was accumulated a week after infection with *Phytophthora infestans*, while the lowest was measured in non-treated tubers (*Figure 2, A and B*). Phytoalexin contents were 3 fold increased in the treated inoculated tubers, followed by un-treated tuber (CPi) (*Figure 2 A and B*). Wounding treatments (PhiW) also showed a 1 fold increase than their controls (*Figure 2, A and B*). The lowest phytoalexin detected in healthy tubers. As shown in *Figure 2*, 48 hours after infection Xinjia No. 2 tubers produced a higher accumulation of phytoalexin than Zhongshu No. 3. The phenol contents were increased 3 fold in KPhi treated plants (PhiPi), followed by untreated plants

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DOI: http://dx.doi.org/10.15666/aeer/1702_44994514

(CPi) 48 h after tubers inoculation (*Figure 2, C and D*). Wounding (CW) increased phenol contents in both Xingjia No. 2 and Zhongshu No. 3 (*Figure 2, C and D*). Pathogen infection (CPi) increased the phenol contents in both Xingjia No. 2 and Zhongshu No. 3 when compared with their controls. Phi treated samples (Phi) also had a significant increase in phenol contents when compared with the controls in both cultivars, but wounding after Phi treatments (PhiW) only increased phenol contents in Zhongshu No. 3 (*Figure 2, C and D*). The highest content of phenols was detected after inoculation of KPhi treated tubers in both cultivars; while the lowest were found in non-inoculated tubers of both cultivars (*Figure 2, C and D*).

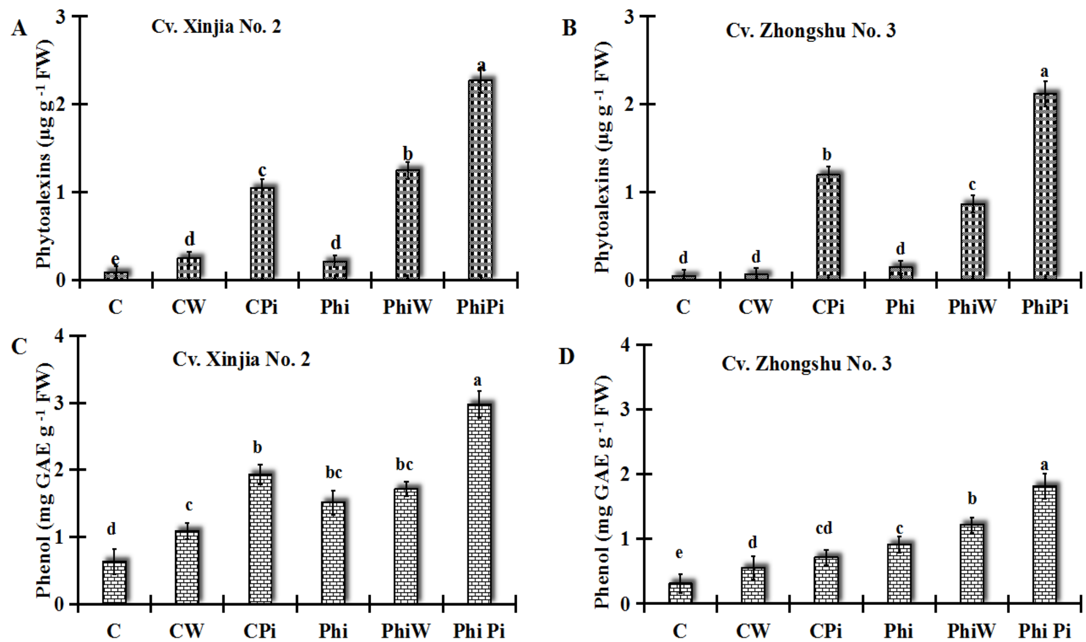

***Figure 2**. Effects of KPhi application on phytoalexin contents of Cv. Xingjia No. 2 (A) and Zhongshu No. 3 (B), and phenol contents of Cv. Xingjia No. 2 (C) and Zhongshu No. 3 (D). Phytoalexin and phenol were quantified in tuber pieces after wounding (W), and inoculation by P. infestans (Pi) ASO isolate. Means not sharing a common letter within the same letters in the graph are significantly different at P < 0.05 according to Tukey's test. The experiments included three biological replications and were performed three times. C, control tubers; CW, control + wounded; CPi, control + inoculation with ASO isolate; Phi, tubers taken from KPhi applied plants; PhiW, tubers treated by KPhi + wounding; PhiPi, tubers from KPhi treated plants + inoculation with ASO isolate. FW, fresh weight*

Effect of KPhi on chitinase and ß-1, 3-glucanase activities in tubers

In this study, the effect of KPhi on chitinase and ß-1, 3-glucanase activities after wounding and inoculation with ASO isolate strain were determined at various time points. Tuber slices from KPhi-treated (Phi) and control plants (C), wounded (W) or inoculated with a pathogen (Pi) were measured at 0, 6, 12, 24 and 48 hours after the treatments (*Figure 3*). A gradual increase with time in chitinase activity after wounding and pathogen infection was observed in control potatoes of both cultivars. The level of chitinase activities in both cultivars without treatments was similar (*Figure 3, A and B*). Wounding

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(CW) increased the chitinase activities in both cultivars, similarly to the Phi treatments (CPhi). The infection treatments (CPi) also showed a similar response as with wounding in both cultivars. Both wounding and pathogen infections in Phi treated tubers had significantly increased the chitinase activities in both cultivars (*Figure 3, A and B*).

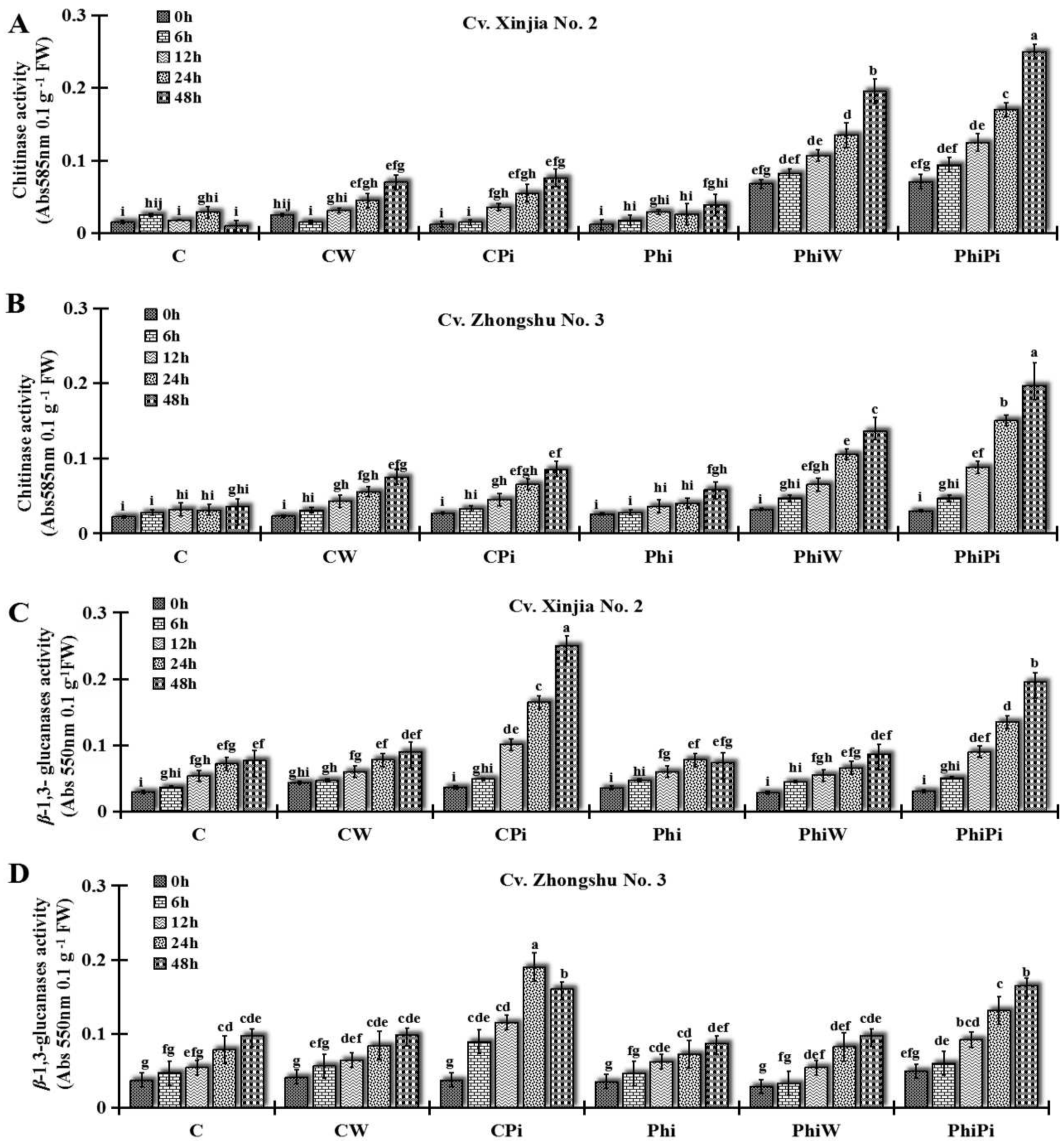

***Figure 3.** Effects of KPhi application on chitinase activities in Xingjia No. 2 (A) and Zhongshu No. 3 (B) tubers, and β-1, 3-glucanase activities in Xingjia No. 2 (C) and Zhongshu No. 3 (D) tubers. Activities of chitinase and β-1, 3-glucanases were analyzed from tuber after wounding or inoculation with P. infestans for 0, 6, 12, 24, and 48 h, respectively. Means not sharing a common letter within the same letter in the graph are significantly different at P< 0.05 according to Tukey's test The experiments included three biological replications and were performed three times. C, control tubers; CW, control + wounded; CPi, control + inoculation with ASO isolate; Phi, tubers taken from KPhi applied plants; PhiW, tubers treated by KPhi + wounding; PhiPi, tubers from KPhi treated plants + inoculation with ASO isolate. FW, fresh weight*

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In wounded tubers taken from plants applied with KPhi, the rate of chitinase activity was increased 2 folds by 48 hours in KPhi treated tubers in Xinjia No. 2. In infected tubers taken from plants applied with KPhi, the rate of chitinase activity was increased 3 folds by 48 hours in Xinjia No. 2 tubers and then about 2 folds in Zhongshu No. 3. It is noted that the enzyme activities of wounded and inoculated tuber slices from Xingjia No. 2 after Phi treatments were higher when compared to that of Zhongshu No. 3 (*Figure 3, A and B*).

In contrast to chitinase, ß-1, 3-glucanase activities did not change significantly in potatoes tubers of KPhi treated plants when compared with that of the controls in both cultivars (*Figure 3, C and D*). Wounding treatment did not significantly alter the enzymatic activities in these samples either. The ß-1, 3-glucanase activity was increased sharply 48h after *P. infestans* infection in untreated tubers (CPi) more dramatic of cultivar Xingjia No. 2 (*Figure 3C*). An approximately two-fold increase in ß-1, 3-glucanase activity was recorded 24 hours after pathogen infection in Xingjia No. 2 cultivars when compared to control (*Figure 3C*). In Zhongshu No. 3, approximately 3 folds increase were noted at 24 hours after pathogen infection in untreated samples (CPi) and about 2 folds increase from the KPhi treatment plants and wounded tubers (PhiW) (*Figure 3D*) when compared to their corresponding controls. The ß-1, 3-glucanase activity increased significantly in potato tubers 24 hours after infection with a pathogen (CPi) (*Figure 3D*) followed by treated (KPhi) tubers and control after infection, compared to healthy tubers (*Figure 3, C and D*).

Effect of KPhi on POD and PPO in tubers

Inoculation by ASO late blight strain caused an increase in reactive oxygen species (ROS) response and up-regulation of antioxidant enzymes in the defense system to alleviate ROS-mediated damages. The POD and PPO activities were measured spectrophotometrically at various time points in tubers after wounding or inoculation by a pathogen (*Figure 4*). The highest POD activities values were recorded 48 hours after the pathogen inoculation, both in control (CPi) and KPhi treated plants (PhiPi). At 48 hours, the enzymatic activities increased up to 4 folds in Xingjia No. 2 and about 4 folds in Zhongshu No. 3 in the inoculated potatoes taken from tubers applied with KPhi compared with the tubers from the untreated plants (*Figure 4, A and B*). Meanwhile, the PPO activities showed similar increases, but the highest activities were detected at 12 hours after the inoculation (CPi) in Xingjia No. 2, and 24h after infection (CPi) in Zhongshu No. 3 (*Figure 4, C and D*). At these time points, the tubers obtained from the plants treated with KPhi presented increases in the PPO activities, approximately 4.8 times in Xingjia No. 2 and 4-times in Zhongshu No. 3 in comparison to the inoculated potatoes collected from untreated plants (*Figure 4, C and D*).

Effect KPhi on SOD and CAT response after inoculation by late blight

SOD and CAT activities were analyzed in a spectrophotometer at 0, 6, 12, 24 and 48 hours after pathogen inoculation with ASO isolate on KPhi and control tubers. As shown in *Figure* 5 (A and B), the lowest SOD activity was found at 0 hours while the highest value of SOD was found at 48h after inoculation (CPi) in Xingjia No. 2 (*Figure 5A*) and 24 hours after inoculation (CPi) in Zhongshu No. 3 (*Figure 5B*). The KPhi treated samples 48 hours after infection (PhiPi) had increased the SOD activity by about 3 folds in Xingjia No. 2, while it has increased by around about 2 folds in the Zhongshu No. 3 24hours after infection (*Figure 5, A and B*). Wounding did not significantly increase the SOD activities in

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Phi treated samples. The CAT activity was found to be the lowest at 0 h in both cultivars and increased in the KPhi treated at 48 hours after inoculation (PhiPi) to about 4 folds in Xingjia No. 2 (*Figure 5C*) and only about 3 folds after inoculation in Zhongshu No. 3 cultivar (*Figure 5D*). Control samples at 24 hours had an increase of about 2 folds after inoculation (CPi), in contrast to the activities of controls inoculated by the late blight pathogen (*Figure 5, C and D*).

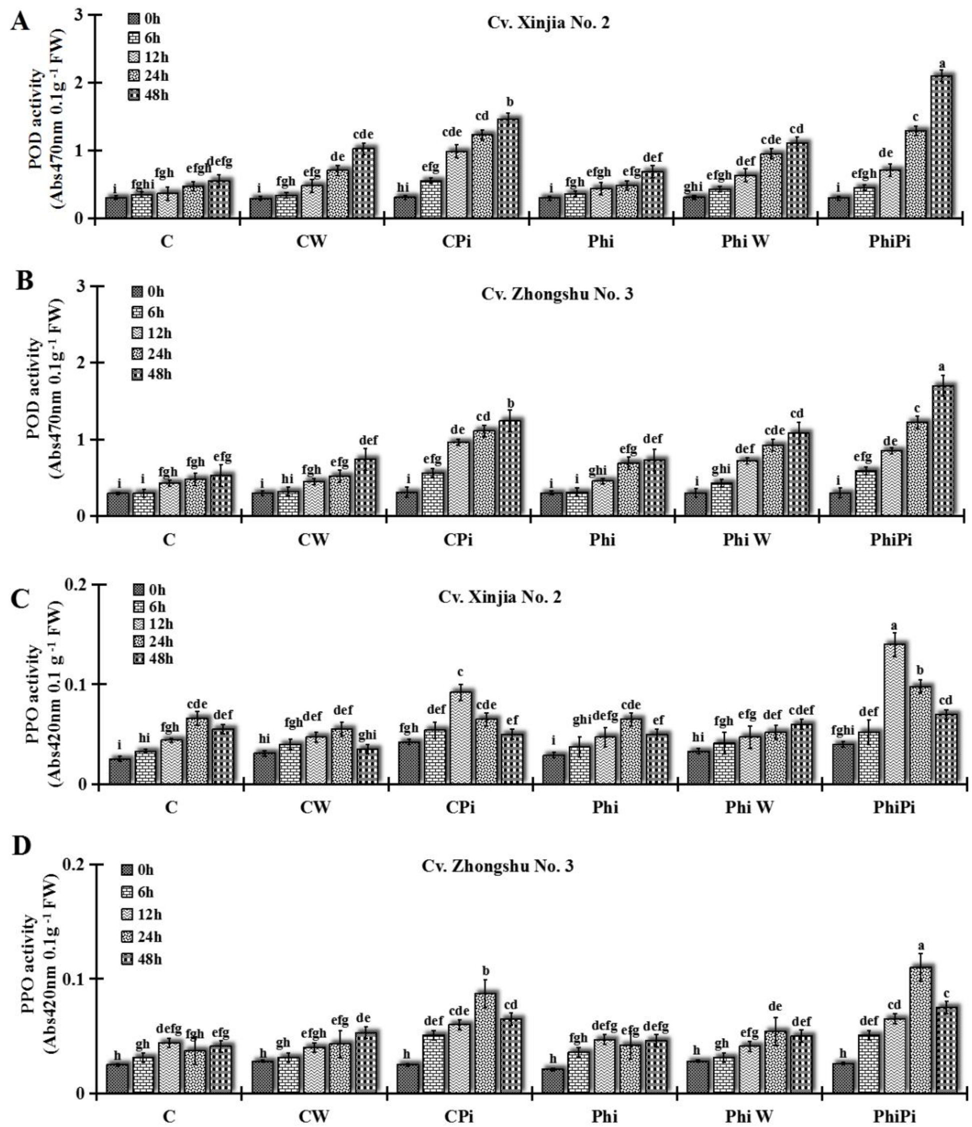

Figure 4. *Effects of KPhi application on peroxidase (POD) activities in Xingjia No. 2 (A) and Zhongshu No. 3 (B) tubers, and polyphenol oxidase (PPO) activities in Xingjia No. 2 (C) and Zhongshu No. 3 (D) tubers. Measures not sharing a common letter within the same letter in the graph are significantly different at P < 0.05 according to Tukey's test. The experiments included three biological replications and were performed three times. C, control tubers; CW, control + wounded; CPi, control + inoculation with ASO isolate; Phi, tubers taken from KPhi applied plants; PhiW, tubers treated by KPhi + wounding; PhiPi, tubers from KPhi treated plants + inoculation with ASO isolate. FW, fresh weight*

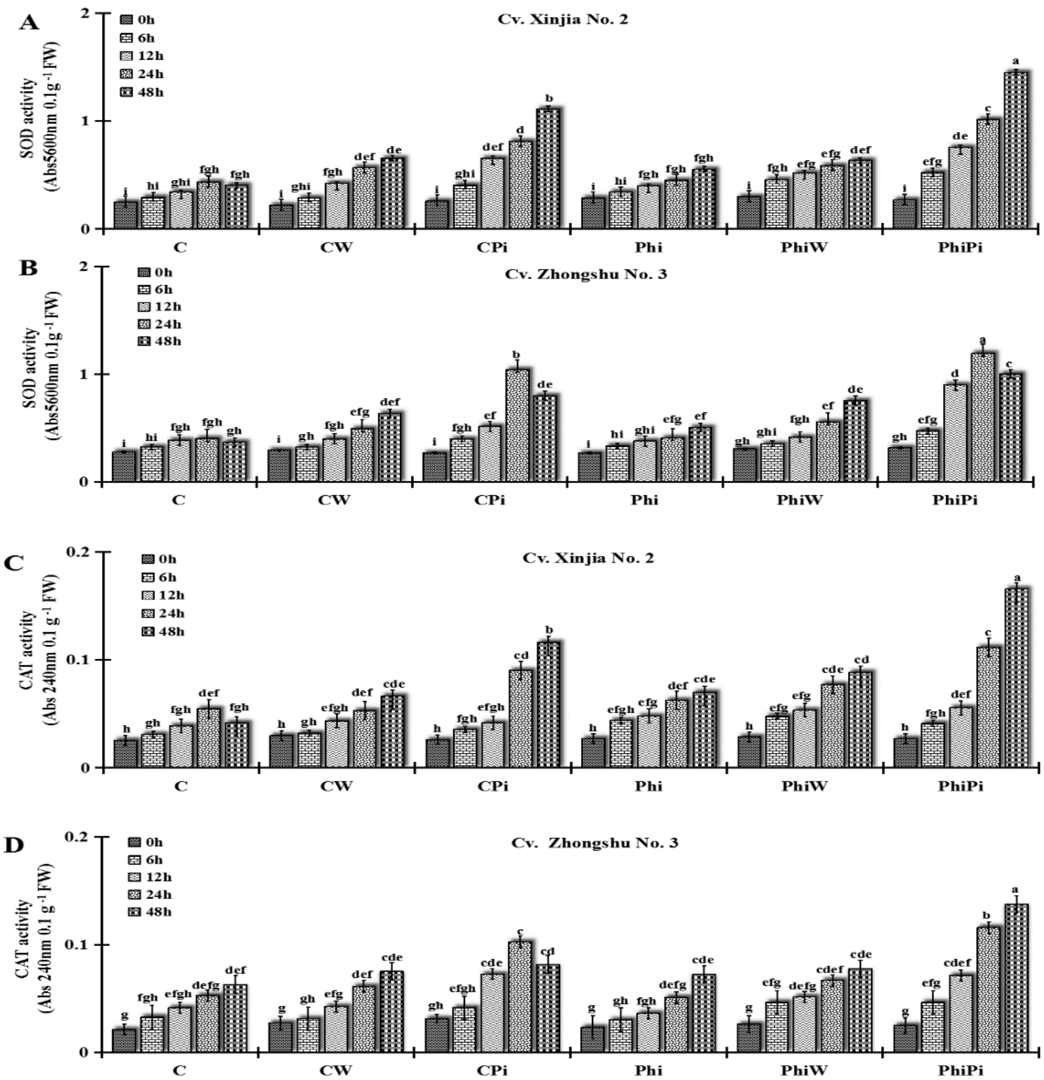

Figure 5. *Effects of KPhi application on superoxide dismutase (SOD) activity in Xingjia No. 2 (A) and Zhongshu No. 3 (B) tubers, and catalase (CAT) activity in Xingjia No. 2 (C) and Zhongshu No. 3 (D) tubers. Measures not sharing a common letter within the same letter in the graph are significantly different at P < 0.05 according to Tukey's test. The experiments included three biological replications and were performed three times. C, control tubers; CW, control + wounded; CPi, control + inoculation with ASO isolate; Phi, tubers taken from KPhi applied plants; PhiW, tubers treated by KPhi + wounding; PhiPi, tubers from KPhi treated plants + inoculation with ASO isolate. FW, fresh weight*

Discussion

In the present study, the action of KPhi on physio-biochemical contents and the enzyme activities of the potato plant in response to *Phytophthora. infestans* and wounding was studied, using two potato cultivars with different degrees of horizontal resistance against the two late blight isolate strains (*Figure 1*). It is demonstrated that the applications of KPhi on leaves results in the postharvest tubers with notably decreased disease symptoms after infection with two *P. infestans* pathogen strains. The significant protection was observed in

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moderately resistant Xinjia No. 2, after postharvest. These results were consistent with previous studies in potato. A study reported by Mayton et al. (2008) who analyzed various compounds of Phi on the protection against *P. infestans* in leaves and tubers of postharvest potato. Their experiments showed that *P. infestans* infection on leaves was well controlled by Phi as effective as a conventional fungicide against tuber late blight. Comparable to our results, Lobato et al. (2011) showed that the use of KPhi on the leaves resulted in a series of postharvest defense reactions at field conditions. Phytoalexin and phenol accumulations were related to defense reactions of the potatoes and our results showed great increase phytoalexins and phenols in potatoes tuber treated with KPhi compared to untreated slices from the control plants and wounded samples. This effect was relatively high in moderately resistant cultivar Xingjia No. 2 (*Figure 2, A and C*), and lower in the more susceptible cultivar. Zhongshu No. 3 (*Figure 2, B and D*). This means that KPhi has been able to activate a general defense response in the potato plants and the response is cultivar dependent. The involvement of phytoalexins and phenols in response to Phi were reported and various works had been reviewed by Gray et al. (2018). Plants produce enzymes, such as chitinases and ß-1, 3-glucanases (Silva et al., 2011) which can break down cell wall compounds of pathogens. These enzymes are essential determinants of plant resistance to fungi attack (Funnell and Phillips, 2004). Since these enzymes are PR proteins and their stimulations in the tubers after wounding or inoculation have been reported (Alexandersson et al., 2016; Lobato et al., 2017). Chitinases play essential roles in activating defense response in plants, alone or in combination with ß-1,3-glucanases (Mauch et al., 1988). Our results represented an increase in chitinase activity after infection by late blight pathogen. KPhi treated tubers at 48 hours after infection, clearly indicated the presence of the increased enzymatic activities (*Figure 3, A and B*). This result also confirms the efficacy of anti-fungal function of KPhi, as pointed out by Deliopoulos et al. (2010).

In the present study, ß-1, 3-glucanase was not significantly induced by KPhi after inoculation by the pathogen in Xingjia No. 2, and the highest enzymatic activity was found in untreated tubers 48 h (*Figure 3C*). Likewise, KPhi had a less effect on Zhongshu No. 3, with the highest activities of this enzyme found at 24 hours after inoculation in untreated tubers (*Figure 3D*). Compared to our results, cucumber plants inoculated with *Pseudoperonospora cubensis* showed a quick increase in chitinase and ß-1, 3-glucanase activities, which causes the degradation of the cell wall of fungi (Moazzameh et al., 2018). Greater induction of ß-1, 3-glucanase in the treated plants indicates that the activity of ß-1, 3-glucanase can weaken the fungi cell wall and prevent hypha colonization (Menu-Bouaouiche et al., 2003). Lim et al. (2013) used iTRAQ-based quantitative proteomics to identify significant changes in defense proteins including pathogenesis-related, stress-responsive and detoxification-related proteins in potato leaves after treatment with Phi. They identified that 93 (62 up-regulated and 31 down-regulated) differentially regulated proteins were identified in the leaf proteome of Phi-treated plants. To find out whether oxidation enzymes caused by stress can participate in the KPhi-induced defense mechanism, the activity of POD, PPO, SOD and CAT were measured in tubers after harvest. The increases in POD and PPO activities were highlighted that these enzymes could be a part of KPhi defense responses.

The antioxidant POD enzyme is necessary for inducing systemic resistance and could be used as a biomarker of induced resistance in plants (Kuć, 2001). The POD and PPO

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activities is increased after wounding or inoculation with a pathogen in tubers taken from plants applied with KPhi (Lobato et al., 2011). In their studies, KPhi application on infected plants showed a direct relation between disease reduction and the enzyme activity. Similarly, in our result, the POD and PPO activities were increased significantly in contrast to the control plants 12 and 48 and hours after inoculation in both cultivars (*Figure 4*). Foundation PPO enzymes to wounding and enzymatic browning had been investigated in many plants (Demiİr and Kocaçalişkan, 2001). This enzyme plays a significant role in confirm of alkaloids under biotic and abiotic stress (Bilková et al., 2005). PPO activity in this study had increased at 12 hours in inoculated tubers collected from KPhi used plants in Xingjia No.2 (*Figure 4C*) and 24 hours after infection in Zhongshu No. 3 (*Figure 4D*). Similar to our results, Lobato et al. (2011) also showed that KPhi promoted POD and PPO activities after plants were infected by late blight.

It is known that SOD plays a substantial role in stress tolerance of plants and it is the first path of protection against damaging effects of high levels of ROS. Several environmental stresses usually lead to an increase in ROS production. As showed in *Figure 5 (A and B)*, the highest rate of SOD was found at 48 hours in Xingjia No. 2 (*Figure 5A*), followed by Zhongshu No. 3 (*Figure 5B*) in both varieties. Similar to our results, Mofidnakhaei et al. (2016) showed that potassium phosphite affected plant growth, and enzymatic activities of POD, SOD and CAT in cucumber plants challenged with *Pythium ultimum*.

Furthermore, CAT is necessary for ROS detoxification under stress situations by degrading H_2O_2 into H_2O and O_2 (Debnath et al., 2018a). In this study, CAT activity was increased in the plants treated with KPhi after inoculation by *P. infestans* in cv. Xingjia No. 2 (*Figure 5C*) and Zhongshu No. 3 (*Figure 5D*) in the potato tubers, respectively, compared to the wounded or control samples (*Figure 5C and 5D*). This is the first report demonstrating the responses of CAT and SOD to KPhi.

Conclusions

Plant defense pathways use some physiological and biochemical means to enhance resistance against pathogen invasions. KPhi induced the biochemical compounds and enzymes activity. Increased contents of phytoalexins and phenols were needed for plant defense processes. These metabolites will help plants to improve their resistance to pathogens.

Additionally, KPhi can increase the defense enzyme activities, inducing chitinase, ß-1, 3-glucanase activities and boosting production of antioxidants enzymes. The detailed analysis of cell wall composition and structure will elucidate and confirm our ideas of Phi involve in inducing resistance. KPhi treatment considerably mitigated POD, PPO, SOD and CAT activities which leads to late blight tolerance in potato plants. Finally, our data showed a partial characterization of some biochemical markers and certain phytopathological features involved in the response by KPhi in two cultivars with different degree of multigenic resistance to *Phytophthora infestans*. We believe this increased tolerance triggered by KPhi is belong to cell wall amplification and increased productions of biochemicals and a group of enzymatic antioxidants activities.

Furthermore, our data supported the application of KPhi as a priming inducer to enhance cell defense mechanisms. This suggests that the response to foliar KPhi treatment

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could have a useful effect on physio-biochemical events and promoting tuber resistance against *Phytophthora infestans* if used in an IPM program. Overall, our data support the fact that KPhi improves resistance by improving cellular defense biochemical compounds, PR enzymes and key antioxidants enzymes activities.

Authors' contributions. M. A. Mohammadi conceived, performed, designed and conducted the research and wrote the manuscript. Yupi Xi and Beibei Zhang assisted in performing experiments. Xiaoyun Han analyzed the data. Faxiu Lan contributed reagents/ materials/ analysis tools. Gefu Wang-Pruski and Zhizhong Zhang conceived, instructed research work, supported financially and administratively and final approval of the manuscript. All of the authors revised, discussed and commented on the manuscript.

Acknowledgments. This experiment conducted in the Joint FAFU-Dalhousie lab, College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou, 350002, China. The financial support for this research is from the Foundation for Fostering Young Talents and Scientific Research Starting Foundation for Mingjiang Scholars from College of Horticulture, FAFU. Additional funding from Ming Jiang Scholar fund from Fujian Province and FAFU (116-114120019) and Special Innovation Foundation (CXZX2016108, CXZX2017168) from FAFU, China also contributed to this work. We want to express our appreciation to all lab mates, Abdul Waheed Ph.D. candidate college of plant protection, Fujian Agriculture and Forestry University for his valuable suggestions.

Competing Interests. The authors state that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_44994514

IMMUNOHISTOCHEMICAL EVALUATION OF CARDIAC ANGIOGENESIS OF MICE EXPOSED TO SODIUM DICHROMATE AND LINDANE

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(Received 30th Jan 2019; accepted 28th Feb 2019)

Abstract. The immunohistochemical distribution and expression of the three markers in cardiomyocytes were evaluated in three groups of mice: a control group, mice exposed to 5 mg/kg b.w./day sodium dichromate (group A) and mice exposed to 100 mg/kg b.w./day lindane (group B). All mice were sacrificed after 2 months of chronic exposure to the chemicals. Hematoxylin eosin staining revealed thickening of the anterior walls and thinning of the posterior walls of the left ventricle. Anterior and posterior vessel wall densities increased by a factor of about 1.5 in group A and about 2 in group B compared to the control group. Posterior wall vessel diameters declined by a factor of 3.7 in group A and 2.9 in group B. Immunohistochemical staining showed that sodium dichromate was associated with significantly lower expression of CD117 ($p = 0.019$) and p53 ($p = 0.002$) than in controls, whereas VEGF expression increased ($p = 0.03$). In mice exposed to lindane, the results showed an opposite, though not significant trend, namely increased levels of CD117 and p53 and decreased levels of VEGF expression with respect to the control group. We observed significant changes in ventricular wall thickness, vessel density, vascular growth factor expression and mast cell apoptosis, directly correlated with exposure to lindane.
Keywords: *VEGF, CD117, p-53, aral sea area pollutants, chromium industry*

Introduction

Among global environmental contaminants with pathogenic effects on the human body, those associated with the atmosphere, water, plants and soil are particularly prominent. Despite recent scientific and technological advances, the state of the environment continues to deteriorate year by year with negative impacts on human health.

Widespread use of chemical compounds in non-ferrous metallurgy and agriculture have increased the chemical burden of the human body. Chemicals including organochlorine pesticides are widely used to increase soil fertility, protect gardens and crops from insects, prevent infectious diseases and so forth. Chromium (VI) microelements and organochlorine pesticides, despite their different chemical nature, have toxic effects and trigger irreversible processes that affect organ histology. Structural changes in organs can lead to functional disorders in the same organs (Sánchez-Bayo et al., 2011; Cholewa et al., 2015).

The toxicity of hexavalent chromium and organochlorine pesticides has been shown to inhibit metabolic processes through genotoxic, embryotoxic, teratogenic and carcinogenic effects.

Sodium Dichromate is an orange to red colored, crystalline, inorganic compound that emits toxic chromium fumes upon heating. This chemical is used in drilling muds, in metal treatments, in wood preservatives, in the production of dyes and organic chemicals and as a corrosion inhibitor (PubChem, 2019a).

Lindane is the gamma-isomer of benzene hexachloride, a colorless to white colored, synthetic, crystalline solid. It emits toxic fumes of hydrochloric acid and other chlorinated compounds when heated to decomposition. Lindane is used as an insecticide for hardwood logs, lumber, and crops (PubChem, 2019b).

Intensive production of various substances and products is accompanied by release of many residues, the harmful environmental effects of which have now reached a critical level. These residues may contain organochlorine compounds classified as persistent organic pollutants. Residues of pesticides and herbicides are particularly significant. Their toxicity to humans ranks them near the top in the hierarchy of global problems, while their intense and long-term effects lead to various diseases (Yunbo et al., 2016; Thompson et al., 2011; Yazicioglu et al., 2013; Seidler et al., 2013).

These substances have acute and chronic effects on human and animal health, depending on the type of pesticide, its route of intake, the dose, the moment and duration of exposure, the subject's age, gender and genotype, and geographic differences (Reid et al., 2013; Mehrpour et al., 2014).

The geographical location of our research team gives it a special interest in environmental health, since the Aktobe region includes part of the Aral Sea disaster area (Palmerini et al., 2017) and a chromite mining and smelting industry (Zhumalina et al., 2018). Environmental deterioration caused by Aral Sea disaster has created a hazardous situation for the health of approximately 3.5 million people. Due to this ecelogical circumstance, high levels of dichlorodiphenyltrichloroethane and other persistent organic pollutants appeared in the soil, air and water, as well as at every level of the food chain, including humans (Ataniyazova et al., 2001).

There is increasing evidence that exposure to these pollutants and chemicals can increase the risk of cardiovascular disease. Studies into the effects of pesticides on myocardial tissue reported cardiomyocyte decompensation, circulatory and lymph flow disturbances and changes in myofibrils (Stojanovic et al., 2016; Liu et al., 2017). In particular, complex irreversible processes in blood microcirculation and myocardial ultrastructure were found (Rückerl et al., 2007). Immunohistochemistry is an important tool in experimental research, in which monoclonal and polyclonal antibodies are applied to detect the distribution of an antigen in blood vessels and cardiac tissue (Duriyan et al., 2012).

Due to the high prevalence of cardiovascular disease in ecologically impaired regions, we focus here on the morphofunctional state of heart tissue. Our aim was to evaluate morphological changes in cardiac muscle tissue through an experiment involving chronic exposure of mice to sodium dichromate and lindane.

Materials and methods

Ethics

The design of the study was approved by the Local Ethical Commission of the Marat Ospanov West Kazakhstan State Medical University on 30th November 2015 (protocol no. 11) in line with the European Convention for the Protection of Vertebrates used for experimental and other purposes (Strasbourg, 1986).

Exposure to sodium dichromate and lindane

Sixty outbred white mice weighing 20-35 grams (females and males) (Laboratory of the Scientific and Practical Center of Marat Ospanov West-Kazakhstan State Medical

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

University, Aktobe, Kazakhstan) were kept in the laboratory under a 12:12 h light-dark schedule (lights on 7 am to 7 pm) at 27 °C ± 2 and 70% humidity with free access to unlimited water and food. The mice were divided into control (20) and experimental (40) groups. The experimental groups were divided into subgroups: group A: 20 mice exposed to 5 mg/kg b.w. per day sodium dichromate ($Na_2Cr_2O_7$) (Rasool et al., 2014) (Sigma-Aldrich (MERCK, Darmstadt, Germany) and group B: 20 mice exposed to 100 mg/kg b.w. per day lindane ($C_6H_6Cl_6$) (Vijaya Padma et al., 2013) (Sigma-Aldrich, MERCK, Darmstadt, Germany). The chemicals were administered orally through a smooth plastic tube attached to a syringe. The mice were sacrificed under ether anesthesia after 2 months of exposure to the chemicals.

Histology and immunohistochemistry

Paraffin embedded histological samples were fixed for hematoxylin eosin (HE) (Van Gieson) staining. Immunohistochemistry was conducted by a commercial laboratory subcontractor according to Thermo Fisher Scientific and Keele et al. (2008) protocols.

Three commercial monoclonal antibodies were used for immunohistochemical study: CD117/c-Kit/SCF-receptor rabbit monoclonal antibody, VEGF (SP28) rabbit monoclonal antibody and p53 monoclonal antibody (DO-7), all from Thermo Fisher Scientific.

To identify heart MCs, proto-oncogene c-Kit encoding a transmembrane tyrosine kinase receptor protein, c-Kit (CD117), is used. This antibody recognizes the extracellular domain and is expressed by a variety of normal and abnormal cell types, including cardiac MCs (Lammie et al., 1994).

VEGF expression levels are indeed directly correlated with endothelial status. This vascular layer contains a variety of receptors for interactions with bacteria, chemical particulate, vasoactive substances, hemostatic factors, immune factors, complement components and more (Berendt et al., 1989). The endothelium is therefore very sensitive and easily damaged.

Considering the importance of the above factors, we selected CD117 and p53 as markers to identify mast cells and apoptosis.

Another criterion for evaluating heart tissue ageing is the level of apoptosis. Apoptosis is a cell response to a wide range of toxic substances and leads to necrosis (Neuman et al., 2001, 2002). In vitro studies have proven that exposure to chemicals can induce oxidative stress and trigger apoptosis by activating apoptosis-initiator caspase-9 and apoptosis-effectors caspases-3 to 7 (Zerin et al., 2015). Since tumor suppressor Trp53 (p53) inhibits cell growth after acute stress by regulating gene transcription, it is an appropriate marker to study apoptosis in heart tissue (Mak et al., 2017).

Sections 3 µm thick were applied to high-adhesive glass slides, treated with L-polylysine and dried at room temperature for 24 hours. The staining was performed manually and using an AUTOSTAINER-360 (THERMO, UK) with NovoLinc polymer imaging systems (“NovoCastra”, Great Britain). Controls for reaction sensitivity and specificity were unimmunized rabbit and mouse sera, and sections of control heart tissue.

Morphometric analysis of anterior and posterior walls of the left ventricle was performed with an Olympus model BX41 microscope at 40X magnification and “ImageJ” software. VEGF, CD117 on mast cells and apoptosis marker p53 were evaluated to determine toxic effects on heart tissue. Fluorescence microscopy was used

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

to calculate the percentage of cells expressing VEGF, CD117 and p53 in representative frontal serial sections of the left ventricle. Cells were counted in three sections of similar sizes of the left ventricle of each mice heart.

Statistical analysis

The data was analyzed using the MedCalc software (MedCalc Software, Mariakerke, Belgium). The t-test was applied to the parameters of morphometric analysis. The Mann-Whitney test for independent samples was used to test for cell count differences in tissue sectionsbetween the control population and the population of exposed mice. Differences were considered statistically significant at $p < 0.05$.

Results

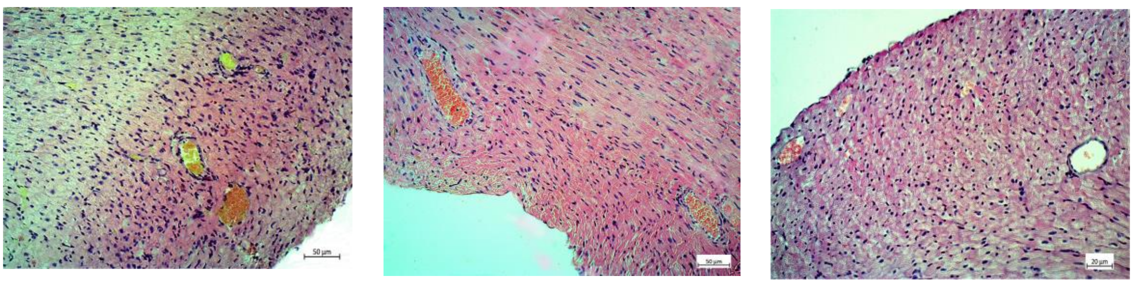
Hematoxylin-eosin staining

HE staining showed significantly higher thicknesses of the anterior left ventricular wall in experimental groups A and B than in the control group. Inversely, the posterior left ventricular wall was thinner (*Fig. 1; Table 1*). Vessel diameters were also significantly smaller. On the other hand, anterior and posterior wall densities increased in both experimental groups, reaching almost double the control density in group A ($p < 0.0001$) (*Table 1*). Regarding the localization of vascularization, in the control group, vascular density was higher close to the epicardium, whereas in the experimental groups it was intense in the myocardium and endocardium. In group A, vessel walls were thinner and more compressed than in group B.

Immunohistochemistry

Expression of immunohistochemical markers varied in relation to chemical exposure. Results are summarized in *Table 2*.

Immunohistochemical staining showed that sodium dichromate significantly lowered expression of CD117 ($p = 0.019$) and P53 ($p = 0.002$) with respect to the control population and increased VEGF expression ($p = 0.03$). In mice exposed to lindane, the results showed the opposite sign, with non-significant increases in CD117 ($p = 0.08$) and P53 expression ($p = 0.76$) and a non-significant decrease in VEGF ($p = 0.62$) with respect to the control group (*Figs. 2* and *3*).



A B C

Figure 1. *Structural changes in left ventricle wall. A. Control group. Vessel density, HE staining (x400). B. Group A. Vessel density. HE staining (x400). C. Group A. Vessel density. HE staining (x400)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

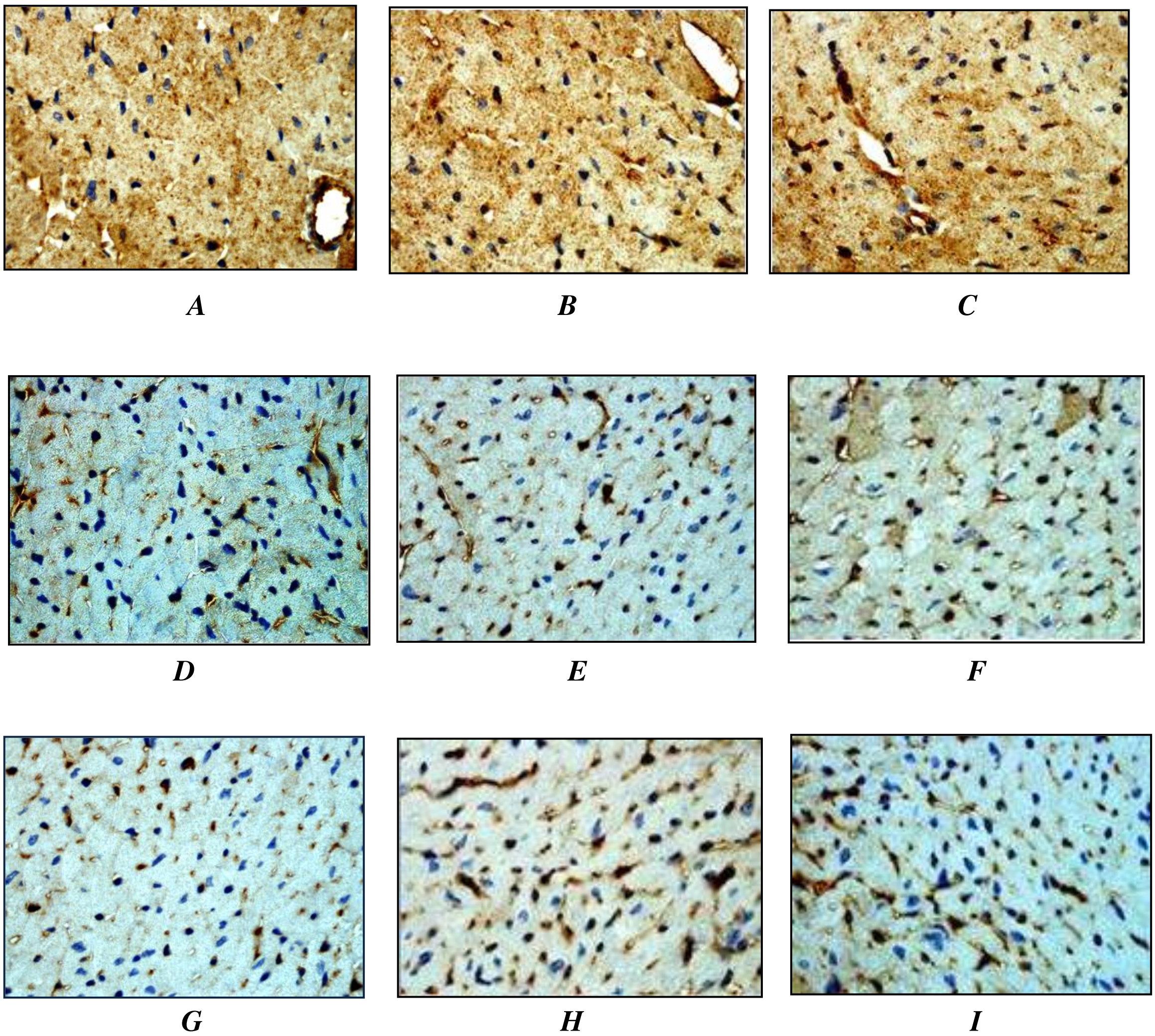

***Figure 2.** Immunohistochemical staining of left ventricle tissue. A. VEGF marker expression in Control group (x800). B. VEGF marker expression in group A (x800). C. VEGF marker expression in group B (x800). D. CD117 marker expression in Control group (x800). E. CD117 marker expression in group A (x800). F. CD117 marker expression in group B (x800). G. p53 marker expression in Control group. H. p53 marker expression in group A (x800). I. p53 marker expression in group B (x800)*

***Table 1.** Parameters of morphological changes in heart tissue*

Parameters		Wall thickness (pixel)	t-test	Vessel wall density (pixel)	t-test	Vessel diameters (pixel)	t-test
Anterior wall	Control Group	1186.1±0.02		8.2±0.1		727.14x225.32	
	Group A Sodium dichromate	1797.9±0.3	p<0.0001	16.01±0.3	p<0.0001	279.66×102.18	p<0.0001
	Group B Lindane	1550.24±0.3	p<0.0001	11.6±0.02	p<0.0001	256.04×75.25	p<0.0001

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

Posterior wall	Control Group	1359.39±0.05		6.1±0.04		910.8×153.51	
	Group A Sodium dichromate	1121.06±0.02	p<0.0001	10.03±0.2	p<0.0001	172.32×66.87	p<0.0001
	Group B Lindane	996.76±0.01	p<0.0001	8.3±0.4	p<0.0001	219.81×86.34	p<0.0001

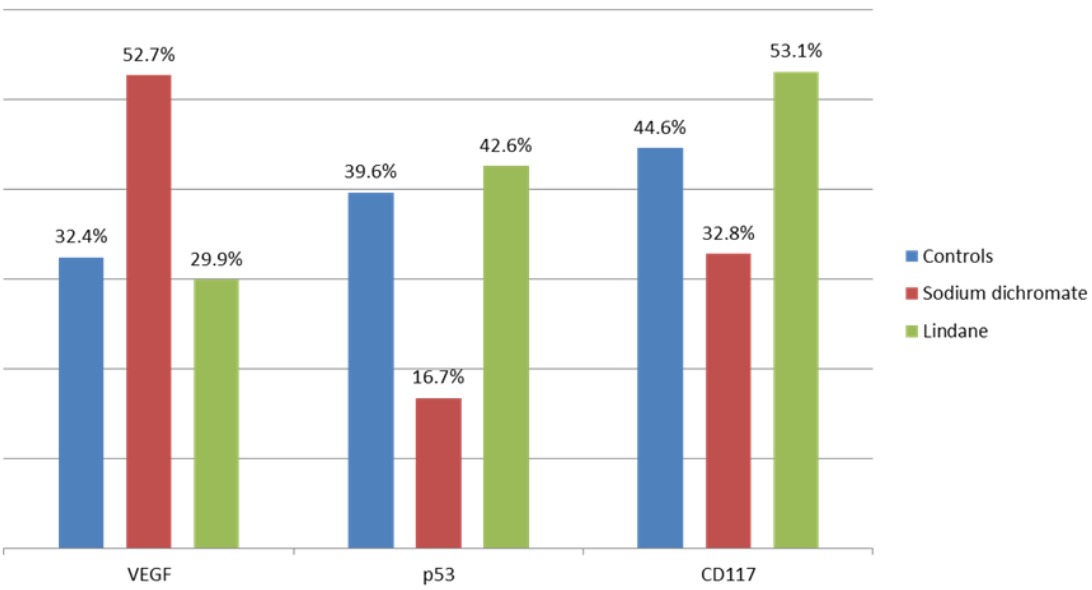

***Figure 3.** Histogram comparing expression of immunohistochemical markers in heart tissue of control and treated mice. Percentage of positivity in cells expressing VEGF, CD117 and p53, calculated as the sum of strongly and weakly antibody-positive cells in representative frontal serial sections of the left ventricle, showing that sodium dichromate significantly increased VEGF levels in confront to the control population; on the contrary, lowered the expression of P53 and CD117. Immunohistochemical markers in mice exposed to lindane showed comparable results to the control group*

***Table 2.** Immunohistochemical results with cell counts in mice*

Mice group/antibody	**Percentage of positive cells**			
	Strong	**Weak**	**Total positive**	**Negative**
Controls/VEGF	11.95 ± 8.3	20.48 ± 10.1	32.42 ± 15.3	67.10 ± 15.3
Controls/p53	19.35 ± 12.1	20.28 ± 10.6	39.63 ± 13.4	60.37 ± 13.4
Controls/CD117	10.35 ± 3.9	34.24 ± 8.9	44.59 ± 9.9	55.41 ± 9.9
Sodium dicrhomate/VEGF	18.39 ± 11.7	34.34 ± 16.6	52.72 ± 18.6	47.28 ± 18.6
Sodium dicrhomate/p53	4.71 ± 2.6	12.03 ± 4.4	16.74 ± 5.9	83.26 ± 5.9
Sodium dicrhomate/CD117	24.84 ± 24.3	7.98 ± 5.0	32.81 ± 22.2	67.19 ± 22.2
Lindane/VEGF	18.39 ± 11.7	34.34 ± 16.6	52.72 ± 18.6	47.28 ± 18.6
Lindane/p53	20.73 ± 9.1	21.85 ± 8.5	42.58 ± 11.8	57.42 ± 11.8
Lindane/CD117	16.50 ± 6.1	36.62 ± 6.3	53.12 ± 11.1	46.88 ± 11.1

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

Discussion

A major criterion for evaluating aging processes is the biological age of blood vessels, in turn linked to the microstructural properties of the vascular wall. This is why it is important to study aging of heart vessels, i.e. the level of angiogenesis and apoptosis, particularly under conditions of exposure to chemicals (Saghiri et al., 2015; Haberzettl et al., 2012; Chin, 2015).

Researchers have confirmed that stress caused by exposure to chemicals and other factors predisposes to coronary heart disease and impairs heart function through stimulation of mast cells (MCs), leading to local inflammation (Alevizos et al., 2014). MCs contain mediators, such as cytokines, histamine, proteases and leukotrienes, and play an important role in inflammatory reactions, neovascularization and repair after myocardial injury (Galli and Wershil, 1995; Church and Levi-Schaffer, 1997; Welle, 1997; Hara et al., 1999; Zhang et al., 2006; Marone et al., 1995; Kennedy et al., 2005). Mast cell number and density are reported to be elevated in patients with cardiomyopathy (Patella et al., 1998). Acute stress of experimental myocardial infarction in rats was associated with increased MC density in affected areas during tissue remodelling. Researchers (Hara et al., 1999) reported that MCs caused apoptosis of cardiomyocytes and proliferation of non-myocardial cells, thus aggravating pathological heart conditions (Engels et al., 1995).

Chemical exposure causes pathological conditions in the vascular wall and affects vascular wall matrix remodeling.

CD117 plays an essential role, among others, in the regulation of cell survival and proliferation. Importantly, c-kit expression had been associated with heart repair after myocardial infarction (Cimini et al., 2007), where it establishes a proangiogenic milieu in the infarct border zone by increasing VEGF (Fazel et al., 2006).

The main distinguishing sign of endothelial aging is increasing vascular permeability, VEGF migration and reduction of regenerative capacity (Brandes et al., 2005). Our observation of increasing numbers of VEGF-expressing cells and their uneven concentrated distribution in the endothelium are polymorphic changes that indicate cell migration and damage to the vascular wall.

Gogiraju et al. revealed that p53-rich endothelial cells contribute to scarcity of blood vessels and development of fibrosis during cardiac hypertrophy and that apoptotic endothelial cell death plays an important role in chronic heart disease (Gogiraju et al., 2015). The present study showed that expression of CD117 and p53 decreased in the sodium dichromate experimental group with respect to controls, while VEGF expression is increased. It is known that lindane can cause abnormal heart rhythm (Sauviat and Pages, 2007) as well as histological alterations mainly in the left ventricular wall (Sauviat and Pages, 2002) and our morphological analyses confirms significant changes in heart tissue. However we did not observe any significant change in the three markers evaluated in the present study.

In healthy tissues, p53 function is inactive until induced by toxic or oncogenic stress (Sauviat and Pages, 2007). Biochemical stress activates beneficial gene sets, including proteins involved in excitation-contraction coupling, energy metabolism and response to oxidative stress through inhibition of hypertrophic signaling and apoptosis (Lammie et al., 1994). An increased rate of endothelial cell apoptosis reduces the regenerative capacity of the endothelium and leads to endothelial senescence (Barnabas et al., 2013).

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

Chronic exposure to sodium dichromate and lindane modified the micro-structure of the left ventricle of the heart by increasing vessel wall density, narrowing the vessel lumen and increasing the number of small capillaries.

Conclusions

Exposure to lindane fosters aging processes in heart tissue due to an increase in the number of mast cells, which leads to apoptosis, cardiomyocyte necrosis and proliferation of non-myocardial tissue. Further research is needed to fully understand the effects of chronic exposure to chemicals and find out possible solutions for mitigating pollutants' effect by continuing experiment with protective pharmaceuticals against the risk of the occurance and progression of cardiovascular diseases. These prelimary findings justify the necessity of taking special preventive actions targeted to the zones exposed to chemical hazards.

Acknowledgements. We thank Professor Jürgen Hescheler, Dr. Marek Molcanyi and Mrs. Suzanne Wood of the Institute of Neurophysiology, University of Cologne, for their advice and for administration of the initial stage of the research, Dr. Matteo Bertelli Dr. Paolo Enrico Maltese and Dr. Yeltay Rakhmanov from MAGI, International Institute for Diagnostics and Research of Genetic and Rare Diseases, Rovereto, Italy for their help in writing this article, commercial laboratory subcontractor "Avizzena Medical Center", Novosibirsk, Russian Federation, for providing laboratory facilities, and Helen Ampt for revising the English.

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DOI: http://dx.doi.org/10.15666/aeer/1702_45154525

STUDY OF SOME BIOLOGICAL PARAMETERS OF THE RED CALIFORNIAN EARTHWORM *EISENIA FOETIDA* (SAVIGNY, 1826) IN VERMICOMPOST FOLLOWING THE APPLICATION OF WOOD VINEGAR

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(Received 28th Jan 2019; accepted 27th Feb 2019)

Abstract. This study was carried out with the purpose to assess some biological parameters of the red Californian worm (*Eisenia foetida*) in vermicompost, to which two types of wood vinegar, manufactured from poultry manure (A) and nutshell (B), have been applied. The study was implemented under laboratory conditions with Coincidence Parcels Trial Layout and 5 repetitions in 2018. Profile Analysis Technique was used in the in the investigation of wood vinegar types and the effect of various doses on the weight gain and number of worms on a weekly basis, while Factorial ANOVA was used in the investigation of their effects on cocoon numbers. The Profile Analysis proved that the interaction between Week × Dose × Vinegar type was important in regards to both the weight and number of worms (P = 0.00). The Factorial ANOVA carried out to investigate the effects of the vinegar type and doses on the cocoon number showed that the Dose × Vinegar type interaction was important (P = 0.00). Therefore, the effects of the vinegar type on the number and weight of worms differed depending on the dosage. In general, it was noted that 3%- and 5% dosages of vinegars had positive effects on *Eisenia foetida,* while the dosage of 100% had toxic effects. Based on the findings, it is possible that wood vinegars have a potential use with various purposes including bio-pesticides or worm manure.
Keywords: *agricultural, bio-economy, bio-pesticides, cocoon, pyrolysis liquids, worm*

Introduction

Although earthworms are not abundant in number, they are probably significant factors for sustaining soil productivity in various ways (Edwards and Bohlen, 1992). These living beings are accepted as important indicators of chemical toxicity in the soil ecosystem (Bustos-Obregón and Goicochea, 2002; Yasmin and D'Souza, 2010). Wood vinegar (WV) is a liquid produced through carbonization (pyrolysis liquids) that has been found to had been produced as early as the time of the Neanderthals (Tiilikkala et al., 2010; Cai et al., 2012). Kim et al. (2008) stated with reference to Jang that WV is a clear liquid containing 80-90% water with the rest being water-soluble organic compounds exceeding 200 in number. Jothityangkoon et al. (2008) stated that the main components of WV were acetic acid paired with organic acids and phenolic compounds, alkanes, alcohols and esters. This product is used as manure, insect repellant or organic fungicide in agriculture. Furthermore, there are studies showing that WV has effects that improve the soil and promote plant growth. As examples of such studies, Jothityangkoon et al. (2008), Yin (2008), Baimark and Niamsa (2009), Velmurugan et al. (2009), Chalermsan and Peerapan (2009), Oramahi and Yoshimura (2013), Hagner (2013), Namlı et al. (2014), Koc et al. (2017), De Souza Araújo et al. (2018) and Oramahi et al. (2018) have performed studies concerning the antifungal activities of WV. Studies of Yatagai et al. (2002), Oramahi and Yoshimura (2013) are notable on the issue of anti-termite effects of WV. It was stated by the studies of Shi (2003), Jothityangkoon et al. (2008), Yin (2008), Rakmai (2009), Cai et al. (2012) that WV affects plant growth. Regarding the antibacterial effectiveness of WV, positive remarks of Chalermsan and Peerapan (2009), Eric et al. (2012), Duan et al. (2016), Yang et al. (2016), Chukeatirote et al. (2018) and De Souza Araújo et al. (2018) can be noted. Koç et al. (2018) have found significant relations between treatments with WV and nematodes in the soil. Shi (2003) and Rakmai (2009) have obtained findings indicating the activities of WV that increase the edaphon and the number of bacteria in the root area of plants. There are also studies by Inoue et al. (2000), Yatagai et al. (2002), Jothityangkoon et al. (2008), Diba et al. (2009), Kiarie-Makara et al. (2010), Pangnakorn et al. (2011), Wititsiri (2011), Hagner (2013), Oramahi and Yoshimura (2013) explaining the insecticide or insect-repellant effects of WV. Cai et al. (2012) have reported that WV promotes the development of reactive oxidative species (ROS)-sensitive mutant worms, the lifetime of worms is prolonged and their body size increased. Mu et al. (2003) have stated that WV is a good resource for organic agriculture and is widely used in Japan, both in agriculture and daily life. Kim et al. (2008) have stated that the annual production of WV is around 14.000 tons. Hagner (2013) has stated in his study that there is little information on the toxic effects of WV, while Rakmai (2009) stated referring to Ikeshima that this substance has no toxic effects on humans or animals. Tiilikkala et al. (2010) have reported that pyrolysis liquids have the potential of replacing pesticides and synthetic chemicals in biocide form in the future, and this is based on bio-economic knowledge and depends on its development. As seen in the studies cited above, is it clear that WV has multiple effects.

This study has been carried with the purpose of determining some biological parameters of the red Californian earthworm (*Eisenia foetida*) in vermicompost after application of two types of wood vinegar obtained from poultry manure (A) and nutshell (B).

Materials and methods

This study was carried out under laboratory conditions in 2018. The vermicompost required for the experiments was obtained from 100% cow manure, and cocoons were obtained through reproduction from stock cultures of *Eisenia foetida* individuals. Wood vinegar (WV), was obtained from a firm developing WV products from poultry manure (A) and nutshell (B) using a gasification machine and bio-coal (Namlı et al., 2014). Vinegar obtain from nutshell (B) is light brown in color, and is characterized by a burning smell. Vinegar obtained from poultry manure (A) however, is darker in color as compared to vinegar B, is more consistent and has a stronger odor. The study was implemented using the Coincidence Parcels Trial Layout with 5 repetitions (*Fig. 1*). After placing 100 g vermicompost in 300 cm^3 plastic containers, the solution was treated with WV using a 10-mL injector. One percent, 3%, 5%, 10%, 25% and 100% WV doses were used in the treatments, and only tap water was injected into the control group. WV treatment was performed only once. Following these procedures, 10 cocoons were placed in each container. The ambient temperature of the study was kept between 20 and 29 °C and the humidity was kept between 70 and 90% (Gunadi et al., 2002). With the purpose of maintaining the ambient humidity, 10 mL tap water was administered to the samples periodically with 2 day intervals. Live weight, number of cocoons laid, and the number of young individuals coming out of cocoons were determined weekly until the young individuals laid cocoons themselves. Profile

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Analysis was used during the investigation of the effects of different doses and vinegar types on weekly body weight gain and number of worms (Mendeş et al., 2007). Factorial ANOVA was used to investigate the effects of the factors mentioned above on the number of cocoons, and Tukey's Multiple Comparison Test was used in determining the different groups (Winer et al., 1991; Mendeş, 2012). The abovementioned analyses were carried out using the SPSS (Ver. 21.0) statistical package programs.

***Figure 1.** Photo of the experimental culture*

Results

Findings related to the weights of worms

Profile Analysis results for WV(A) and WV(B) are presented in *Figures 2* and *3,* respectively. Results of Profile Analysis indicated that the Week × Dose × Vinegar type interaction is significant (Wilks' Lambda = 0.253; F = 3.61; P = 0.00). Meaning that the effect of vinegar type on weekly body weight gain of worms varied based on the doses (*Figs. 2* and *3; Table 1*). When only week 5 is taken into consideration in comparison with the controls, treatments with WV (B) have not resulted in much difference between the body weights of worms (*Fig. 3; Table 1*). However, treatments with WV (A) 1%, 25% and 100% had negative effects while treatments with 3% and 5% had positive effects (*Fig. 2; Table 1*).

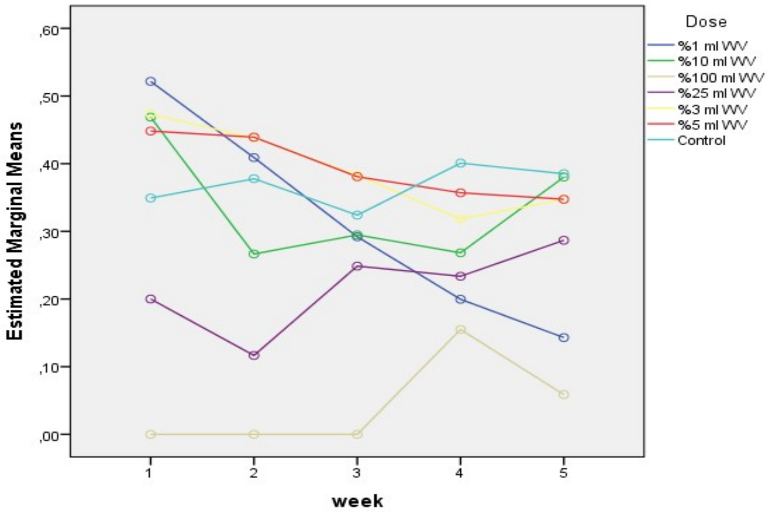

***Figure 2.** Profile analysis for investigating the effect of WV(A) on weekly body weight gain of worms based on doses*

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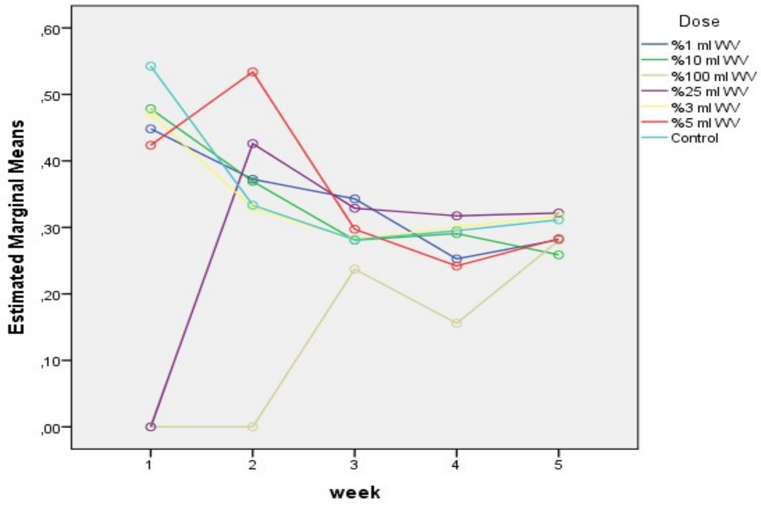

***Figure 3.** Profile analysis for investigating the effect of WV (B) on weekly body weight gain of worms based on doses*

***Table 1.** Descriptive statistics for the body weight of worms*

Week	WV dose	Vinegar type	Mean	Std. deviation	N	Week	WV Dose	Vinegar type	Mean	Std. deviation	N
w3	1%	A	0.522	0.131	5	w4	1%	A	0.409	0.076	5
		B	0.448	0.134	5			B	0.372	0.114	5
		Total	0.485	0.131	10			Total	0.390	0.093	10
	3%	A	0.473	0.043	5		3%	A	0.438	0.145	5
		B	0.471	0.112	5			B	0.329	0.120	5
		Total	0.472	0.080	10			Total	0.384	0.138	10
	5%	A	0.448	0.024	5		5%	A	0.439	0.055	5
		B	0.424	0.050	5			B	0.534	0.072	5
		Total	0.436	0.039	10			Total	0.487	0.078	10
	10%	A	0.469	0.625	4		10%	A	0.267	0.309	4
		B	0.479	0.099	5			B	0.369	0.036	5
		Total	0.474	0.080	9			Total	0.324	0.198	9
	25%	A	0.200	0.187	5		25%	A	0.117	0.162	5
		B	0.000	0.000	5			B	0.426	0.042	5
		Total	0.100	0.163	10			Total	0.271	0.198	10
	100%	A	0.000	0.000	4		100%	A	0.000	0.000	4
		B	0.000	0.000	5			B	0.000	0.000	5
		Total	0.000	0.000	9			Total	0.000	0.000	9
	Control	A	0.349	0.058	5		Control	A	0.378	0.128	5
		B	0.543	0.081	5			B	0.333	0.059	5
		Total	0.446	0.122	10			Total	0.356	0.097	10
	Total	A	0.359	0.191	33		Total	A	0.302	0.209	33
		B	0.338	0.232	35			B	0.338	0.169	35
		Total	0.348	0.212	68			Total	0.320	0.189	68
w5	1%	A	0.292	0.175	5	w6	1%	A	0.200	0.274	5
		B	0.343	0.049	5			B	0.253	0.025	5
		Total	0.318	0.124	10			Total	0.226	0.185	10

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		A	0.383	0.063	5			A	0.318	0.035	5
	3%	B	0.282	0.050	5		3%	B	0.301	0.043	5
		Total	0.333	0.075	10			Total	0.309	0.038	10
		A	0.381	0.080	5			A	0.357	0.024	5
	5%	B	0.297	0.019	5		5%	B	0.242	0.027	5
		Total	0.339	0.070	10			Total	0.300	0.065	10
		A	0.295	0.057	4			A	0.268	0.179	4
	10%	B	0.281	0.023	5		10%	B	0.291	0.034	5
		Total	0.287	0.039	9			Total	0.281	0.113	9
		A	0.249	0.172	5			A	0.234	0.136	5
	25%	B	0.329	0.077	5		25%	B	0.317	0.059	5
		Total	0.289	0.133	10			Total	0.276	0.108	10
		A	0.000	0.000	4			A	0.155	0.232	4
	100%	B	0.237	0.154	5		100%	B	0.156	0.213	5
		Total	0.132	0.166	9			Total	0.155	0.207	9
		A	0.324	0.075	5			A	0.401	0.034	5
	Control	B	0.281	0.049	5		Control	B	0.295	0.021	5
		Total	0.303	0.064	10			Total	0.348	0.062	10
		A	0.282	0.153	33			A	0.280	0.164	33
	Total	B	0.293	0.074	35		Total	B	0.265	0.095	35
		Total	0.288	0.118	68			Total	0.272	0.132	68
		A	0.143	0.198	5			A	0.287	0.055	5
	1%	B	0.282	0.022	5		25%	B	0.322	0.059	5
		Total	0.212	0.152	10			Total	0.304	0.057	10
		A	0.347	0.066	5			A	0.059	0.117	4
	3%	B	0.317	0.016	5		100%	B	0.280	0.256	5
		Total	0.332	0.048	10			Total	0.182	0.227	9
w7		A	0.347	0.021	5	w7		A	0.385	0.016	5
	5%	B	0.283	0.018	5		Control	B	0.311	0.034	5
		Total	0.315	0.039	10			Total	0.348	0.046	10
		A	0.380	0.023	4			A	0.282	0.144	33
	10%	B	0.259	0.033	5		Total	B	0.293	0.095	35
		Total	0.313	0.070	9			Total	0.288	0.120	68

Findings on the number of worms

Profile Analysis results for WV(A) and WV(B) are presented in *Figures 4* and *5,* respectively. Results of Profile Analysis indicated that the Week × Dose × Vinegar type interaction is significant (Wilks' Lambda = 0.05; F = 7.83; P = 0.00). Meaning that the effect of vinegar type on weekly number of worms varied based on the doses (*Figs. 4* and *5, Table 2*).

It can be noted that treatments with 1%, 25% and 100% WV (A) have negative effects on the number of worms in comparison with controls, while 3% and 5% doses have positive effects (*Fig. 4; Table 2*). It was also seen that the 100% dose of WV (B) had negative effects in comparison with the controls, while treatments with 3%, 5% and 10% WV had positive effects (*Fig. 5; Table 2*).

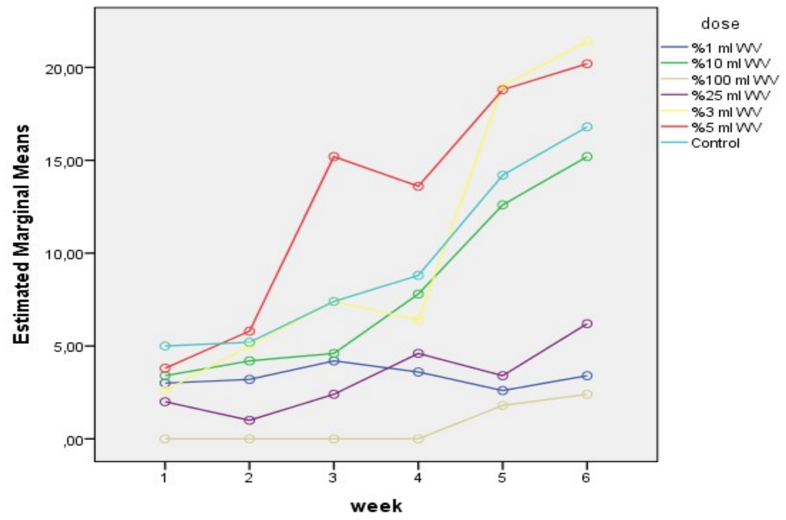

Figure 4. *Profile analysis for investigating the effect of WV (A) on weekly number of worms based on doses*

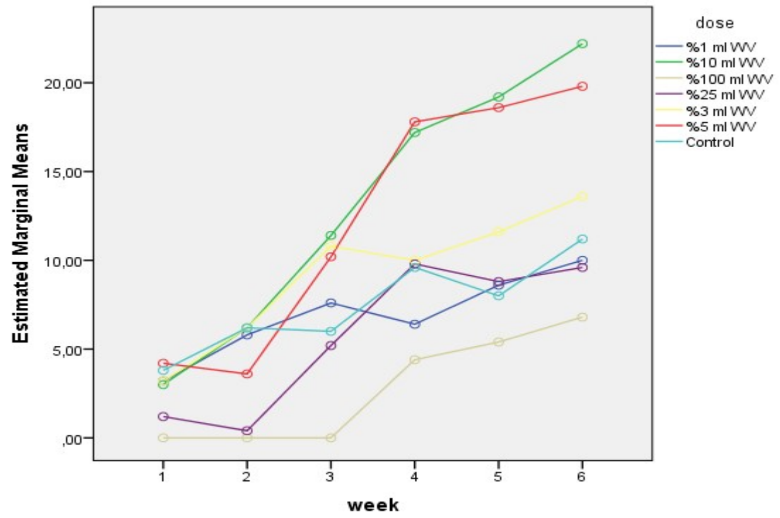

Figure 5. *Profile analysis for investigating the effect of WV (B) on weekly number of worms based on doses*

Table 2. *Descriptive statistics for number of worms*

Week	WV Dose	Vinegar type	Mean	Std. deviation	N	Week	WV Dose	Vinegar type	Mean	Std. deviation	N
w2	1%	A	3.000	1.581	5	w3	1%	A	3.200	1.304	5
		B	3.200	1.304	5			B	5.800	0.836	5
		Total	3.100	1.370	10			Total	4.500	1.716	10
	3%	A	2.600	0.894	5		3%	A	5.000	1.000	5
		B	3.200	0.447	5			B	6.200	0.837	5
		Total	2.900	0.738	10			Total	5.600	1.075	10
	5%	A	3.800	1.304	5		5%	A	5.800	0.837	5
		B	4.200	0.447	5			B	3.600	1.140	5
		Total	4.000	0.943	10			Total	4.700	1.494	10

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DOI: http://dx.doi.org/10.15666/aeer/1702_45274538

	10%	A	3.400	1.673	5		10%	A	4.200	0.447	5
		B	3.000	1.414	5			B	6.200	1.095	5
		Total	3.200	1.476	10			Total	5.200	1.316	10
	25%	A	2.000	1.414	5		25%	A	1.000	1.000	5
		B	1.200	0.447	5			B	0.400	0.548	5
		Total	1.600	1.075	10			Total	0.700	0.823	10
	100%	A	0.000	0.000	5		100%	A	0.000	0.000	5
		B	0.000	0.000	5			B	0.000	0.000	5
		Total	0.000	0.000	10			Total	0.000	0.000	10
	Control	A	5.000	1.000	5		Control	A	5.200	0.447	5
		B	3.800	0.447	5			B	6.200	1.304	5
		Total	4.400	0.966	10			Total	5.700	1.059	10
	Total	A	2.829	1.855	35		Total	A	3.486	2.215	35
		B	2.657	1.589	35			B	4.057	2.754	35
		Total	2.743	1.717	70			Total	3.771	2.497	70
w4	1%	A	4.200	1.924	5	w5	1%	A	3.600	2.191	5
		B	7.600	0.894	5			B	6.400	1.342	5
		Total	5.900	2.283	10			Total	5.000	2.261	10
	3%	A	7.400	0.548	5		3%	A	6.400	0.894	5
		B	10.800	1.304	5			B	10.000	1.225	5
		Total	9.100	2.025	10			Total	8.200	2.150	10
	5%	A	15.200	1.789	5		5%	A	13.600	3.050	5
		B	10.200	2.049	5			B	17.800	1.924	5
		Total	12.700	3.199	10			Total	15.700	3.268	10
	10%	A	4.600	1.517	5		10%	A	7.800	3.493	5
		B	11.400	1.342	5			B	17.200	1.304	5
		Total	8.000	3.829	10			Total	12.500	5.543	10
	25%	A	2.400	0.548	5		25%	A	4.600	2.881	5
		B	5.200	0.447	5			B	9.800	3.962	5
		Total	3.800	1.549	10			Total	7.200	4.264	10
	100%	A	0.000	0.000	5		100%	A	0.000	0.000	5
		B	0.000	0.000	5			B	4.400	2.608	5
		Total	0.000	0.000	10			Total	2.200	2.898	10
	Control	A	7.400	0.548	5		Control	A	8.800	1.304	5
		B	6.000	0.707	5			B	9.600	3.647	5
		Total	6.700	0.949	10			Total	9.200	2.616	10
	Total	A	5.886	4.708	35		Total	A	6.400	4.558	35
		B	7.314	3.917	35			B	10.742	5.271	35
		Total	6.600	4.359	70			Total	8.571	5.358	70
w6	1%	A	2.600	3.578	5	w7	1%	A	3.400	3.507	5
		B	8.600	1.817	5			B	10.000	1.414	5
		Total	5.600	4.142	10			Total	6.700	4.296	10
	3%	A	19.000	1.871	5		3%	A	21.400	2.074	5
		B	11.600	1.517	5			B	13.600	2.191	5
		Total	15.300	4.218	10			Total	17.500	4.577	10
	5%	A	18.800	2.588	5		5%	A	20.200	1.924	5
		B	18.600	2.608	5			B	19.800	3.033	5
		Total	18.700	2.452	10			Total	20.000	2.404	10
	10%	A	12.600	4.278	5		10%	A	15.200	2.280	5

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		B	19.200	1.643	5			B	22.200	2.168	5
		Total	15.900	4.630	10			Total	18.700	4.244	10
	25%	A	3.400	2.408	5		25%	A	6.200	1.095	5
		B	8.800	2.168	5			B	9.600	2.191	5
		Total	6.100	3.573	10			Total	7.900	2.424	10
	100%	A	1.800	1.483	5		100%	A	2.400	2.074	5
		B	5.400	3.050	5			B	6.800	3.899	5
		Total	3.600	2.951	10			Total	4.600	3.748	10
	Control	A	14.200	0.836	5		Control	A	16.800	1.304	5
		B	8.000	2.121	5			B	11.200	3.633	5
		Total	11.100	3.604	10			Total	14.000	3.916	10
	Total	A	10.343	7.550	35		Total	A	12.229	7.807	35
		B	11.457	5.447	35			B	13.314	5.890	35
		Total	10.900	6.559	70			Total	12.771	6.887	70

Findings related to cocoon numbers

Factorial ANOVA was used to analyze the data set. Results of ANOVA showed that the Dose × Vinegar type interaction is significant ($P = 0.00$). Therefore, the effect of vinegar type on number of cocoons varied based on the doses (*Table 3; Fig. 6*). Upon comparison of WV (A) treatments with the controls, doses of 3% and 5% exerted positive effects on the number of cocoons, while treatments of 1%, 25% and 100% doses had negative effects. A smaller numbers of cocoons was produced under treatments with WV (B) compared to the controls (*Table 3*).

Table 3. *Descriptive statistics and Tukey multiple comparison test for the number cocoons*

WV Dose	Vinegar type	Mean	Grouping	Std. error	N
1%	A	0.000	b	0.000	5
	B	1.600	a	1.342	5
	Total	0.800		1.229	10
3%	A	4.000	a	0.707	5
	B	0.400	b	0.548	5
	Total	2.200		1.989	10
5%	A	4.000	a	0.707	5
	B	0.000	b	0.000	5
	Total	2.000		2.160	10
10%	A	3.200	a	1.304	5
	B	1.600	a	0.548	5
	Total	2.400		1.265	10
25%	A	1.800	a	0.447	5
	B	0.800	b	0.837	5
	Total	1.300		0.823	10
100%	A	0.600	a	0.894	5
	B	0.000	a	0.000	5
	Total	0.300		0.675	10

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DOI: http://dx.doi.org/10.15666/aeer/1702_45274538

Control	A	3.200	a	0.836	5
	B	2.600	b	0.548	5
	Total	2.900		0.738	10
Total	A	2.400	a	1.684	35
	B	1.000	b	1.111	35
	Total	1.700		1.582	70

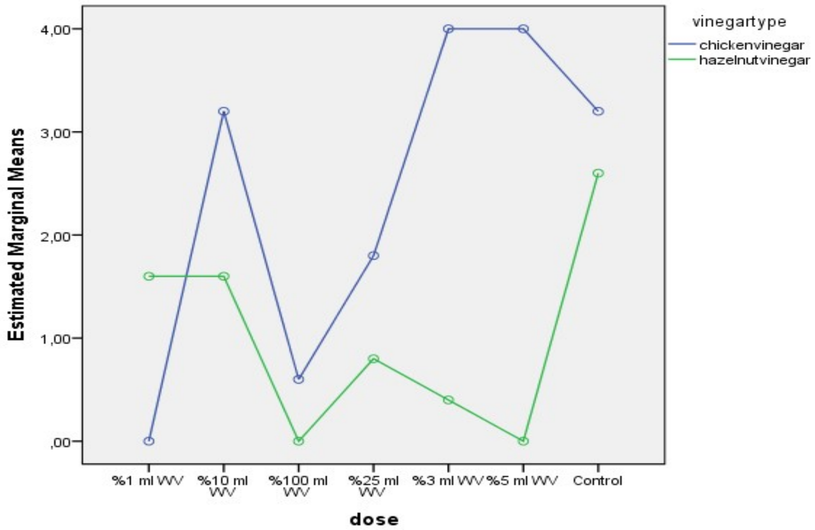

Figure 6. *Changes in the number of cocoons based on dose and vinegar type*

Discussion

Chemical pesticides and fertilizers used in agricultural activities pollute the environment, damage biodiversity and exert negative effects on human health. This situation can be eliminated through the use of alternative methods such as organic pesticides and manures. Wood vinegar (WV) is an organic product that can be degraded in a short time produced from plant and animal waste. Treatments with WV (A, B) have been found to have effects on the body weight, number of worms and number of cocoons depending on the type of vinegar, dose and the week of measurements. Of these vinegar types, WV (A) is thought to have exerted positive effects on the numbers of worms and cocoons particularly in doses of 3% and 5% (*Table 3*), while WV (B) has positive effects particularly on the number of worms in 3%, 5% and 10% doses (*Table 2*). These results are consistent with the findings of Cai et al. (2012) indicating that WV exerts positive effects on the development, lifetime and body size of worms. Furthermore, the fact that WV has positive effects on plants and other living beings in the soil is supported by the findings of Shi (2003), Jothityangkoon et al. (2008), Yin (2008), Rakmai (2009) and Cai et al. (2012). However, studies indicating the negative effects of a 1% dose on worms has been attributed to either an adverse factor in the test apparatus or to some toxic effect. Generally, the 100% dose and partially 25% dose of WV have been determined to exert negative effects on the biological parameters of worms. These results are supported by the findings of Inoue et al. (2000), Yatagai et al. (2002), Jothityangkoon et al. (2008), Yin (2008), Baimark and Niamsa (2009),

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DOI: http://dx.doi.org/10.15666/aeer/1702_45274538

Velmurugan et al. (2009), Chalermsan and Peerapan (2009), Diba et al. (2009), Kiarie-Makara et al. (2010), Pangnakorn et al. (2011), Wititsiri (2011), Eric et al. (2012), Oramahi and Yoshimura (2013), Hagner (2013), Namlı et al. (2014), Duan et al. (2016), Yang et al. (2016), Koc et al. (2017), De Souza Araújo et al. (2018), Oramahi et al. (2018) and Chukeatirote et al. (2018), agreeing that WV can exert bactericide, fungicide, anti-termite and insecticide effects. However, it can easily be seen from the examination of figures and tables of these studies that the effect of types and doses of WV differ weekly. It is estimated that this can be attributed to the statements of Kim et al. (2008) and Jothityangkoon et al. (2008) in their respective studies, stating that vinegar consists of water-soluble substances exceeding 200 in number and the main components are acetic acid paired with organic acids, phenolic acids, alkanes, alcohol and esters.

Conclusion

As a result of this study we can conclude that types and doses of wood vinegar can have positive effects on certain parameters of *Eisenia foetida*. It is thought that the 3% and 5% doses in particular exert positive effects on worms, while the dose of 100% has toxic effects. Vinegar types with all their characteristics may have a potential use in agriculture, or more precisely in organic agriculture and worm manure production.

Acknowledgements. I hereby present my great appreciations to Prof. Dr. Mehmet MENDEŞ for his help in the interpretations of the statistical analyses of this study and to Science Expert Birgül ILIKHAN for her help in laboratory works.

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INVESTIGATING ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES PRODUCED THROUGH GREEN SYNTHESIS USING LEAF EXTRACT OF COMMON GRAPE (*VITIS VINIFERA*)

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(Received 28th Jan 2019; accepted 28th Feb 2019)

Abstract. In this study, a direct approach to fabricating silver nanoparticles (AgNPs) via the leaf extract of common grape (*Vitis vinifera*) has been demonstrated. The produced particles were found with a maximum wavelength of 452.47 nm, spherical shape and the crystal size of 18.53 nm through UV-Visible spectrophotometry, XRD (X-ray diffraction) and SEM (Scanning electron microscopy) characterization methods. Furthermore, the functional groups involved in the reduction were specified with FTIR (Fourier transform infrared spectroscopy), the elemental compounds were identified with EDX (Energy dispersive X-Ray spectroscopy) and the degradation points were determined with TGA-DTA (Thermal gravimetric analysis) methods. AgNPs were found to be effective against hospital pathogens, namely Gram-negative *Escherichia coli* ATCC 25922, Gram-positive *Staphylococcus aureus* ATCC 29213 and *Candida albicans* fungus at the concentrations of 0.314, 0.078 and 0.334 µg mL^{-1}, respectively.
Keywords: *green synthesis, metal nanoparticles, grape leaf extract, characterizations*

Introduction

Nowadays, nanoparticles are widely used. These particles have found their use in different areas such as electronics, photography, material science, dye removal, cosmetics, biomedicine, bioremediation etc. (Saha et al., 2017; Swamy et al., 2015; Chaudhry et al., 2018). Green synthesis, which includes biological methods, is highly advantageous compared to physical and chemical methods due to it being environmentally friendly and not using toxic chemicals during any step (Sinsinwar et al., 2018; Ramkumar et al., 2017; Pantidos and Horsfall, 2014). Particles between the sizes of 1-100 nm are defined as nanoparticles. These nanoparticles can be obtained through biological, physical and chemical techniques (Ahmed et al., 2017). Since it is inexpensive and easy to obtain, the use of plants for the synthesis of nanoparticles is becoming increasingly common. Due to the strong antimicrobial activity of silver (Ag) against fungi, viruses, and bacteria, AgNPs have been a subject of study by researchers from many different fields in recent years, and it has been stated that AgNPs have anti-inflammatory properties in addition to their antimicrobial properties (Shah et al., 2017). Silver nanoparticle synthesis and phytochemicals in plant leaf extracts create AgNPs by reducing Ag^+ ions present in the medium to Ag^0 and also provide stability (Geethalakshmi and Sarada, 2010; Prakash et al., 2013; Ahmed et al., 2018). Green synthesis is an important phenomenon that must be taken into consideration, especially since it supports the use of non-toxic chemicals, and environmentally friendly, renewable materials (Kaushal et al., 2016). Plant-based green synthesis generally uses different parts of plants (leaves, roots, seeds and fruits) (Majeed et al., 2018).

In this research, the green synthesis, which is an environmentally friendly, easy and simple method, was used to synthesize AgNPs from the leaf extract of the common grape (*Vitis vinifera*) and its antimicrobial effect on microorganisms was investigated.

Materials and methods

Preparation of Vitis vinifera extract and silver nitrate ($AgNO_3$) solution

Collected grape leaves from Mardin region in Turkey were initially washed with tap water, then washed with distilled water and finally dried under room conditions. After the drying process, the dried leaves were grounded, then boiled with distilled water and filtered at room temperature using coarse filter paper followed by Whatman 1 filter paper and the extract was obtained for the synthesis of AgNPs. Afterwards, 1 mM solution was prepared using solid $AgNO_3$ (purity 99.8%, Sigma-Aldrich).

Synthesis and characterization

The leaf extract and the $AgNO_3$ solution were mixed in a ratio of 1: 4, and after the color change in a few minutes, subsequently the formation and presence of silver nanoparticles were observed with Perkin Elmer one UV Visible spectrophotometer. After centrifugation with OHAUS FC 5706 at 10.000 rpm for 5 min, the resulting nanoparticles were dried. Then the RadB-DMAX II computer controlled X-ray diffractometer for XRD, the scanning electron microscope EVO 40 LEQ for SEM-EDAX, the Perkin Elmer Spectrum One for FTIR, and the Shimadzu TGA-50 for TGA-DTA were used to determine the characterization of the synthesized AgNPs.

Determining antimicrobial effects of silver nanoparticles

The antimicrobial effects of the obtained AgNPs were examined on pathogenic microorganisms, Gram-negative *Escherichia coli* ATCC 25922, Gram-positive *Staphylococcus aureus* ATCC 29213 bacteria and *Candida albicans* yeast. The minimum inhibitory concentration (MIC) was determined by the micro dilution method. In the experiments, Mueller-Hinton medium, the solutions including a certain amount of microorganism, which were prepared according to 0.5 McFarland standard, and appropriate concentrations of the AgNP solution were added to 96-well microplates and wells were incubated at 37 °C overnight (El-Batal et al., 2018; Vishwasrao et al., 2018; Dhand et al., 2016). The next day, the MIC was determined by the well in which the reproduction ended. To compare the effects of AgNPs, commercially available vancomycin, colistin and fluconazole antibiotics and a 1 mM $AgNO_3$ aqueous solution were used on *S. aureus*, *E. coli*, and *C. albicans*.

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Results and discussion

After a few minutes of mixing the grape plant and the $AgNO_3$ solution, a dark coffee color change was observed indicating the formation of AgNPs by vibrations on the plasma surface (Alruqi et al., 2018). Data with the maximum absorbance of 452.47 nm were obtained (*Fig. 1*). The maximum absorbance in the synthesis with green tea and turmeric extracts was found to be 450 nm (Selvan et al., 2018). In another study conducted with the green synthesis, it was stated that the maximum absorbance was at 460 nm (Begum et al., 2009).

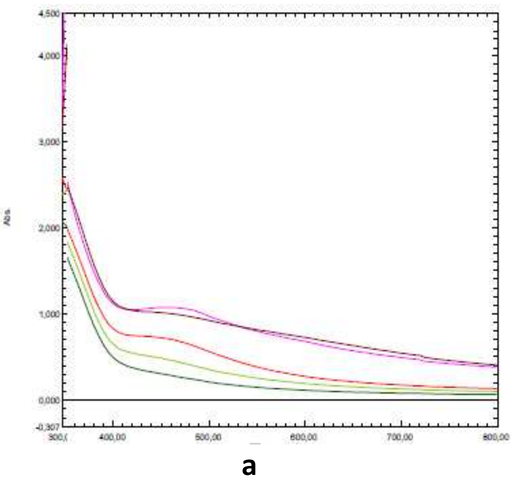

***Figure 1. a.** Time-dependent formation of AgNPs in UV spectroscopy. **b.** Maximum absorbance of synthesized AgNP in UV spectrophotometry*

Functional groups involved in the reduction were evaluated via FTIR analysis. It was thought that the shifts at 3332, 2127 and 1635 cm^{-1} were caused by the active role of -OH, -CN and C = O groups in the reduction (*Fig. 2*). Similar groups were evaluated in the nanoparticle synthesis study with *Matricaria chamomilla* extract (Dadashpour et al., 2018). Other study results also support these findings (Selvakumar et al., 2018).

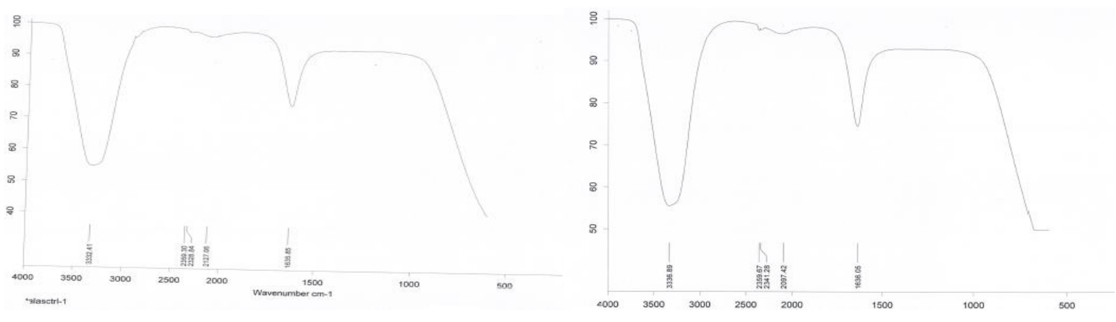

***Figure 2.** UV-vis spectrum of plant extract and UV-vis spectrum of synthesized AgNPs*

The crystal structure of the obtained AgNPs particles was found by the 2θ values of 38.52°, 44.71°, 64.85° and 77.72° from the peaks which were located at (111), (200), (220) and (311) (*Fig. 3*). In the study with *Melissa officinali*, the peaks at (111), (200), (220) and (311) were associated with AgNPs (de Jesús Ruíz-Baltazar et al., 2017). The

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DOI: http://dx.doi.org/10.15666/aeer/1702_45394546

orientation at (111), (200), (220) and (311) were also connected to AgNPs in the nanoparticle synthesis research of *Coffea arabica* extract (Dhand et al., 2016).

The crystal dimensions of AgNPs were determined as 18.53 nm using Debye-Scherrer equation [D = Kλ / (β cos Sch)].

In other studies on the synthesis of AgNPs, the crystal dimensions of the nanoparticles were calculated using Debye-Scherrer equation (Pugazhendhi et al., 2018; Rajesh et al., 2018; Jogaiah et al., 2017).

It was seen that AgNPs were spherical in SEM images, and according to EDAX, there were peaks of Ag metal in the elemental composition (*Fig. 4*). It was reported that the AgNPs obtained in a study with *Physalis angulata* extract also have a spherical shape (Nishanthi et al., 2018). Many researchers have reported having synthesized AgNPs in spherical shapes (Premkumar et al., 2018; Alam et al., 2018; Ibrahim et al., 2016).

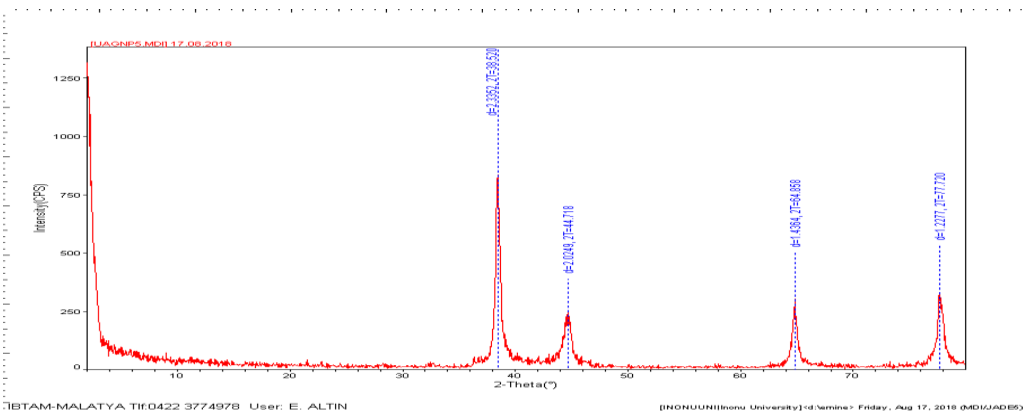

Figure 3. *X-ray diffraction (XRD) analysis*

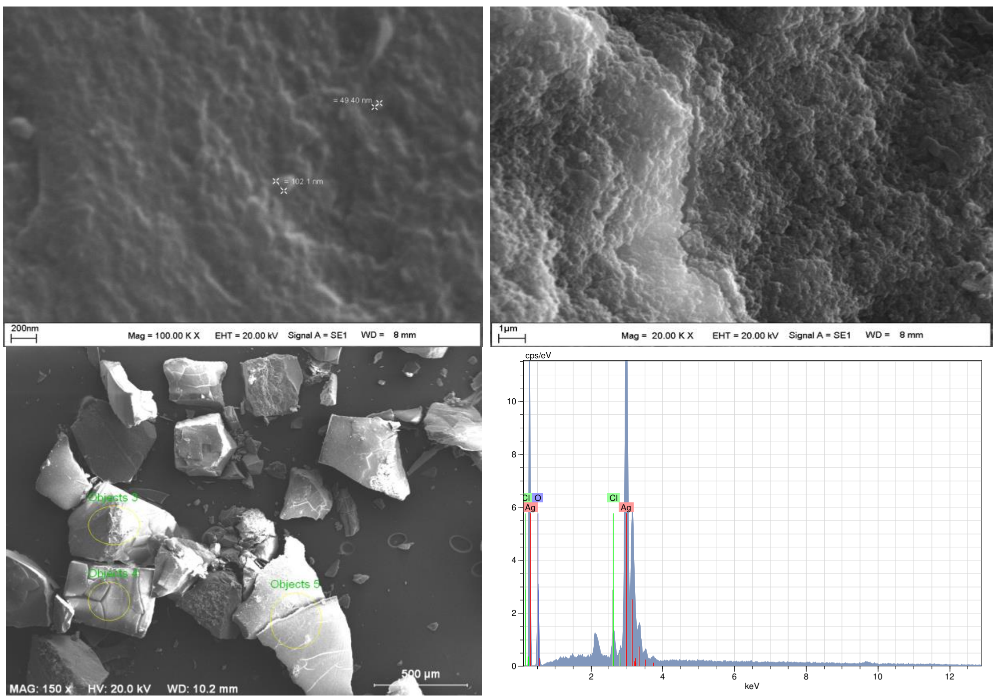

Figure 4. *SEM-EDAX results of AgNPs*

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DOI: http://dx.doi.org/10.15666/aeer/1702_45394546

The TGA and DTA analyses of the AgNPs were evaluated between 30-900 °C with a heating rate of 10 °C min^{-1} and a flow rate of 20 mL min^{-1} in N_2 (g) atmosphere. The TGA curve indicates the mass loss of the nanoparticles versus the temperature, and the DTA curve shows the highest decomposition temperature at all levels of the degradation (Baran et al., 2018).

The loss of mass at 30-201 °C was caused by moisture, while the loss of mass at 201-515 °C was due to phytochemicals in the plant extract and the green synthesized AgNPs were gradually degraded between 515-810 °C (*Fig. 5*).

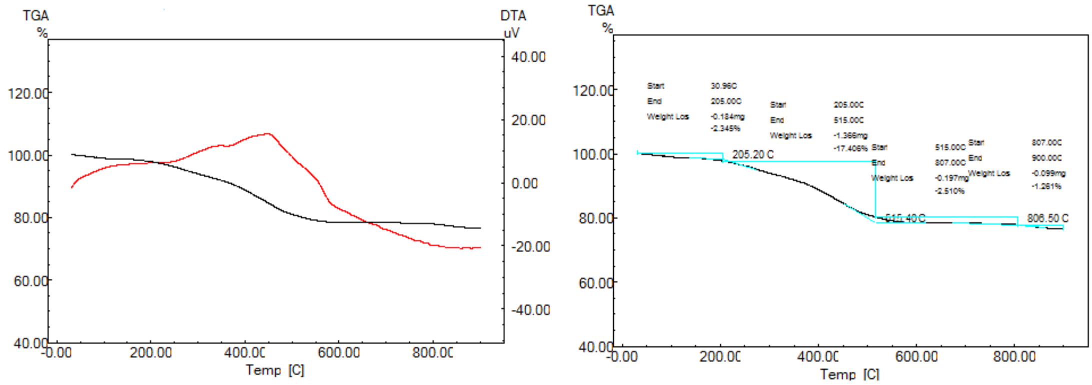

Figure 5. *TGA-DTA results of synthesized AgNPs*

The minimum inhibitory concentrations (MIC) of AgNPs on Gram-negative *Escherichia coli* ATCC 25922, Gram-positive *Staphylococcus aureus* ATCC 29213 bacteria and *Candida albicans* microorganisms were found to be 0.078, 0.314 and 0.331 µg mL^{-1}, respectively (*Table 1*). In the green synthesis and characterization of AgNPs from *Abelia grandiflora*, MIC values of Gram-negative and Gram-positive bacteria were found to be 3.12 µg mL^{-1} for *E. coli* and 12.5 µg mL^{-1} for *S. aureus* (Sharma et al., 2014).

Table 1. *MIC values of synthesized silver nanoparticles (AgNPs), silver nitrate, vancomycin, fluconazole and colistin antibiotics on S. aureus, C. albicans and E. coli*

Organism	AgNP (µg mL^{-1})	Antibiotic (µg mL^{-1})	Silver nitrate (µg mL^{-1})
S. Aureus ATCC 29213	0.314	0.50	0.50
E. coli ATCC 25922	0.078	0.12	1.00
C. albicans	0.331	0.06	0.50

Conclusion

In this research, synthesis of AgNPs was performed using the common grape (*Vitis vinifera*) leaf extract. It was determined that AgNPs had spherical shapes with sizes around 75 nm according to the data obtained from the SEM images, and also had a crystal size of 18.53 nm according to XRD results. FTIR results showed that the functional groups involved in the reduction were -OH, -CN and C=O. The antimicrobial effect of nanoparticles was determined to be significant on *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 strains. In the light of these results, the usage of the nanoparticles

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DOI: http://dx.doi.org/10.15666/aeer/1702_45394546

obtained by an environmentally friendly synthesis can be improved for the medical industry due to their biocompatibility as antimicrobial agents. With their antimicrobial effect, AgNPs are thought to be effective in bioremediation of wastewater due to their rapid and efficient synthesis, as well as their being used in many areas such as shelf life extension, cosmetics, and others.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_45394546

COMPREHENSIVE ASSESSMENT OF THE WATER ECOLOGICAL SECURITY OF THE XIANGJIANG RIVER BASIN BASED ON PHYSICO-CHEMISTRY AND ORGANISM INDICES

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(Received 19th Oct 2018; accepted 28th Jan 2019)

Abstract. The water ecological security of a river basin affects the vigorous growth of the regional economy and the healthy development of the ecological environment. Based on river physics, chemistry, and biological indicators, this study constructed a comprehensive assessment index system for the water ecological security of the Xiangjiang River Basin in China. The system consisted of 6 elements, and 18 indicators. This study used the analytic hierarchical process (AHP) to evaluate the water ecological security of the upstream, midstream, and downstream environments of the Xiangjiang River Basin. The results show that the overall ecological security of the upper and midstream portions of the Xiangjiang River Basin is relatively good, while the downstream section is at a general level. The three indicators that are given the most weight in the indicator system are the fish bio-loss index, the degree of ecological flow satisfaction, and the compliance rate of the water function zone. The less significant indicators are the natural wetland retention rate, riparian vegetation coverage, riparian human activity, and riverside connectivity. The research results of this study can provide a reference for the ecological restoration of regional rivers and the protection of Xiangjiang River.
Keywords: *ecological security, water environment, indicator system, AHP, Xiangjiang River Basin*

Introduction

Xiangjiang River, the mother river of Hunan Province, is an important tributary of the Yangtze River and the largest tributary of the Dongting Lake water system. The location of Xiangjiang River Basin is both in the radiating zone of the Yangtze River Economic Belt and in the South China Economic Circle. This basin is located in the most densely populated and economically developed region in Hunan Province and is an important grain production base in China. In the past two decades, with the increase in water consumption due to urbanization as well as industrial water and agricultural irrigation, some wastewater has been directly discharged into the Xiangjiang River, causing serious pollution concerning heavy metals, fecal coliforms, ammonia nitrogen, total phosphorus and chemical oxygen demand. When the indicators of the presence of these pollutants exceed standard values, aquatic biodiversity is reduced, which brings about problems with watershed resources and the ecological environment, and the pressure of sustainable development increases dramatically. In 2013, in order to protect the Xiangjiang River, the Government of Hunan Province issued the “Regulations on the Protection of the Xiangjiang River in Hunan Province” and launched the “No. 1 Key Project” for implementing the protection and treatment of the Xiangjiang River, which was then comprehensively carried out.

A river is the core of a river basin ecosystem. Rivers are often regarded as ecological corridors to provide good habitats for animals and plants. They are also sources of matter and energy (He, 2017). A river ecosystem is one of the most complex ecosystems on the planet. Water ecological security has evolved from environmental changes and ecological risk analysis. Since the International Institute for Applied Systems Analysis (IIASA) was first proposed in 1989 (Ji and Long, 2016; Pan et al., 2017), the concept of water ecological security has not been scientifically defined. At present, there are two levels of understanding of water ecological security: the generalized concept and the narrow concept (Chen and Zhou, 2005; Chen et al., 2013). According to the generalized concept, water ecological security is a complex ecosystem safeguard composed of natural water ecological security, economic-ecological security, and social-ecological security (Zhang et al., 2013; Chen et al., 2016). According to the narrow concept, water ecological security concerns the safety of the “human” aspect, which includes the safety of natural and semi-natural ecosystems (Wang and Hu, 2013). The foundation of water ecological security assessment involves establishing a scientific indicator system and evaluation criteria. At present, there are some typical evaluation systems, such as the indicator species ecosystem evaluation method (Zhang et al., 2017), biological integrity index (Schnier et al., 2016), and the PSR (Pressure-State-Response) model (Sun et al., 2016). The PSR model was proposed by Canadian statisticians David J. Rapport and Tony Friend in 1979 (Rapport et al., 1979) and later developed by the Organisation for Economic Co-operation and Development (OECD) and the United Nations Environment Programme (UNEP) in the 1980s and 1990s. Based on the PSR framework, in order to better characterize the role of non-environmental indicators in ecosystem health assessment, the United Nations Commission on Sustainable Development (UNCSD) established the Driver-State-Response (DSR) framework in 1996 (Doran, 1996).

The assessment of water ecological security involves the analysis of numerous factors using various methods. The existing water ecological safety assessment framework, however, often attaches importance to ecological health, while ignoring the assessment of ecosystem service functions and ecological risks. Due to different interpretations of various indicators, the selected evaluation indicators differ. Even the same evaluation indicators can be given different evaluation connotations. Liu et al. (1989) used the cultivated land area as an indicator to characterize the ecological state. Other researchers believe that agricultural and urban land represent the pressure of human production and lifestyle on aquatic ecosystems (Li et al., 2012). In addition, existing evaluation indicators are mostly based on the entire basin, administrative divisions, fixed wide grid, and different ecosystem types (Nödler et al., 2016). These indicators have the advantage of reflecting the integrity of the basin through convenient data collection, but they also

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

have shortcomings, such as the one concerning the spatial differences that cannot adequately reflect ecological security. Therefore, a series of indicators are needed to measure the water ecological security statusIn this paper, based on the comprehensive assessment of the water ecological security in the Xiangjiang River Basin, the water ecological characteristics of the Xiangjiang River Basin are understood. The research results provide basic support for watershed water ecological protection and restoration, and provide scientific basis for the formulation of water resources and water ecological security planning by the national and local governments in China.

Materials and methods

Overview of the study area

The Xiangjiang River Basin (110°31′E–114°15′E, 24°31′N–29°52′N) is located south of the Yangtze River, and north of the Nanling Mountains in China. It has a subtropical humid climate and is greatly affected by monsoons. The average annual temperature of the basin is 17.4°C. The average annual evaporation of the basin is 1275.5 mm. The average annual precipitation in the basin is 1490 mm. The spatial and temporal distribution of rainfall is uneven. From April to June, the rainfall was concentrated, and the average annual rainfall is 617.9 mm, accounting for 42.9% of the whole year; from July to September, the average annual rainfall is 318.2 mm, accounting for 22.1% of the whole year; from October to March, the rainfall is only 504.7 mm, accounting for 35.0% of the whole year. In terms of geographical distribution, there is a trend of more rainfall in the north and south, and less rainfall in the central areas.The main stream of the Xiangjiang River is 856 km long, with a drainage area of 94,600 km^2. A total of 85,400 km^2 of the drainage area is in Hunan Province, accounting for 90% of the total basin area. To be specific, the Xiangjiang River Basin refers to the section of the basin in Hunan Province. The Xiangjiang River Basin includes important urban centers such as Yongzhou City, Chenzhou City, Hengyang City, Shaoyang City, Loudi City, Zhuzhou City, Xiangtan City, Changsha City, Yueyang City, and Yiyang City. The elevation of the basin is high in the southwest and low in the east. The Xiangjiang River flows into Dongting Lake from the south. The water system in the basin is complicated, consisting of many tributaries. There are 2,157 tributaries in the basin with lengths greater than 5 kilometers. The two sides of the main stream of the Xiangjiang River are in the form of asymmetrical feathers. The right bank covers an area of 67,316 km^2, accounting for 71.2% of the total area of the basin. The main tributaries of the Xiangjiang River (the Xiaoshui River, Chonglinshui River, Leishui River, Mishui River, Lushui River , and Liuyang River) flow from the right bank into the main stream. The area of the left bank is 27,433 km^2, accounting for 28.8% of the total area of the basin. The main tributaries (the Qishui River, Zhengshui River, Juanshui River, Lianshui River, and Weishui River) flow from the left bank. The distribution of water system and hydrological stations as shown in *Figure 1*.

Division of evaluation units

Based on the boundaries of municipal administrative divisions within the basin, this study comprehensively considered the spatial overlapping relationships among natural geographic units, administrative divisions, and watershed environmental management

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

units of the basin and the consistently divided evaluation unit of the combination. Using the ArcHydro hydrological analysis module of ArcGIS software, the ASTER GDEM V2 (30 m resolution) data jointly measured by National Aeronautics and Space Administration (NASA) and Ministry of Economy, Trade and Industry (METI) was used as the basic DEM data for river network information extraction in the Xiangjiang River Basin. Finally, areas in Yongzhou City and Chenzhou City were designated as the upstream evaluation units; areas in Hengyang City and Zhuzhou City were designated as the mid-stream evaluation units; and areas in Changsha City, Xiangtan City, and Loudi City were designated as the downstream evaluation units. *Figure 2* is a interpretation map of the catchment area of the Xiangjiang River Basin.

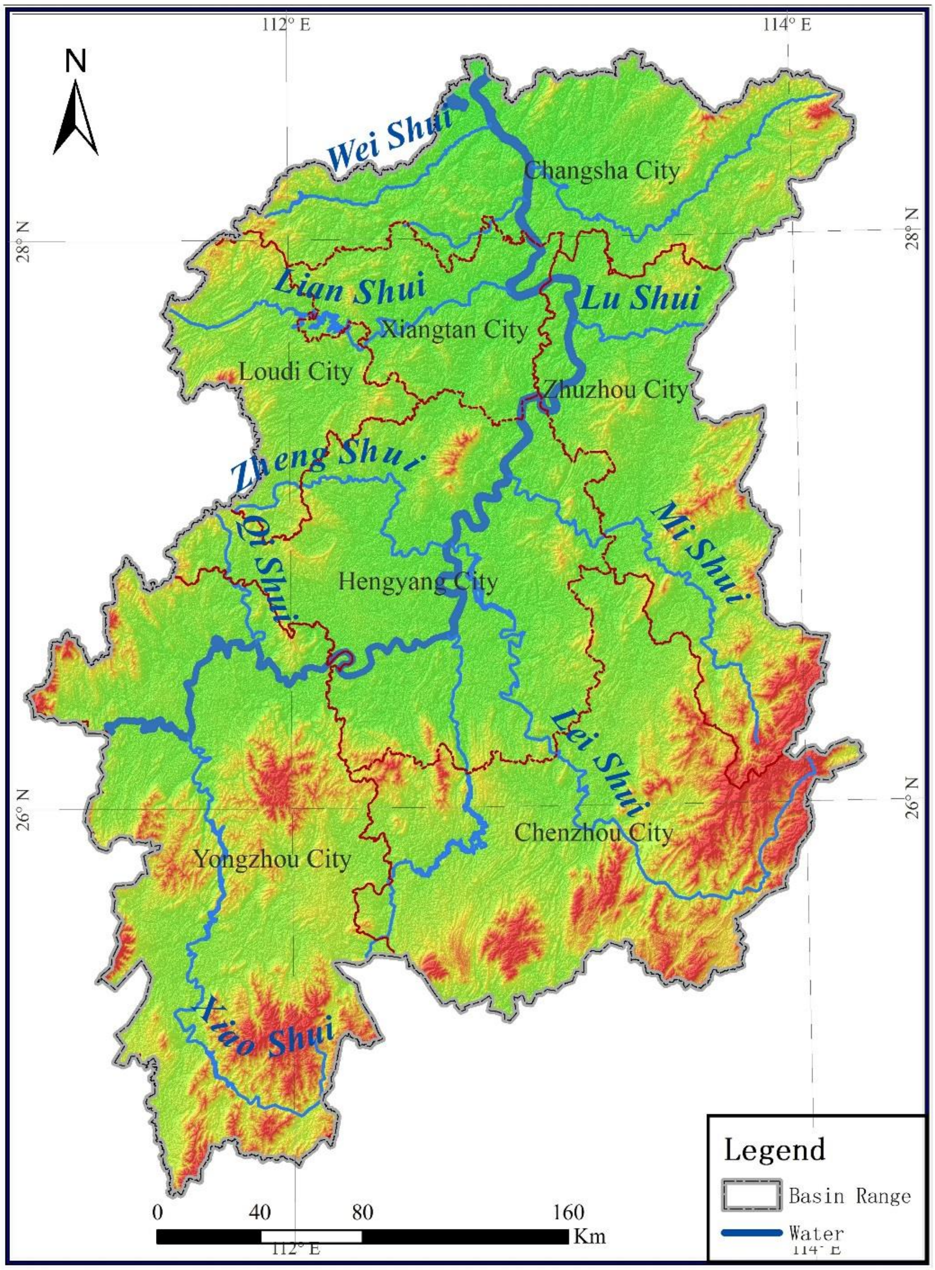

Figure 1. *Distribution of the water system and hydrological stations in the Xiangjiang River Basin*

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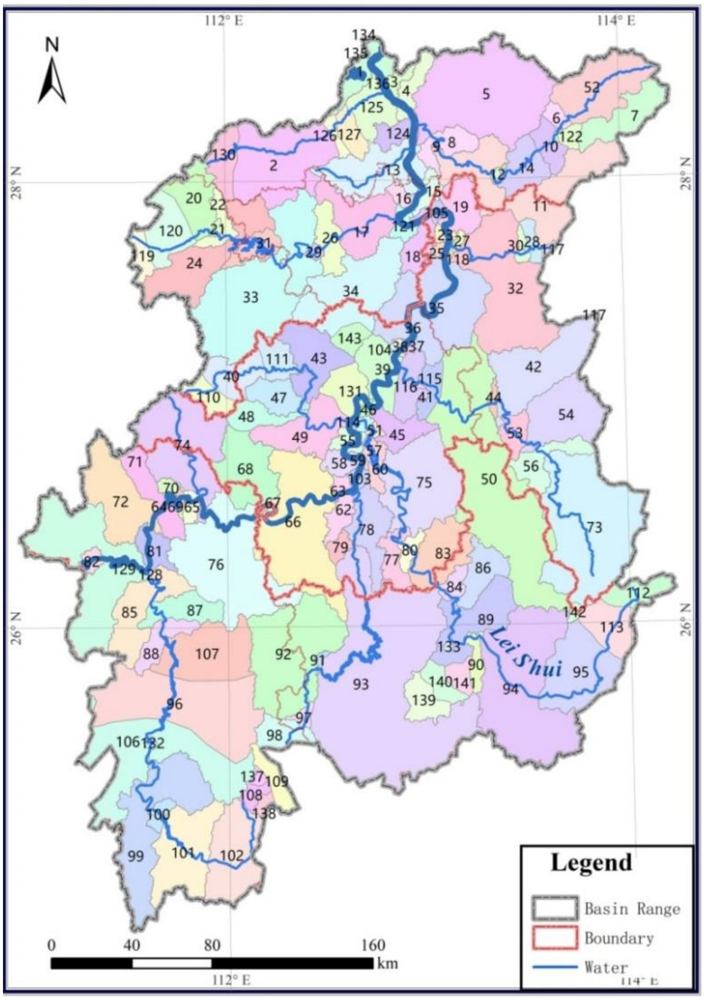

***Figure 2.** Interpretation map of the catchment area of the Xiangjiang River Basin*

Construction of a comprehensive evaluation index system

According to previous research, the water ecosystem safety of a river basin means that the basin ecosystem is in a stable and sustainable state, maintaining physical, chemical, and biological integrity, providing humans with different ecological products or service functions, and having a certain resilience with respect to natural and human interference, as well as adjustment and repair capabilities. While assessing the water ecosystem safety through a series of indicators, we often tend to the principles of science, systems, conciseness, and operability. There are many water ecosystem safety assessment methods according to the previous studies (Dai et al., 2015; Lin et al., 2016; Wang et al., 2016). Among them, the AHP is a useful tool for providing qualitative and quantitative assessment to decision makers, which enables managers to evaluating the complicated indicator system (Yoshimatsu and Abe, 2006; Haji et al., 2016). In this study, therefore, the AHP was used to construct a comprehensive evaluation index system for water ecological security in Xiangjiang River Basin. This indicator system contains 18 indicators related to six aspects: hydrology and water resources, physical structure, water quality, biology, water ecological management, and social service functions (*Table 1*).

Table 1. *Comprehensive evaluation index system for water ecological security in the Xiangjiang River Basin*

Target layer	Factor layer	Indicator layer	Indicator description	Data Sources
Water ecological security	Hydrology and water resources	The flow process variation degree	Reflects and evaluates the influence of water resources development and utilization above the monitoring section of river reach on the hydrological situation of river reach	Hunan Hydrological Network (http://61.187.56.156/wap/index_sq.asp) The flow and water level data of all hydrological stations in the basin of the Xiangjiang River Basin from 1996 to 2015
		The degree of ecological flow satisfaction	Reflects the degree of ecological flow satisfaction of the main section	Hunan Hydrological Network (http://61.187.56.156/wap/index_sq.asp) Three stations from Guiyang, Hengshan and Xiangtan from 1996 to 2017
	Physical structure	Riverbank Stability	Shows whether the indicators reflect the stability of the river bank , i.e., is readily eroded	The main river course is counted by Google Earth software. 12 sampling points are selected randomly and evenly in the main river course within the scope of the river basin. The statistical time is 2017.
		Vegetation coverage on river banks	Reflects whether the structure and function of the riparian zone are in good condition	NDVI product data using the official MODIS website (https://modis.gsfc.nasa.gov/) MOD13Q1 satellite.
		Degree of human activity in the riparian zone	The focus of the survey is to assess nine types of human activities in the riparian zone and its adjacent area: riverbank rigid masonry, sand mining, coastal buildings (houses), roads (or railways), landfills or dumps, riverside parks, degree of impact on water ecosystems, pipelines, mining, agricultural farming, livestock farming, etc.	Monitoring of riverside belts on the left and right sides of the river in an on-site survey (2017)
		River connection barrier condition	The main survey assesses river migration of biological species such as fish and the blocking of water flow and nutrient transport, which can be used to characterize river water ecological security.	Statistics of the main river channel using Google Earth software. The statistical time is 2017.
		The natural wetland retention rate	Reflect the pros and cons of the state of the river's ecological environment	Land use classification product data using the MODIS website (https://modis.gsfc.nasa.gov/) MOD12Q1 satellite.
	Water quality	Dissolved Oxygen Concentration	Dissolved oxygen is very important for aquatic animals and plants. DO values that are too high or too low are harmful to aquatic organisms, and the suitable range is 4-12 mg/L.	Observation data of hydrological station from Hunan Hydrological and Water Resources Survey Bureau in 2015
		Oxygen-poor organic pollution status	Reflects the content of organic matter in water, based on the permanganate index, chemical oxygen demand, five-day biochemical oxygen demand, ammonia nitrogen and four other items to evaluate the oxygen consumption of rivers	Observation data of hydrological station from Hunan Hydrological and Water Resources Survey Bureau in 2015
		Heavy metal pollution status	Five items , i.e., arsenic, mercury, cadmium, chromium (hexavalent) and lead were selected to assess the metal pollution status of water.	Concentration data of measured heavy metal pollutants at hydrological stations from Hunan Hydrological and Water Resources Survey Bureau in 2015

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Target layer	Factor layer	Indicator layer	Indicator description	Data Sources
		Water function area compliance index	Reflects the level of ecological environmental protection and resource utilization	Assessment of the monitoring sections of the upstream, midstream and downstream of the Xiangjiang River Basin. The proportion of the number of compliance points in the water functional zones during the year of 2015.
	Biology	Fish Bio-Loss Index	Reflects the quality of aquatic ecosystems	Survey data on fishery resource flows obtained from 8 sampling points in the Xiangjiang River Basin (November-December 2008, February-December 2009 and February-September 2010, 30 times in total)
	Water ecological management	Sewage centralized treatment efficiency index	Reflects the ability of water ecosystems to treat wastewater	Sewage treatment plant operation survey data
		Soil erosion control index	Reflects the intensity of soil erosion and the implementation of control measures	Urban soil erosion area survey data, the third remote sensing survey of soil and water loss in hunan province organized by Hunan Water Resources Department in 2015.
		Hazard Source Risk Index	Reflects the extent to which the source of the hazard affects the ecosystem	Hazard source survey and evaluation data of major industrial parks (2017)
	Social service function	Flood Control Indicators	Reflects the safe discharge capacity of the river	Data sourced from the "Xiangjiang River Basin Comprehensive Planning Report"
		Public Satisfaction Indicator	Reflects the public's satisfaction with river landscapes, aesthetic values, etc.	Collected and processed relevant information through questionnaires
		Centralized drinking water source safety guarantee compliance rate public satisfaction index	Reflects the safety of water	National Safe Drinking Water Source Safety Assessment Indicators

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Indicator calculation method and indicator assignment

Based on the river water environment and ecological characteristics of the Xiangjiang River Basin, the standard values for river water ecology evaluation were determined. The calculation and assignment of each indicator utilized the following principles: (a) If there were specific applicable standards and industry norms in the country, the national standards and norms were preferred; (b) the relevant research results in China and abroad were used for reference; (c) indicators were standardized. The closer a detected indicator is to the natural state or the less a detected indicator is affected by human activities, the higher the score given to the indicator.

The flow process variation degree (C11) indicator can be calculated by the following equation (*Eq. 1*):

$$FD = \left\{\sum_{m=1}^{12}\left[\frac{q_m - Q_m}{\bar{Q}_m}\right]^2\right\}^{1/2}, Q_m = \frac{1}{12}\sum_{m=1}^{12} Q_m \qquad \text{(Eq.1)}$$

where q_m is the measured annual monthly runoff and Q_m is the annual natural monthly runoff. The annual average natural runoff is then evaluated. And the final score is shown in *Table 2*.

Table 2. *Flow process variation degree indicator score*

Flow process variation index	Score
0.05	100
0.1	75
0.3	50
1.5	25
3.5	10
5	0

The degree of ecological flow (EF) satisfaction (C12) can be calculated by the following equation (*Eq. 2*):

$$EF1 = min\left[\frac{q^d}{\bar{Q}}\right]_{m=4}^{m=9}, \mathrm{EF2} = \min\left[\frac{q^d}{\bar{Q}}\right]_{m=10}^{m=3} \qquad \text{(Eq.2)}$$

where q^d is the estimated annual runoff, which is the average annual runoff; EF1 is the lowest percentage of the daily average flow from April to September; and EF2 is the lowest percentage of the daily average flow from October to March. Based on the hydrological method to determine the ecological base flow, the EF1 and EF2 assignment values were calculated according to *Table 3*, with the minimum value of the assignment taken as the final assignment of the indicator.

Riverbank Stability (C21): The Riverbank Stability Index (BKSr) was assessed based on the current status of riverbank erosion, including erosive erosion that has occurred or is likely to occur.

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

The calculation of BKSr can be expressed as follows (*Eq. 3*):

$$BKSr = \frac{SAr + SCr + SHr + SMr + STr}{5} \quad \text{(Eq.3)}$$

where BKSr is defined as the bank slope stability index score, SAr is the bank slope dip score, SCr is the bank slope coverage score, SHr is the bank slope height score, SMr is the river bank matrix score, and STr is the slope foot scour strength score. The specific scoring criteria are shown in *Table 4*.

Table 3. *Staging base flow standard and score*

Grading	Qualitative description	Recommended base flow standard (annual average flow percentage)		Score
		EF1: General water period (October-March)	EF2: Fish spawning period (April-September)	
1	maximum	200%	200%	100
2	optimal	60-100%	60-100%	100
3	excellent	40%	60%	100
4	very good	30%	50%	100
5	good	20%	40%	80
6	general	10%	30%	40
7	poor	10%	10%	20
8	very poor	<10%	<10%	0

Table 4. *Evaluation criteria for river bank stability indicators*

Bank slope characteristics	Stable	Basically stable	Secondary unstable	Unstable
Score	90	75	25	0
Slope inclination (degrees) (<)	15	30	45	60
Vegetation coverage (%) (>)	75%	50%	25%	0%
Slope height (m) (<)	1	2	3	5
Matrix (category)	Bedrock	Rocky bank	Clay river bank	Non-clay river bank
Riverbank scouring	No flushing signs	Mild scouring	Moderate scouring	Severe scouring
General characterization	In the near future, the river bank will not be deformed and destroyed, and no soil loss will occur.	The riparian structure has signs of loosening development and soil erosion, but it will not be deformed and destroyed in the near future.	The development trend of loose cracks on the river bank is obvious. Under certain conditions, the river bank can be deformed and destroyed, with moderate soil erosion.	The water and soil loss on the river bank is serious, and large deformation and damage may occur at any time, or damage may have already occurred.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Vegetation coverage on river banks (C22): Also known as changes in biomass indicators; vegetation can be separated from water and soil. This process is closely related to plant transpiration, interception of sunlight, photosynthesis, and net primary productivity. This coverage is calculated as follows (*Eq. 4*):

$$NDVI = \frac{Band2 - Band1}{Band2 + Band1} \quad \text{(Eq.4)}$$

where Band2 and Band1 are derived from MOD13Q1 satellite band data. And the final score is shown in *Table 5*.

Table 5. *Riverbank vegetation coverage index score table*

NDVI	Score
0.4	100
0.3	74.9
0.2	47
0.1	24.6
0.05	9.7
0	0

Degree of human activity in the riparian zone (C23): A riparian human impact assessment was performed in the assessment of the river section using a method for reducing the corresponding score for each human activity. A river section with all 9 types of activities listed below was given 100 points, with corresponding points being deducted according to the type of human activity and its position, with a lowest score of 0. The specific scoring criteria are shown in *Table 6*.

Table 6. *Standards for human activities in the riparian zone*

NO.	Type of human activity	Location		
		Inside the river (within the waterline)	Riparian zone	The riparian zone is adjacent to the land (within 10 m of a small river and within 30 m of a large river)
1	Rigid masonry		-5	
2	Sand mining	-30	-40	
3	Coastal buildings (houses)	-15	-10	-5
4	Highway (or railway)	-5	-10	-5
5	Landfill or dumping		-60	-40
6	Riverside park		-5	-2
7	Pipeline	-5	-5	-2
8	Agricultural cultivation		-15	-5
9	Livestock farming		-10	-5

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River connection barrier condition (C24): The final score of C24 is from *Table 7*.

Table 7. *Dam barrier identification table*

Fish migration barrier	Water quantity and material circulation barrier characteristics	Score
Unobstructed	No regulation of runoff	0
There is a fish channel and it is running normally	Adjusting the runoff, the discharge flow meets the ecological base flow	-1
No fishway, blocking the migration of some fish	The runoff is regulated, and the discharge flow does not satisfy the ecological base flow	-2
The migration channel is completely blocked	Partial time leads to interruption	-20

The calculation of the natural wetland retention rate (C25) can be expressed as follows (*Eq. 5*):

$$NWL = \frac{AW}{AWR_n} \quad \text{(Eq.5)}$$

where NWL is the natural wetland retention rate, AW is the natural wetland area (km^2) for the assessment base year, and AWR_n is the historical wetland area (km^2). The final score of C25 is from *Table 8*.

Table 8. *Natural wetland retention rate assignment standard table*

Natural wetland retention rate	Score	Description
93%	100	Close to reference conditions
86%	75	Small difference from reference conditions
72%	50	Moderately different from reference conditions
44%	25	A large difference from reference conditions
16%	0	Significantly different from the reference status

Dissolved Oxygen Concentration (C31): The monthly average concentration of 12 months was used to classify the data into 2 groups, the flood season and the non-flood period. The scores of the flood season and the non-flood period were then evaluated. The specific scoring criteria are shown in *Table 9*.

Table 9. *DO water quality indicator score criteria*

DO (mg/L) (>)	Saturation rate 90% (or 7.5)	6	5	3	2	0
DO indicator assignment	100	80	60	30	10	0

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Oxygen-poor organic pollution status (C32): Permanganate index, 5-day biochemical oxygen demand, and ammonia nitrogen were assigned separately. The monthly average concentration of the 12-month evaluation year was selected and the average value was calculated based on the flood season and the non-flood period. The scores of the flood season and the non-flood period were separately assessed. The average of the scores of the three water quality projects was taken as the oxygen-consuming organic pollution status. The indicator calculation can be expressed as *Eq. 6*:

$$OCPr = \frac{(CODMNr + BODr + NH3Nr)}{3} \quad \text{(Eq.6)}$$

The specific scoring criteria of C32 are shown in *Table 10*.

Table 10. *Oxygen consumption index*

Permanganate index (mg/L)	2	4	6	10	15
5-day biochemical oxygen demand (BOD5) (mg/L)	3	3.5	4	6	10
Ammonia nitrogen (NH3-N) (mg/L)	0.15	0.5	1	1.5	2
Score	100	80	60	30	0

Heavy metal pollution status (C33): The average monthly concentrations of mercury, cadmium, chromium, lead, and arsenic concentrations were determined according to 12 months of assessment. The averages were determined based on the flood season and non-flood period. The scores of the flood season and non-flood period were then evaluated. The minimum score was divided into the scores of the water quality project, and the lowest scores of the 5 water quality projects were assigned as the indicators of the heavy metal pollution status. The calculation can be expressed as *Eq. 7*:

$$HM\ Pr = Min(Arr, Hgr, Cdr, Crr, Pbr) \quad \text{(Eq.7)}$$

where $HM\ Pr$ is the heavy metal pollution index factor and Arr, Hgr, Cdr, Crr, and Pbr are factors assigned to the corresponding metals. The specific scoring criteria are shown in *Table 11*.

Table 11. *Standards for assigning indicators of heavy metal pollution status*

Arsenic	0.05	0.025	0.1
HG	0.00005	0.0001	0.001
Cadmium	0.001	0.005	0.01
Chromium (hexavalent)	0.01	0.05	0.1
Lead	0.01	0.05	0.1
Score	100	60	0

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Water function area compliance index (C34): The water function area that met the number of assessments during the year at least 80% of the time was defined as the water quality standard water function area. The calculation method for the water quality compliance index of the water function area was that 20 points were deducted when the river section was not up to standard.

Fish Bio-Loss Index (C41): This index refers to the assessment of the difference between the current number of fish species in a river segment and the number of fish species in the historical reference system. The surveyed fish species did not include alien species. The fish bio-loss index is calculated as follows (*Eq. 8*),

$$\mathrm{FOE} = \frac{\mathrm{FO}}{\mathrm{FE}} \quad \text{(Eq.8)}$$

where FOE is the fish bio-loss index, FO is the number of fish species obtained from the survey of a particular river segment, and FE is the number of fish species in the section based on historical assessment.

Sewage centralized treatment efficiency index (C51): From a basic survey of the operational status of sewage treatment plants in the Xiangjiang River Basin, the scores of the sewage centralized treatment efficiency indicators could be determined. The specific scoring criteria are shown in *Table 12*.

Table 12. *Sewage centralized treatment efficiency index score*

Sewage centralized treatment efficiency index	Score
1	100
0.95	80
0.8	70
0.75	50
0	0

Soil erosion control index (C52): This index indicates the intensity of soil erosion and the implementation of control measures throughout the river. The calculation of this index can be expressed as follows (*Eq. 9*):

$$\mathrm{SECI} = \frac{\mathrm{SEC}}{\mathrm{SE}} \quad \text{(Eq.9)}$$

where SECI represents the soil erosion control index, SE represents the area of soil erosion in the region, and SEC represents the planned area of key treatment of soil erosion in the region.

Hazard Source Risk Index (C53): This index refers to assessed sources of heavy metal pollution and sources of toxic and hazardous substances in the riparian zone and its adjacent land areas. For the assessment of river segments, a hazard source risk assessment was carried out using a method in which each hazard source reduces the total number of points by its corresponding score. River segments without danger

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sources were assigned a value of 100 points, and the corresponding points were deducted according to the type of danger source and its position, untila minimum value of 0 points. Each state-level industrial park in a river segment has an association deduction of 5 points was reached. Each provincial industrial park results in a deduction of 3 points.

Flood Control Indicators (C61): River Flood Control Indicators (FLD) are used to assess the safe discharge capacity of a river course. The calculation of this index can be expressed as follows (*Eq. 10*):

$$FLD = \frac{\sum_{N=1}^{NS}(RIVL_n \times RIVWF_n \times RIVB_n)}{\sum_{N=1}^{NS}(RIVL_n \times RIVWF_n)} \quad \text{(Eq.10)}$$

where FLD is the river flood control index and $RIVL_n$ is the length of river segment nthe number of river segments divided by the river according to the flood control plan is evaluated. $RIVB_n$ is assigned according to whether the flood control project of a given river segment meets the planning requirements, where $RIVB_n = 1$ when the standard is met and $RIVB_n = 0$ when the standard is not met. $RIVWF_n$ is the recurrence period of the planned flood control standard for a given river segment (e.g., 100 years). The scoring criteria are shown in *Table 13*.

Table 13. *Flood control index assignment standard*

Score	100	75	50	25	0
FLD	100%	75%	50%	25%	0%

Public Satisfaction Indicator (C62): This indicator is calculated based on the public participation survey statistics. The public questionnaire included many parameters: the basic information of the participants, the relationship between the participants, the participants' assessment of the river's water volume, water quality, river beach conditions, fish status and the river suitability, the participants' understanding of the above aspects, and an overall assessment of the river conditions.According to the *Technical Guidelines for River and Lake Health Evaluation*, scores are assigned according to the total number of responses for options in the table, with the assignment criteria shown in *Table 14*.

Table 14. *River safety assessment public survey indicator assignment criteria*

Option type	Option	Score
Option 1	A	100
	B	75
	C	50
	D	0
Option 2	A	100
	B	50
	C	0
Option 3	A	100
	B	50

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

Centralized drinking water source safety assessment index (C63): The drinking water source refers to the water source area that provides residents' living and public service water intake projects. This index was calculated using the centralized drinking water source safety assessment index system from the *National Important Drinking Water Source Safety Assessment Guide*. This guide includes a water quality assessment of 30 points, a water quality assurance assessment of 40 points, a monitoring assurance assessment score of 15 points, and a management assurance assessment score of 15 points. The maximum evaluation of the centralized drinking water source is 100 points. The average value was taken as the safety assurance assessment index of the centralized drinking water source.

Index weight calculation

This study selected the current mature and general AHP method to calculate the index weights, which can make this process clear, quantifying qualitative problems and requiring fewer data. There are three basic steps. The first step was the establishment of a hierarchical structure. The second step concerned the construction of a pairwise comparison judgment matrix. The third step involved the hierarchical ranking calculation and consistency test, followed by an n index weight calculation. The AHP method generally uses a scale of 1–9 and its reciprocal when constructing the pairwise comparison judgment matrix (*Table 15*).

Table 15. *Judgment matrix scale and its corresponding meanings*

Scaling	Implication
1	Equally important to a function
3	Slightly more important relative to a certain function
5	Significantly important relative to a function
7	Strongly important relative to a function
9	Extremely important relative to a function
2,4,6,8	Intermediate conditions between two adjacent judgments (Reciprocal, results of geminate contrast and reversal, namely index *i* is relative to index *j*)

Building a judgment matrix

Experts in three related fields (#1, #2, #3) determined the importance of the indicators. The importance judgment matrices of the factor layer and the indicator layer were obtained. The final weight determination depended on the average values of the three experts (*Tables 16,17,18*).

There are many methods for normalizing the eigenvectors of the judgment matrix. The maximum eigenvalues and eigenvectors of the judgment matrix were calculated using the square root method. The judgment matrix is $U = (u_{i,j})_{n\times n}$, where $u_{i,j}$ indicates that the importance of factor *i* is higher than that of factor *j* relative to the previous level. n is the order of the matrix.

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

Table 16. *Judgment matrix of the water ecological security indicator system of the Xiangjiang River Basin (#1)*

A	B_1	B_2	B_3	B_4	B_5	B_6
B_1	1	7	1	5	5	1
B_2	1/7	1	1/7	1/3	1/5	1/7
B_3	1	7	1	7	5	1
B_4	1/5	3	1/7	1	1/3	1/2
B_5	1/5	5	1/5	3	1	1/5
B_6	1	7	1	2	5	1

B_1	C_{11}	C_{12}
C_{11}	1	1/5
C_{12}	5	1

B_2	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}
C_{21}	1	5	5	3	5
C_{22}	1/5	1	1	1/2	1
C_{23}	1/5	1	1	1/2	1/2
C_{24}	1/3	2	2	1	2
C_{25}	1/5	1	2	1/2	1

B_3	C_{31}	C_{32}	C_{33}	C_{34}
C_{31}	1	1	1/3	1/7
C_{32}	1	1	1/3	1/7
C_{33}	3	3	1	1/7
C_{34}	7	7	7	1

B_4	C_{41}
C_{41}	1

B_5	C_{51}	C_{52}	C_{53}
C_{51}	1	7	5
C_{52}	1/7	1	1/3
C_{53}	1/5	3	1

B_6	C_{61}	C_{62}	C_{63}
C_{61}	1	5	2
C_{62}	1/5	1	1/5
C_{63}	1/2	5	1

Table 17. *Judgment matrix of the water ecological security indicator system of the Xiangjiang River Basin (#2)*

A	B_1	B_2	B_3	B_4	B_5	B_6
B_1	1	3	1/3	1/3	2	2
B_2	1/3	1	1/4	1/4	1/3	1/2
B_3	3	4	1	2	2	2
B_4	3	4	1/2	1	2	2
B_5	1/2	3	1/2	1/2	1	1/2
B_6	1/2	2	1/2	1/2	2	1

B_1	C_{11}	C_{12}
C_{11}	1	1/2
C_{12}	2	1

B_2	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}
C_{21}	1	3	2	2	4
C_{22}	1/3	1	1/2	2	2
C_{23}	1/2	2	1	2	2
C_{24}	1/2	1/2	1/2	1	2
C_{25}	1/4	1/2	1/2	1/2	1

B_3	C_{31}	C_{32}	C_{33}	C_{34}
C_{31}	1	1/3	1/3	1/3
C_{32}	3	1	1/2	1/2
C_{33}	3	2	1	2
C_{34}	3	2	1/2	1

B_4	C_{41}
C_{41}	1

B_5	C_{51}	C_{52}	C_{53}
C_{51}	1	4	3
C_{52}	1/4	1	2
C_{53}	1/3	1/2	1

B_6	C_{61}	C_{62}	C_{63}
C_{61}	1	4	1/2
C_{62}	1/4	1	1/3
C_{63}	2	3	1

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

***Table 18.** Judgment matrix of the water ecological security indicator system of the Xiangjiang River Basin (#3)*

A	B_1	B_2	B_3	B_4	B_5	B_6
B_1	1	5	1/3	1	3	9
B_2	1/5	1	1/7	1/5	1	5
B_3	3	7	1	2	3	9
B_4	1	5	1/2	1	3	7
B_5	1/3	1	1/3	1/3	1	3
B_6	1/9	1/5	1/9	1/7	1/3	1

$\mathbf{B_1}$	C_{11}	C_{12}
C_{11}	1	1/3
C_{12}	3	1

$\mathbf{B_2}$	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}
C_{21}	1	1/5	1/3	1/5	1
C_{22}	5	1	1	1/2	3
C_{23}	3	1	1	1	5
C_{24}	5	2	1	1	3
C_{25}	1	1/3	1/5	1/3	1

$\mathbf{B_3}$	C_{31}	C_{32}	C_{33}	C_{34}
C_{31}	1	3	3	3
C_{32}	1/3	1	2	2
C_{33}	1/3	1/2	1	2
C_{34}	1/3	1/2	1/2	1

$\mathbf{B_4}$	C_{41}
C_{41}	1

$\mathbf{B_5}$	C_{51}	C_{52}	C_{53}
C_{51}	1	7	2
C_{52}	1/7	1	1/2
C_{53}	1/2	2	1

$\mathbf{B_6}$	C_{61}	C_{62}	C_{63}
C_{61}	1	1/3	1
C_{62}	3	1	3
C_{63}	1	1/3	1

The specific calculation steps can be expressed as follows:

(1) The calculation of the product of each row element of the judgment matrix can be expressed as (*Eq. 11*):

$$M_i = \prod_{j=1}^{n} u_{ij} \qquad \text{(Eq.11)}$$

(2) The calculation of the nth root of M_i can be expressed as (*Eq. 12*):

$$\overline{W_i} = \sqrt[n]{M_i} \qquad \text{(Eq.12)}$$

(3) The normalization can be expressed as (*Eq. 13*)

$$W_i = \frac{\overline{W_i}}{\sum_{j=1}^{n} \overline{W_j}} \qquad \text{(Eq.13)}$$

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Then, $W=(W_i, i=1,2,\cdots,n)$ indicates the relative priority of each factor, which is the element that constitutes the feature vector W of the judgment matrix, namely, the ranking weight of the corresponding factor of the same level for the relative importance of the previous level factor.

Consistency calculation

(1) The calculation of the maximum eigenvalue of the judgment matrix can be expressed as (*Eq. 14*):

$$\lambda_{max} = \sum_{i=1}^{n}\left(\frac{\sum_{j=1}^{n} U_{ij} W_j}{n W_i}\right) \quad \text{(Eq.14)}$$

(2) The calculation of the computational judgment matrix consistency index can be expressed as (*Eq. 15*):

$$\mathrm{CI} = \frac{\lambda_{\max} - n}{n-1} \quad \text{(Eq.15)}$$

(3) The calculation of the consistency check coefficient of the judgment matrix can be expressed as (*Eq. 16*):

$$CR = \frac{CI}{RI} \quad \text{(Eq.16)}$$

When CR is 0.1, the maximum eigenvalue can be calculated as: $\lambda'_{max} = CI(n-1)+n = 0.1RI(n-1)+n$. RI is the average random consistency indicator, which is related to the order n of the judgment matrix. When $i = 1,2,\cdots,n$, the corresponding RI value is shown in *Table 19*.

Table 19. *Average stochastic consistency indicators*

n	1	2	3	4	5	6	7	8	9
RI	0.00	0.00	0.58	0.90	1.12	1.24	1.32	1.41	1.45
$\lambda'_{\max}$	-	-	3.116	4.27	5.45	6.62	7.79	8.99	10.16

In the *Table 19*, n=1 or 2 and RI=0, because the 1st- and 2nd-order judgment matrices are always consistent.

When n ≥ 3, CR < 0.1, and $\lambda_{max} < \lambda'_{max}$, the consistency of the comparison judgment matrix is considered to be acceptable; otherwise, the judgment matrix should be properly corrected until λ_{max} is smaller than λ'_{max} in order to pass the consistency test and ensure the obtained W is valid.

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

Hierarchical sorting calculation

Calculating the relative importance scale (also known as the weight vector) of all elements in the same hierarchy to the highest level (total target) is called the total ranking of the hierarchy. The steps for total hierarchical ordering are shown below.

A. The first step is to calculate the weight vector of the importance of all factors at the same level relative to the highest level. This process is carried out from top to bottom and layer by layer.

B. The second step is to calculate the weight vector of nk-1 elements relative to the total target for the k-1th layer:

$$W^{(k-1)}=(W_1^{(k-1)}, W_2^{(k-1)}, \ldots, Wn_{(k-1)}^{(k-1)})^T \tag{Eq.17}$$

C. The k^{th} layer has nk elements. The single criterion weight vector for an element j of the previous level (k-1) is

$$P_j^{(k)}=(W_{1j}^{(k)}, W_{2j}^{(k)},. .., W_{nj}^{(k)})T \tag{Eq.18}$$

(for the unqualified relationship with the jth element of the k-1 layer, the corresponding Wij takes a value of 0);

D. The weight vector of the k^{th} layer relative to the total target is

$$W^k=(P_1^{(k)}, P_2^{(k)},. . . , P_{k-1}^{(k)})W^{(k-1)} \tag{Eq.19}$$

Determination of safety assessment criteria

Based on domestic and foreign research results (Jain et al., 2016; Sun et al., 2016; Zhang et al., 2017; Lyu et al., 2018)[22–25], as well as consultation with experts, the safety assessment grades were determined. The safety assessment scores can be obtained after the standardization of each level of indicators. They are then divided into 5 grades (*Table 20*).

Table 20. *Classification of comprehensive assessment of water ecological security in river basins*

Security Level	Very unsafe	Unsafe	General	Safe	Very safe
Indicator score interval	[0, 10)	[10, 30)	[30, 70)	[70, 90)	[90, 100]
Mean score	5	20	50	80	95

Results

Indicator comprehensive value

Through the calculation of indicators and according to the criteria for the corresponding indicators, the evaluation results for each indicator were determined, as shown in *Table 21*.

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***Table 21.** Evaluation results of water ecological security indicators for the Xiangjiang River Basin*

Target layer	Factor layer	Indicator layer	Evaluation result		
			Upstream	Midstream	Downstream
Water ecological security	Hydrology and water resources	Flow process variation degree	43.10	49.57	49.68
		Degree of ecological flow satisfaction	84.23	75.61	51.68
	Physical structure	Riverbank stability	76.06	72.06	64.33
		Vegetation coverage on river banks	53.54	48.67	49.61
		Degree of human activity in the riparian zone	53.00	48.00	13.00
		River connection barrier condition	72.00	67.00	99.00
		Natural wetland retention rate	100.00	47.16	17.42
	Water quality	Dissolved oxygen concentration	93.36	90.51	77.57
		Oxygen-poor organic pollution status	97.05	92.78	90.15
		Heavy metal pollution status	80.47	81.80	82.68
		Water function area compliance index	100.00	93.33	62.22
	Biology	Fish bio-loss index	54.33	43.45	23.74
	Water ecological management	Sewage centralized treatment efficiency index	74.52	66.80	79.26
		Soil erosion control index	77.94	89.52	71.97
		Hazard source risk index	56.00	74.00	49.00
	Social service function	Flood control indicators	48.61	54.38	59.93
		Public satisfaction indicator	76.10	84.23	78.14
		Centralized drinking water source safety guarantee compliance rate public satisfaction index	70.30	88.43	88.05

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

Indicator weights

The average of the weights of the three experts was comprehensively calculated. The results are shown in *Table 22*.

***Table 22.** Weights of water ecological security indicators for the Xiangjiang River Basin*

Target layer	Factor layer	Indicator layer	Index weight
Water ecological security	Hydrology and water resources (0.2166)	Flow process variation degree (0.2340)	0.0507
		Degree of ecological flow satisfaction (0.7655)	0.1658
	Physical structure (0.0500)	Riverbank Stability (0.2660)	0.0133
		Vegetation coverage on river banks (0.1880)	0.0094
		Degree of human activity in the riparian zone (0.2280)	0.0114
		River connection barrier condition (0.2320)	0.0116
		Natural wetland retention rate (0.088)	0.0044
	Water quality (0.3302)	Dissolved Oxygen Concentration (0.2426)	0.0801
		Oxygen-poor organic pollution status (0.1753)	0.0579
		Heavy metal pollution Status (0.2414)	0.0797
		Water function area compliance index (0.3407)	0.1125
	Biology (0.1762)	Fish Bio-loss Index (1.0000)	0.1762
	Water ecological management (0.0931)	Sewage centralized treatment efficiency index (0.6617)	0.0616
		Soil erosion control Index (0.1418)	0.0132
		Hazard Source Risk Index (0.1944)	0.0181
	Social service function (0.1339)	Flood Control Indicators (0.4720)	0.0632
		Public Satisfaction Indicator (0.1337)	0.0179
		Centralized drinking water source safety guarantee compliance rate public satisfaction index (0.3951)	0.0529

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Comprehensive assessment results of water ecological security in the Xiangjiang River Basin

The comprehensive assessment values of the hydrological and water resources, physical structure, water quality, biological, aquatic ecological management, and social service functions of the upstream, midstream, and downstream portions of the Xiangjiang River Basin were calculated separately. On this basis, overall assessments for the upstream, midstream, and downstream portions of the Xiangjiang River Basin were obtained. The comprehensive evaluation index values of ecological security status are shown in *Table 23* for upstream assessment results, *Table 24* for midstream assessment results, and *Table 25* for downstream assessment results.

Table 23. *Results of comprehensive assessments of water ecological security in the upstream portion of the Xiangjiang River Basin*

Objective	Health status of the upstream portion of the Xiangjiang River Basin	
Function	Composite indicator score	Evaluation result
Hydrology and water resources	74.6	Safe
Physical structure	67.9	Average
Water quality	93.2	Very safe
Biology	54.3	Average
Water ecological management	71.2	Safe
Social service function	60.9	Average
Upstream comprehensive health level	74.7	Safe

Table 24. *Results of comprehensive assessments of water ecological security in the midstream portion of the Xiangjiang River Basin*

Objective	Health status of the midstream portion of the Xiangjiang River Basin	
Function	Composite indicator score	Evaluation result
Hydrology and water resources	69.5	Average
Physical structure	59	Average
Water quality	89.8	Safe
Biology	43.5	Average
Water ecological management	71.3	Safe
Social service function	71.9	Safe
Midstream comprehensive health level	71.6	Safe

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

***Table 25.** Results of comprehensive assessments of water ecological security in the downstream portion of the Xiangjiang River Basin*

Objective	Health status of the downstream of the Xiangjiang River Basin	
Function	Composite indicator score	Evaluation result
Hydrology and water resources	51.2	Average
Physical structure	53.9	Average
Water quality	75.8	Safe
Biology	23.7	Unsafe
Water ecological management	71.2	Safe
Social service function	73.5	Safe
Downstream comprehensive health level	59.6	General

Discussion

Through the study of water ecological security assessment at home and abroad, it can be seen that the index system is the key point of water ecological security assessment in river basins, and it is worth further study.

Weight comparison of evaluation indicators

The calculation results of the weights of each indicator from the comprehensive assessment index system for water ecological security are shown in *Figure 3*.

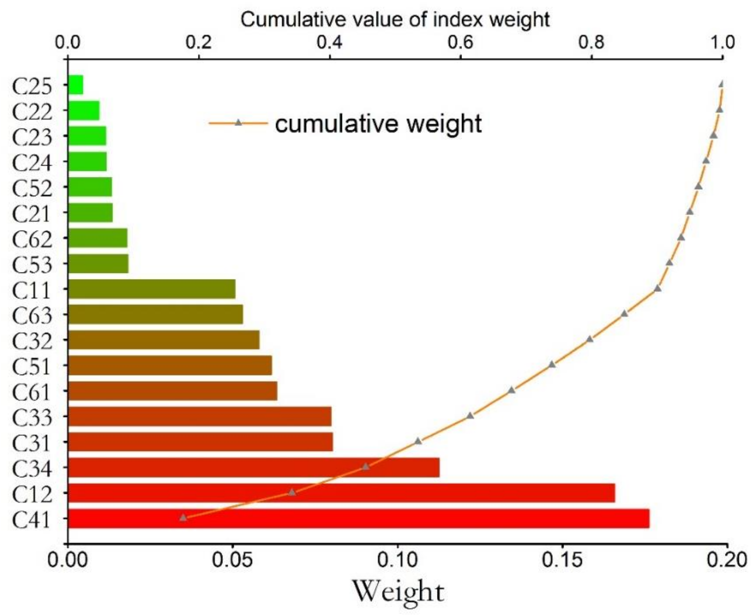

***Figure 3.** Comparison of various indicator weights of water ecological security*

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The figure reflects the importance of indicators in terms of water ecological security assessment. The three most significant indicators are the fish biological loss index, the ecological flow satisfaction level, and water function area compliance, which are important indicators of biological, hydrological, and water resources in the factor layer. The three least important indicators are natural wetland retention, riparian vegetation coverage, and riparian human activity. These indicators reflect the physical structure of the factor layer. Because the indicators with larger weights have a greater impact on the water ecological security of the Xiangjiang River Basin, it is necessary to pay more attention to these indicators.

A comparative analysis of the current status of evaluation indicators

Figure 4 shows the status of the indicators of the upstream, midstream, and downstream portions of the Xiangjiang River Basin after investigation, reflecting the current safety status of each indicator. The comparison shows that the safety status of the upstream water ecosystem is significantly better than those of the middle and downstream ecosystems, and the comprehensive assessment result of the water ecological security in the midstream is better than that in the downstream section. In terms of specific indicators, i.e., the upstream ecological flow satisfaction level, river bank stability, riparian vegetation coverage, riparian human activity, natural wetland retention, dissolved oxygen concentration, oxygen-consuming organic pollution, water functional zone compliance indicators, and fish life, the scores of the biological loss indicators are higher than those of the middle and downstream sections. In the midstream portion, the soil erosion control index, hazard source risk index, public satisfaction index, and centralized drinking water source safety guarantee compliance rate are better than in other river sections. The downstream river section is superior to the other two river sections in the social service function criterion layer. However, other criterion layers of the downstream section perform poorly. Overall, the natural ecosystem of the upstream portion of the Xiangjiang River Basin is larger, making the water ecology healthier, but there are still some problems in ecological water management. As an important area where soil erosion control has been conducted, the midstream section of the river has done well in terms of ecological water health management, but there are also problems concerning the low retention rate of natural wetlands and the low vegetation coverage on river banks. The ecological quality of the aqueous environment in the downstream reaches of the river is poor. However, this section performs well in terms of water ecological management.

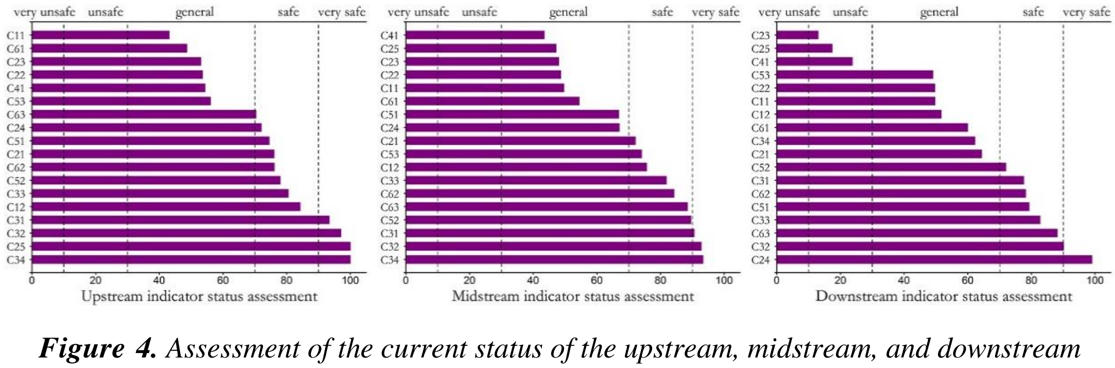

***Figure 4.** Assessment of the current status of the upstream, midstream, and downstream sections of the Xiangjiang River Basin*

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

Variation characteristics of indicators

A comprehensive analysis of the variation of the indicators of the upstream, midstream, and downstream sections of the ecological water security of the Xiangjiang River Basin is shown in *Figure 5*. In terms of health status, there are 8 indicators of the upstream and midstream sections that are better than the those of the downstream section. These indicators include the water function area compliance indicators, natural wetland retention rate, oxygen-consuming organic pollution status, dissolved oxygen concentration, ecological flow satisfaction degree, river bank stability, fish bio-loss index, and degree of human activity in the riparian zone.

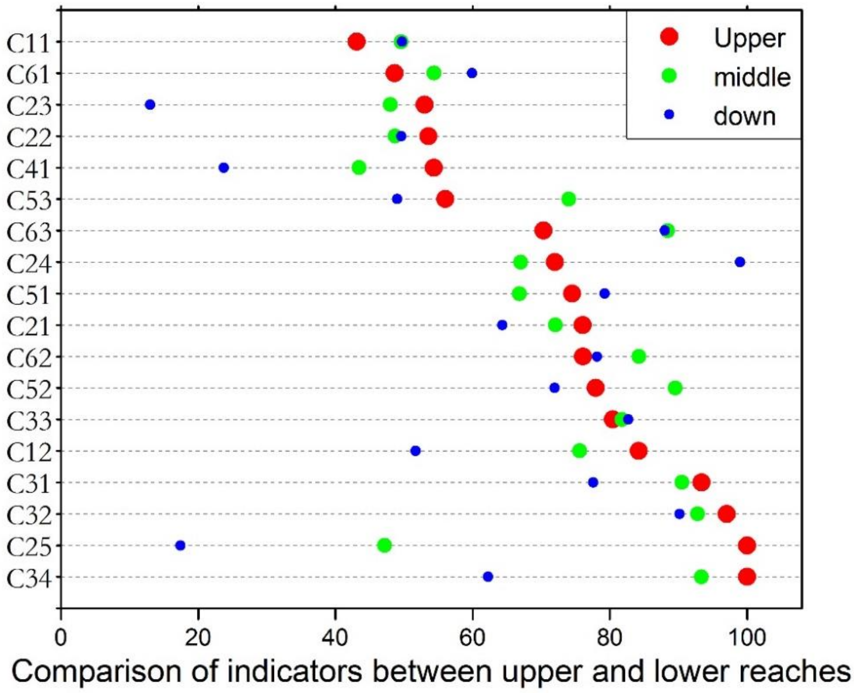

Figure 5. *Variations of current indicators of the upstream, midstream, and downstream sections of the Xiangjiang River Basin*

These indicators are mainly related to hydrology and water resources, physical structure, water quality and biological elements. It is indicated that from the upstream to the downstream sections of the Xiangjiang River Basin that the health status of the indicators transitions from good to bad, and this situation needs to be paid enough attention. It is worth noting that the natural wetland retention rate along the entire river basin ranges from upstream to downstream is good, general and very poor, indicating that the health level of the index has changed dramatically. In addition, with respect to the entire basin of Xiangjiang River, five indicators (the degree of variation in the flow process, the flood control index, human activity intensity in the riparian zone, riparian vegetation coverage, and the fish bio-loss index) are always at unhealthy levels. This fact merits the attention of concerned researchers and citizens.

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Contribution value of indicators

Using the weighted values of each indicator of the Xiangjiang River Basin along with the health status of the selected indicators, the contributions of the upstream, midstream, and downstream components of the Xiangjiang River Basin to the total water ecological security can be obtained, as shown in *Figure 6*. Among them, the top 3 contributing values of the upper reaches are the water function zone compliance index, the ecological flow satisfaction degree, and the fish bio-loss index. The midstream contribution value is consistent with that of the upstream The top 3 contributing values in the lower reaches are water function area compliance, ecological flow satisfaction level and heavy metal pollution status.

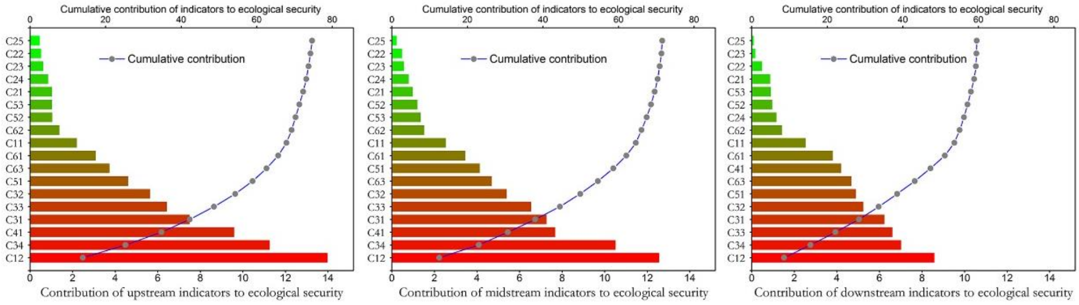

Figure 6. *Contribution of the indicators of the upstream, midstream and downstream sections of the Xiangjiang River Basin to water ecological security*

Conclusions

This study considered the effects of physical, chemical, and biological indicators on the stability and sustainability of watersheds from the perspective of water ecological security. The AHP method was used to calculate the comprehensive evaluation index system of the water ecology of the Xiangjiang River Basin. The top 3 indicators reflecting the health of the river are the fish bio-loss index, the degree of ecological flow satisfaction, and the water function area compliance. These are important indicators of biological, hydrological and water resources, as well as water quality in the factor layer. The last three indicators of importance are the natural wetland retention rate, riparian vegetation coverage, and riparian human activity. These indicators represent the physical structure in the factor layer. A comprehensive assessment of the water ecological security of the Xiangjiang River Basin showed that the water quality in the upstream portions of the Xiangjiang River Basin is satisfactory, and the hydrological water resources and water ecological management have been improved, while the physical structure and biological and social service functions are at an average level. The overall ecological security status of the upstream portion of the river is good. The water quality, water ecological management, and social service functions of the midstream are satisfactory. The hydrology and water resources, physical structure and biology are at an average level, and the overall situation of the midstream is safer than the downstream. The water quality, water ecological management and social service functions of the downstream section of the Xiangjiang River Basin are good. The hydrology and water resources and physical structure are at a general level, while the biological health status is unsafe. The overall ecological safety and health status of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

downstream is at an average level. Based on the health status of the upstream, midstream, and downstream portions of the Xiangjiang River Basin, it can be said that the upstream ecological security level is better than the middle reaches, and that the middle reaches better than the downstream portions.

Acknowledgements. This study was supported by the following Hunan Water Conservancy Science and Technology Project: Research on the water ecological security index system of the Xiangjiang River Basin, contract number: Xiangshui Science and Technology [2015] 186-21; Research on the performance evaluation and benign operation mechanism of urban water ecological civilization construction, contract number: Xiangshui Science and Technology [2016] 194-37.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_45474574

INFLUENCES OF HILLSLOPE WETNESS CONDITIONS ON THE TEMPORAL STABILITY OF SOIL MOISTURE

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(Received 26th Jan 2019; accepted 28th Feb 2019)

Abstract. Knowledge of soil moisture temporal stability under different wetness conditions is critical for hydrological and environmental management decisions. This study analyzed the soil moisture (mean relative difference, MRD) and its associated temporal stability (standard deviation of the relative difference, SDRD) at two depths (10 and 30 cm) during the entire, dry, intermediate and wet periods on a mixed land use (tea garden and forest) hillslope. In addition, the influences of environmental factors on MRD and SDRD were also investigated. Results showed that the MRD of soil moisture had a strong spatial dependence (nugget/sill ratios < 0.25) at each depth during different hillslope wetness periods. The widest range (correlation length) was found during the dry period. In addition, spatial patterns of MRD and SDRD were mainly influenced by topographic factors such as elevation. Correlations between MRD and SDRD were in the order of dry > intermediate > wet conditions. Hillslope wetness conditions had substantial influence on the temporal stability of soil moisture, showing that soil moisture patterns were more stable during wet periods than during dry periods. Therefore, the presentative locations identified with the entire dataset are not always appropriate for estimating hillslope mean soil moisture under all wetness conditions.
Keywords: *soil texture, temporal variability, geostatistics, environmental factors, regression*

Abbreviations: CV, coefficient of variation; DB, depths to bedrock; DEM, digital elevation model; MRD, mean relative difference; PLC, plane curvature; PRC, profile curvature; RF, rock fragment; RMSE, root mean squared error; SDRD, standard deviation of the relative difference; SR, Stepwise regression; TWI, topographic wetness index

Introduction

Soil moisture is an important variable influencing water and solute fluxes in the earth surface (Vereecken et al., 2007; Feng et al., 2017; Liao et al., 2018a, 2018b). It is a major component of the hydrologic cycle, controlling runoff, infiltration and evapotranspiration processes at various scales (Pachepsky et al., 2003). In addition, soil water movement has substantial influence on nutrient loss and availability (Zhu et al., 2009; Schmidt et al., 2011). Therefore, soil moisture variations are critical in hydrological, ecological and environmental management (Fu et al., 2003; Zhu et al., 2017).

Soil moisture variations were influenced by environmental factors, such as soil properties and topography (Lark, 1999; Qiu et al., 2003; Vereecken et al., 2007; Brocca et al., 2007; Zhu and Lin, 2011). The relationships between environmental factors and soil moisture were often modelled using multiple linear regression (Nyberg, 1996; Qiu et al., 2010; van Arkel, 2012). Some studies have shown that there is a significant

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

correlation between soil moisture and environmental factors, while others have indicated that the relationship is insignificant (Famiglietti et al., 1998; Western et al., 1999; Qiu et al., 2001). This may be due to differences in climate, topography, soil, vegetation, scale, time and depth of sampling methods (Famiglietti et al., 1998). The wetness conditions in the study area were also found to affect the relationships between environmental factors and soil moisture content. Previous studies proposed that topography has dominant control on soil moisture distribution under wet soil condition, while soil properties have primary control on soil moisture distribution under dry soil condition (Grayson et al., 1997; Pachepsky et al., 2003; Penna et al., 2013).

Although soil moisture exhibits a high spatio-temporal variability at various scales due to the variations in climate, topography and soil properties, its distribution often shows a similar spatial pattern at different dates (Hu et al., 2010; Penna et al., 2013; Li and Shao, 2014; Qiu et al., 2017). This phenomenon has been called temporal stability by Vachaud et al. (1985), who described it as the time-invariant association between a spatial location and classical statistical parameters. The main purpose of temporal stability analysis of soil moisture was to identify reliable locations that can represent the mean soil moisture content of the entire study area (Grayson and Western, 1998; Jacobs et al., 2004; Zhao et al., 2010).

Relationships between soil moisture temporal stability and environmental factors have often been investigated to identify the best representative locations. Previous studies have found that environmental factors (e.g., soil properties and topography) significantly affected soil water temporal stability (Thierfelder et al., 2003; Vivoni et al., 2008; Brocca et al., 2009; Hu et al., 2010). For example, Vivoni et al. (2008) found that sampling locations with mid elevation tended to have a more pronounced temporal stability. Hu et al. (2010) showed that soil texture can significantly affect the temporal stability of soil water content in the LaoYeManQu watershed, China. In addition, the wetness conditions in the study area had large influence on soil moisture temporal stability. Zhao et al. (2010) observed that the ranked positions of the labelled representative location change with different wetness conditions. This implies that the location with the most pronounced time stability may be different for each wetness condition. Some studies have also demonstrated that soil moisture spatial patterns were more stable during wet periods than during dry periods (Hupet and Vanclooster, 2002; Zhou et al., 2007; Williams et al., 2009; Zhao et al., 2010). This is related to an enhanced capillary movement of water from the subsoil to the topsoil, thereby decreasing temporal stability in topsoil moisture. However, others have shown that a higher degrees of temporal stability in dry conditions than in wet conditions (Martínez-Fernández and Ceballos, 2003; Lin, 2006; Penna et al., 2013). For example, Lin et al. (2006) found more frequent conditions of marked persistence of soil moisture patterns during a long dry-down period in June. Penna et al. (2013) observed a slightly higher degree of temporal stability in dry conditions and for deeper layers. The mixed results suggest that the effect of wetness status on temporal stability was complex and has not been fully understood.

Therefore, the objectives of this study are to provide a comprehensive investigation on the temporal stability of soil moisture content under different hillslope wetness conditions. For this purpose, a typical mixed land-use (tea garden and forest) hillslope was considered for which soil moisture content at two depths (0-20 and 20-40 cm) was repeated measured from January 2013 to December 2015 (a total of 32 sampling days) in 77 sites. The dataset obtained was analyzed for temporal stability analysis. The

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DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

hypotheses of this study are i) the controlling factors of soil moisture and its temporal stability vary with hillslope wetness condition, ii) the temporal stability of soil moisture are different during different hillslope wetness conditions.

Materials and methods

Study hillslope

This study was conducted on a hillslope (31°21′N, 119°03′E) (has an area of 0.6 ha) in the hilly area of Taihu Lake Basin, China (*Fig. 1*). This study area is feature with a north subtropical-middle subtropical transition monsoon climate with four distinctive seasons. The annual mean temperature is 15.9°C and the annual mean precipitation is 1157 mm. Green tea (*Camellia sinensis (L.) O. Kuntze*) and Moso bamboo (*Phyllostachysedulis (Carr.) H. de Lehaie*) are dominant on the hillslope. The elevation of the hillslope ranges from 77 to 88 m and the slope ranges from 0 to 21%. The soil type of the hillslope is shallow lithosols according to the FAO soil classification (Orthents according to Soil Taxonomy). Parent material is quartz sandstone. Soils are described as silt loam texture with silt content > 60%. Surface (0-20 cm) soil organic matter contents were about 2% on both hillslopes. The depth to bedrock varies from <0.3 m at the summit slope position to about 1.0 m at the foot slope position (Liao et al., 2016).

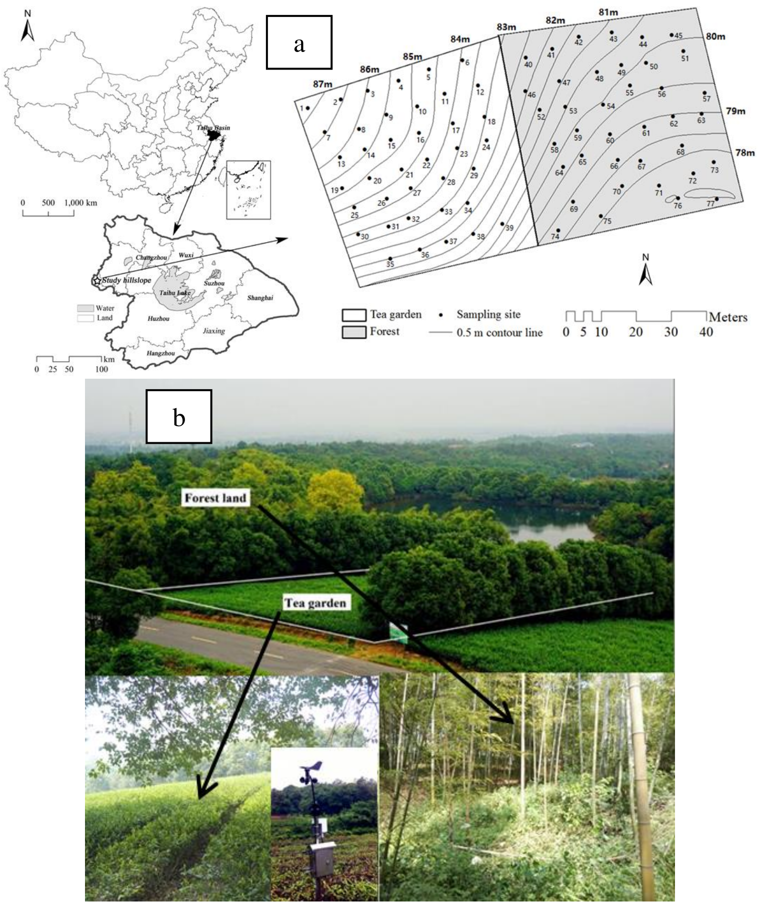

Figure 1. *(a) Location of the study area and sampling sites on study hillslope and (b) photographs from the hillslope*

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DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

Soil moisture measurement

For monitoring volumetric soil water content, access polyvinyl chloride tubes were installed at 77 sites on the hillslope (*Fig. 1*). A portable time-domain reflectrometry TRIME-PICO-IPH soil moisture probe (IMKO, Ettlingen, Germany) was used at 32 dates from January 2013 to December 2015. Volumetric soil water was measured at the depths of 0 to 20 cm (denoted as 10 cm) and 20 to 40 cm (denoted as 30 cm) each time (note that the TRIME-PICO-IPH probe has a length of 18 cm). Due to the shallow soil depths at some locations, only 73 sites had soil moisture readings at 30 cm depth. For each site, the TRIME-PICO-IPH probe was twisted in the access tube to face different directions and 2-3 readings were then taken. The average of these readings was used as the final water content for each site on a specific date. In addition, an outdoor mini weather station was set up to measure rainfall and air temperature. The amounts of precipitation were 889.5 mm, 1296.4 mm and 1617.0 mm in year 2015, 2016 and 2017, respectively.

Soil properties and terrain attributes

Around each soil moisture access tube (within 1-m distance), soil samples at each depth interval were collected using a hand auger. Three subsamples were collected for each site and then fully mixed. These samples were air dried, weighted, ground and sieved through a 2 mm polyethylene sieve. Particles larger than 2 mm (rock fragments) were weighed to determine the rock fragment (RF) content. Soils that passed through the 2 mm polyethylene sieve were used to analyze the particle size distribution using the Malvern Mastersizer 2000 laser analyzer (Malvern Instruments Inc., Worcestershire, UK). The fractions of <0.002 mm (clay), 0.002–0.05 mm (silt), and 0.05–2 mm (sand) were determined for each soil sample. The percentage of the organic matter in the soil was measured by the titration method, which is based on the oxidation of organic matter by $K_2Cr_2O_7$. In addition, the depths to bedrock (DB) of all 77 sites were also determined when installing the access tubes for soil moisture measurements and taking soil samples using a hand auger.

A high-resolution (1 m) digital elevation model (DEM) of the study hillslopes was derived from a 1: 1000 contour map. Terrain attributes including elevation, slope, plane curvature (PLC), profile curvature (PRC), and topographic wetness index (TWI) were determined from this DEM in ArcGIS 10.0 (ESRI, Redlands, CA).

Temporal stability analysis

The temporal stability of soil water content for each soil depth was analyzed using the approach proposed by Vachaud et al. (1985):

$$\theta_j = \frac{1}{N}\sum_{i=1}^{N}\theta_{ij} \quad \text{(Eq.1)}$$

$$\delta_{ij} = \frac{\theta_{ij} - \theta_j}{\theta_j} \quad \text{(Eq.2)}$$

$$MRD = \frac{1}{M}\sum_{j=1}^{M}\delta_{ij} \quad \text{(Eq.3)}$$

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$$SDRD = \sqrt{\frac{1}{M-1}\sum_{j=1}^{M}\left(\delta_{ij} - MRD\right)^2} \quad \text{(Eq.4)}$$

where θ_{ij} is the soil water content at location i in day j; θ_j is the arithmetic mean of soil water content in day j; N is the number of locations; δ_{ij} is the relative difference of soil water content at location i and day j; and M is the number of sampling days, in this case, $M = 17$. MRD is the arithmetic mean relative difference of soil water content at location i; SDRD is the standard deviation of relative difference. The SDRD is the temporal stability of soil water at location i. Smaller SDRD means temporally more stable.

Classical statistics

First soil moisture, soil properties and terrain attributes were investigated using univariate descriptive analysis. The spatial mean soil moisture and corresponding coefficient of variation (CV) were calculated. Correlation analysis was conducted to investigate the relationships between soil moisture contents under different wetness conditions. Stepwise regression (SR) analysis was then conducted to investigate the relationships between environmental factors (e.g., soil properties and topography) and soil moisture and its temporal stability. A backward method regression (Norusis, 1994) was selected and the level for entry in the regression model was set at $p < 0.10$, while a 0.05 significance level was applied to retain the variables in the model. In addition, the *t*-test was used to test the significant of differences in SDRD among different wetness conditions.

Dominance analysis was used to quantify the relative influence of soil properties and topography on MRD and SDRD. Budescu (1993) defined dominance as a pairwise relationship that can be tested for all pairs of variables included in the model. Given a single dependent variable (y) and k explanatory variables (x_1, x_2, …, x_k) (determined by SR analysis), the independent effect of predictor x_1 (I_{x1}) denotes the average contribution of variable x_1 to the variance in y over all 2^k-1 possible submodels. The independent effect of each variable is computed by comparing the fit of all models containing a particular variable to the fit of all nested models lacking that variable, through the process of hierarchical partitioning. Thus, for variable x_1,

$$I_{x_1} = \sum_{i=0}^{k-1}\frac{\sum(R^2_{y,x_1x_h} - R^2_{y,x_h})/\binom{k-1}{i}}{k} \quad \text{(Eq.5)}$$

where x_h is any subset of i predictors, x_1 excluded; R^2 is the coefficient of determination. Because dominance analysis utilizes an all possible models approach, it provides a more robust assessment of variable importance, relative to single-model approaches, by assuring that the contribution of a particular variable is neither enhanced nor masked through its correlation with other explanatory variables (Murray and Conner, 2009). All classical statistics were conducted using the *regress* function of MATLAB software (The MathWorks Inc., USA) and SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA).

Geostatistics

The spatial dependence of soil moisture content was analyzed using semivariograms γ, which were calculated as follows:

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$$\gamma(h) = \frac{1}{2N(h)} \sum_{i=1}^{N(h)} [Z_i - Z_{i+h}]^2 \qquad \text{(Eq.6)}$$

where $N(h)$ is the number of distance pairs within a given distance class, Z_i is the measured variable at location i and Z_{i+h} is the variable at locations separated from i by the distance h that fall within the distance class (Yates and Warrick, 1987). Four semivariogram models (spherical, exponential, linear and Gaussian) were used to describe the semivariograms and the best-fitted models with the largest coefficient of determination (R^2) were selected. Then the geostatistical parameters were obtained, including nugget, sill and effective range. The ratio between nugget and sill was used to characterize the spatial dependencies of soil water content. Smaller nugget/sill ratio indicates stronger spatial dependency. All the geostatistical computations were conducted using GS+ 7.0 (Gamma Design Software LLC., Plainwell, MI, USA).

Results and discussion

Temporal variations of hillslope wetness conditions

From January 2013 to December 2015, a substantial fluctuation of the hillslope mean soil water content was observed for each depth (*Fig. 2*). This fluctuation was influenced by climate factors, such as precipitation and evapotranspiration.

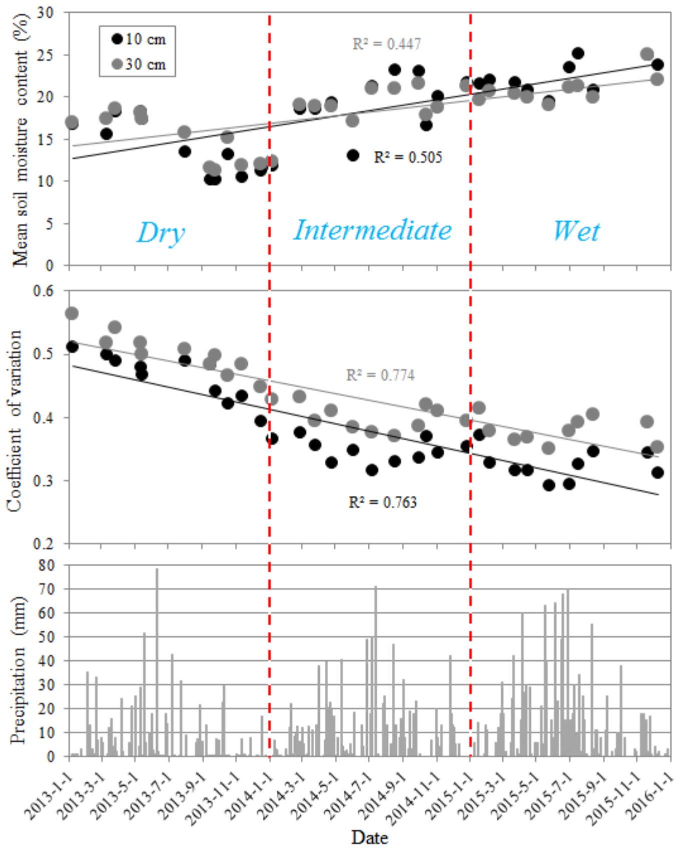

Figure 2. *Time series of precipitation, average soil water contents and corresponding coefficients of variation at two depths*

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The mean soil moisture contents of were 10.18-25.15% and 11.24-24.90% at 10- and 30-cm depths, respectively. An increasing trend of mean soil moisture with time was found at two depths, which can be fitted by a linear function (R^2=0.505 for 10 cm ($P<0.01$) and R^2=0.447 for 30 cm ($P<0.01$)). The reason is that the amount of precipitation increased yearly (890.5, 1296.4 and 1617.0 mm for the year 2013, 2014 and 2015, respectively). Therefore, hillslope wetness conditions can be classified as dry, intermediate and wet periods, corresponding to year 2013, 2014 and 2015, respectively. The mean soil moisture contents were 14.10, 18.82 and 22.38% at 10 cm depth during dry, intermediate and wet periods, respectively, while these values were 15.06, 18.82 and 20.85% at 30 cm depth during dry, intermediate and wet periods respectively (*Table 1*). In addition, seasonal patterns of mean soil moisture were similar from one year to the next year. The highest mean soil moisture content was observed in summer season (from July to September) due to intense and heavy rainfalls occurred, while the lowest value was measured in winter and spring seasons (from November to March) due to the relatively low precipitation.

Table 1. *Statistics of soil moisture at two depths under different hillslope wetness conditions*

	10 cm			30 cm		
	Dry	**Intermediate**	**Wet**	**Dry**	**Intermediate**	**Wet**
No. of locations	77	77	77	73	73	73
No. of sampling times	11	11	10	11	11	10
No. of measurements	847	847	770	803	803	730
Mean (%)	14.10	18.82	22.38	15.06	18.82	20.85
SD (%)	3.29	3.73	1.91	2.85	2.63	1.66
CV	0.23	0.20	0.09	0.19	0.14	0.08

SD: standard deviation; CV: coefficient of variation

The corresponding CV values were 0.293-0.510 and 0.351-0.562 at 10- and 30-cm depths, respectively (*Fig. 2*). The soil moisture at 30 cm depth was found to have stronger variability than that at 10 cm depth. Temporal series of CV for each depth can also be fitted by a linear function (R^2=0.763 for 10 cm ($P<0.01$) and R^2=0.774 for 30 cm ($P<0.01$)). The temporal variations of CV showed the opposite trend as compared to those of the mean soil moisture for each depth (*Fig. 2*). This suggests that the spatial heterogeneity of soil moisture content increases as the soil gets drier. Previous studies also found an increase in spatial variability with decreasing mean soil moisture (Famiglietti et al., 1999; Choi and Jacobs, 2010; Brocca et al., 2012; Korres et al., 2015).

Influencing factors of MRD

At 10 cm depth, the MRD of soil moisture content was mainly influenced by elevation, PLC, DB and RF during the entire period, while at 30 cm depth, it was affected by elevation, PRC, DB, RF and Sand (*Table 2*). Negative coefficients for elevation, PLC, RF and sand and positive coefficients for other factors were observed, indicating that elevation, PLC, RF and sand were significantly ($P<0.05$) negatively correlated with soil moisture, whereas PRC and DB were significantly ($P<0.05$) positively correlated with soil moisture. The results are consistent with previous studies (Tromp-van Meerveld and McDonnell, 2006; Brocca et al., 2007; McMillan and Srinivasan, 2015). The R^2 values of stepwise regression models were 0.649 and 0.643 at

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10 and 30 cm depths, respectively. This suggests that environmental factors can explain nearly 65% of variation in soil moisture at each depth. The accuracies of soil moisture predictions in our study were comparable with those reported by previous studies (Western et al., 1999; Qiu et al., 2010). The importance of the variables was sequenced as elevation>DB>PLC>RF according to standardized regression coefficients at 10 cm depth (*Table 2*). However, the *I* values for elevation, PLC, DB and RF were 0.373, 0.040, 0.120 and 0.116, respectively (*Table 3*). This indicates that elevation is the most important variable, while PLC is the least important variable among the four variables. This suggests that the use of standardized regression coefficients would result in wrong conclusions regarding the relative importance of the variables influencing soil moisture variations.

Table 2. *Results of stepwise regression analysis for environmental factors versus mean relative difference*

Hillslope moisture status	10 cm				30 cm			
	Variables	**Coefficients**	**SC**	***I***	**Variables**	**Coefficients**	**SC**	***I***
Entire period	Constant	6.068			Constant	5.431		
	Elevation	-0.075	-0.565	0.373	Elevation	-0.064	-0.434	0.267
	PLC	-0.018	-0.177	0.040	PRC	0.017	0.205	0.108
	DB	0.006	0.228	0.120	DB	0.006	0.191	0.083
	RF	-0.006	-0.170	0.116	RF	-0.007	-0.205	0.076
	R^2	0.649			Sand	-0.016	-0.180	0.109
					R^2	0.643		
Dry	Constant	7.051			Constant	6.446		
	Elevation	-0.086	-0.528	0.349	Elevation	-0.076	-0.425	0.276
	PLC	-0.019	-0.149	0.036	PLC	-0.025	-0.182	0.104
	DB	0.008	0.231	0.107	DB	0.007	0.192	0.079
	RF	-0.009	-0.201	0.126	RF	-0.008	-0.180	0.073
	R^2	0.618			Sand	-0.024	-0.224	0.112
					R^2	0.644		
Intermediate	Constant	5.730			Constant	4.766		
	Elevation	-0.074	-0.613	0.387	Elevation	-0.056	-0.405	0.237
	PLC	-0.018	-0.193	0.118	PRC	0.017	0.223	0.112
	DB	0.006	0.260	0.110	DB	0.006	0.195	0.078
	R^2	0.615			RF	-0.006	-0.180	0.102
					Sand	-0.016	-0.191	0.077
					R^2	0.606		
Wet	Constant	7.114			Constant	5.073		
	Elevation	-0.085	-0.725	0.343	Elevation	-0.061	-0.456	0.255
	PLC	-0.033	-0.370	0.132	PRC	0.017	0.226	0.120
	PRC	-0.017	-0.266	0.083	DB	0.006	0.194	0.074
	Sand	-0.015	-0.244	0.098	RF	-0.009	-0.272	0.132
	R^2	0.656			R^2	0.581		

SC: standardized coefficient; *I*: independent effect; PLC: plane curvature; DB: depth to bedrock; RF: rock fragment; PRC: profile curvature

The factors influencing the MRD of soil moisture content were slightly different for each period at both depths (*Table 2*). However, topography was always found to explain more variability (49.2-61.5% for 10 cm and 42.7-45.9% for 30 cm) in soil moisture than soil properties (0-12.6% for 10 cm and 13.2-18.5% for 30 cm) under different hillslope wetness conditions. Our results are not consistent with previous studies that indicated a larger influence of soil properties than topography on soil moisture under dry conditions

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(Grayson et al., 1997; Pachepsky et al., 2003; Penna et al., 2013) or wet (Laio et al., 2002; Baroni et al., 2013) conditions. This may be related to relatively homogeneous soil properties on study hillslope.

Table 3. *Influence of environmental factors on mean relative difference at 10-cm depth during the entire period by using dominance analysis*

Submodels	R^2	Increase in R^2			
		E	PLC	DB	RF
Contribution (*k*=0)	0	0.550	0.049	0.225	0.238
E	0.550	-	0.024	0.051	0.025
PLC	0.049	0.525	-	0.224	0.247
DB	0.225	0.376	0.048	-	0.142
RF	0.238	0.337	0.058	0.129	-
Contribution (*k*=1)	-	0.413	0.043	0.135	0.138
E+PLC	0.574	-	-	0.053	0.030
E+DB	0.601	-	0.026	-	0.017
E+RF	0.575	-	0.029	0.043	-
PLC+DB	0.273	0.354	-	-	0.150
PLC+RF	0.296	0.308	-	0.127	-
DB+RF	0.367	0.251	0.056	-	-
Contribution (*k*=2)	-	0.304	0.037	0.074	0.066
E+PLC+DB	0.627	-	-	-	0.022
E+PLC+RF	0.604	-	-	0.045	-
E+DB+RF	0.618	-	0.031	-	-
PLC+DB+RF	0.423	0.226	-	-	-
Contribution (*k*=3)	-	0.226	0.031	0.045	0.022
E+PLC+DB+RF	0.649	-	-	-	-
Independent effect (*I*)	-	0.373	0.040	0.120	0.116
Standardized coefficient	-	-0.505	-0.177	0.228	-0.170

E: elevation; PLC: plane curvature; DB: depth to bedrock; RF: rock fragment

Spatial patterns of MRD

The MRD dataset under different hillslope wetness conditions had low skewness (0.181-0.383) and kurtosis (-0.620--0.145), thus meeting the requirement of a normal distribution for kriging prediction. The semivariogram of MRD provided a clear description of its spatial structure with some insight into possible processes influencing its spatial distribution (*Table 4*). The semivariograms of MRD at 10 cm depth were well fitted with the spherical and Gaussian model under the entire and wet periods, respectively, whereas the remaining semivariograms were well fitted with an exponential model. The nugget/sill ratios of the fitted semivariogram models for MRD under different wetness conditions were less than 0.25, indicating that soil moisture had a strong spatial dependence on study hillslope. Range can reflect some information about spatial dependency of environmental variables (Wu et al., 2009). The semivariogram of MRD had 95.1-115.8 m of range at 10 cm depth, while the semivariogram of MRD had 38.7-62.1 m of range at 30 cm depth. This means that soil moisture at 10 cm depth had stronger spatial structure than at 30 cm depth. In addition, for each depth, the largest range was found during the dry period. This is related to the fact that when soil is dry the soil moisture is relative uniform (Lv et al., 2016).

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Table 4. *Semivariance analysis of mean relative difference at two depths under different hillslope wetness conditions*

Variable	Model	Nugget	Sill	Nugget/sill ratio	Range (m)	R^2
MRD-10cm-Entire	S	0.018	0.138	0.130	84.7	0.900
MRD-10cm-Dry	E	0.014	0.223	0.061	115.8	0.865
MRD-10cm- Intermediate	E	0.002	0.115	0.016	95.1	0.878
MRD-10cm-Wet	G	0.033	0.161	0.202	109.5	0.955
MRD-30cm-Entire	E	0.000	0.133	0.001	46.8	0.795
MRD-30cm-Dry	E	0.008	0.212	0.035	62.1	0.747
MRD-30cm-Intermediate	E	0.000	0.119	0.001	38.7	0.798
MRD-30cm-Wet	E	0.000	0.111	0.001	42.0	0.892

S-spherical model; E-exponential model; G-Gaussian model

From the maps of predicted MRD developed by kriging (*Fig. 3*), we found that the MRD had strong spatial variability on study hillslope.

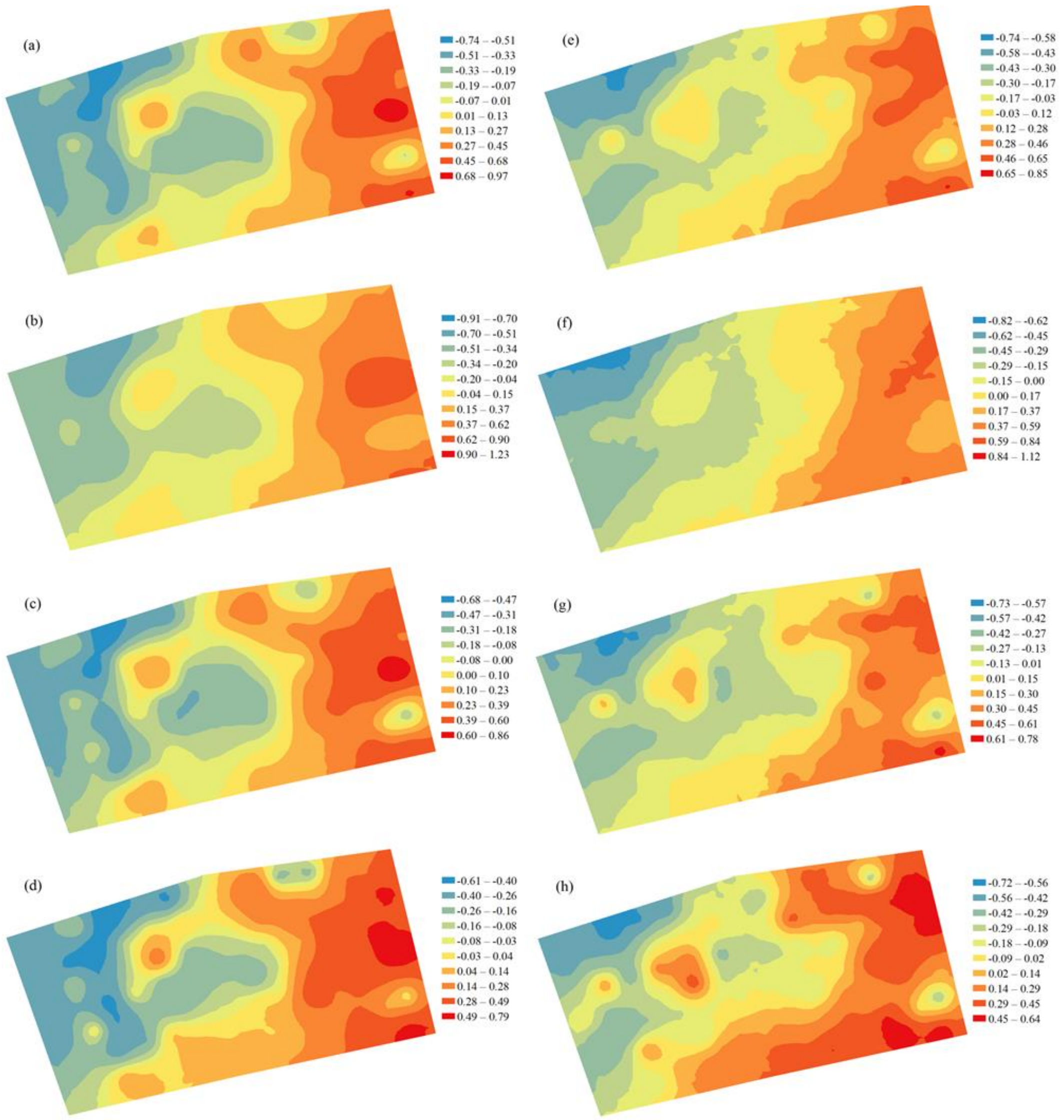

Figure 3. *Spatial distribution of mean relative difference at depths of 10 (a-d) and 30 cm (e-h) during the entire (a,e), dry (b,f), intermediate (c,g) and wet (d,h) periods*

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The MRD values in the northwestern region of the study hillslope were generally lower, whereas the MRD values in the southeastern region were generally higher. Spatial patterns of MRD were similar to that of elevation (*Fig. 1*). This suggests that elevation had large influence on soil moisture variations, which is consistent with the results of stepwise regression (*Table 2*). In addition, the distributions of soil moisture presented a similar spatial pattern for each depth under different hillslope wetness conditions. Correlation matrix of MRD is shown in *Fig. 4*, including all possible combinations, even between different soil depths. Interestingly, all correlation coefficients were larger than 0.7, indicating the pronounced stability of soil moisture at two depths. Generally, lower values of correlation (but still fully above the statistical significance level, $p<0.01$) were associated to comparisons of MRD between two soil depths. In addition, for each depth, the correlation between MRD during wet period and MRD during dry period was weakest among all cases, indicating that soil moisture patterns during dry and wet periods were different to some extent.

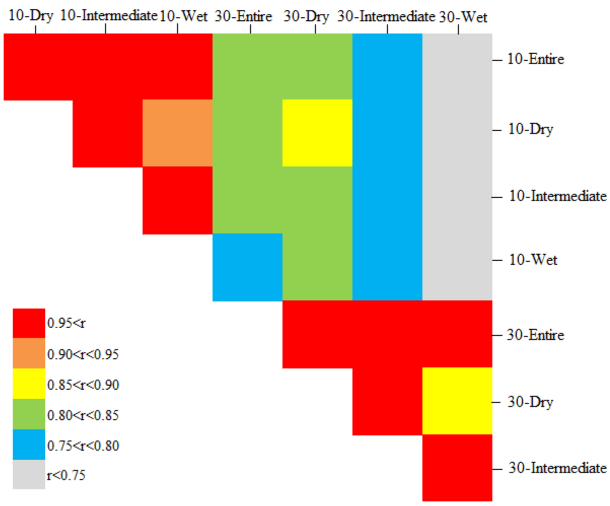

Figure 4. *Correlation matrix of mean relative differences under different hillslope wetness conditions at two depths*

Relationships between MRD and SDRD

During the entire period, MRD was positively significantly ($P<0.01$) correlated to SDRD for each depth (*Fig. 5*), which implies that the value of SDRD tended to be lower for the drier locations. This is consistent with the results of Martínez-Fernández and Ceballos (2003) and Hu et al. (2010). Correlation coefficients between MRD and SDRD at 10 cm depth during dry, intermediate and wet periods were 0.659 ($P<0.01$), 0.468 ($P<0.01$) and 0.320 ($P<0.01$) respectively, while these values at 30 cm depth during dry, intermediate and wet periods were 0.582 ($P<0.01$), 0.277 ($P<0.05$) and 0.231 ($P<0.05$), respectively. This indicates that the correlations between MRD and SDRD at each depth were in the order of dry > intermediate > wet conditions. Hillslope wetness conditions had substantial influence on temporal stability of soil moisture. This is different from the finding of Martínez-Fernández and Ceballos (2003) that the amount of rainfall was not seen to modify the patterns of temporal stability. In addition, stronger correlations were found at 10 cm depth than at 30 cm depth. This is probably due to stronger variability of soil moisture at 30 cm depth than that at 10 cm depth.

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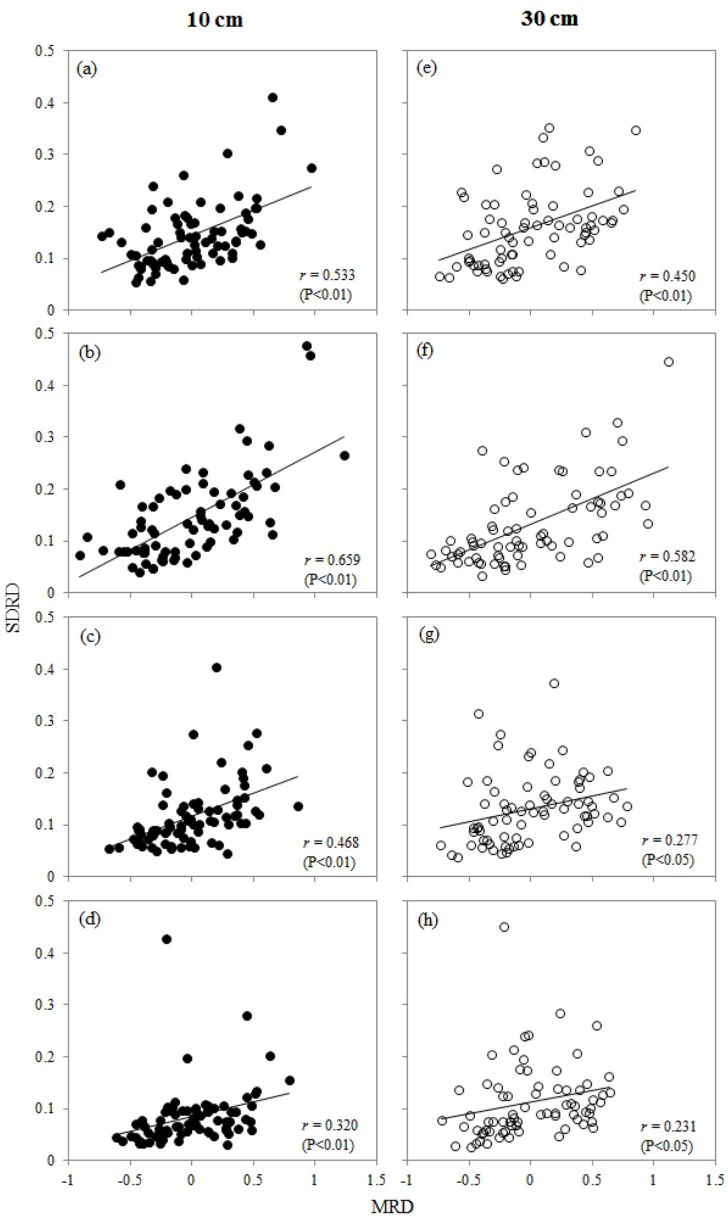

***Figure 5.** Relationships between mean relative difference and standard deviation of relative difference at depths of 10 (a-d) and 30 cm (e-h) during the entire (a,e), dry (b,f), intermediate (c,g) and wet (d,h) periods*

From *Fig. 6*, it can be seen that there is a significant difference (P<0.05) in hillslope mean SDRD at 10 cm depth between dry (0.145), intermediate (0.117) and wet (0.085) periods. For 30 cm depth, hillslope mean SDRD under wet condition (0.112) was significantly less than those under dry (0.133) and intermediate (0.131) conditions. As a result, soil moisture patterns were more stable during wet period than during dry period. This is consistent with most previous studies (Gómez-Plaza et al., 2000; Williams et al., 2009; Zhao et al., 2010; Penna et al., 2013). Conversely, the results by Martínez-Fernández and Ceballos (2003) and Lin (2006) reveal a constant higher degree of temporal stability during dry conditions. This suggests that relatively homogeneous soil properties favor the temporal persistence of soil moisture patterns in wet conditions.

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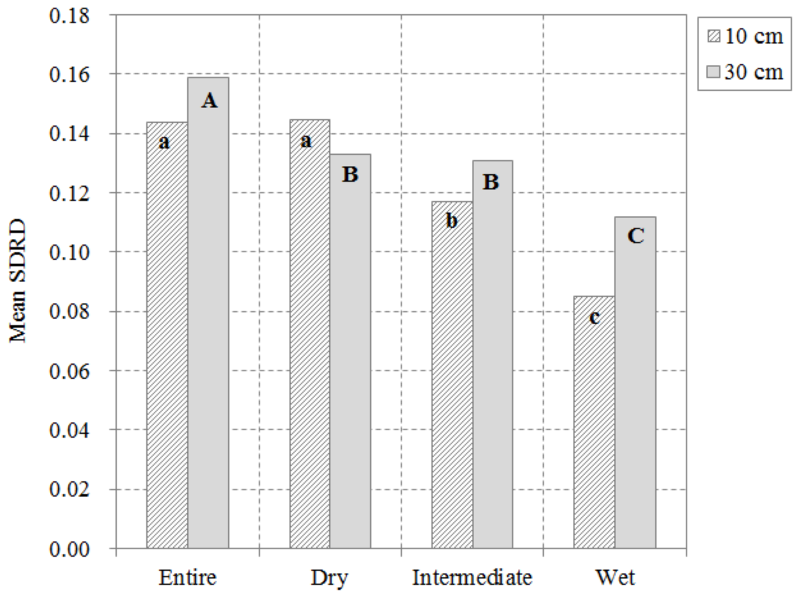

Figure 6. *Standard deviation of relative difference at different hillslope wetness conditions. The same lowercase and capital do not show significant difference at p<0.05*

Identification of representative locations

The rank ordered MRD and its associated SDRD, as well as locations with absolute MRD less than 5% and the driest and wettest 10 locations are shown in *Fig. 7*. Obviously, the rank of MRD varied with soil depth. This is consistent with previous studies (Starks et al., 2006; Hu et al., 2010). During the entire period, locations 4 and 9 can be representative of dry conditions at 10 cm depth, and locations 77 and 63 of wet conditions. In addition, the absolute MRD of locations 11, 10, 46, 59 22, 54, 12, 65, 53 and 34 were less than 5% at 10 cm depth. Among these 10 locations, the SDRD of location 22 was smallest. Therefore, location 22 can directly represent the hillslope mean soil moisture content at 10 cm depth. Likewise, location 60 can be representative of 30 cm depth. As can be seen in *Fig. 8*, there is a close linear regression between the measured moisture contents at the representative locations and the hillslope mean values (R^2=0.882 and root mean squared error (RMSE) =1.55% for 10 cm; R^2=0.729 and RMSE=2.62% for 30 cm). This indicates that locations 22 and 60 are appropriate for estimating hillslope mean soil moisture with an acceptable degree of accuracy at depths of 10 and 30 cm, respectively, regardless of the hillslope wetness conditions.

At 10 cm depth, locations 35, 22 and 22 can best represent the hillslope mean soil moisture content under dry, intermediate and wet conditions respectively, while locations 60, 60 and 19 can be representative of 30 cm depth under dry, intermediate and wet conditions respectively (*Fig. 7*). This implies that the presentative locations identified with the entire dataset are not always appropriate for estimating hillslope mean soil moisture under all wetness conditions. Therefore, location 22 was replaced by location 35 for predicting hillslope mean soil moisture content at 10 cm depth under dry period, while location 60 was replaced by location 19 at 30 cm depth under wet period. It is found that the accuracy of the linear regression was substantially improved after correction (R^2=0.926 and RMSE =1.28% for 10 cm; R^2=0.794 and RMSE =1.90% for 30 cm) (*Fig. 8*).

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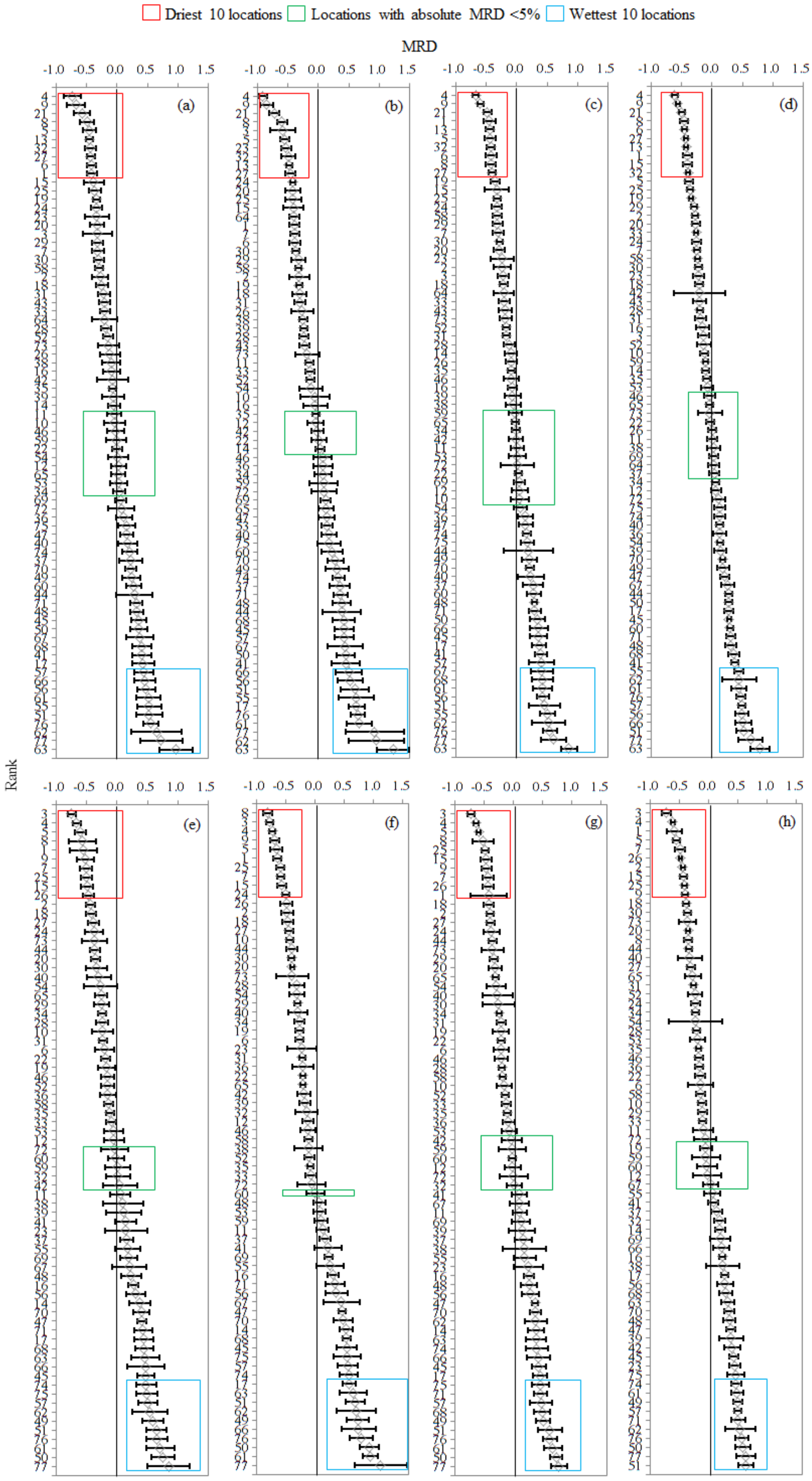

***Figure 7.** Rank ordered mean relative differences (MRD) at depths of 10 (a-d) and 30 cm (e-h) during the entire (a,e), dry (b,f), intermediate (c,g) and wet (d,h) periods. Vertical bars correspond to ±standard deviation of the relative difference over time. Sampling locations are presented orderly according to the MRD*

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DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

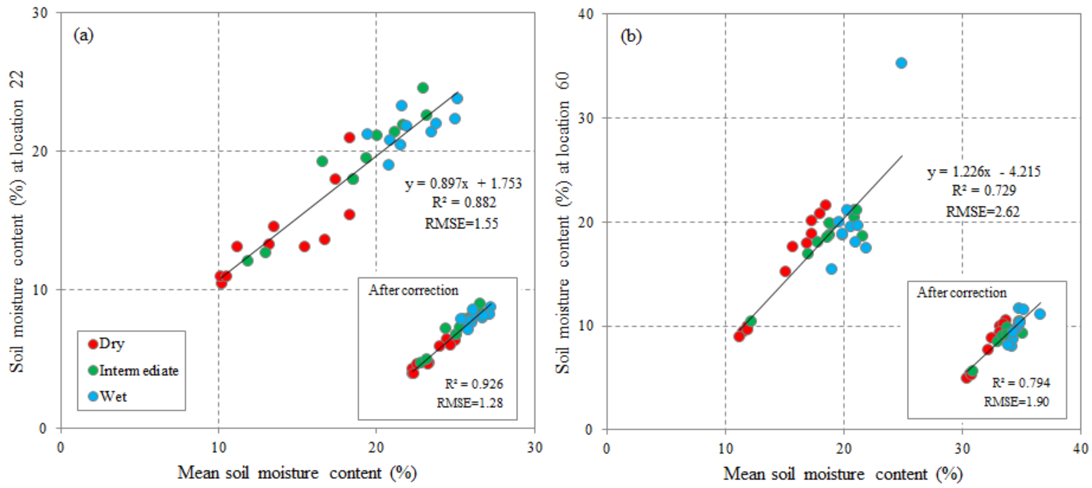

Figure 8. *Hillslope mean soil moisture content versus the representative point moisture content at the depths of 10 (a) and 30 cm (b). Results after correction (location 22 was replaced by location 35 for predicting hillslope mean soil moisture content at 10 cm depth under dry period, while location 60 was replaced by location 19 at 30 cm depth under wet period) are shown in the lower right corner of the figure*

Influencing factors of SDRD

At 10 cm depth, the SDRD was mainly influenced by PLC, slope and RF during the entire period, while at 30 cm depth it was affected by only elevation (*Table 5*). Negative coefficients for all variables were observed, indicating a significant (P<0.05) negative correlation between these variables and SDRD.

Table 5. *Results of stepwise regression analysis for environmental factors versus standard deviation of relative difference*

Hillslope moisture status	10 cm				30 cm			
	Variables	Coefficients	SC	*I*	Variables	Coefficients	SC	*I*
Entire period	Constant	0.298			Constant	1.038		
	PLC	-0.004	-0.232	0.095	Elevation	-0.011	-0.394	0.155
	Slope	-0.004	-0.261	0.093	R^2	0.155		
	RF	-0.002	-0.358	0.101				
	R^2	0.289						
Dry	Constant	0.859			Constant	1.460		
	Elevation	-0.006	-0.207	0.092	Elevation	-0.016	-0.520	0.274
	Slope	-0.007	-0.336	0.175	Slope	-0.005	-0.263	0.152
	RF	-0.003	-0.294	0.103	R^2	0.426		
	R^2	0.370						
Intermediate	Constant	0.692			a			
	Elevation	-0.007	-0.283	0.103				
	Slope	-0.004	-0.262	0.090				
	R^2	0.193						
Wet	Constant	0.676			Constant	0.973		
	Elevation	-0.007	-0.343	0.117	Elevation	-0.011	-0.403	0.155
	R^2	0.117			PRC	-0.004	-0.305	0.091
					R^2	0.246		

SC: standardized coefficient; *I*: independent effect; PLC: plane curvature; RF: rock fragment; PRC: profile curvature.
a: No sound stepwise regression model was found

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DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

The R^2 values of stepwise regression models were 0.289 and 0.155 at 10 and 30 cm depths, respectively. This suggests that environmental factors can only explain 28.9% and 15.5% of variation in SDRD at depths of 10 and 30 cm depths, respectively, which is comparable to the finding of Hu et al. (2010). Topography explained more variability (18.8% for 10 cm and 15.5% for 30 cm) in SDRD than soil properties (10.1% for 10 cm and 0 for 30 cm). Therefore, topography had dominant control on soil moisture temporal stability on study hillslope. This is consistent with the study by Grayson and Western (1998) and Vivoni et al. (2008).

The R^2 of regression equations for SDRD at 10 cm depth were 0.370, 0.193 and 0.117 under dry, intermediate and wet periods, respectively. This indicates that environmental factors can explain the most variability (37.0%) in SDRD during the dry period. This may be attributed to the fact that the strongest correlation between MRD and SDRD was found during the dry period. Similarly, topography can explain the most variability (42.6%) in SDRD at 30 cm depth during the dry period. However, topography did not significantly affect the SDRD during the intermediate period. This may be attributed to the relatively weak correlation between MRD and SDRD at 30 cm depth during this period. Overall, hillslope wetness conditions can modify the effects of environmental factors on temporal stability.

Conclusions

Topography had larger influence on soil moisture content denoted as MRD than soil properties at each depth under different hillslope wetness conditions. The MRD of Soil moisture had a strong spatial dependence on study hillslope. However, soil moisture at 10 cm depth had stronger spatial structure than at 30 cm depth in terms of effective range. In addition, for each depth, the largest effective range was found during the dry period.

The value of SDRD tended to be lower for the drier locations. Correlations between MRD and SDRD at each depth were in the order of dry > intermediate > wet conditions. Therefore, hillslope wetness conditions had substantial influence on temporal stability of soil moisture. Soil moisture patterns were more stable during wet period than during dry period.

At 10 cm depth, location 35 can best represent the hillslope mean soil moisture under dry condition, while location 22 can be representative under intermediate and wet conditions. However, at 30 cm depth, location 60 can be representative under dry and intermediate conditions, while location 19 can be representative under wet condition. In addition, topography had dominant control on soil moisture temporal stability at each depth on study hillslope.

Acknowledgements. This study was financially supported by the National Natural Science Foundation of China (41571080 and 41801169), the Natural Science Foundation of Jiangsu Province (BK20180819), and the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (18KJB170004).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_45754593

EFFECTS OF OCCUPATIONAL HEALTH AND SAFETY LAW ON FORESTRY EMPLOYEES

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(Received 24th Jan 2019; accepted 8th Mar 2019)

Abstract. Forest covering 28.6% of Turkey is an important area of employment. In this study, the awareness of the employees of Erzurum Forest Nursery Directorate has been attempted to be investigated by applying the Law No. 6331 on occupational health and safety. In the study, a questionnaire consisting of 36 independent questions, 8 independent and 28 dependent variables were prepared. The data obtained from the questionnaires was evaluated in the SPSS 20.0 program, and frequency distributions were determined. In addition, Chiropractic Analysis was performed to see if there is a statistical relation between dependent and independent variables in the questionnaires. According to the results; the ratio of General Occupational Health and Safety Education is 82.3%. However, the percantage of those using Personel Protective Equipment was lower 70.5%. Nearly all of those who stated that they had health problems in the institution were married women over the age of 40. If these groups are generally considered to be physically active and do not have job descriptions, the measures to be taken are: To ensure that dangerous situations affecting the health of employees are identified and removed from the scene by means of the risk assessment team established in accordance with Law No. 6331 on occupational health and safety.
Keywords: *forest workers, nursery workers, Law No. 6331, occupational health and safety, personal protective equipment*

Introduction

Providing legal protection to all employees to protect their rights that arise from work life is a requirement of the principle of social law state (Kılıç, 2006). Forestry employees working in forestry sector have been deprived of the legal protection that is normally provided by the Labor Law for many years.

Although partly, forestry employees have been included in the Turkish Labor Law for the first time with the Law 4773, which is also known as the Law on Job Security. However, since emerging new concepts that have appeared with the international commitments of Turkey and since the Law 1475 remained insufficient in covering today's needs in work life, the Law 4857, which was enacted in 2003, has taken its place as the basic law of labor legislation. The Law 4857 expanded the scope of Law 1475 by significantly changing it, and now it is a modern Labor Law with new principles and amendments it brought.

With some limitations, forestry employees were also included in Labor Law 4857 and in the regulations issued in accordance with this Law. Although there are limitations, it must be considered as an important step taken in this area for the legal protection of forestry employees (Engür, 2014).

In forestry sector, compared to other sectors, because of the fact that seasonal employment is excessive and employment status is temporary, many problems

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DOI: http://dx.doi.org/10.15666/aeer/1702_45954606

preventing the establishment and management of an insurance system have become inevitable; and the social security of the employees in this sector has not been established completely. This situation has been an obstacle for the necessary care to be taken for the implementation of relevant measures in terms of occupational accidents and diseases (Tunay and Emir, 2015).

The occupational health and safety issue constitute a very important aspect of today's business life and labor law. Occupational health and safety measures intend to ensure that occupational accidents and diseases do not occur or are reduced to an acceptable level.

The most important factor making businesses successful or unsuccessful is the human factor. Based on this understanding, an increasing interest has emerged in the psychological and physical conditions of employees and their behaviors related to their jobs. People spend most of their time at work; and the productivity increases as much as they can be happy at work. For this reason, the importance of the occupational health and safety concept is increasing with each passing day (Zorlu, 2008).

Parallel to the industrialization of countries, the problem of ensuring that employees work in health and safety has emerged as one of the most important problems that need to be solved. The concern for not making employees pay for the cost of industrialization and technological developments that are benefited by all people in the society constitutes one of the main aims of contemporary societies (Ekin, 2009).

The purposes of the studies conducted on occupational health and safety may be listed as follows;

- Protecting Employees.
- Contributing to Create Safe Working Environments.
- Ensuring the Productivity of Working is Achieved.
- Providing Opportunities for Employees to Sustain Their Work Lives (Bayılmış, 2013).

For this reason, investments made on the measures intended to ensure occupational health and safety will help to protect the health and safety of employees, avoid occupational risks, eliminate the risk and accident factors, and improve the health and safety conditions continuously at workplaces (Oğuz, 2010).

It is a fact that occupational health and safety measures can only become effective with joint understanding of employees, employers and the state. However, when forestry sector is compared with other sectors, it is clear that this triple mechanism has not gone beyond creating awareness. This situation stems from the different characteristics of forestry business in terms of work life, and prevents the implementation of the provisions of the Law on Occupational Health and Safety in this field (Tunay and Emir, 2015).

According to Poschen (1993), ILO defined forestry activities as "3-D" profession, in other words, a dirty, difficult, and dangerous work (Menemencioğlu, 2006). When this is considered, it becomes compulsory for forest employees to be careful, practical, intelligent, think in a sophisticated manner, be healthy, endure with effective body structure, have appropriate equipment at work, love the nature and walking, and be responsible people (Acar and Şentürk, 1997).

In this study, the purpose was to evaluate the effect of Occupational Health and Safety Law 6331 on forest employees who work under difficult conditions. For this reason, Erzurum Forest Tree-nursery Directorate was chosen as the Study Area; and the effects of the Occupational Health and Safety law on the awareness levels of the nursery employees were examined.

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DOI: http://dx.doi.org/10.15666/aeer/1702_45954606

Material and Method

The case study area

The material of the study consisted of 102 nursery personnel who worked at the 4 Forest Nurseries in Erzurum Forest Nursery Directorate, and Questionnaire forms that consisted of 36 questions. In addition, previous studies in the literature that were conducted in this field and the records of Erzurum forest nursery directorate were also made use of.

Erzurum Regional Directorate of Forestry of which the Erzurum Forestry Nursery Directorate is the sub-unit, and which is selected as the Study Area, covered the cities of Erzurum, Erzincan, Kars, Ardahan, Iğdır and Ağrı consisting of 10 forest management directorates of Erzurum, Oltu, Şenkaya, Erzincan, Refahiye, Kars, Sarıkamış, Ardahan, Iğdır and Ağrı. Nearly 2.6% of Turkey’s population lives in this region (TUİK, 2018). Acording to General Directorate of Forestry (GDF) records, the total area of the region is 8.849.193 ha, about 5% of which forested (GDF, 2018).

In the study area, the high amount of the area that is the subject to forestation is noteworthy. In the area, 5000 ha forestation and 8000 ha erosion control works are performed. These activities constitute the most important forestry activity of the area. To cover the need of seedlings that are used in the forestation and erosion control works in the area, there are 4 nursery engineering units (Sarıkamış, Ağrı, Erzincan and Erzurum), with their headquarters in Erzurum with a capacity of 20 million seedlings a year. A total of 102 employees work in these units.

Sampling method

The data obtained from the face-to-face questionnaires that were carried out with 102 employees in the nurseries, which were included in the study, were converted into a matrix in computer medium. This matrix was made by encoding the answers that were given to each question in numbers. The matrix that was generated from the encoding was analyzed in the SPSS 20.0 Program; and the frequency distributions were determined. As the data did not show normal distribution, the Chi Square (χ^2) Analysis was made to determine whether there were statistically significant relations between the dependent and independent variables in the questionnaires. The analyzes were carried out by employing the SPSS 20.0 Program.

Results

Erzurum Forestry Directorate has nursery engineering units in Erzurum, Erzincan, Erzincan, Ağrı and Sarıkamış. As it may be understood in the data given in *Table 1* below, the number of people who participated in the questionnaire was 102. It was understood that there were no employees in Ağrı Engineering Unit. It was also understood that many of the employees (66%) were employed in Erzurum Nursery Engineering Unit.

It was understood in the analyses of the questionnaires that a total of 9.8% of the nursery employees were female; and 90.2% were male. In addition, it was also determined that 6.9% of the employees were literate, 26.5% were primary school graduates, 45.1% of were secondary education graduates, 21.6% had undergraduate and post-graduate education levels. When the ages of the nursery employees were examined, it was determined that the highest rate was 39.2% in the 41-50 age group.

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Table 1. *Demographic situations of forest nursery workers*

		Forest Nursery Engineering				
		Erzurum	**Erzincan**	**Sarıkamış**	**Others**	**Total**
Gender	Female	2	7	-	1	**10**
	Male	65	12	4	11	**92**
Marital status	Single	8	4	2	2	**16**
	Married	59	15	2	10	**86**
Age group	15-18	1	-	-	-	**1**
	19-30	9	2	1	-	**12**
	31-40	7	4	2	5	**18**
	41-50	30	7	-	3	**40**
	50+	20	6	1	4	**31**
Education status	Literate	5	2	-	-	**7**
	Primary	16	11	-	-	**27**
	Secondary	38	3	-	1	**46**
	Undergraduate	7	3	4	-	**17**
	Postgraduate	1	-	-	2	**5**
Title	Manager	-	-	-	1	**1**
	Engineer	3	2	1	1	**9**
	Officer	8	1	-	-	**12**
	Worker	52	16	2	1	**74**
	Others	4		1	2	**6**
Work done *de facto*	Body worker	31	10	1	-	**44**
	Fertilization, spraying	1	-	-	-	**1**
	Driver	20	5	1	-	**27**
	Office worker	15	4	2	-	**30**

The fact that the rate of the participants saying "Yes" to the question "Is OHS training necessary?" at a rate of 95% clearly shows that these trainings are necessary for the employees (*Table 2*). All women believe that training is necessary. When the physical conditions of the workplace and the OHS culture of the employees were compared before and after the enacted law, it is possible to claim that the OHS culture improved at a significant level.

The nursery employees who thought that the trainings on the OHS Law 6331 was necessary believe that it would be adequate if these trainings were organized once a year at a rate of 20.6%; and 6.9% believed that there is no need for these trainings; 69.6% believed that these trainings should be organized every 6 months; 2.0% believed that these trainings should be organized every 3 years; and 1% believed that these trainings should be organized every 5 years. Such trainings should be organized in each month of the year; and the OHS issue should be kept active on the agenda of employees. It is considered that in this way, a contribution will be made to the development of OHS culture; and the demands of the employees will be covered.

Nursery employees were asked whether the OHS trainings they participated in covered the nursery issues or general OHS issues; and the employees stated that the issues were on nursery issues at a rate of 32.4%. In this context, 15.7% stated that the issues were on general forestry issues; 39.2% stated that the issues were on basic OHS issues; and 12.7% answered as "None" to this question. The distribution of the answers in this context shows that the employees have demands on the work they do, which is also a sign that they care about this issue.

After the Law No. 6331 on Occupational Health and Safety was enacted, and after the trainings, the employees were asked "Have you experienced any changes in your work life?". The answer to this question was "Yes" at a rate of 80%. A total of those

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DOI: http://dx.doi.org/10.15666/aeer/1702_45954606

who said "Yes" to this question said "Our working environment became safer" at a rate of 21.6%; 19.6% of the participants said "We were given more care by our employers"; 17.6% said "Our work started to be more regular and systematic"; and 17.6% said "We started to use personal protective equipment". The fact that there were very positive answers at a rate of 80%to the questionnaire shows that positive and valuable feedbacks are received about the law. Here, we see that male employees said "Yes" at a higher percentage than female employees.

Table 2. *Occupational health and safety knowledge level of forest nursery workers*

	Yes		**No**		**Total**	
	Female	**Male**	**Female**	**Male**	**Yes**	**No**
Do you have a written job description with clear and defined boundaries?	2	51	8	41	**53**	**49**
Is your opinion applied in the arrangements regarding the working place and working conditions?	2	52	8	40	**54**	**48**
Are adequate security measures taken?	5	76	5	14	**81**	**19**
Do you participate in studies outside the nursery?	2	10	8	82	**12**	**90**
Do you have a transport limit during operation?	1	40	9	52	**41**	**61**
Do you take personal protective measures when working with substances that threaten human health?	2	12	8	80	**14**	**88**
Do you think that the way you transport and store the chemical substances you use is done in accordance with the occupational safety rules?	1	24	9	68	**25**	**77**
Have the necessary training been given to transport, storage and use of chemicals?	2	28	8	64	**30**	**72**
Do you have any health problems that you think arises from nursery work?	8	13	2	79	**21**	**81**
Do you have any currently diagnosed chronic diseases?	7	9	3	77	**16**	**86**
Are personal protective equipment suitable for your work?	4	56	6	36	**60**	**42**
Do you use the personal protective equipment that you use to maintain and maintain the work properly?	2	80	8	12	**82**	**20**
Did you experience any occupational accidents or diseases after you entered work?	2	23	8	69	**25**	**77**
Have you received basic training in Occupational Health and Safety?	10	8	-	84	**18**	**84**
Do you think OHS trainings are necessary?	10	85	-	7	**95**	**7**
Have you been informed about how to safely and correctly use equipment equipment in the nursery in OHS trainings?	10	77	-	15	**87**	**15**
Is training on effective use of personal protective equipment provided?	10	62	-	30	**72**	**30**
After the entry into force of the Occupational Health and Safety Law No. 6331, and after the trainings, have you experienced any changes in your working life?	6	74	4	18	**80**	**22**
Do you think there is a decrease in the number of occupational accidents and occupational diseases in the nursery after the entry into force of the Occupational Health and Safety Law No. 6331?	10	80	-	12	**90**	**12**

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The relation between gender, workplace and working conditions

In the answers that were given to the question “Is your opinion asked about the regulations about your work place and working conditions?”, it was examined whether there were statistically significant differences between the genders (female/male); and it was determined that there were significant differences (*Table 3*). According to this result, there is a statistically significant relation between working conditions and gender variable.

Table 3. *Relationship between gender and workplace and working conditions*

Workplace and Conditions	Gender			
	Female		Male	
	Number of people	%	Number of people	%
Yes	2	20.0	52	56.5
No	8	80.0	40	43.5
Total	10	100.0	92	100.0

Chi Square =13.812; sd 2; 0.01<0.05

The relation between the work done de facto and security measures

The employees were asked “Are adequate security measures taken at the unit where you work?”. It was examined whether there were statistically significant differences between the work done *de facto* (body employees, fertilization, drug-spraying employees, packaging and sales employee, drivers and office employees) in the answers given to this question, and significant differences were detected as a result of the analyses (*Table 4*). A statistically significant relation was detected between the dependent variable “Are adequate safety measures taken at the unit where you work?” and the independent variable “the work done *de facto*”.

Table 4. *The relation between work done de facto and security measures*

Security Measures	Work Done *De Facto*							
	Body worker		Fertilization, spraying		Packaging sales employee		Driver and office worker	
	Number of people	%	Number of people	%	Number of people	%	Number of people	%
Yes	30	68.2	0	0.0	23	85.2	28	93.3
No	14	31.8	1	100.0	4	14.8	2	6.7
Total	**44**	**100.0**	**1**	**100.0**	**27**	**100.0**	**30**	**100.0**

Chi Square =13.896; sd 6; 0.031<0.05

The relation between the work done de facto and health problems

The employees were asked “Do you have any health problems that you think stem from the nursery work?”. It was examined whether there were statistically significant differences between the work done *de facto* (body employees, fertilization, drug-spraying employees, packaging and sales employee, drivers and office employees) in the answers given to this question, and significant differences were detected as a result of the analyses (*Table 5*). In the answers given by the employees to the question, “Do you have any health problems that you think stemming from the nursery work?” which

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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was under the title of "the health conditions of the employees", a statistically significant relation was detected between this dependent variable and the independent variable (the work done *de facto*).

Table 5. *The relation between work done de facto and health problems*

Health Problems	Work Done *De Facto*							
	Body worker		Fertilization, spraying		Driver		Office worker	
	Number of people	%	Number of people	%	Number of people	%	Number of people	%
Yes	13	29.5	0	0.0	7	25.9	1	3.3
No	31	70.5	1	100.0	20	74.1	29	96.7
Total	**44**	**100.0**	**1**	**100.0**	**27**	**100.0**	**30**	**100.0**

Chi Square =16.849; sd 6; 0.01<0.05

The relation between the educational status and OHS basic trainings

It was examined in the answers given to the question whether there was a statistically significant difference between the educational status (literate, elementary, secondary, faculty and postgraduate or doctorate level) and it was found that there was a significant difference in the answers given to the question "Have you received basic training on occupational health and safety?" (*Table 6*). A statistically significant relation was detected between the dependent variable "Have you received OHS Basic Training?", which is under the title "Evaluation of Existing OHS Trainings", and the independent variable "educational status", which is under the "general information about employees" heading.

Table 6. *The relationship between educational status and OHS basic trainings*

OHS Basic Training	Educational Status									
	Literate		Primary		Secondary		Under graduate		Post graduate	
	Number of people	%	Number of people	%	Number of people	%	Number of people	%	Number of people	%
Yes	3	42.9	25	92.6	37	60.9	14	82.4	5	100
No	4	57.1	2	7.4	9	19.6	3	17.6	0	0,0
Total	**7**	**100**	**27**	**100**	**46**	**100**	**17**	**100**	**5**	**100**

Chi Square=16.386; sd 8; 0.037<0.05

Discussion

According to the study results, it was observed that the Nursery Directorate did not employ women over 40 years of age who had relatively low educational levels at jobs that required muscle strength; and instead, women were generally employed in more physical works like removing weeds or hoeing preferably in the nursery field, which do not relatively require training and qualifications to increase their productivity. In addition, the fact that there were no job definitions for women is considered as a condition that needs correction by the nursery directorate.

It was observed that single employees who were under the age of 40 and who had low education levels were preferably employed in physical works outside the nursery. In addition, it was also determined as a negative situation in terms of work efficiency and safety that married women who were older than 40 years of age and who had low

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educational levels thought that they could not rest with regular intervals. Here, it may be understood that the provisions stated in the labor legislation and the current Collective Labor Agreement (CLA) are not covered in the workplace. When the working years of these groups were examined, it was observed that many of them had been doing the same work at the same workplace for more than 15 years.

It was also observed that since the drivers and office employees worked in closer positions to the management staff, their job descriptions were made in general. It is considered that since the drivers and office employees share the same medium with the administrators, and as they are close and necessary for the job they do, they read and understand the business legislation and CLA, their job descriptions are made, and they are given more place in the management and in the organization. It is also considered that they are more satisfied with their jobs because of their proximity to decision-makers and due to the more comfortable working environment compared with other groups. Unfortunately, this comfort is relatively seen in the work environment where employees work with their physical strengths. This situation is the opposite in employees who work in the field, and creates dissatisfaction. To eliminate this, more trainings, seminars and information meetings must be held. In addition, the work done by employees must be defined, in other words, the work must not be in an open-ended structure.

When the health status of the employees working at the nursery was examined, it was considered that women face more health problems compared to men, which is considered to be due to the fact that they work in more bodily works. It is necessary that women employees are made to use the personal protective equipment that is provided to them and their working conditions are improved.

When the surgery status of the employees was considered, it was seen that the employees who had higher education levels had more surgeries. It is considered that employees who had high education levels took both reactive and proactive measures and treatments, and those who had low education levels might not have reached the adequate level in terms of awareness in this respect.

It was noteworthy that female employees who were above the age of 40 stated that they were dissatisfied with the suitability of their KKDs to the work, and that they had very low occupational accident rates; however, they had health problems. Although their work involves bodily work, it is possible that they have occupational diseases rather than occupational accidents. They might especially have diseases like muscle and joint disorders. In addition to this, the employees who had primary and secondary education levels also considered that the KKD that was provided to them was inappropriate. It is considered that this group has a relatively higher rates in terms of work accidents. The high risk of occupational accidents in this group might be associated with the fact that they do work that require physical strength. The groups that have high work accident rates may be listed as follows; the male employees who had been working at Erzurum and Sarıkamış nurseries for more than 15 years. Many of these employees work with physical strength.

According to the mandatory provision of the OHS Law 6331, it is compulsory to provide employees with basic OHS trainings in accordance with the danger class of their works; however, the rate of those who declared that they were not provided with any trainings in the institution was 17.6%. It was determined in the files that the training programs were organized previously. It was seen that those who were married and who

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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were over 40 years of age complained more about the inadequacy of training on tools, equipment and correct use of KKD.

The entire master and doctorate education groups and most of the faculty group said "Yes" to the question on whether the basic trainings of OHS were received. The highest rate of those who said "No" is secondary education and literate group. The rate of saying "No" in secondary education is 50%. It was seen in the distribution of the training subjects that the employees made evaluations on the general OHS and the work they did. This demand complies with the legislation. In the answers that were given to the question about the correct use of tools, equipment and KKD, it was seen that as the education level increased, the satisfaction also increased. In general, the employees who were elderly, women, married and had low education levels were dissatisfied with this issue.

The office employees and drivers of Erzurum Forest Nursery Directorate face dangerous situations less in terms of working environments than physical workers. Here, the group that must be examined is the body workers. The dangerous situations body workers face in the work environment are more than the other groups. For example, the risk levels of a person who works in pruning and the risk level of a person who works at a desk in an office are different. For this reason, the fact that a significant relation was detected between the work done *de facto* and the safety measures in the Chi-Square Test, which shows that this issue should be paid attention. The answer that was given by the employees to the question "Are adequate security measures taken in the unit where you work?" comes to the forefront in this respect. The rate of the employees who did body works and who said "No" was 66.7% among the employees that said "No". This rate is very high compared to the 20.6% rate in the overall average.

The relation between the work done and health problems was examined, and a statistically significant relation was detected. In this respect, 79.4% of the employees said "No" to the question "Do you have any health problems that you think stem from nursery work?"; and 20.6% said "Yes". Here, 20.6% of 84.6% who said "Yes" were physically employed. The high rates of the employees who did body works and who said "Yes" to the question "Do you have any health problems that you think stem from nursery work?" means that this is a negative situation that is worth to investigate. This is a very important finding for determining and following-up the occupational diseases at workplaces by doctors who work in these workplaces.

The relation between educational status and OHS basic education was examined; and a statistically significant relation was determined. In this respect, it was determined that 82.4% of the participants said "Yes" to the question "Have you received basic education on occupational health and safety?", and 17.6 said "No". Here, the entire postgraduate and doctorate groups and most of the faculty groups said "Yes". The highest rate in the respondents is in the secondary education and literate group. The rate of those who said "No" at secondary education level was 50%. It is considered that the reason may be that the employees who were from secondary education level and who were literate were preferred in jobs other than nursery; and that there were employees who were off duty on the given date caused this situation. Organizing the training programs in a planned and announced manner can reduce this rate. One of the main mandatory provisions of the OHS Law 6331 is to provide OHS basic training in hours and periods that are proper for the danger class of all employees without considering the danger class.

As a result of the present study, it was determined that approximately half of the employees did not have job descriptions. This situation poses a contrary situation to the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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labor legislation and CLS in the workplace. It is a legal liability that administrations make the job descriptions of all employees as soon as possible, and make a notification to relevant employees concerned.

It was observed that those who had worked for more than 15 years had more health problems compared to those who had worked less. It is considered that this is because of the fact that employees face more health problems with their furthering age. It is stated in the relevant article of the CLS that is in force that those who work bodily for more than 15 years must be employed in lighter jobs. In case this is applied, it is considered that such employees will be less affected by health problems.

It may be seen more probable that nursery employees experience occupational diseases rather than occupational accidents. They may especially face disorders like muscular joint disorders. It is possible to reduce these diseases to acceptable levels with relevant preventive measures. The measures that will be taken must be determined by a risk assessment report that will be prepared by a commission that will be formed by employers or their representatives. For example, ergonomics trainings may be provided to employees for the solution of this issue. Mechanical and automatic systems may be established to improve thermal comfort conditions. Suitable personal protective equipment may be provided to employees in this context. Such measures may be diversified and multiplied by the abovementioned commission. In addition, it is considered that the KKDs of the employees at the primary and secondary level are unsuitable; and that the rate of occupational accidents will be higher for this group. This situation may be associated with the fact that such employees work in jobs that require body strength, which constitutes the risk of occupational accidents. Again, it may be possible to decrease the risk to an acceptable level with preventive measures in this respect. Dangerous situations faced by employees included in this category must be eliminated with priority. If such situations cannot be eliminated, the source of the danger must be eliminated. Here, engineering and managerial methods must be used. And finally, employees must be made to use appropriate KKD.

In the forestry sector, it is extremely important that legal arrangements are made to ensure that occupational health and safety services are performed in a conscious manner, and that relevant regulations that will cover all the regulations and working conditions are issued and implemented (Tunay and Emir, 2015).

It is considered that the fact that the rate of those who considered that the OHS trainings had positive contributions in the work life and that the rate of those who thought that work accidents and occupational diseases decreased after the implementation of Law 6331 was determined to be 88.2% shows that the implementations of the law was perceived well by employees and had a good impact on their conscious levels.

Conclusion

The aim of the present study was to evaluate the effects of Occupational Health and Safety Law (OHS) on forest nursery workers in Erzurum Forest Nursery Directorate. OHS is aimed to permanently improve the health and safety of workers. This matter is supported by the state with national strategy and policies.

Participation of workers to OHS administration (OHSA) was included in many OHSA standards and guidance. OHSA models should emphasize the active participation of workers as a critical factor to improve OHS performance (Walter and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Frick, 2000). Empowering of workers and giving them feeling of autonomy and control are the important factors in terms of OHSA success (Hale and Hovden, 1998). Nevertheless, properly training and informing of the workers, and giving them a chance to investigate the problems and communicate with each other’s to provide active participation is necessary.

The study showed that the workers in forest nursery directorate had enough knowledge about OHS and OHS applications were properly conducted based on laws. The use of personal protective equipment and periodical training of the workers are also in important matters regarding OHS. Raising the awareness of workers with regard to OHS culture, precautions improving their safety and health, and encouraging workers to communicate with administrators regarding OHS problems and risks which they observe and encounter are suggested. All demands from workers should be evaluated and taken due precautions.

As a result, it should be concentrated on training and educations regarding OHS to get better results and provide the workers a better environment not adversely affect their physical and mental health.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_45954606

STIMULATION OF BARLEY (*HORDEUM VULGARE* L.) GROWTH WITH LOCAL *TRICHODERMA* SP. ISOLATES

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(Received 24th Jan 2019; accepted 27th Feb 2019)

Abstract. *Trichoderma* species are used as biological control agents against plant pathogenic fungi. In addition, some isolates of *Trichoderm*a species produce metabolites that promote plant growth. In this study, it was aimed to determine the effect of local isolates of *Trichoderma* sp. and a commercial microbial fertilizer containing *Trichoderma harzianum* on the growth of barley. The experiment was carried out according to the randomized plots experimental design. Barley seeds were inoculated with spore solutions of local isolates and then compared with controls (without inoculation and commercial fertilizer). Plant height, root length, root dry weight, shoot dry weight and chlorophyll content were determined. The effects of isolates were different. In this study, plant height was increased with the application of local isolates. The difference between isolates was statistically significant. Isolates were determined to have positive effects on barley growth.
Keywords: *Trichoderma sp., plant growth promoting, barley, biostimulant, photosynthetic pigment*

Introduction

One of the alternatives used in fertilization programs in environmentally friendly production techniques is microbial fertilization. Microbial fertilizers are defined as commercial formulations of live microorganisms which are involved in the removal of plant nutrients necessary for the plant used in agricultural production (Akladious and Abbas, 2014). Microbial fertilizers are used in agriculture for many purposes, such as increasing plant growth, yield, and nutrient intake, controlling soil-borne diseases, decomposing organic residues, improving soil structure and productivity and providing resistance to diseases and pests (de Santiago et al., 2011; Ousley et al., 1993). This strengthening effect in plants can also lead to a decrease in the use of pesticides and fertilizers. In many developed and developing countries, intensive studies have been carried out to obtain biological or microbial fertilizer formulations considering clean environment and healthy crop production (Kucharski et al., 1996; Vessey, 2003). Microorganisms such as *Bacillus* spp., *Azotobacter* spp., *Trichoderma* spp., *Rhizobium* spp., *Azospirillum* spp. and *Saccharomyces* spp. are used as microbial fertilizers (Sharma et al., 2003; Vazquez et al., 2000).

Trichoderma is a soil fungus found in many parts of the world (Hoyos-Carvajal et al., 2009). *Trichoderma* spp. are the most studied microorganisms which are used as microbial fertilizers and biological control agents against fungal pathogens (Hoyos-Carvajal et al., 2009).

In addition to its ability to promote plant growth, *Trichoderma* spp. isolates have been used for many years in the biological control of many fungal induced plant diseases (Woo et al., 2006). It is known that *Trichoderma* spp., which is colonized in the root, stimulates

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46074614

resistance to plant diseases as well as it promotes shoot and root growth, increases yield, resistance to abiotic stress conditions, promotes nutrient intake and use and increases photosynthesis (Inbar et al., 1994; Yedidia et al., 2001; Harman et al., 2004; Harman, 2006).

Datnoff and Pernezny (2001) reported that *Trichoderma* spp. preparates were increased the growth of tomato seedlings in greenhouse and field conditions. In recent years the use of microorganisms that promote plant growth has increased. Therefore, studies are carried out to reduce environmental pollution by reducing the use of chemical fertilizers (Kucharski et al., 1996; Hermosa et al., 2012). Some *Trichoderma* isolates stimulated the plant against abiotic and biotic factors, increased the germination rate and percentage of the seeds, the fertility of the fertilizers, the nutrient intake from the soil and directly affected the plants (Shoresh ve ark., 2010).

de Santiago et al. (2011) and Zhang et al. (2013) were reported that the application of *Trichoderma* species in various plants were increased the root growth of the plants, yield, leaf area, the weight of fresh seedlings. Dissolution of insoluble minerals for plant growth (Altomare, 1999; Küçük et al., 2008; Rawat et al., 2011), indole acetic acid, siderophore, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production (Viterbo et al., 2010) as effective factors in plant growth were studied.

It is known that nitrogenous fertilizers accumulate in agriculture crops which increase nitrate ratio in drinking and irrigation waters and adversely affect human and animal health (Bashan, 1998; Rennie and Heffer, 2003). The use of microorganisms in agriculture has been gaining importance in recent years in order to eliminate these problems and to reduce the use of chemical fertilizers. Barley has an important in the agriculture of Turkey and has an important role in the reduction of fallow land (Karakaya et al., 2016).

The cultivation and production of barley is done in all regions of Turkey. While most of the barley produced in Turkey is used in feed industry, some are used directly in animal feed and some in beer industry. Since barley is mostly used as animal feed, the seeds are inoculated with microorganisms in order to increase the protein content in barley (Karakaya et al., 2016). In this study was carried out in greenhouse conditions to investigate the effects of three different *Trichoderma* sp. isolates and commercial microbial fertilizer containing *T. harzianum* applied to seed on the growth of barley plant.

Materials and methods

The local isolates (T4, T7 and T11 isolates) of *Trichoderma* sp., were obtained from the Microbiology laboratory of Harran University. The isolates (*Fig. 1*) were stored in Potato Dextrose Agar (PDA) media and stocked at +4 °C until use. *Trichoderma harzianum* Rifaii KRL-AG2 was used as a commercial microbial fertilizer. Commercial microbial fertilizer was received from Biyoglobal A. Ş., Turkey.

The effect of isolates on plant growth

Barley (*Hordeum vulgare* L.) seeds were immersed in 70% ethanol for 3 minutes, 2% NaOCl for 2 minutes and washed several times with sterile distilled water. *Trichoderma* sp. isolates were incubated in PDA containing petri dishes for 7 days at 25 °C. Spore solutions of isolates (10^6 cfu/ml) were then prepared. Sterilized seeds were incubated for 3 hours in spore solution and sown in sterile soil. Soils were taken from Osmanbey Campus Harran University, Turkey where no adaptation was made before. The soil

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46074614

samples were sterilized in autoclave for consecutive three days. Steriled soils were filled in 2 kg pots. The diamonium phosphate fertilizer (10 kg/da) was given to the soil. Plants were grown in natural light greenhouse for 1 month. Akhisar varieties were used as barley varieties. The seeds were obtained from GAP Research Institute.

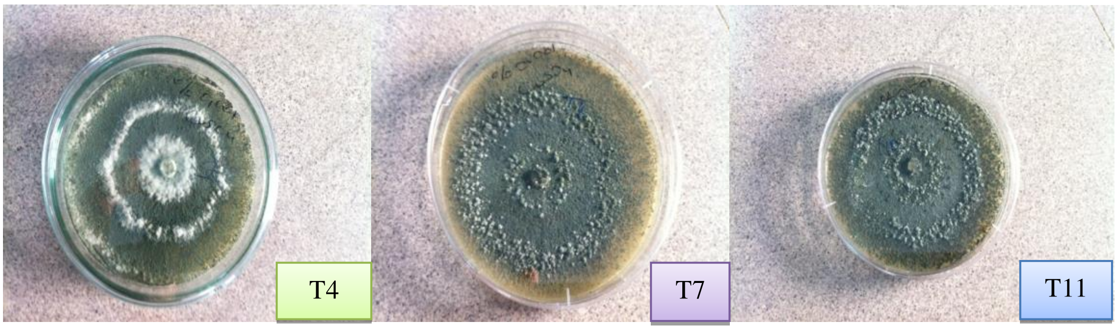

Figure 1. *Trichoderma sp. isolates used in the study*

In each pot, plant height, shoot dry weight, root dry weight and root lenght were determined. The photosynthetic pigments extracted from the leaves (chlorophyll a, b, carotenoid) were made according to Akladious and Abbas (2014).

The soil pH was determined by pH meter in a 1: 2.5 soil mixture. The organic matter content of the soil was made according to the method determined by Walkley (1964). The distribution of soil particles was determined by the Bouyoucours hydrometer (Bouyoucos, 1951) and the total nitrogen content was determined by the Kjeldahl method (Bremner, 1982).

Indole acetic acid (IAA) production of isolates

Spore solutions of *Trichoderma* isolates (10^6 conidia / ml) were prepared. Spore solutions (0.1 ml) were inoculated onto the agar surface and coated with a nitrocellulose membrane, petri dishes were incubated at 25 °C for 7 days. Then, the membrane disc was removed, the filter paper (Whatman no 2) saturated with the salkowski reagent was placed. After 5-10 minutes, IAA production by isolates was determined by color change (Hoyos-Carvajal et al., 2009).

Solubility of phosphorus

The isolates were inoculated into the medium containing 0.1 g/l bromocresol purple using tribasic calcium phosphate as the phosphorus source. 48 hours after incubation, the color of the medium from purple to yellow showed that the isolates can dissolve the phosphate (Vazquez et al., 2000).

Statistical analysis

The data obtained from the study were analyzed in JMP 11 statistics program.

Results and discussion

In this study, the effect of three isolates of *Trichoderma* sp. on the growth of barley and some plant growth-promoting metabolites were investigated. Phosphate solubility

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and indole acetic acid production as a metabolite were evaluated. Phosphorus is a very hard to reach nutrient. Its low amount adversely affects plant growth (Raghothama, 1999). All of our isolates were able to dissolve phosphorus.

Siderophores are iron chelating ligands which may be useful to plants by increasing the solubility of ferric iron (Fe III) not present for plant nutrition. This element is assimilated by stem cells in reduced form (Fe II). Fe III is dominant especially in the aerated soils. *Trichoderma* may reduce Fe (III) by chelators such as siderophores (Altamore et al., 1999; Jalal et al., 1987; Zhao and Zhang, 2015). Jalal et al. (1987) reported that *T. virens* produced hydroxamate siderophore as a Fe III chelating mechanism. According to these researchers, *Trichoderma* sp. transformation into solvable forms by chelation and reduction. Both mechanisms play a role in the stimulation of growth and biocontrols of plant pathogens (Qi et al., 2012; Vessey, 2003; Woo et al., 2006). Siderophores can support rhizosphere colonization and plant growth in synergy with other substances and are the effective mechanism of some microorganisms to increase plant growth (Sharma et al., 2003).

Production of plant growth hormones is another mechanism that increases the growth of plants. The tested isolates produced auxin hormone, which increased plant growth. It has been reported that *Trichoderma* isolates synthesize auxin hormones in their interaction with both plant pathogens and plants (Hermosa et al., 2012; Shayakhmetov, 2001). In this study, it was determined that isolates produced indole acetic acid.

In pot experiment, the pH of the soil used is 7.8. The clay ratio is 53.12%, silt ratio is 24% and sand ratio is 22.8%. The organic matter content of the soil is 1.71%, total nitrogen is 0.18% and K_2O content is 97.2 kg/da. The effects of *Trichoderma* applications on barley plants are given in *Table 1*. When *Table 1* is examined, the effects of *Trichoderma* applications differed. It was determined that the applications increased the plant height statistically ($p < 0.05$).

Table 1. *Effects on barley shoot and root dry weight, root lenght, plant height of treatments*

Treatment	Plant height (cm)	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Root lenght (cm)
T4	18.3a	14.6b	2.66a	15.5a
T7	16.7ab	13.9bc	2.78a	14ab
T11	16.3ab	16.4a	2.05ab	13.1b
Commercial fertilizer	16.2ab	12.5c	2.69a	10.8c
control	14.7b	10.6d	1.33b	10.3c

Trichoderma sp. T4 application was the longest plant height (18.3 cm) cm, followed by T7 and T11 isolates respectively (*Fig. 2*). The shortest plant height was obtained from untreated control application (14.7 cm). The effect of *Trichoderma* applications on root length in barley plants was statistically significant ($p < 0.01$). The highest root length was taken from T4 application. The effects of isolates on barley shoot and root dry weight were different. The highest shoot dry weight was obtained from T11 application. All of the applications increased root weight relative to control (*Table 1*). *Trichoderma* isolates were found to be effective on chlorophyll content in barley plants ($p < 0.01$) (*Table 2*). A significant increase in plant growth of cucumber plants inoculated with *Trichoderma harzianum* was determined (Yedidia et al., 2001).

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DOI: http://dx.doi.org/10.15666/aeer/1702_46074614

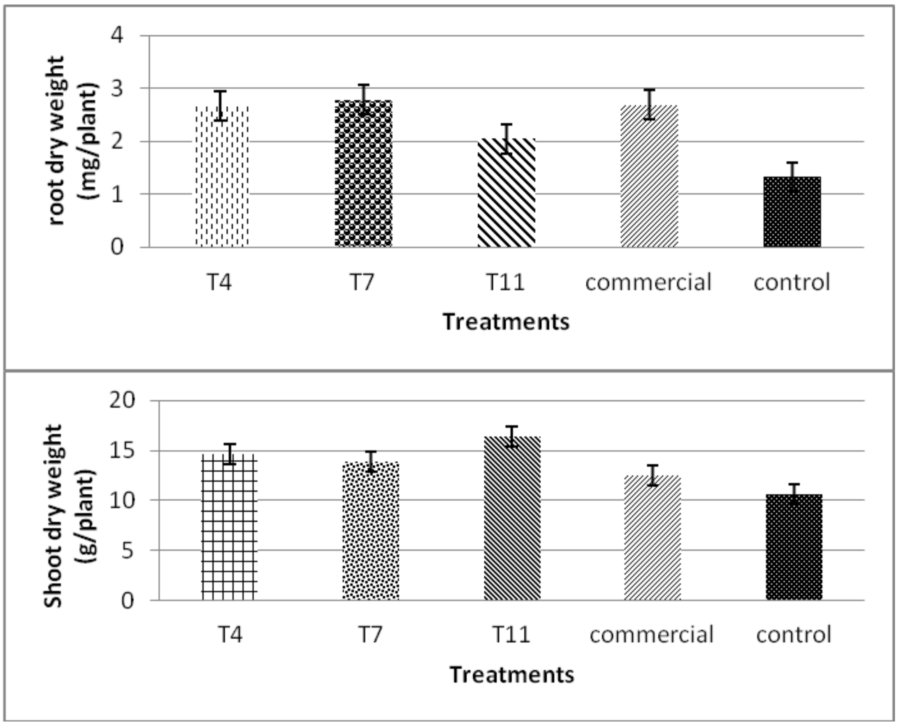

Figure 2. *Effects of Trichoderma applications on root and shoot dry weights of barley*

Table 2. *Effects of Trichoderma applications on photosynthetic pigments of barley plants*

Treatment	(A663 and A645) chlorophyll-a (mg/ fresh wt.)	(A645 and A663) chlorophyll-b (mg/ fresh wt.)	(A645 and A663) total chlorophyll (mg/ fresh wt.)	(A480 and A510) carotenoid (mg/ fresh wt.)
T4	1.554b	2.705c	2.705c	1.214b
T7	1.580a	2.946a	2.946a	1.201c
T11	1.530d	2.669d	2.669d	1.192d
Commercial fertilizer	1.541c	2.842b	2.842b	0.857e
control	1.227e	2.241e	2.241e	1.250a

A: absorbance

Compared to control, *Trichoderma* sp. isolates increased plant height by 24.4% (T4), 13.6% (T7) 10.8% (T11 and commercial preparation). These results are consistent with the studies of the researchers (Hoyos-Carvajal et al., 2009; Kaveh et al., 2011; Ousley et al., 1993). In plants inoculated with *Trichoderma* sp., the amount of root were increased (Ousley et al., 1993). Therefore plants receive more water and nutrients. In this study the applications with *Trichoderma* sp. were increased the root length of plant between 5.8 and 50.4%. Datnofff and Pernezy (2001) explained that *Trichoderma* sp. increased the growth of tomato seedlings. In this study the the applications with *Trichoderma* sp. isolates were increased the root weight increased between 52 and 109% (*Table 1*). The contents of photosynthetic pigment were increased in plants treated with *Trichoderma* sp. isolates (*Table 2*). The results are similar to those of the investigators (Ousley et al., 1993).

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DOI: http://dx.doi.org/10.15666/aeer/1702_46074614

Trichoderma isolates transformed minerals such as phosphorus, manganese, and copper iron into the soluble form (Altomare et al., 1999). Thus, roots can easily get the nutrients they need from the soil and the plant growth rate has increased (Küçük et al., 2008; Altomare et al., 1999). Yedidia et al. (2001) reported that *T. harizanum* applied cucumber; increased the 90% of phosphorus intake and 30% increase in iron intake iron intake.

Conclusion

In this study, the effect of *Trichoderma* sp. isolates and commercially available *Trichoderma harzianum* on barley growth was evaluated. As a result; the establishment of the *Trichoderma*-plant interaction can support the biological control activities by revealing the defense mechanism of the host plant. *Trichoderma* sp. isolates may contribute to increasing plant growth by affecting the microbial population in the rhizosphere. In this study, it was determined that *Trichoderma* sp. isolates could be used in the growth of barley plants. The effects of the isolates tested on plant growth has been different. It was observed that the isolates had a positive effect on the plant growth characteristics of the barley plant. *Trichoderma* isolates have shown that they play a role as plant growth promoters. In future studies, it is necessary to determine the effects of *Trichoderma* sp. isolates on plant growth in stress conditions. Thus, the isolate or isolates of *Trichoderma* sp. that are effective can be an alternative to chemical fertilizers.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46074614

SIMULATION BASED RANKING OF VEGETABLE CASH CROPS FOR SUSTAINABLE GREENHOUSE FARMING PRACTICES

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(Received 23rd Jan 2019; accepted 27th Feb 2019)

Abstract. Choosing vegetable crops in greenhouse farming plays a significant role in the feasibility of sustainable farming practices. The selected crop affects the economic and environmental conditions which in turn influence the sustainability of farming practices. Therefore, a systematic approach is needed to prioritize the most suitable vegetable crop for sustainable farming practices. The current research focuses on prioritizing vegetable crops grown in green house farming in the environment of the Kingdom of Saudi Arabia (KSA), which takes into account comprehensive criteria related to sustainable farming practices. Five major criteria including crop field area, crop field production, cost of production, price of sale and the usage of water have been selected for the five major vegetable crops grown in the green chamber farming of KSA to determine the most sustainable one. The multiple attribute-based decision making (MADM) model has been developed taking into account the KSA Green House Chamber farming system. The research work reported in this study provides useful information for greenhouse farming in Saudi Arabia in particular and also helps policymakers to formulate effective regional policies to improve the gross domestic product and make farmers self-reliant.
Keywords: *Crop Prioritization; Multiple Attribute Decision Making (MADM); Sustainable Farming Practices; Fuzzy Delphi Method (FDM); Nonlinear Programming (NLP)*

Introduction

In the greenhouse farming system vegetable cash crop prioritization is significant for sustainable farming practices which help the economic development of the nation and promote self-reliance (Nambiar et al, 2001). Due to technological advancement in the farming sector in terms of farm instruments, availability of seeds, manure and irrigation facilities there is considerable increase in farm yields, but as a result of the high rate of population growth the achieved benefit is not sufficient (Pramanik, 2016). There is a misbalance between supply and demand due to the exponential growth of the local and global population. Sustainable greenhouse farming can provide a better solution to bridge the gap between supply and demand (Roy and Chan, 2012). Sustainable greenhouse farming combines the environmental, economic and social factors to achieve the growth and upliftment of humanity, its surrounding life and the environment (Cocklin, 1995).

Greenhouse sustainable farming practices include environmental, economic and social aspects, which are linked to farmers with improved equity, self-sufficiency, reduced risk and good quality of life (Peacock and Sherman, 2010). The economic factors include the crop yield, the system profitability, the economic gain of the farmers, the security of the food for the society. The environmental conditions in semi-arid and arid climatic zones are not favorable for farming because of harsh and extreme temperature. Many agricultural programs were started at different times to ensure food security and rural development (Bailey and Willoughby, 2013). The environmental factors include the nutrient content of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46154629

agricultural soil, the market value of the yield and its quality, the quality of the irrigation water as per the FAO and the carbon credit of the farm and greenhouse gases contribution. The main limiting factor in greenhouse farming is soil and water therefore it is necessary to promote the innovative technologies at the same time spreading the awareness to greenhouse farming community, among the innovative technologies promotion of vegetable cash crops, hydroponics, seawater harvesting also promotion of bio-salinity research and rainwater harvesting.

The selection of a particular set of crop patterns depends on many criteria that can vary from place to place, so that it is difficult to decide on an optimal crop pattern (Qureshi et al., 2018). This may be treated as a system where we have the system input then change within the system and system output the farming vegetable cash crop system requires all the essential parts to produce certain cash crop with relation to surrounding environmental conditions. In the greenhouse farming system largely depends on the tools (Management Policies, Optimum use of Manpower, Mechanizations, Farming Materials, Farming Methods, Economy, Production and Sale).

In the greenhouse farming system management aspects is the important component of the farm if we emphasis this based on system engineering then the components are policies related to farm management, utilization of optimum manpower, the mechanization of the system, the materials of the farming, the farming techniques, the economic aspects of the farm and yield with market value. The second aspect is utilization of sustainable greenhouse farming activities such as economic use of natural resources such as irrigation water, natural soil, wind, sun light and manure, use of natural pesticides (Garg and Dadhich, 2014). The last component is production of cash crops for the benefit of the farmers with focus on environment, economic and social. For achieving the sustainable farming the system input, the system output and things happening within the system should all should be integrated. The employed manpower should be aware with the sustainable farming activities. Mechanized greenhouse farms and equipment viz. plougher, tractors, crusher are used at various stages of the farming activity starting from the preparation of land up to cultivation in the greenhouse the care is also taken to maintain the internal environment for the maximum possible yields it also include the process of plowing, seedling, manure, water and pests applications etc. In Saudi Arabia, water as an important natural resource specially used in sophisticated irrigation system such as drip irrigation and sprinkler systems. Availability of sufficient funds to run the activities and marketing strategies to get the better return are the important aspects of management practices. The prioritization of vegetable cash crops in greenhouse farm can be considered as a strategic decision which may benefit to different stake holders. The climatic factors, economic factors, sustainability, the vegetable crop selection practice in greenhouse are considered to be an important decision which helps the decision makers. Therefore, the crops selection practice is vital for sustainability and better socio-economic conditions.

Review of literature

The concept of farming sustainability criteria was inducted by Gowda and Jayaramaiah (1998) also extended by Qureshi and Singh (2017) using system approach and management tools. The concept of index was introduced to get the sustainability indicators for the selected criteria and prevailing climatic conditions by Dillon et al. (2009). In order to measure the farm sustainability for the temporal variation (Nambiar et

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DOI: http://dx.doi.org/10.15666/aeer/1702_46154629

al, 2001). The concept of farming sustainability assessment was performed by Zhen et al. (2005). Also a crucial study on the field level management of soil nutrient was carried out by Zhen et al. (2006). The environmental and socio-economic factors were considered and the analysis was carried out by Sydorovych and Wossink (2008; 2009) classified sustainability indicators based on the comprehensive data. A study carried out in the Bangladesh related to farming sustainability indicators by Roy and Chan (2012). A study was carried out for the crop selection process using the agricultural landscape by Sorensen et al. (2015).

The different criteria which may be non-dimensional and dimensional in nature can be efficiently handled in the decision making process using the multiple attribute decision models (MADM) this modeling method is used in the prioritizing the alternate solutions even in the case of complicated criteria involved (Rao and Patel, 2010; Venkata Roa, 2008; Geng and Wardlaw, 2013). The diffusion and flow of trace metal in river Ganga a study using MCDM was reported by Srinivas et al. (2017). In the two districts in the Uttar Pradesh state of India viz. Raebareli and Unnao in India, groundwater quality assessment was carried out using GIS and MCDM by Agarwal and Garg (2016) at the same time an spatial vulnerability of flood mapping keeping in view the effect of climate change using MCDM was reported by Song and Chung (2016).

The MADM methods were applied in the various fields of Environmental Engineering and Water Resources Management such as agricultural irrigation water management and water tariff, flood related problems and decision making, at the basin level integrated water resources management, management of city water supply scheme, planning of water quality and quantity at the sustainable level, reduction of water loss due to major and minor reasons in water supply lines, ground water and surface water contamination due to anthropogenic activities and control using modeling techniques by various researchers (Raju et al., 2000; Chitsaz and Banihabib, 2015; Raju and Vasan; Azarnivand et al., 2015; Geng and Wardlaw, 2013; Mutikanga et al., 2011; Carroll et al., 2013; Roozbahani et al., 2012; Behzadian et al., 2010; Latinopoulos, 2008; Srdjevic et al., 2004). The MADM methods are also applied in the area of waste management choices (Karagiannidis and Perkoulidis, 2009), interventions in anaerobic digestion process to manage the feed stock (Rao and Baral, 2011), in the area of manufacturing systems (Rao and Patel, 2010), energy sector such as power plants (Garg et al., 2007) and in the area of robotics for robots selections (Bhangale et al., 2004).

In present study MADM methods are used to prioritize the vegetable cash crops in greenhouse farming system using techniques such as WPM,SAW,TOPSIS and PROMETHEE and the results were compared using optimization package LINGO (LINGO, 2006). The objective of the research as per these backgrounds discussed here are the prioritization of vegetable cash crops in greenhouse farming system taking in account the important criteria for the sustainable farming practices. The development of multi attribute based decision model (MADM) to prioritize the vegetable cash crops in greenhouse farming system. The overall paper is arranged in five parts first part deals with the introduction, the second part deals with the materials and methods, the third part deals with multi attribute based decision models, fourth part deals with the results and discussions and the fifth and last part deals with the conclusions.

In the coming parts the construction of decision matrix is carried out to prioritize the vegetable cash crops grown in the Greenhouse for sustainable farming practices and maximum returns for five most preferred vegetable crops viz. (Cucurbitaceae, *Solanum lycopersicum, Capsicum annuum, Solanum melongena, Abelmoschus esculentus*) with

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respect to different dimensional and no dimensional criteria (crop field area, crop field production, cost of production, price of sale, usage of water) based on the importance of the objective under study. The constraints of minimum available farming area has been fixed as per the percentage of system of pattern of cropping (Cucurbitaceae 35%, *Solanum lycopersicum* 36%, *Capsicum annuum* 10%, *Solanum melongena* 11%, *Abelmoschus esculentus* 7%) of farming area in Greenhouse system. In order to control the dominance of most profitable crop the farm area constraints for certain crops are fixed as minimum in the total farming area. The type of the soil in the farming area varies farm are sandy clay loam, clay loam and clay. The water production function (WFP) is referred as the ratio of crop production and water use that is the influencing factor of the model. The price of the crop product is decided based on the guideline of the Ministry of Agriculture Saudi Arabia.

The constraints such as farming area, crop yield, cost of crop production per unit area, price of crop product per unit mass. The cost of farming was qualitative (value based) and irrigation water use is quantitative. Depending on the expert opinion of farming area the inter relationships between the attributes has been established. The beneficial attributes are the farm area and farm yield whereas the non-beneficial attributes are the price of sale and cost of production. In the present study the qualitative attributes were considered and put under the ranked value judgement with a fuzzy conversion scale. The crisp score were obtained using the fuzzy set theory (Chen and Hwang, 1992). The decision matrix shown in *Table 1* is formed based on the data collected through different departments, experts' opinion.

Table 1. *Model Attribute Simulation of Green House Vegetable Cash Crops used*

S. No.	Cash Crops used (Alternatives)	Crop Field Area (CFA) (m^2) '000	Crop Field Production (CRF) (Kg/m^2)	Cost of Production (CP) (USD/m^2)	Price of sale (PS) USD per Kg
1.	Cucurbitaceae (A1)*	109800	15.4000	4.04	1.07
2.	Solanum lycopersicum (A2)*	110000	16.8000	4.68	1.07
3.	Capsicum annuum (A3)*	35000	13.4000	4.80	1.07
4.	Solanum melongena (A4)*	34000	9.7000	4.40	0.80
5.	Abelmoschus esculentus (A5)*	21000	1.6000	3.20	2.13

* Cucurbitaceae (A1) = Cucumber; Solanum lycopersicum (A2) = Tomato; Capsicum annuum (A3) = Green pepper; Solanum melongena (A4) = Eggplant; Abelmoschus esculentus (A5) = Okra

Materials and methods

Study area

In the Kingdom of Saudi Arabia greenhouse farming is promising venture. Recognizing its yields per unit area of the farm and the effective use of scare natural resource viz. water and soil. Saudi Arabia is putting the sincere efforts to enhance the greenhouse farming activities. The trend in increase in the greenhouse farm in past twenty

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years double in the numbers and more than eighteen times in yields. The number and production in greenhouse farms has increased from 104 and 26 tons in the year 1984 to 295 and 142 tons in 1999. In the Saudi Arabia Greenhouse chambers mostly produce vegetables which is around 42% of the total production the Cucurbitaceae and *Solanum lycopersicum* are the most produced vegetable crops producing more than 85% of the total production (Ministry of Agriculture and Water, 2001).There are many factors which affects the prioritization of crop type in Greenhouse farming. The criteria like available irrigation water in terms of quality and quantity, nutrient value of soil, financial conditions, climatic conditions, skill level of workers etc. play important role in agricultural farming.

In this paper the effort is made for the sustainable greenhouse farming for efficient greenhouse farming expansion is the need of the hour and this should be exploited and utilized in precise and most efficient way in Saudi Arabia. The figure below shows the green chamber farms located at 18°06'25.2"N, 42°53'45.3" E (DMS WGS 84) in the Asir region (*Fig. 1*).

Figure 1. *Green Chamber Farms in Aseer Region in the Kingdom of Saudi Arabia*

The MADM methods

Weighted Product Method (WPM) method

In this method all possible alternative is compared with the other alternative by taking the product of number of ratios, one for every selected criterion. The relative weight of the concern criterion is taken as the power to the ratio, (Chen and Hwang, 1992) as mentioned below (*Eq.1*):

$$P_i = \prod_{j=1}^{M} \left[(m_{ij})_{normal} \right] w_j \qquad \text{(Eq.1)}$$

The (m_{ij}) normal is normalized value of an alternative with respect to an attribute is raised to the with the relative weight of the concern attribute. The best alternative is selected with the value with highest P_i.

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Simple Additive Weighting Method (SAW) method

This method was first developed by (Edwards et al., 1982). The mathematical form of this method (*Eq. 2*) can be written as follows:

$$P_i = \prod_{j=1}^{M} \left[(m_{ij})_{normal} \right] w_j \quad \text{(Eq.2)}$$

The different terms in the above equation can be defined as, P_i is overall score of the crop alternative (A_i), W_j is the weight of importance of jth criterion, m_{ij} is the measure of the performance of the jth performance criterion and the (m_{ij}) normal represents the normalized value of m_{ij}. This method can be also used for non-identical units of measure when the values of decision matrix is normalized.

The Technique for Order of Preference by Similarity to Ideal Solution method (TOPSIS)

The TOPSIS process is carried out in six steps as follows:

Step 1. Creation of evaluation matrix:

Create an evaluation matrix consisting of m alternatives and n criteria, with the intersection of each alternative and criteria given as x_{ij} we therefore have a matrix $(x_{ij})_{mxn}$. This matrix is considered as a decision matrix with the dimensions mxn. This matrix formation takes care of the dimensional and non-dimensional units.

Step 2. Creation of relative importance matrix:

The relative importance of one attribute is compared with the other attribute. This also helps in calculating the weight of each criteria (Saaty, 1980).

Step 3. Development of normalized decision matrix (*Eq.3*):

The matrix $(x_{ij})_{mxn}$ is then normalized to form the matrix

$$R = (r_{ij})_{mxn} \quad \text{(Eq.3)}$$

using the normalization method (*Eq.4*):

$$r_{ij} = \frac{x_{ij}}{\sqrt{\sum_{i=1}^{m} x_{ij}^2}}, = 1,2,\ldots,m, j = 1,2,\ldots,n \quad \text{(Eq.4)}$$

Step 4. Development of weighted normalized matrix:

Calculate the weighted normalized decision matrix (*Eqs.5*):

$$T = (t_{ij})_{mxn} = (w_j r_{ij})_{mxn}, i = 1,2,\ldots,m \quad \text{(Eq.5a)}$$

$$w_j = W_j / \sum_{j=1}^{n} W_j, j = 1,2,\ldots,n \quad \text{(Eq.5b)}$$

Where

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$\sum_{j=1}^{n} w_j = 1$

So that, and W_j is the original weight given to the indicator $v_j, j = 1,2, \ldots, n.$

Step 5. Determination of best and worst conditions (*Eq.6*):

$$A_w = \{\langle max(t_{ij}|i = 1,2, \ldots, m|)j \in J_-\rangle, \langle min(t_{ij}|i = 1,2, \ldots, m|)j \in J_+\rangle\} \equiv \{t_{wj}|j = 1,2, \ldots, n|\} \quad \text{(Eq.6)}$$

Determine the worst alternative (A_w) and the best alternative (A_b) (*Eq.7*):

$$\mathrm{A_b}=\{\langle \min(\mathrm{t_{ij}}|\mathrm{i} = 1,2, \ldots, \mathrm{m}|)\mathrm{j} \in \mathrm{J_-}\rangle, \langle \max(\mathrm{t_{ij}}|\mathrm{i} = 1,2, \ldots, \mathrm{m}|)\mathrm{j} \in \mathrm{J_+}\rangle\}\equiv\{\mathrm{t_{wj}}|\mathrm{j} = 1,2, \ldots, \mathrm{n}|\} \quad \text{(Eq.7)}$$

Where,

$J_+ = \{j = 1,2, \ldots, n|j|\}$

associated with the criteria having a positive impact, and

$J_- = \{j = 1,2, \ldots, n|j|\}$ associated with the criteria having a negative impact.

Calculate the distance between the target alternative i and the worst condition A_w as below mentioned (*Eq.8*):

$$d_{iw} = \sqrt{\sum_{j=1}^{n} (t_{ij} - t_{wj})^2}, i = 1,2, \ldots, m \quad \text{(Eq.8)}$$

and the distance between the alternative i and the best condition A_b (*Eq.9*):

$$d_{ib} = \sqrt{\sum_{j=1}^{n} (t_{ij} - t_{bj})^2}, i = 1,2, \ldots, m \quad \text{(Eq.9)}$$

Where d_{iw} and d_{ib} are L2-norm distances from the target alternative i to the worst and best conditions, respectively.

Step 6. Euclidean distance:

Calculate the similarity to the worst condition as mentioned below (*Eq.10*):

$$S_{iw} = d_{iw}/(d_{iw} + d_{ib}), 0 \leq S_{iw} \leq 1, i = 1,2, \ldots, m \quad \text{(Eq.10)}$$

$S_{iw} = 1$ if and only if the alternative solution has the best condition; and

$S_{iw} = 0$ if and only if the alternative solution has the worst condition.

Step 7. Prioritization of alternatives:

Rank the alternatives according to $S_{iw}(i = 1,2, \ldots, m)$

Fuzzy Delphi Method (FDM)

The general Delphi method and the fuzzy set theory was clubbed together for the first time by (Ishikawa et al., 1993) and developed the method known as Fuzzy Delphi Method (FDM). In order to solve the fuzziness of general understanding of professional ideas (Noorderhaben, 1995) used the FDM. The fuzziness of the general opinion of the professional could be solved using FDM, and can be evaluated on a more flexible scale.

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The questionnaires can be improved for its quality and efficiency thus more parameters could be screened through the fuzzy set theory. The fuzzy numbers used for this purpose is shown in *Table 2* and the fuzzy scale is shown in *Figure 2*.

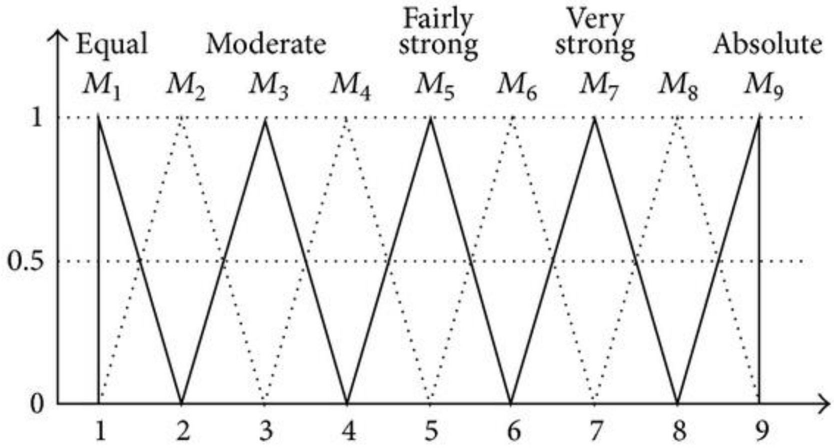

Figure 2. *The fuzzy number scale (Li et al., 2013)*

Table 2. *Fuzzy numbers and definition*

Fuzzy numbers	Definition
1 (1,1,1)	Equally important
2 (1,2,3)	Equally to moderate important
3 (2,3,4)	Moderately more important
4 (3,4,5)	Moderately and strongly important
5 (4,5,6)	More strongly important
6 (5,6,7)	Strongly and very strongly important
7 (6,7,8)	More very strongly important
8 (7,8,9)	Very strongly to extremely important
9 (8,9,9)	Extremely more important

The steps involved in Fuzzy Delphi Method (FDM) are as follows:

a. The decision group opinion data base generation:

The linguistic variables in the questionnaire is used to generate the score of factors important in the study as per the expert opinion.

b. The triangular fuzzy numbers set up:

As per the experts opinion triangular fuzzy number generated are used to get the evaluated values and their significance triangular fuzzy number for the alternate factors are generated. In this study the geometric mean model proposed by (Klir and Yuan, 1995) is used for FDM to get the general understanding of group decision. The formula used is given as follows. Assuming n number of professional whose element is given by number i and the significance of number j then the fuzzy weight.

$\widetilde{w_{ij}} = (a_{ij}; b_{ij;}\ c_{ij}), i = 1,2; \dots\dots\dots; n; j = 1,2; \dots\dots\dots; m$ then the $\widetilde{w_j}$ fuzzy weighting of no. j element is presented as $\widetilde{w}j = (a_{j;}\ b_{j;}\ c_j); j = 1,2; \dots\dots, m.$

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Among which $a = Max_i a_{ij}, b_j = \frac{1}{n\sum_{n-1}^{n} bij}, c = Max_i C_{ij}$

c. Defuzzification

The absolute value S_j is obtained by using the ordinary center of gravity method the process is called defuzzification and the parameter used is fuzzy weight w_j is calculated as follows in *Equation 11*.

$$S_j = \frac{(a_j + b_j + c_j)}{3} \quad where\ j = 1,2,3, \dots \dots \dots, m \qquad \text{(Eq.11)}$$

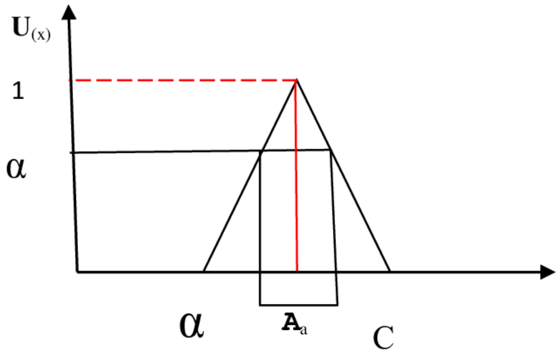

Figure 3. *Fuzzy Delphi method diagram (Moradi et al. 2014)*

If $S_j \geq \alpha$, then the no. j factor is the evaluation index

If $S_j < \alpha$ then delete no. j factor

The solution obtained using FDM is reported in *Table 6* and the important criteria with their definition is mentioned in *Table 3*.

Table 3. *Important criteria and definition*

Criteria	Abbreviation	Definition
Crop Field Area	CFA	The study taken here is the Greenhouse chamber so the land area located, under this category around 42% of the land out of total vegetable crops grown in KSA is put.
Crop Field Production	CRF	This is the yield of crops in ton per unit area of the land in ha for example in the case of Cucurbitaceae 154 ton/ha.
Cost of Production	CP	This include all type of the expenditures involved per unit production of the crop for example in the case of Abelmoschus esculentus it is SR 21562/ha.
Price of sale	PS	This the rate received in the market per unit produce of the crop on average in the case of Cucurbitaceae it is around SR4/Kg.
Usage of water	US	This is amount of water required per unit area of the land per cropping period it is quantitative value (ha-m) but if we compare crops with each other it can be taken as qualitative.

The Nonlinear Programming (NLP) model and solution

In the NLP model the objective function is to maximize the profit and the constraints were set up on the availability of water quantity using the Lingo (Shrivastava et al., 2012). The defined objective function of the problem is as follows (*Eq.12*).

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$$MaximiseF = (PY - C)A \tag{Eq.12}$$

In the above equation the function F stands for the net profit, SR (Kingdom of Saudi Arabia, Currency); P is the market value of vegetable in SR/kg; C is the cost of cultivation in SR/ha; Y is the yield of vegetable per unit area of the land in Kg/ha; A is the total area of cultivation in ha. The model took in account the various constraints. The water availability for the groundwater source is considered as a constraint. The total area is divided in sub area on the basis soil and land availability constraint. The agricultural land area to some crops is fixed as minimum so that the crop with high market value should not dominant over the complete agricultural area. The cultivated area and irrigation water depth is considered as positive decision variable.

Results and discussion

In TOPSIS model the formulation matrix developed as shown in Table 1 is utilized in this section to develop the normalized matrix as written by *Equation 13* in order to remove the dimensions the entry in the matrix from 0 to 1.

$$\begin{bmatrix} \downarrow^{\rightarrow} & CFA & CFP & CP & PS & UW \\ A1 & 0.998 & 0.917 & 0.790 & 0.750 & 0.817 \\ A2 & 1.00 & 1.00 & 0.683 & 0.750 & 0.670 \\ A3 & 0.318 & 0.798 & 0.666 & 0.750 & 0.568 \\ A4 & 0.309 & 0.577 & 0.728 & 1.00 & 0.761 \\ A5 & 0.191 & 0.095 & 1.00 & 0.375 & 1.00 \end{bmatrix} \tag{Eq.13}$$

From the decision matrix in *Equation 13*, the normalized matrix is developed as shown in *Equation 14*.

$$\begin{bmatrix} 0.670 & 0.546 & 0.451 & 0.446 & 0.470 \\ 0.671 & 0.596 & 0.390 & 0.446 & 0.386 \\ 0.213 & 0.475 & 0.381 & 0.446 & 0.327 \\ 0.207 & 0.344 & 0.416 & 0.595 & 0.438 \\ 0.128 & 0.057 & 0.571 & 0.223 & 0.576 \end{bmatrix} \tag{Eq.14}$$

The relative importance matrix developed is shown in *Equation 15* this is based on analytic hierarchy process first developed by (Saaty, 1980), then the weight of all the criteria is computed as shown in *Equation 16*. The consistency ratio (CR) is 0.08 which is less than 0.1 hence it depicts good consistency with the decision.

$$\begin{bmatrix} \downarrow^{\rightarrow} & CFA & CFP & CP & PS & UW \\ CFA & 1 & 4 & 3 & 2 & 5 \\ CFP & 1/4 & 1 & 1/3 & 1/2 & 2 \\ CP & 1/3 & 3 & 1 & 4 & 1/3 \\ PS & 1/2 & 2 & 1/2 & 1 & 1/2 \\ UW & 1/5 & 1/2 & 1/3 & 2 & 1 \end{bmatrix} \tag{Eq.15}$$

As per the method proposed by (Saaty, 1980) the weight for all the criteria is calculated and reported in the following *Equation 16*.

$$\left(W_j = (0.47240 \;\; 0.11030 \;\; 0.19210 \;\; 0.11960 \;\; 0.10550)\right) \tag{Eq.16}$$

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In *Equation 17* the weighted normalized matrix is written which is obtained by multiplying weights into normalized matrix.

$$\begin{bmatrix} 0.316 & 0.060 & 0.087 & 0.053 & 0.049 \\ 0.316 & 0.066 & 0.075 & 0.053 & 0.041 \\ 0.101 & 0.052 & 0.073 & 0.053 & 0.034 \\ 0.098 & 0.038 & 0.079 & 0.071 & 0.046 \\ 0.060 & 0.006 & 0.109 & 0.027 & 0.061 \end{bmatrix} \qquad \text{(Eq.17)}$$

In the next step the positive-ideal (best) and the negative-ideal (worst) is calculated (*Eq. 18*) as depicted in the *Table 4* subsequently the positive and the negative separation measure were calculated as shown in *Table 5* the relative closeness value is calculated for all the options and is shown in *Table 3*.

$$\begin{bmatrix} A11 & A12 & A13 & A14 & A15 \\ B11 & B12 & B13 & B14 & B15 \\ C11 & C12 & C13 & C14 & C15 \\ D11 & D12 & D13 & D14 & D15 \\ E11 & E12 & E13 & E14 & E15 \end{bmatrix} \qquad \text{(Eq.18)}$$

Table 4. *For different attributes best (+ve) and worst (-ve) Results*

S. No.	Ideal Best	Ideal Worst
1.	$I_{CFA}^{+} = 0.316$	$I_{CFA}^{-} = 0.060$
2.	$I_{CFP}^{+} = 0.066$	$I_{CFP}^{-} = 0.006$
3.	$I_{CP}^{+} = 0.109$	$I_{CP}^{-} = 0.073$
4.	$I_{PS}^{+} = 0.071$	$I_{PS}^{-} = 0.027$
5.	$I_{UW}^{+} = 0.061$	$I_{UW}^{-} = 0.061$

Table 5. *For the positive ideal and negative ideal solution separation measure*

S. No.	Positive Separation Measure	Negative Separation Measure	Relative Closeness
1.	$P_{Cucurbitaceae}^{+} = 0.031$	$P_{Cucurbitaceae}^{-} = 0.264$	$P_{Cucurbitaceae} = 0.895$
2.	$P_{Solanumlycopersicum}^{+} = 0.043$	$P_{Solanumlycopersicum}^{-} = 0.264$	$P_{Solanumlycopersicum} = 0.859$
3.	$P_{Capsicum\ annuum}^{+} = 0.221$	$P_{Capsicum\ annuum}^{-} = 0.067$	$P_{Capsicum\ annuum} = 0.233$
4.	$P_{Solanum\ melongena}^{+} = 0.222$	$P_{Solanum\ melongena}^{-} = 0.068$	$P_{Solanum\ melongena} = 0.235$
5.	$P_{Abelmoschus\ esculentus}^{+} = 0.267$	$P_{Abelmoschus\ esculentus}^{-} = 0.554$	$P_{Abelmoschus\ esculentus} = 0.554$

Table 5 shows the calculation details of relative closness for the crops , $P_{Cucurbitaceae}$, $P_{Solanumly\ copersicum}$, $P_{Capsicum\ annuum}$, $P_{Solanum\ melongena}$ *and* $P_{Abelmoschus\ esculentus}$. The Fuzzy Delphi Method (FDM) model formulation is discussed as above. The detail calculations and results obtained are in *Table 6*.

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***Table 6.** The Result of the FDM*

Preference values	D1	D2	D3	α	β	γ	U	M	L	Best non fuzzy performance [(U-L)+(M-L)]/3+L
Cucurbitaceae	(8,9,9)	(8,9,9)	(7,8,9)	7.7	8.7	9.0	9.0	8.6	7.6	8.445
Solanum lycopersicum	(7,8,9)	(8,9,9)	(7,8,9)	7.7	8.3	9.0	9.0	8.3	7.3	8.222
Abelmoschus esculentus	(7,8,9)	(8,9,9)	(6,7,8)	7.0	8.0	8.7	8.6	8.0	7.0	7.889
Solanum melongena	(7,8,9)	(6,7,8)	(8,9,9)	7.0	7.7	8.7	8.6	7.7	7.0	7.000
Capsicum annuum	(6,7,8)	(6,7,8)	(6,7,8)	6.0	7.0	8.0	8.0	7.0	6.0	7.777

The MADM techniques are used here to prioritize the vegetable cash crops grown in Greenhouse chamber in local climatic and socio-economic conditions which prevails the different criteria for the different attributes in the Kingdom of Saudi Arabia. The MADM techniques adopted here are the (WPM, SAW, TOPSIS and FDM). In order to analyze the alternatives, four methods (SAW, WPM, TOPSIS and FDM) of MADM approach is used in the present study. The selection index values computed by all the techniques are shown in *Table 7* the crop prioritization is carried out by the highest index value for first and the lowest index value for the last crop. This is observed that some variations are there when we apply the different techniques due to its different mathematical formulation for the similar parameters. The WPM and SAW methods give the Cucurbitaceae is on top priority followed by Solanum lycopersicum same trend was found by TOPSIS and FDM. The variation is found in the case of Abelmoschus esculentus and Solanum melongena may be due to inclusion of logical interrelationship among the criteria's are taken in account by estimating the separation measure to the pairwise comparison and ideal solution. The *Table 6* depict the result of MADM approach used. In comparison with the NLP model results TOSIS and FDM performance was satisfactory. In NLP model and all other MADM techniques the Cucurbitaceae followed by Solanum lycopersicum is most preferred crop, the NLP is solved using LINGO model. For most sustainable Greenhouse practices the importance should be given to land availability, social-economic and environmental factors.

***Table 7.** Tabulated values of index using WPM, SAW, TOPSIS and FDM method*

Index values	WPM	SAW	TOPSIS	FDM
PCucurbitaceae	0.894	0.900	0.895	8.445
PSolanum lycopersicum	0.861	0.874	0.859	8.222
PCapsicum annuum	0.569	0.516	0.233	7.777
PSolanum melongena	0.494	0.549	0.235	7.000
PAbelmoschus esculentus	0.314	0.443	0.554	7.889

Ranking of alternative crops

WPM Cucurbitaceae-Solanum lycopersicum-Capsicum annuum-Solanum melongena-Abelmoschus esculentus, SAW Cucurbitaceae-Solanum lycopersicum -

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DOI: http://dx.doi.org/10.15666/aeer/1702_46154629

Solanum melongena- Capsicum annuum-Abelmoschus esculentus, TOPSIS Cucurbitaceae-Solanum lycopersicum-Abelmoschus esculentus-Capsicum annuum-Solanum melongena, FDM Cucurbitaceae-Solanum lycopersicum-Abelmoschus esculentus-Capsicum annuum-Solanum melongena.

Conclusions

In this research work the prioritization of most commonly grown vegetable cash crop is made based on the MADM modeling in The Kingdom Saudi Arabia for the sustainable agricultural practices and local climatic and socio-economic conditions this will help the policy maker and Greenhouse farmers in sustainable agricultural practices. The results obtained were analyzed and compared with LINGO model results. The different major criteria selected in the prioritization are the farming area, irrigation water required per unit area, market value of crop yield per unit mass, cost of farming per unit area were decided for each vegetable cash crop grown in the Greenhouse from the collected data source. The methodologies used in simulation modeling are WPM, SAW, TOPSIS and FDM were employed for ranking to each selected crops. The results calculated by WPM and the SAW were little different may be because of weightages chosen to each criteria may not be in comparison of it. The output from the TOPSIS and FDM helped strongly in prioritization of the vegetable cash crops. These two methods permit the policy maker and farmers (decision makers) inclusion or removal of any attribute based on the importance of the criteria with respect to goal of the study. The best possible close hypothetical solution is obtained measuring separation measure, ideal best and ideal worst using TOPSIS method. The prioritization of vegetable cash crops was carried out by FDM method. The results were compared with LINGO model results and found to be satisfactory. Since the computation time required in NLP is more so the quick decision can be taken by MADM. The recommendations for future work are that the system-based process need to be adopted by incorporating multi-criteria methods and using these methods more in the process of crop selection, the other aspect takes into account the more sustainability-based criteria and ecological aspects in order to improve decision making process.

Acknowledgements. Authors thankfully acknowledged the Deanship of Scientific Research for proving administrative and financial supports. Funding for this work has been provided by the Deanship of Scientific Research; King Khalid University, Ministry of Education, Kingdom of Saudi Arabia under award numbers R.G.P.1/28/38.

Conflict of Interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46154629

A LARGER ROOT SYSTEM IN OAT (*AVENA NUDA* L.) IS COUPLED WITH ENHANCED BIOMASS ACCUMULATION AND HORMONAL ALTERATIONS UNDER LOW NITROGEN

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(Received 8th Jan 2019; accepted 14th Feb 2019)

Abstract. Nitrogen (N), an essential macronutrient for crop growth and development, is well known for its economic losses and environmental hazards due to imbalanced application. Oat (*Avena nuda* L.), a food and fodder crop, is well adapted to marginal soils and requires minimum water and nutrients to produce biomass. A hydroponic experiment was conducted in China Agricultural University, Beijing, China to evaluate the morphological and physiological adaptations of oat to organic N (ON) and low N (LN) with respect to chemical N (CK as control). The results have shown that oat responded to different nitrogen supplies with response to LN having been the dominant one. Lowest SPAD value, net photosynthesis rate, transpiration rate, gas exchange and stomatal conductance showed that the oat was under LN stress. Highest level of root dry mass, as indicated by increased R/S ratio and C/N ratio, was at the cost of minimum nitrogen utility as depicted by increased nitrogen utilization efficiency in roots under LN. Accumulation of total soluble proteins and sugars elaborated the carbon allocation to nitrogen deprived roots. As a result of sufficient biomass provision in LN root, total root length increased which was coupled with increased concentrations of indole-3-acetic acid, gibberellic acid and zeatin riboside whereas decreased concentration of abscisic acid. Overall, oat is found to be a nitrogen use efficient crop which responded to LN through enhanced root system through biomass accumulation by the provision of nitrogen metabolites.
Keywords: *root architecture, photosynthesis, nitrogen utilization efficiency (NUtE), nitrogen metabolites, hormonal accumulation*

Introduction

Nitrogen (N) is an important macronutrient required for the biosynthesis of chlorophyll, nucleic acids, amino acids, proteins and some organic acids. Its essentiality is obvious from its involvement in most of the biological and physiological processes such as photosynthesis, biomass production, hormonal accumulation, carbohydrate allocation, plant root architectural growth and development of reproductive organs of plants (Zhao et al., 2005; Miller and Cramer, 2005). To produce 1 kg biomass most of the non-legume plants absorb 20 to 50 g of N from the soil (Robertson and Vitousek, 2009). Nitrogen is present in soils in a patchy manner either as inorganic form (nitrate or ammonium) or organic form (amino acids, peptides and proteins). Plants uptake different nitrogen forms from the soil through roots with nitrate being the most preferential form (Yuan et al., 2007; Puangfoo-Lonhienne et al., 2008 and Wang et al., 2012). Being highly mobile element, nitrogen is subjected to losses once applied to the soil as inorganic form whereas nitrogen released by organic forms is slow which cannot meet the plant requirement.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Hence, farmers are compelled to use chemical nitrogen fertilizers which, more often than not, which damages soil physical and chemical health, and toxifies the environment. Approximately 10^{11} kg N per annum is applied in the soil globally with the aim to get highest crop production (Glass, 2003; Tilman et al., 2011; Liu et al., 2013; Chandna et al., 2011; Rawat et al., 2012). Hence, high levels of soil N are susceptible to losses (Zhu et al., 2002; Ju et al., 2004) which ultimately can reduce the N use efficiency (NUE) (Ata-Ul-Karim et al., 2014). On the other hand, deficiency of N reduces the biosynthesis of chlorophyll, affects plant organ development and finally results in reduced crop yield (Marschner, 1995). Plants have developed morphological and physiological alterations over evolution to cope with heterogeneous environmental conditions. Nitrogen is present in soil solution in mineral as well as organic form (Nasholm and Persson, 2001); thus, it depends on the adaptability of a plant to dwell under the conditions of its high or low availability (Glass, 2003; Garnett et al., 2009). The primary response of a plant to low external nitrogen availability is to modify its root system. Most plants expand their root system to capture N by exploring as much rhizosphere as possible (Palta, et al., 2011). Leguminous crops form root nodules to fix atmospheric N but non-leguminous crops like maize, rice produce longer roots (Wang et al., 2003; Chun et al., 2005a) whereas, foxtail millet produces shorter and thicker roots facilitating carbohydrate allocation perhaps through wider xylem vessels (Nadeem et al., 2018). Hence, the diversity is observed in the adaptive measures of different crops under different rhizosphere condition.

Naked oat (*Avena nuda* L.) and hulled oat (*A. sativa* L) are two types of oats cultivated in China. The fast growing nature of oat capacitates itself to produce ample quantity of fresh fodder within short period of time with high nutritional values. The high protein and β-glucone content along with the presence of hypo-glycemic fiber content in oat grain increase its value as a human food. Generally, rice, wheat, maize and other high yielding cereal crops generally occupy most of the agricultural land but crops like oat find very limited area and interest to be cultivated worldwide. Current study was aimed to characterize root morphological and physiological adaptations of oat in order to maximize its nitrogen utilization efficiency (NUtE) in terms of biomass accumulation under different nitrogen supplies. The findings revealed oat as an extremely large root crop under low nitrogen (LN) condition with remarkable nitrogen use efficiency and biomass accumulation.

Materials and methods

The hydroponic experiment was carried out during April-July in a standard growth chamber with 28/22 °C alternate day night temperature, 14/10 h (day/night) photoperiod, 65% relative humidity and 300 µmol photons m^{-2} s^{-1} illumination in China Agricultural University, Beijing, China. High quality seeds of oat (*Avena nuda* L.) variety Baiyan 2 were collected from Baicheng Academy of Agricultural Science, Jilin, China. Deionized H_2O was used to wash the seeds followed by sterilization with 10% H_2O_2 solution for 30 min and soaking in saturated $CaSO_4$ solution for 5 h with continuous aeration. Seeds were germinated on moist tray in the growth chamber. After emergence of approximately 2 cm primary roots, seedlings were wrapped with two layers of deionized water saturated filter paper, enfolded in cylinder shape and placed vertically in a growth holder containing distilled water to assure a continuous water supply to the seedlings. 12 uniform and consistent seedlings with two fully emerged leaves were selected and transplanted into well aerated 5-L vessels containing nutrient solution. The total nutrient solution (as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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control or CK) consisted of 2 mM NH_4NO_3, 0.75 mM K_2SO_4, 0.25 mM KH_2PO_4, 0.1 mM KCl, 0.2 mM Fe-EDTA, 0.65 mM $MgSO_4$, 2 mM $CaCl_2$, 1×10^{-3} mM $ZnSO_4$, 1×10^{-3} mM MnSO4, 5×10^{-6} mM $(NH_4)_6Mo_7O_{24}$, 1×10^{-4} mM $CuSO_4$, and 1×10^{-3} mM H_3BO_3. 2 mM NH_4NO_3 was replaced by 4 mM glycine ($C_2H_5NO_2$) as a source of organic nitrogen (ON). Low nitrogen (LN) treatment contained 10% of 2mM NH_4NO_3 used in control (CK). Every treatment had six replicates. Each container had 12 plants. Oat seedlings were grown in nutrient solution for 21 days in total having 25% of CK solution for 3 days, 50% of CK solution for 4 days, 100% of CK solution for 7 days followed by the treatment application (CK, ON and LN) for 7 days. The pH of the nutrient solution was adjusted to 5.8-6 and the nutrient solution was changed every 2 days. The whole experiment was repeated three times.

Plant roots and shoots were harvested, frozen in liquid N and stored in -80 °C for amino acids, soluble proteins, soluble sugars and hormone analysis. Root samples were, washed with deionized water 3 times, surfaced dried using a blotting paper prior to storage. For other physiological analysis, shoot and root samples were harvested and dried in an oven at 105 °C for 30 min and then at 70 °C until constant weight.

Gas exchange and SPAD values (Soil and plant analyzer developer) measurements

On the morning of 21st day, between 8.00 to 9.00 am, the 3rd fully expanded leaf of every plant of every replicate was measured in three points carefully with the help of a portable chlorophyll meter (SPAD-502, Minolta, Japan). Every pot had 12 seedlings. The three SPAD reads of every seedling were averaged to get 12 SPAD reads from every pot which served as technical replicates. On the same day a portable gas exchange system (LI-6400, LI-COR Biosciences, Lincoln, NE, USA) was used to measure photosynthetic rate (Pn), gas exchange (Ci), stomatal conductance (gs) and transpiration rate (Tr). Measurements were taken on 6 cm^2 leaf area segment next to the mid-rib of the 4th leaf. Readings from 12 seedlings per pot served as 12 technical replicates and 6 pots of every treatment served as 6 biological replicates. Photosynthetic photon flux density provided by a red/blue LED light source (6400-02B, LI-COR Biosciences, Lincoln, NE, USA) was fixed to 600 μmol m^{-2} s^{-1} and the concentration of ambient CO_2 was adjusted to 400 μmol mol^{-1} by CO_2 injection as reported by Geilfus et al. (2011).

Root length analysis

The root system was separated into crown and lateral roots. Each kind of roots was counted manually and crown roots were measured with a ruler. Roots were scanned using a scanner (Epson 1680, Indonesia). WinRHIZO software (version 5.0) (Regent Instruments Inc., Quebec City, QC, Canada) was used to analyze the scanned images to get total root length and average diameter following previously described methods (Peng et al., 2010). Lateral root length was obtained by subtracting crown root length from total root length. Lateral root density was defined as the number of lateral roots per unit length of crown root containing lateral roots. Each sample had three technical and six biological replicates.

N concentration analysis in the shoot and root

The dried plant samples were weighed and ground into fine powder. 0.3 g of ground sample was taken for digestion by H_2SO_4–H_2O_2 followed by total N analysis using a modified Kjeldahl digestion method (Baker and Thompson, 1992).

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Analysis of mineral concentration

For the analysis of mineral contents of the plant using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent Technologies 7700 Series, Böblingen, Germany), the dried (60 °C) plant tissues were ground into fine powder. 100 mg of fine powder was digested with 10 mL of 69% HNO_3 (ROTIPURAN Supra for ICP, 69%) in an 1800 W microwave oven (MARS 6 Xpress; CEM, Matthews, MC, USA) at 100 °C for 2 min, at 120 °C for 1 min and at 180 °C for 20 min followed by 20 min of cooling. Samples were diluted to 100 mL using sterilized deionized water (18.2 MΩ cm conductivity) and stored at 4 °C till analysis. Similar procedure also followed for blank digestions. A certified reference material NCSDC 73350 from the Beijing vegetable research institute (Beijing, China) was used for standardized analytical technique. Internal macro- and micronutrient standards and reference material samples were tested as internal control.

Hormone extraction and quantification by enzyme linked immunosorbent assay (ELISA)

Approximately 0.5 g fresh plant sample was ground into powder and homogenized by using 2 mL of 80% methanol (containing 40 mg l^{-1} butylated hydroxytoluene as an antioxidant), incubated 48 h at 4 °C, and then centrifuged at 1900 × g for 15 min at 4 °C. The supernatant was passed through C18 Sep-Pak cartridges (Waters Corp.), and the hormone fraction was eluted with 10 mL of 100% (v/v) methanol and then 10 mL ether. The elute was N_2-dried at 20 °C. The N_2 dried extracts were dissolved in 2.0 mL phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 and 0.1% (w/ v) gelatin (pH 7.5) to analyze the concentration of free indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA) and zeatin riboside (ZR) by enzyme linked immunosorbent assay (ELISA) following a well-established protocol (Weiler et al., 1981).

Extraction of metabolite and analyses

The soluble proteins were extracted and analyzed by using a standard kit (Coomassie Protein assay reagent; Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a reference. Chlorophyll was extracted from 0.2 g fresh leaf disks using 25 mL mixture of alcohol and acetone (1:1 v/v). The absorbance of the extract was measured at 663, 645, and 470 nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu, Japan) to estimate chlorophyll a, chlorophyll b and total carotenoids according to the previously described methods Wellburn and Lichtenthaler (1984). The concentration of total free amino acids was measured according to the Rosen ninhydrin colorimetric method using leucine as a standard (Rosen, 1957). The concentration of soluble sugars was determined using a commercially available kit (Boehringer Mannheim, Germany). Individual amino acids profile was analyzed using Waters e2695 HPLC (Waters Corp, Milford, MA, USA) by incubating 100 mg of fresh tissue powder with 1.5 mL of the 5% (w/v) Trichloroacetic acid (TCA) solution for 1 h at room temperature. ~50 mg plant tissues were loaded into an Elementar vario Macro CN (Elementar Technologies, Hanau, Germany) analyzer for the C: N ratio analysis.

Statistical analysis

Statistical analysis was performed using a windows software package (IBM SPSS Statistics 22). Means of different treatments were compared using the least significant

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

difference at a 0.05 or 0.01 level of probability. Correlation analysis was performed using Pearson correlation function of SPSS. Graphical rendering was carried out using sigma plot 13.0 windows software. All the data were presented as means ± standard errors (SE) of six replicates.

Results

Root and shoot morphology of oat and biomass accumulation

Oat seedlings were subjected to control (CK), organic nitrogen (ON) and low nitrogen (LN) treatments for one weak. At harvest, the seedlings showed obvious symptoms of various nitrogen regimes having green, mild green and light green (yellowish) shoot color in CK, ON and LN respectively (*Fig. 1A, B*).

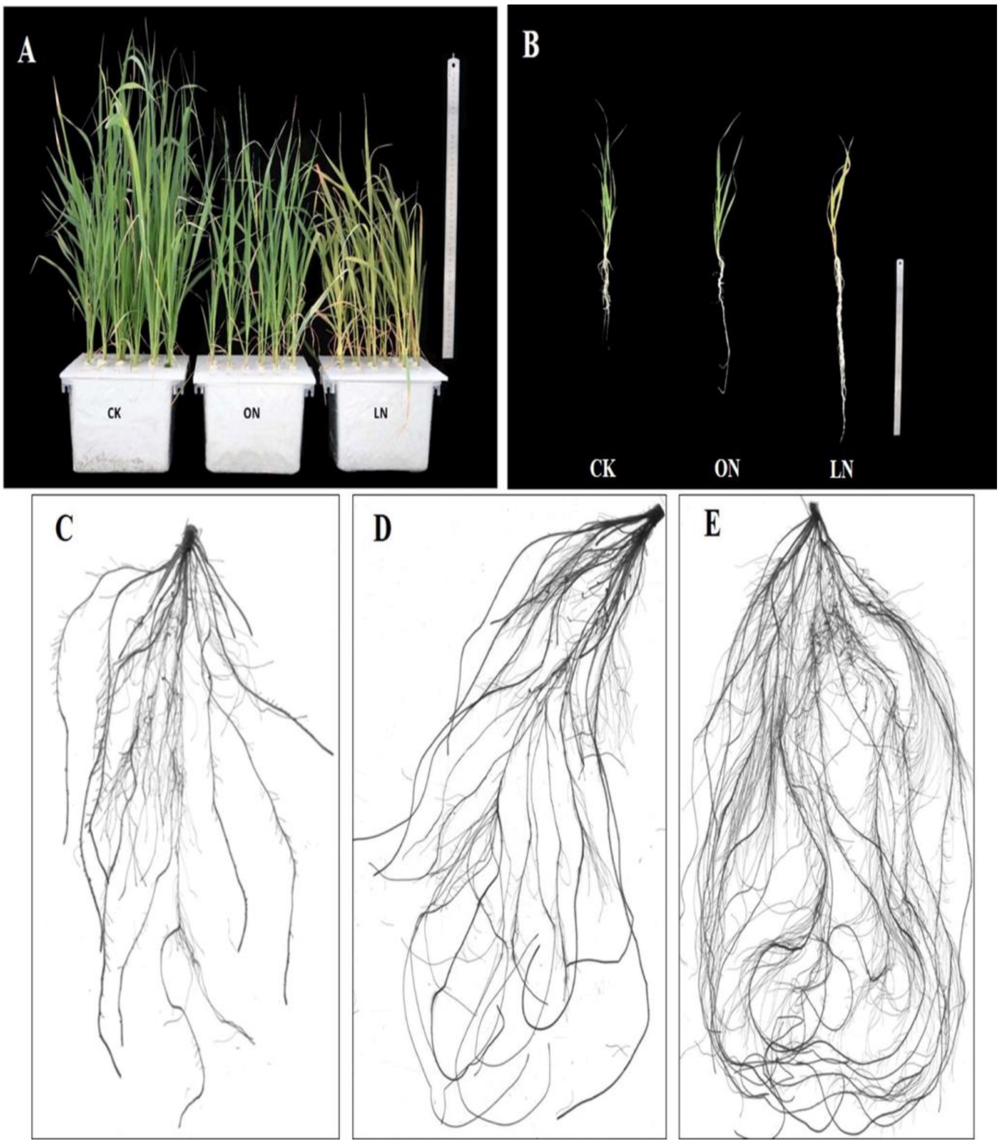

Figure 1. *Shoot and root responses of oat to different form and level of N. Plants grown under CK, ON and LN condition in hydroponic pots (A), plants with the intact root and shoot (B), The whole scan picture of root under Ck (C), ON (D) and LN (E). Treatment CK = control, ON = organic N, LN = low N*

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

During the harvesting time, each seedling under LN condition revealed the deficiency symptoms of N. Limitation of N generally hinders oat plant growth. In current study, shoot length, leaf area and shoot dry weight decreased whereas root dry weight and root to shoot ratio increased in ON and LN treatments (*Fig. 2A-E*). Reduction in plant height was observed in the ON and LN treatment which was almost 14% and 28%, respectively, in comparison with CK (*Table A1* in the *Appendix*). Compared to CK and ON treatment 45.77% and 35.77% less shoot dry weight and 82.61% and 31.25% higher root DW was produced under LN condition, respectively, (*Table A2*). Interestingly, root to shoot ratio resulted in 241.27% and 109.76% increment in LN treatment with respect to CK and ON respectively. Similar was the case in ON treatment where 62.7% increase in root to shoot ratio was observed as compared to CK (*Table A2*). Talking about root architectural traits (*Fig. 1C-E*), LN treatment increased total root length (112.3% and 64.65%), crown root length (116.27% and 75.91%), lateral root length (111.57% and 63.46%), crown root number (96.67% and 40.47%), and lateral root number (34.1% and 16.39%) and density (128.21% and 67.92%) with respect to CK and ON whereas, ON treatment only increased lateral root number (15.22%) and length (29.43%) and total root length (28.78%) significantly as compared to CK (*Table 1; Table A3* and *A4*). Average diameter of roots decreased in LN significantly (*Table 1*).

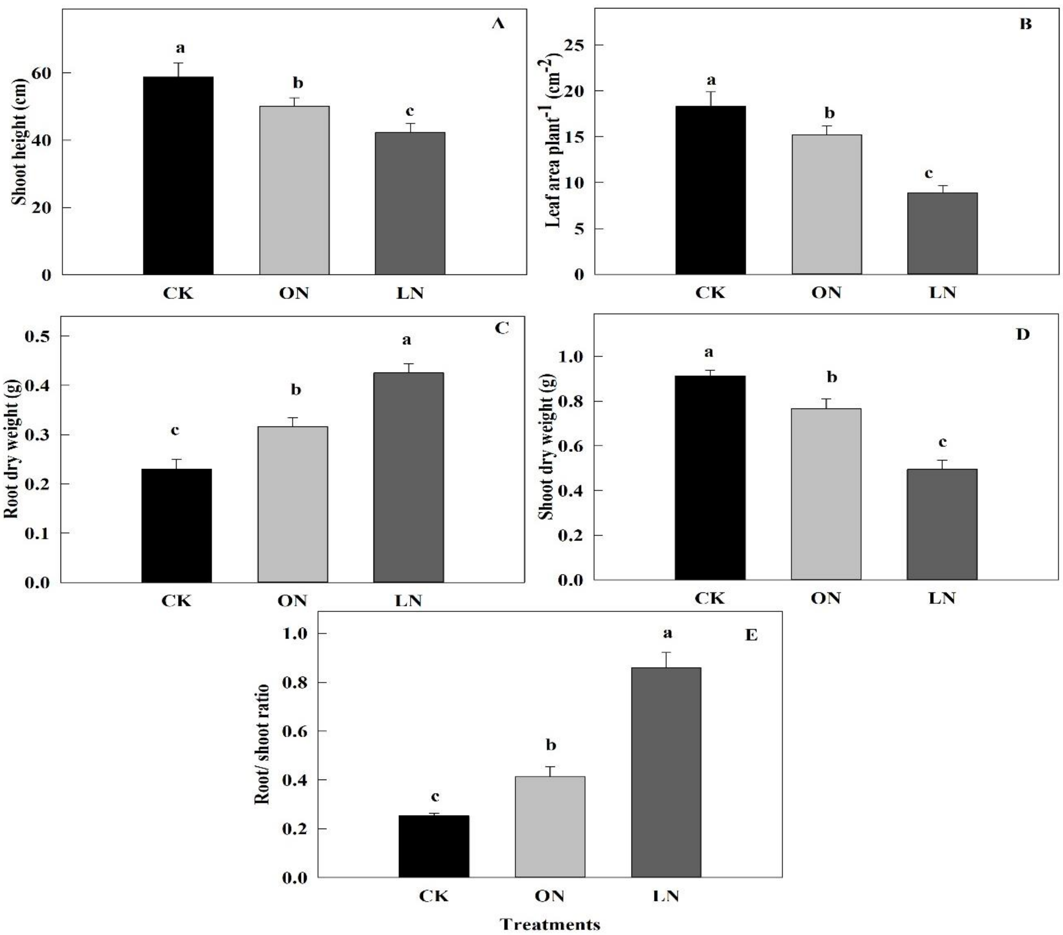

Figure 2. *The oat plant responses to different form and level of N. Shoot height (A), leaf area plant*$^{-1}$*(B), root dry weight (C), shoot dry weight (D) and root/shoot ratio (E). Error bars represented standard error of six biological replicates, different letters indicate significant differences (P < 0.05). Treatment CK = control, ON = organic N, LN = low N*

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Table 1. *Root morphological characteristics under CK, ON and LN condition during harvesting*

Treatment	Crown root number	Lateral root number	Lateral root density	Crown root length (cm)	Lateral root length (cm)	Total root length (cm)	Average diameter (cm)
CK	7.5±0.87b	1736.17±15.2c	7.8±0.98b	143.34±10.56b	1288.16±82.2c	1431.5±97.36c	0.073±0.0039a
ON	10.5±1.08b	2000.4±123.3b	10.6±1.76b	176.23±13.98b	1667.22±102.1b	1843.45±131.23b	0.078±0.27a
LN	14.75±2.22a	2328.17±155.8a	17.8±2.01a	310±17.76a	2725.3±178.3a	3035.33±198.3a	0.052±0.45b

In each column lower case lettering is used to show the significant differences between different types of treatments at $P < 0.05$ level. Values show Standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N

Attributes of photosynthesis and nitrogen accumulation

We found a continuous and significant decrease in SPAD value, Chlorophyll a, b and total carotenoids from CK to LN (*Fig. 3A-D; Table A5*). Moreover, net photosynthesis (Pn), intercellular carbon dioxide (Ci), transpiration rate (Tr) and stomatal conductance (g_s) followed the same pattern across all three treatments (*Fig. 4A-D*). Shoot and root nitrogen concentrations were decreased in the order of CK>ON>LN (*Fig. 5A, B; Table A6*), and vice versa for whereas for carbon to nitrogen (C/N) ratio and nitrogen utilization efficiency (cumulative biomass per unit of nitrogen; g DW g^{-1} N in the plant; NUtE) in both shoot and root (*Fig. 5C-F*).

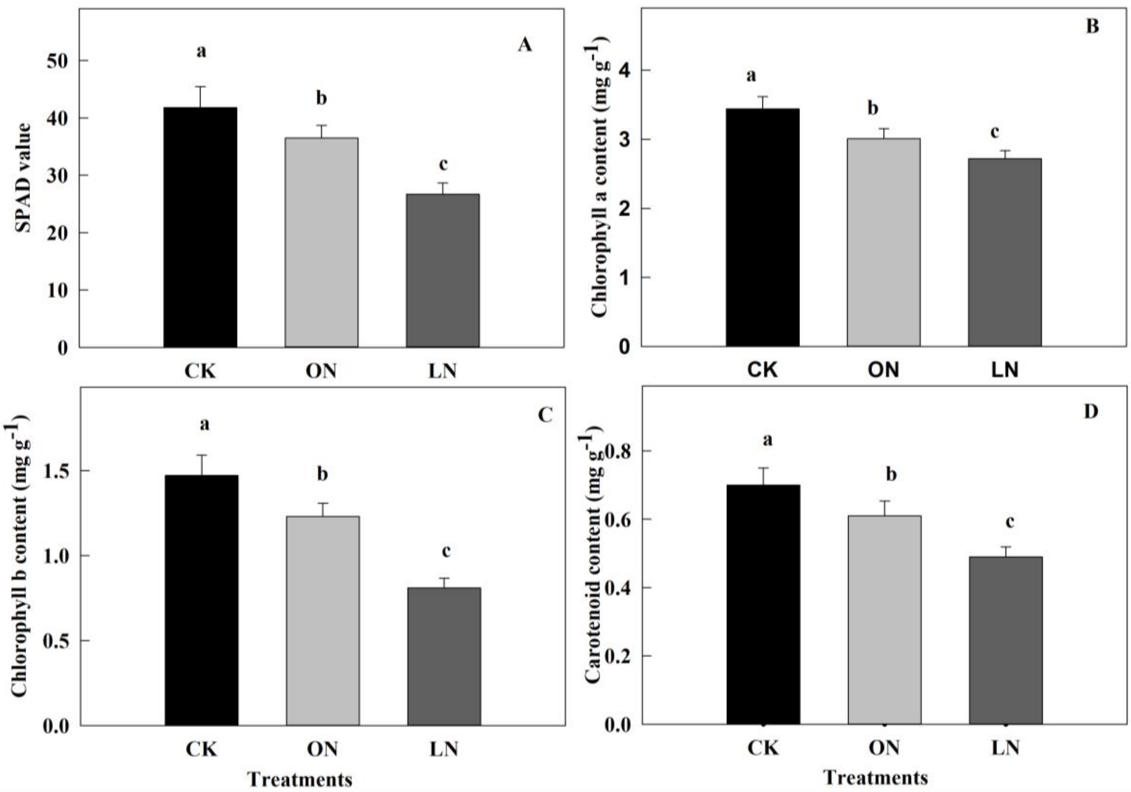

Figure 3. *Comparison in chlorophyll contents of oat leaf grown under different sources and doses of N. SPAD value (A), chlorophyll a content (B), chlorophyll b content (C), carotenoid content (D). Error bars represented standard error of six biological replicates, different letters indicate significant differences ($P < 0.05$). Treatment CK = control, ON = organic N, LN = low N*

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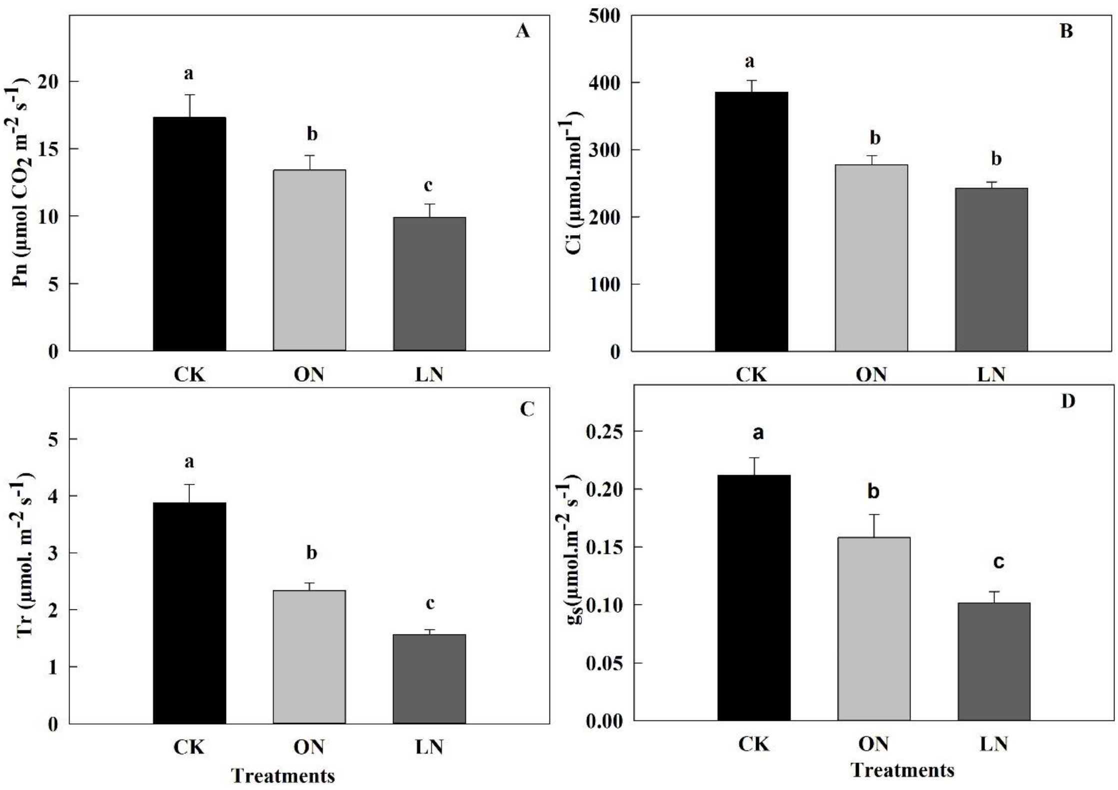

Figure 4. *Trend of photosynthetic parameters in oat leaf under different treatment. Net photosynthetic rate Pn (A), inter cellular CO2, Ci (B), transpiration rate, Tr (C), stomatal conductance, g_s (D) in response to N treatments. Error bars represented standard error of six biological replicates, different letters indicate significant differences (P < 0.05). Treatment CK = control, ON = organic N, LN = low N*

Free amino acids, total soluble proteins, total soluble sugars, amino acid profile and mineral status of oat

Low nitrogen treatment reduced total free amino acids (-26.78% and -18.97%) in shoot as well as root (-36.03% and -25.76%) with respect to CK and ON however, no significance was found between CK and ON (*Fig. 6A, B; Table A7*). Total soluble proteins and sugars in shoot of oat decreased significantly (CK>ON>LN) and vice versa in roots without any significance between CK and ON (*Fig. 6C-F*). Total soluble protein synthesis in shoot was found to be the least under LN treatment having decrements of -73.42% and -48.78% from CK and ON respectively, however, in roots they were increased up to 195% and 99.71% from CK and ON respectively under LN treatment (*Table A7*). Similarly, total soluble sugars in shoot of oat seedling were found to be minimal (-57.73% and -33.87%) whereas highest in roots (159.68% and 80.9%) under LN treatment with respect to CK and ON (*Table A7*). In current study amino acids profile of shoot and root of oat seedlings under CK, ON and LN treatments was generated and found that 18 amino acids (Glu, ser, Gln, Ala, β-Ala, Val, Asp, Asn, Lys, Ile, Gly, Tyr, Trp, His, Leu, Orn, Cit, Tau) in shoot and 15 amino acids (Glu, Ser, Gln, Arg, Ala, β-Ala, Asp, Lys, Gly, Tyr, Leu, Orn, Cit, Tau) in root were decreased in LN treatment as compared to CK; however, Thr increased in both shoot and root of LN seedlings (*Table 2*). Among these amino acids Ala, β-Ala and Lys are the amino acids

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which co-decreased significantly in shoot and root following the order CK>ON>LN (*Table 2*) As far as mineral accumulation in shoot and root of oat seedlings is concerned, P, Mg, Na, Cu, B, Mn and Zn concentration increased while that of K and S decreased in LN root and shoot as compared to CK and ON (*Table 3*).

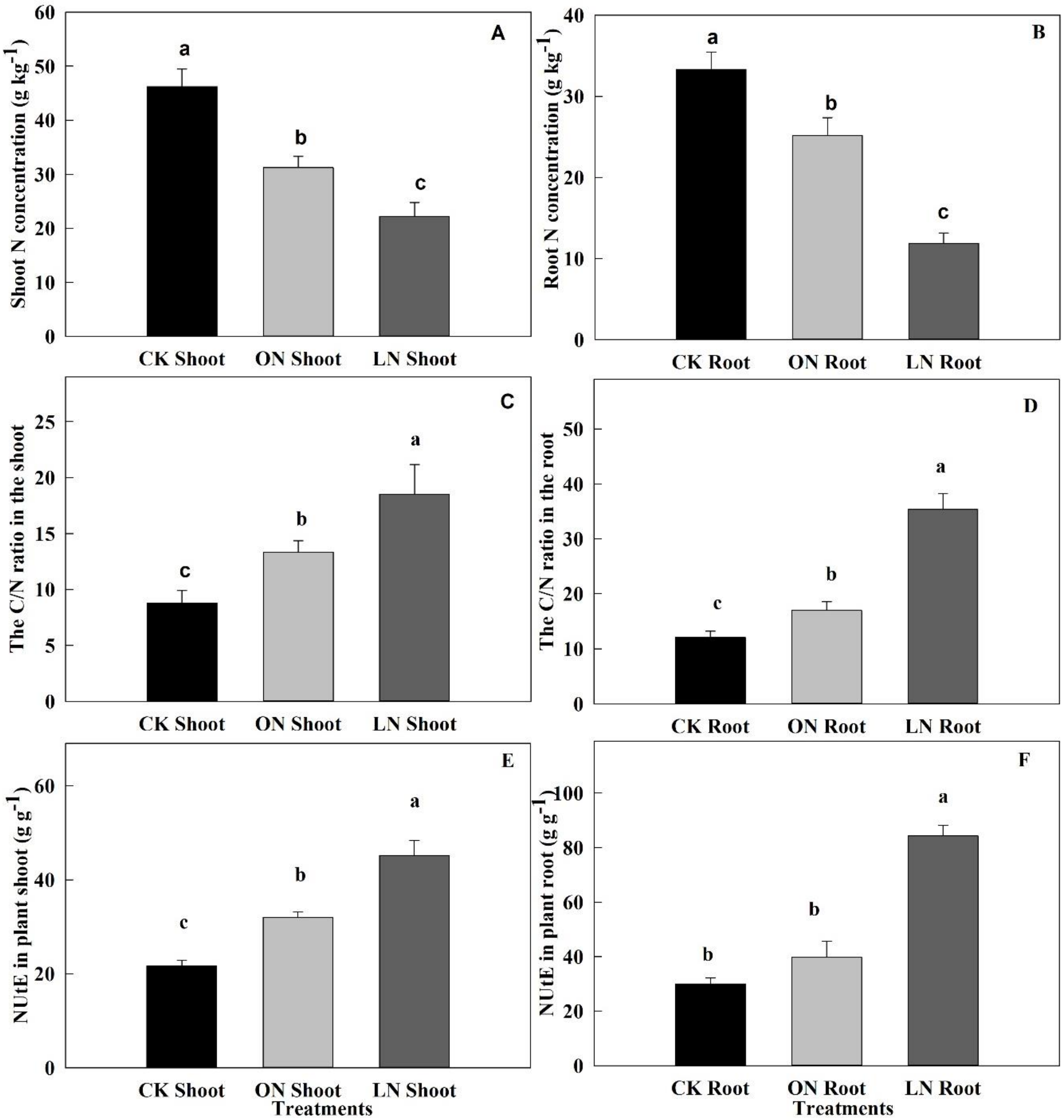

Figure 5. *N accumulation in oat plant parts under different treatment. Shoot N concentration (A), root N concentration (B), C/N ratio in the shoot (C), C/N ratio in the root (D), NUtE in the shoot (E), and NUtE in the root (F) in response to LN. Error bars represented standard error of six biological replicates, different letters indicate significant differences (P < 0.05). Treatment CK = control, ON = organic N, LN = low N*

Alterations in hormone accumulation

Plant growth and development is regulated by plant hormones (Beveridge et al., 1997; Marsch-Martinez and de Folter, 2016). In this study, the concentrations of indole-

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3-acetic acid (IAA/auxin), gibberellic acid (GA3) and zeatin-riboside (ZR; a kind of cytokinins) decreased in shoots of ON as well as LN treated oat seedlings (*Fig. 7A, C, G*). In contrast to shoot, the concentration of aforementioned hormones reversed and increased in roots of LN treated seedlings as compared to CK and ON (*Fig. 7B, D, H*). On the other hand, abscisic acid concentration increased in both ON and LN treated shoots as compared to CK (CK < ON < LN) with LN treated shoots depicting the highest number than the rest and vice versa in roots (*Fig. 7E, F*).

Discussion

Nitrogen is an important macronutrient for plant growth and development. As an imperative component of chlorophyll, it is required for proper functioning of photosynthesis apparatus whereas being a building block of peptides, it is required for protein biosynthesis (Hirel et al., 2007; Wang et al., 2012). Owing to the heterogeneous availability of nitrogen in soil, the adaptation of plants to uptake nitrogen from its immediate vicinity becomes vital (Wang et al., 2007), hence different plants respond to external N availability differentially through physiological, morphological and molecular adaptations. Legumes form nodules to fix atmospheric nitrogen whereas non-legumes like maize, rice and foxtail millet may alter their root architecture in order to explore the nitrogen patches in soil (Postgate, 1998; Wang et al., 2003; Chun et al., 2005a; Nadeem et al., 2018). Long-term nitrogen deficiency hinders plant root and shoot growth (Chun et al., 2005b; Guo et al., 2005; Goron et al., 2015). It is well established that rice and wheat crops produced longer root system under nitrogen deprivation in order to explore as much soil surface as possible to reach nitrogen rich patches (Cai et al., 2012; Guo et al., 2014). Oat can be cultivated in marginal lands with little nutrient and/or water requirements. The morphological and physiological adaptation of oat to chemical nitrogen (CK), organic nitrogen (ON) and low nitrogen (LN) remains unknown to date. In this study, oat produced the longest root system, shortest shoot and smallest leaf area under LN as compared to CK and ON whereas it produced the longer root system, shorter shoot and smaller leaf area under ON with respect to CK (*Figs. 1A-E* and *2A, B*) as a primary response to various nitrogen regimes.

Table 2. *Amino acid concentrations (µmol g^{-1} FW) in shoot and root of oat grown hydroponically low N, organic N and Control (high chemical N) condition*

Amino acid	Shoot			Root		
	CK	ON	LN	CK	ON	LN
Glutamic acidGlu	2·72 ± 0·80a	1·39 ± 0·20b	1·27 ± 0·11b	0·76 ± 0·24a	0·54 ± 0·13b	0·29 ± 0·15c
Serine (Ser)	1·42 ± 0·04a	1·25 ± 0·25b	1·09 ± 0·17c	1·02 ± 0·24a	0·91 ± 0·16a	0·75 ± 0·15b
Glutamine (Gln)	1·12 ± 0·80a	0·78 ± 0·27b	0·63 ± 0·25b	3·61 ± 0·97a	1·85 ± 0·18b	1·68 ± 0·64b
Arginine (Arg)	0·06 ± 0·01a	0·06 ± 0·01a	0·07 ± 0·01a	0·04 ± 0·01c	0·07 ± 0·02b	0·13 ± 0·06a
Alanine (Ala)	1·70 ± 0·04a	1·67 ± 0·66ab	1·17 ± 0·14c	1·25 ± 0·81a	0·62 ± 0·13b	0·45 ± 0·03c
βAlanine (β-Ala)	0.62 ± 0.02a	0.51 ± 0.01b	0.40 ± 0.03c	0.33 ± 0.03a	0.17 ± 0.01b	0.09 ± 0.01c
Valine (Val)	0·27 ± 0·01a	0·18 ± 0·04b	0·18 ± 0·00b	0·69 ± 0·26a	0·47 ± 0·20b	0·61 ± 0·18a
Aspartic acid (Asp)	2.88 ± 0.28a	1.98 ± 0.28b	1.49 ± 0.13c	0·43 ± 0·06a	0·36 ± 0·13ab	0·21 ± 0·01b
Asparagine (Asn)	1·55 ± 0·66a	1·53 ± 0·75a	1·15 ± 0·68b	1·18 ± 0·70a	1·18 ± 0·34a	0·74 ± 0·43b
Lysine (Lys)	0·09 ± 0·00a	0·07 ± 0·01ab	0·03 ± 0·00c	0·06 ± 0·01b	0·09 ± 0·02a	0·03 ± 0·01c
Isoleucine (Ile)	0·07 ± 0·00a	0·05 ± 0·00b	0·023 ± 0·01c	0·45 ± 0·16a	0·43 ± 0·24a	0·32 ± 0·20b
Methionine (Met)	0·03 ± 0·00	0·00 ± 0·00	0·01 ± 0·00	0·1 ± 0·07a	0·03 ± 0·04c	0·07 ± 0·02b
Threonine (Thr)	0·51 ± 0·10c	0·71 ± 0·16b	1.49 ± 0.01a	0·70 ± 0·36c	0·95 ± 0·45b	1·14 ± 0·36a
Glycine (Gly)	0·10 ± 0·01b	0·19 ± 0·05a	0·08 ± 0·02b	0·23 ± 0·06b	0·48 ± 0·05a	0·18 ± 0·02b

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Tyrosine (Tyr)	0·04 ± 0·01a	0·01 ± 0·01b	0·02 ± 0·00b	0·16 ± 0·04a	0·12 ± 0·06b	0·09 ± 0·04b
Tryptophan (Trp)	0·11 ± 0·03a	0·06 ± 0·02b	0·04 ± 0·00b	0·17 ± 0·09a	0·15 ± 0·08b	0·14 ± 0·02b
Phenylalanine (Phe)	0·07 ± 0·00a	0·03 ± 0·01b	0·02 ± 0·01b	0·04 ± 0·02a	0·04 ± 0·02a	0·03 ± 0·00a
Histidine (His)	0·20 ± 0·03a	0·10 ± 0·02b	0·09 ± 0·01b	0·27 ± 0·12a	0·17 ± 0·09b	0·15 ± 0·03b
Leucine (Leu)	0·04 ± 0·01a	0·02 ± 0·001ab	0·01 ± 0·01b	0·61 ± 0·35a	0·45 ± 0·26b	0·06 ± 0·21c
Ornithine (Orn)	0.14 ± 0.01a	0.09 ± 0.02b	0.06 ± 0.00b	0.12 ± 0.01a	0.10 ± 0.02a	0.04 ± 0.00b
Citrulline (Cit)	0.12 ± 0.01a	0.11 ± 0.01a	0.07 ± 0.00b	0.13 ± 0.01a	0.09 ± 0.01b	0.06 ± 0.00b
Taurine (Tau)	0.02 ± 0.00a	0.01 ± 0.00b	0.009 ± 0.00b	0.04 ± 0.01a	0.02 ± 0.001ab	0.01 ± 0.00b

Tissue samples were taken just prior to harvesting. In each column lower case letters indicated the significant differences between different types of treatments at P < 0.05 level. Values show Standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N

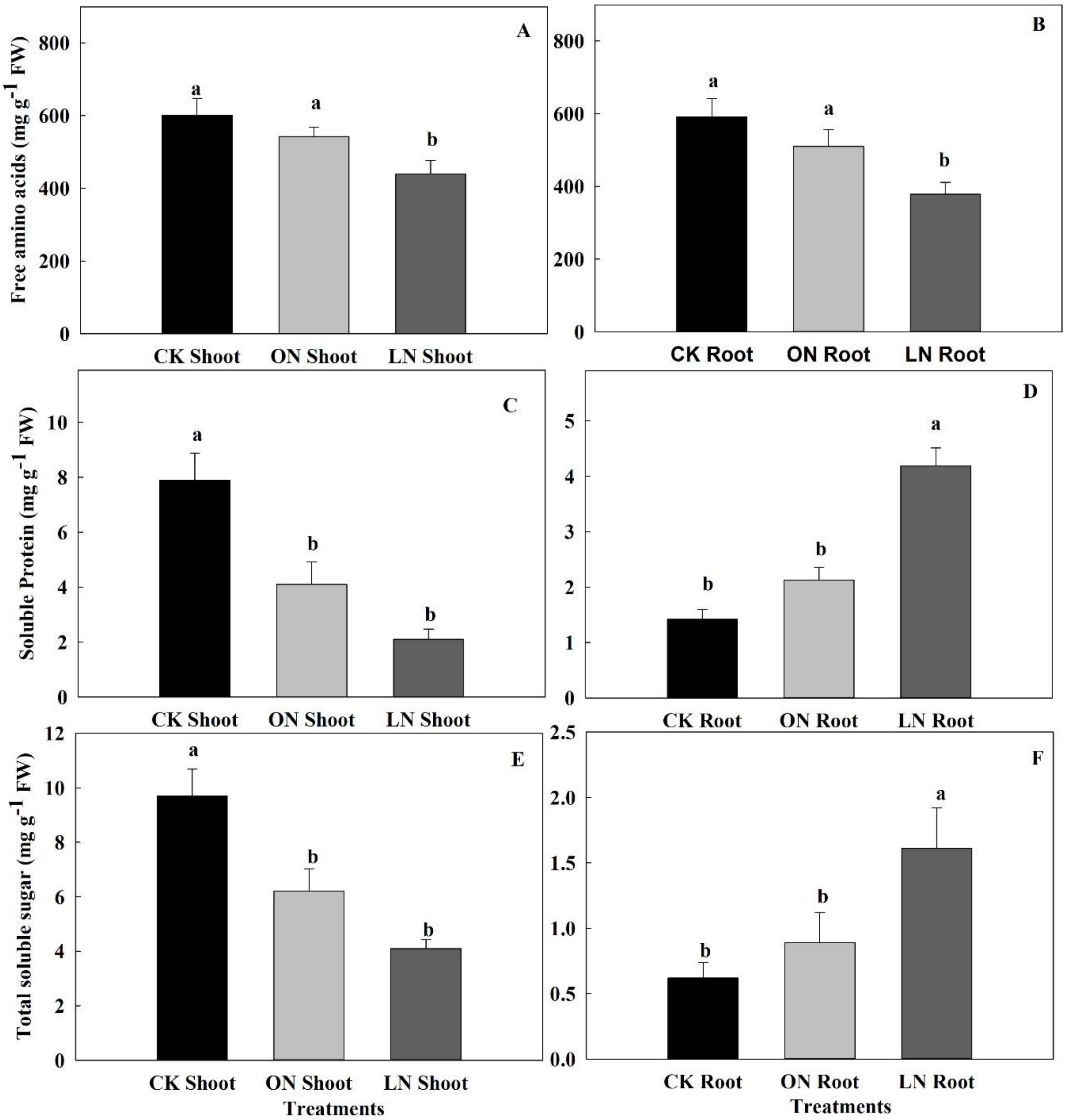

Figure 6. *Concentrations of N metabolites in shoot and root of oat plant under different N treatment, free amino acids (A, B), soluble proteins (C, D), and soluble sugars (E, F). Error bars represented standard error of six biological replicates, different letters indicate significant differences (P < 0.05). Treatment CK = control, ON = organic N, LN = low N*

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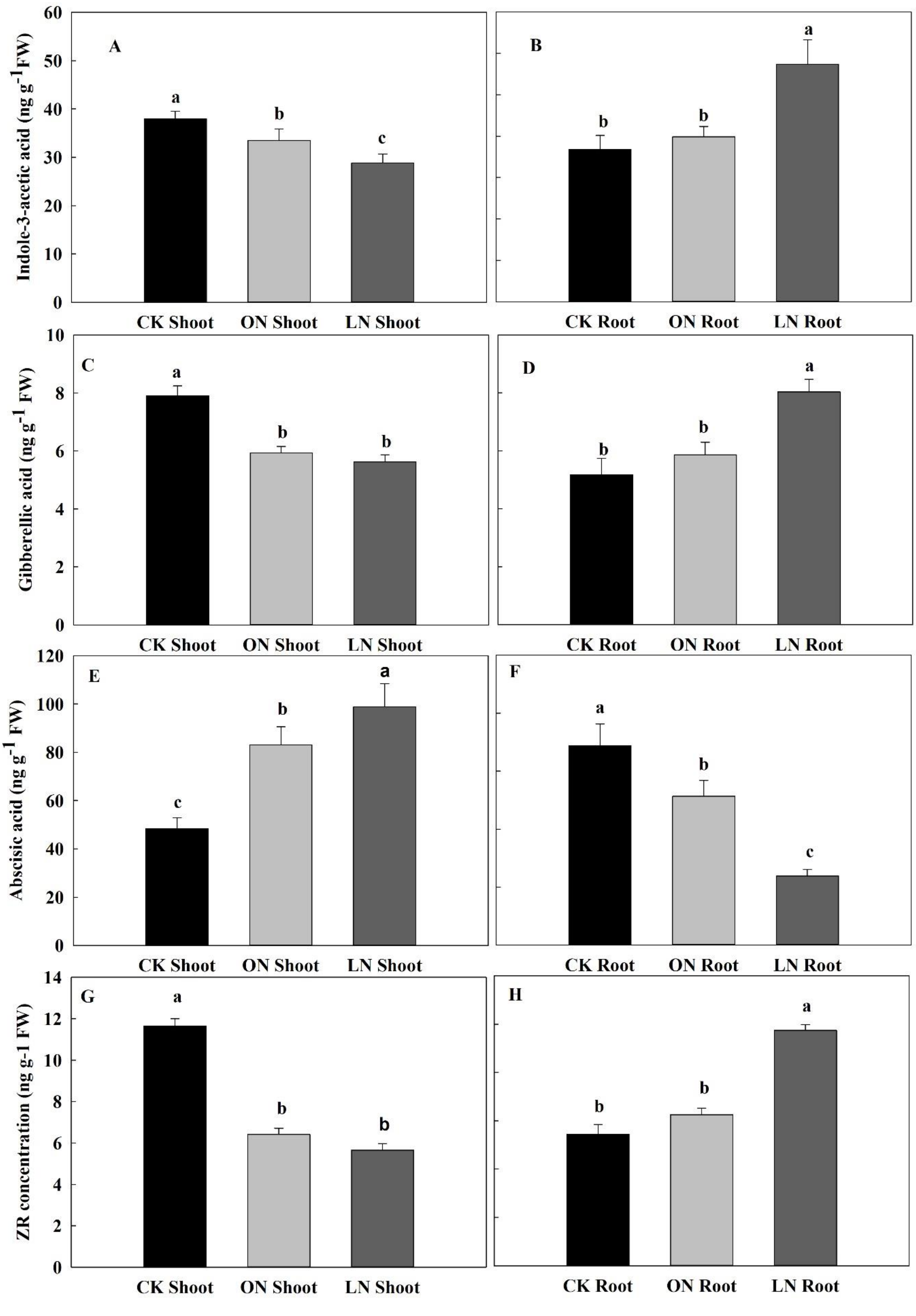

Figure 7. *Effects of N deficiency on hormone accumulation in the oat shoot and root. (A, B) IAA. (C, D). GA. (E, F). ABA, (G, H). ZR. Error bars represented standard error of six biological replicates, different letters indicate significant differences (P < 0.05). Treatment CK = control, ON = organic N, LN = low N*

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Table 3. *Minerals concentration in Shoot and root after harvesting of oat under different treatment*

	Treatment	P	K	Ca	Mg	Fe	Na	Cu	Zn	Mn	S	B
		(mg kg^{-1})										
Shoot	CK	6750c	31900a	2463.3c	1345c	115.83c	131.5c	3.88c	12.82c	10.85c	2698.33a	3.85c
	ON	8016.67b	29583.3b	5300a	1865b	229.5a	330.5b	5.73b	15.27b	18.53b	2451.66b	5.48b
	LN	9133.33a	25600c	5116.67b	1983.3a	170.5b	687.5a	6.25a	20.3a	39.17a	2263.33c	6.91a
Root	CK	5676.54b	17733.15a	2457.52c	1432.9c	256.57c	1508.51c	4.7c	39.66b	14.1c	3915.43a	3.26c
	ON	5838.3ab	12400b	5075a	2592.5b	1105a	3000b	6.65b	42.7b	46.75b	2997.5b	3.91b
	LN	6500a	10625b	2750b	5766.67a	438.83b	5877.5a	13.775a	60a	56.63a	1938.33c	5.84a

In each column lower case lettering is used to show the significant differences between different types of treatments at $P < 0.05$ level. CK = 100% chemical N, ON = 100% organic N and LN = Low N

Large root system, hormonal variations and subsequent biomass accumulation in oat

Nitrogen starvation produced larger root system in maize (Han et al., 2015). Roots are the means by which plants uptake nutrients and these are the organs directly sensing their availability. In current study, oat seedlings exposed to LN treatment produced largest root system in terms of crown root number and length, lateral root number, length and density, and total root length. In ON treated seedlings, lateral root number, length and total root length increased as compared to CK only (*Table 1*). The enhancement of total root system of oat under low nitrogen and lateral root system under ON could be due to increased carbon allocation from shoot towards the root as shown by subsequently decreased shoot dry weight and increased root dry weight and root to shoot ratio under ON and LN treatments (*Fig. 2C-E*). Consistent with the previous studies (Han et al., 2015; Nadeem et al., 2018) the specific root length (SRL) of oat was found to be 6227 cm g^{-1}, 5760 cm g^{-1}, and 7145 cm g^{-1} under CK, ON and LN treatments respectively. Extremely large SRL of oat under LN indicated its adaptation and primary response strategy to explore nitrogen under nitrogen limitation conditions. On one hand the increased total root length under LN was due to increased crown root number and length, and lateral root number and length whereas on the other hand it could also be due to decreased average root diameter (*Table 1*) which illustrated the longitudinal growth of roots. It is well established now that if only NO_3^- used as a source of N (no NH_4^+) LN caused reduced total root length including crown and lateral root length and number in maize (Wang et al., 2005; Tian et al., 2008; Gao etal.,2015). Hence the differential response of oat to ON treatment in terms of root system architecture remained consistent with previous findings and it could be the innate response of oat where different plant species respond to different nitrogen sources differentially. A significant positive correlation was revealed between concentration of P, Mg, Cu, Mn, Zn, B and Na in root tissue with root morphological traits (*Table A8*). These attributes possibly influenced the ion balance within oat root leading to alterations in root morphology which needs to be further investigated in future. The most crucial role played by hormones is to regulate the speed of growth and development of the individual parts of the plant to adapt the surrounding environment (Wolters and Jürgens, 2009). Auxin is the most essential hormone which modulates different physiological processes of the plants, i.e. cell division, elongation and differentiation of different parts of plant (Reed, 2001) including root growth and development regulation (Jiang and Feldman, 2003). Gibberellic acids have crucial role of plant organ differentiation and development, regulation of the tall phenotypic character of plants (Yamaguchi, 2008), shoot internode extension, enlargement of leaf surface area and enhancement of apical dominance. Cytokinins help to regulate the mobilization of carbohydrate and N signaling (Ashikari et al., 2005; Sakakibara, 2006). On the other hand, the special cytokinin "zeatin-riboside" helps in the bio-synthesis and maintenance of auxin (Jones et al., 2010). IAA, GA3 and ZR decreased in ON and LN shoot whereas increased in respective roots in this study (*Fig. 7A-D, G-H*). This finding is in accordance to shorter shoot and larger root system observed in ON and LN treated oat seedlings (*Fig. 2A; Table 1*). ABA stimulates plant capacity to cell division or intermediates adaptive responses to the stress environment as well as N starvation (Chin and Beevers, 1970; Santner et al., 2009). This study found increased ABA concentration in LN shoot followed by ON and CK shoot whereas, in total contrast to shoot, the concentration of ABA totally reversed in roots of oat seedlings having least concentration under LN followed by ON and CK (*Fig. 7E, F*). This finding illustrated that oat roots were not under stress in LN condition and can act as the basis to develop plant varieties which can adapt to stress conditions efficiently.

Nitrogen concentration, mineral accumulation and photosynthesis

Chlorophyll (Chl) concentration in plant leaves affects photosynthesis by regulating photosynthetic capacity and ultimately plant growth (Li et al., 2018). The most important source of energy required for plant growth is photosynthesis (Baker, 2008). A photosynthetic reaction is generally split into three steps: primary reaction, electron transport and photophosphorylation, and carbon assimilation. Chl a and Chl b are the most essential pigments to complete primary reaction. Chl a can absorb red to orange light whereas Chl b absorbs blue to purple light. SPAD value, Chl a, Chl b and total carotenoids decreased under ON and LN treatment in current study (*Fig. 3A-D*) which resulted in subsequent decreases in net photosynthesis (Pn), intercellular carbon dioxide (Ci), transpiration rate (Tr) and stomatal conductance (gs) (*Fig. 4A-D*). The reason to this could be the decreased N concentration in shoot of oat under ON and LN treatment (*Fig. 5A*) which could have hindered chlorophyll biosynthesis and disturbed the functioning of photosynthesis apparatus. These findings are consistent with previous study in sorghum (Muchow and Sinclair, 1994) and corn (Zhao et al., 2003; Long et al., 2004). Another possibility could have been the gradually reduced leaf area due to less N accumulation under ON and LN (*Fig. 1B*) which provided less surface area for light absorption and hence resulted in malfunctioned photosynthetic bio-machinery similar to maize (Zhao et al., 2003). Another reason which could have led to decreased photosynthesis was the reduction of potassium (K) concentration and increment in sodium (Na) concentration in LN shoot (*Table 3*). Lower the ratio of K/Na, lower will be the opening of stomata (Willmer et al., 1983) which could have reduced stomatal conductance in current study gradually from CK to LN conditions.

Nitrogen metabolism, carbon accumulation and re-allocation

In the process of inorganic N assimilation amides and amino acids are the first stable products (Yang et al., 2015) and act as the building blocks for proteins. The changes in several amino acids concentration or total free amino acids accumulation regulates many processes in plants including the N metabolism process and storage (Miller and Cramer, 2005; Cañas et al., 2009). The free amino acids concentration in the plant shoot

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

and root tissues has been reported to fluctuate significantly with different N levels and sources. A higher N dose can boost the concentration of total free amino-acid in leaves of many plant species (Yang et al., 2015). The total free amino acids concentration in wheat root was dramatically decreased due removal of the N from the nutrient solution (Rodgers and Barneix, 1989) but drastically increased upon resupply of NH_4^+ or NO_3^- the N-deficient plants (Causin and Barneix, 1993). Free amino acids concentration in shoot and root of oat seedlings in current study decreased in ON and LN treatments (*Fig. 6A, B*) which is consistent with related low nitrogen concentrations in shoot and root under ON and LN (*Fig. 5A, B*) resulting in lesser biosynthesis of amino acids. Reduced free amino acids concentration resulted in reduced soluble proteins biosynthesis in shoot under ON and LN treatments respectively (*Fig. 6C*). Similar trend was observed for total soluble sugars concentration in shoot under ON and LN respectively (*Fig. 6E*). In contrast to shoot, total soluble sugars and total soluble proteins concentrations increased in root of oat seedling under both ON and LN treatment (*Fig. 6D, F*) probably due to the co-transport of carbon and amino acids to nitrogen deficient root. This phenomenon resulted in increased carbon and nitrogen metabolites accumulation in nitrogen deficient root leading to a larger root system (*Fig. 1B, D, E*) to cope nitrogen limitation. There occurred a positive correlation of total soluble proteins and total soluble sugars with shoot and root dry weight (*Fig. 8B, C, E, F*) whereas the correlation of free amino acids with shoot dry weight was positive but that of with root dry weight remained negative (*Fig. 8A, B*) which again emphasized our argument of possible co-transport of carbon and N-metabolites to N-deficient root as an adaptive response to cope low nitrogen.

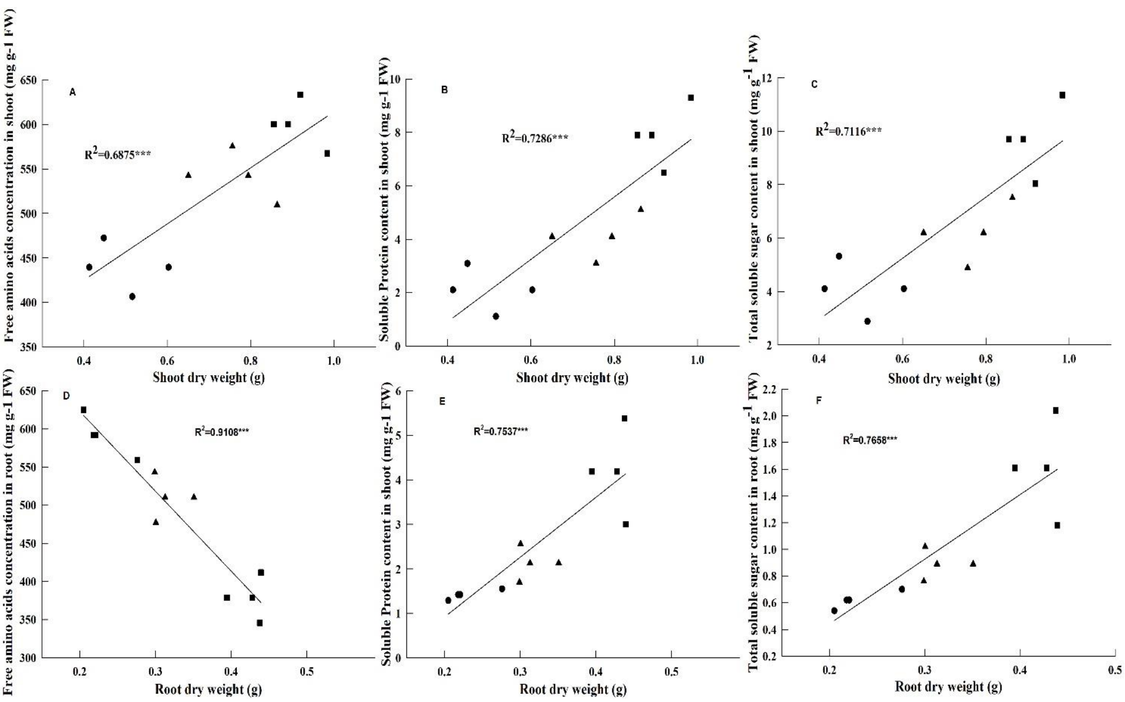

Figure 8. *Relation between N metabolites and root and shoot dry weight of oat. Free amino acids and shoot dry weight (A), SOLUBLE protein and shoot dry weight (B), soluble sugar and shoot dry weight (C), free amino acids and root dry weight (D), soluble protein and root dry weight (E) and soluble sugar and root dry weight (F). Values were obtained from four replicates*

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Furthermore, free amino acids to N concentration ratio, total soluble proteins to N concentration ratio and total soluble sugars to N concentration ratio significantly increased in LN roots (*Table 4*) which further elucidated the shoot to root transport of carbon and nitrogen metabolites in order to let oat roots acclimatize to N-deprivation. In the mechanism of ammonium assimilation, the first product is glutamine and glutamate is its precursor whereas asparagine, aspartate and serine generally form the dominant pool of the amino acids in the leaves of many plants (Yang et al., 2015). Sometimes other amino acids may also become dominant depending on the plant species and N concentration of the surrounding environment (Xu et al., 2012; Pratelli and Pilot, 2014). Particularly, Gln and Asn are the most crucial mediators for synthesis and integration of different amino acids and also storage proteins (Miller and Cramer, 2005; Seebauer et al., 2004). Lower N concentration in shoot and root might have contributed to decreased biosynthesis of Gln (*Table 2*). Similar was the case with Asn in both shoot and root which could have altered protein synthesis negatively as revealed by decreased soluble proteins concentration in the LN shoot (*Table 2, Fig. 6C*). Altogether, the amino acid profile depicted a complicated regulation of N-metabolism which needs to be further dissected in future studies. shoot due to Carbon to nitrogen ratio was the highest in shoot as well as root under LN followed by ON treatment (*Fig. 5C, D*). Similar was the trend of nitrogen utilization efficiency (NUtE) (*Fig. 5E, F*). These results further suggested the higher accumulation of carbon in shoot and root and subsequent re-allocation within the oat tissues as the N availability shifted from CK to LN conditions.

Table 4. *Nitrogen metabolites/ N concentration ratio in oat shoot and root after harvesting*

	Treatment	Free amino acids/N concentration ratio	Soluble protein/N concentration ratio	Soluble sugar/N concentration ratio
Shoot	CK	12.99 ± 0.5c	0.17 ± 0.01a	0.21 ± 0.01a
	ON	17.37 ± 0.84b	0.13 ± 0.01ab	0.2 ± 0.01a
	LN	19.88 ± 1.83a	0.096 ± 0.01b	0.19 ± 0.02a
Root	CK	17.76 ± 0.75b	0.043 ± 0.001b	0.019 ± 0.001b
	ON	20.52 ± 1.61b	0.085 ± 0.001b	0.04 ± 0.002b
	LN	31.95 ± 1.4 a	0.35 ± 0.04a	0.14 ± 0.01a

In each column lower case lettering is used to show the significant differences between different types of treatments at $P < 0.05$ level. Values show Standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N

Conclusion

Nitrogen, a vital macronutrient for crop growth and development, is often subjected to losses through various means and causes environmental hazards through its excessive deposition in the ecosystem. Hence, nitrogen use efficient crops can minimize the use of chemical nitrogen and thus reverse the environmental deteriorations. In this study, Oat (a cereal crop) was studied for its adaptation to different nitrogen regimes (CK, ON, LN). Root dry weight, R/S ratio, C/N ratio and NUtE increased under LN treatment which indicated the allocation of carbon to continuously growing root as suggested by increased crown root number and length, lateral root number and length, and total root length under low nitrogen. Highest levels of total soluble proteins and total soluble sugars were in total contrast to lowest N concentration in roots under LN which further emphasized the carbon provision of roots at the cost of minimum photosynthesis.

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Furthermore, highest levels of IAA, GA3 and ZR in Low nitrogen roots was perhaps the innate adaptive strategy of oat allowing the roots to expand under low nitrogen stress as shown by highest level of abscisic acid. Altogether, these findings suggested that oat is an extremely nitrogen use efficient crop which can be well adapted to N-poor soils.

Acknowledgements. We thank Lili Chen, Yubin Zhao, Nurun Nabi, Ashiqur Rahman, Faisal Hayat Maken and all the lab-fellows and workers for their kind help throughout the experiment. This study was supported by the China Agriculture Research System (CARS-08-B-1)

Author contributions. TAK, FN, ZZ, and YH designed the experiment. TAK, FN, YG, and YY, performed the research. TAK and FN analyzed the data and wrote the manuscript. XW, ZZ and YH revised the manuscript. All authors approved the final manuscript.

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

APPENDIX

Table A1. *Percentage changes in the shoot height and leaf area of oat*

Treatment	Shoot length (cm)	Percentage change (%)			Leaf area plant^{-1} (cm^{-2})	Percentage change (%)		
		CK to ON	CK to LN	ON to LN		CK to ON	CK to LN	ON to LN
CK	58.75 ± 4.11a				18.3 ± 1.6a			
ON	50 ± 2.58 b	-14.89	-28.09	-15.5	15.2 ± 0.97b	-16.94	-51.37	-41.45
LN	42.25 ± 2.63c				8.9 ± 0.81c			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

Table A2. *Percentage changes in the shoot dry weight, root dry weight and root shoot ratio*

Treatment	Shoot dry weight (g)	Percentage change (%)			Root dry weight (g)	Percentage change (%)			Root/shoot ratio	Percentage change (%)		
		CK to ON	CK to LN	ON to LN		ON to CK	LN to CK	LN to ON		CK to ON	CK to LN	ON to LN
CK	0.91 ± 0.03a				0.23 ± 0.02c				0.252 ± 0.01c			
ON	0.77 ± 0.04b	15.38	-45.77	-35.75	0.32 ± 0.02b	39.13	82.61	31.25	0.41 ± 0.04b	62.7	241.27 (3.41 fold more)	109.76 (2.1 fold more)
LN	0.49 ± 0.04c				0.42 ± 0.02a				0.86 ± 0.06a			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

Table A3. *Percentage changes in the N concentration C/N ratio, N utilization efficiency in the shoot and root of oat*

	Treatment	N concentration (g kg^{-1})	Percentage change (%)			C/N ratio	Percentage change (%)			NUtE	Percentage change (%)		
			CK to ON	CK to LN	ON to LN		CK to ON	CK to LN	ON to LN		CK to ON	CK to LN	ON to LN
	CK	46.2 ± 3.23a				8.76 ± 1.12c				21.65 ± 1.19c			
Shoot	ON	31.23 ± 2.09b	-32.4	-52.01	-29.01	13.32 ± 1.04b	52.05	111.42	39.04	32.02 ± 1.16b	47.9	108.36	40.88
	LN	22.17 ± 2.64c				18.52 ± 2.64a				45.11 ± 3.19a			
	CK	33.33 ± 2.12a				12.08 ± 1.12c				30 ± 2.19c			
Root	ON	25.2 ± 2.19b	-24.39	-64.39	-52.9	16.97 ± 1.57b	40.48	193.23	108.78	39.68 ± 5.97b	32.27	180.9	112.37
	LN	11.87 ± 1.32c				35.43 ± 2.78a				84.27 ± 3.82a			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

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DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Table A4. *Percentage changes in the crown root number, lateral root number and lateral root density under different treatment*

Treatment	Crown root number	Percentage change (%)			Lateral root number	Percentage change (%)			Lateral root density	Percentage change (%)		
		CK to ON	CK to LN	ON to LN		ON to CK	LN to CK	LN to ON		CK to ON	CK to LN	ON to LN
CK	7.5 ± 0.87b	40	96.67	40.47	1736.17 ± 145.2c	15.22	34.1	16.39	7.8 ± 0.98b	35.9	128.21	67.92
ON	10.5 ± 1.08b				2000.4 ± 123.3b				10.6 ± 1.76b			
LN	14.75 ± 2.2a				2328.17 ± 155.8a				17.8 ± 2.01a			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

Table A5. *Percentage changes in the crown root length, lateral root length and total root length under different treatment*

Crown root length (cm)	Percentage change (%)			Lateral root length (cm)	Percentage change (%)			Total root length (cm)	Percentage change (%)		
	CK to ON	CK to LN	ON to LN		ON to CK	LN to CK	LN to ON		CK to ON	CK to LN	ON to LN
143.34 ± 10.56b	22.95	116.27	75.91	1288.16 ± 82.2c	29.43	111.57	63.46	1431.5 ± 97.36c	28.78	112.3	64.65
176.23 ± 13.98b				1667.22 ± 102.1b				1843.45 ± 131.23b			
310 ± 17.76a				2725.3 ± 178.3a				3035.33 ± 198.3a			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

Table A6. *Percentage changes in the chlorophyll a, chlorophyll b and carotenoid in oat leaf under different treatment*

Treatment	Chlorophyll a content/mg g^{-1} FW	Percentage change (%)			Chlorophyll b content/mg g^{-1} FW	Percentage change (%)			Carotenoid content/mg g^{-1} FW	Percentage change (%)		
		CK to ON	CK to LN	ON to LN		ON to CK	LN to CK	LN to ON		CK to ON	CK to LN	ON to LN
CK	3.44 ± 0.09a	-12.5	-20.93	-9.63	1.47 ± 0.12a	-16.36	-44.9	-34.15	0.7 ± 0.02a	-12.86	-30	-19.67
ON	3.01 ± 0.07b				1.23 ± 0.08b				0.61 ± 0.02b			
LN	2.72 ± 0.05c				0.81 ± 0.06c				0.49 ± 0.01c			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] × 100, CK to LN = [(value under LN – Value under CK)/Value under CK] × 100, ON to LN = [(value under LN – Value under ON)/Value under ON] × 100

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

Table A7. *Percentage changes in the concentration of free amino acids, total soluble protein and soluble sugars in the shoot and root of oat*

	Treatment	Free amino acids (mg g^{-1} FW)	Percentage change (%)			Total soluble protein (mg g^{-1} FW)	Percentage change (%)			Total soluble sugar (mg g^{-1} FW)	Percentage change (%)		
			CK to ON	CK to LN	ON to LN		CK to ON	CK to LN	ON to LN		CK to ON	CK to LN	ON to LN
Shoot	CK	600.29 ± 47.4a	-9.6	-26.78	-18.97	7.9 ± 0.99a	-48.1	-73.42	-48.78	9.7 ± 0.98a	-36.08	-57.73	-33.87
	ON	542.46 ± 26.3a				4.1 ± 0.82b				6.2 ± 0..83b			
	LN	439.56 ± 37.3b				2.1 ± 0.38b				4.1 ± 0.33b			
Root	CK	592.04 ± 49.25a	-13.83	-36.03	-25.76	1.42 ± 0.18b	50	195	99.71	0.62 ± 0.12b	43.54	159.68	80.9
	ON	510.14 ± 45.96a				2.13 ± 0.2b				0.89 ± 0..23b			
	LN	378.71 ± 32.66b				4.19 ± 0.32a				1.61 ± 0.31a			

Different letters after the values within the same column indicated significant differences (P < 0.05). Values show Standard errors (SE) ± mean of four replicates. CK = 100% chemical N, ON = 100% organic N and LN = Low N**.** Percentage change CK to ON = [(value under ON – Value under CK)/Value under CK] * 100, CK to LN = [(value under LN – Value under CK)/Value under CK] * 100, ON to LN = [(value under LN – Value under ON)/Value under ON] * 100

Table A8. *Correlation coefficient between root morphological traits and nutrient concentration in oat plant root*

Nutrient	Crown root number	Seminal root number	Seminal root density	Crown root length	Lateral root length	Total root length	Average root diameter
P	0.656*	0.54	0.619*	0.579*	0.662*	0.651*	0.56
K	-0.713**	-0.785**	-0.718**	-0.731**	-0.749**	-0.759**	-0.853**
Ca	-0.12	0.05	-0.15	-0.22	-0.16	-0.17	0.001
Mg	0.907**	0.877**	0.902**	0.956**	0.967**	0.977**	0.963**
Fe	-0.017	0.14	-0.04	-0.13	-0.06	-0.07	0.10
Cu	0.925**	0.868**	0.922**	0.958**	0.976**	0.983**	0.953**
Mn	0.764**	0.803**	0.759**	0.776**	0.804**	0.814**	0.905**
Zn	0.808**	0.706*	0.819**	0.918**	0.886**	0.904**	0.897**
B	0.925**	0.877**	0.916**	0.938**	0.969**	0.973**	0.941**
S	-0.817**	-0.773**	-0.822**	0-.886**	-0.891**	-0.907**	-0.942**
Na	0.878**	0.899**	0.866**	0.927**	0.941**	0.954**	0.954**

Tissue samples were taken just prior to harvesting. Significant decreases or increases were indicted by ‘*’or ‘**’. *P < 0.05; **P < 0.01

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46314653

THE IMPORTANCE OF SOIL CONDITIONERS CONTROLLING BACTERIAL BIODIVERSITY OF VEGETABLE FIELDS UNDER CONTINUOUS CROPPING

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(Received 17th Dec 2018; accepted 20th Feb 2019)

Abstract. A soil-borne fungal parasite (*Plasmodiophora brassicae Woron*) has regularly caused 50-60% reductions in the seasonal yield of a 35 years old cabbage plantation under garlic revaccination in China. We investigated the impact of soil conditioners on soil chemical properties and bacterial communities in order to recommend actions to address increasing frequencies of root-resistant cabbage infections. The results showed urease and polyphenol oxidase activities increased by 10.4-15.8% in the topsoil after two-years of quicklime and organic fertilizer application. High throughput sequencing data showed significant increases in the relative abundance of bacteria with potential for biocontrol (*Xanthomonadales*, *Pseudomonas*, and *Bacillus*) under lime and bioorganic fertilizer. Also, according to a forward-selection CANOCO analysis, in this case the two most important variables contributing to the variation in bacterial communities' composition were soil organic matter and total nitrogen.
Keywords: *soil-borne parasites, antibiotic bacteria, high throughput sequencing, lime, soil enzyme activities*

Introduction

Agricultural cropping systems are intensive and involve crop rotations among a limited number of species under specific climatic conditions in China (Chen et al., 2015). Extensive intensification of cropping has been achieved through high inputs of chemical fertilizers, which, in turn, seriously degraded soil physicochemistry and reduced the diversity and abundance of soil bacteria communities (Xiong et al., 2015). Continuous cropping can decrease soil pH from neutral to acidic levels (Wang et al., 2014), and can increase root exudates, such as amino acids, phenolic acids, root cap cells, cellular debris, etc. (Wei et al., 2015b). These changes to agricultural soils and their bacterial communities increase the vulnerability of crops to pests and diseases (Yin et al., 2010). Consequently soil-borne parasites become particularly difficult to control with alternative pesticide techniques due to the long-term survival of resistant spores and eggs in the soil (Fukuta et al., 2013). Manipulation of soil conditioners is one potential approach to address soil-borne parasites. As the capacity to process soil nutrients varies among groups of microorganisms, soil fertilization and acidity manipulation can influence growth and competitiveness of microorganism in different ways, and therefore affect the diversity, biomass, and activity of the soil microbial community. Chagnon and Bradley (2013) provide evidence that the relative availability of soil nitrogen (N) and phosphorus (P; i.e., soil nutrient stoichiometry) controls the competitive balance between these two fungal guilds. Species richness and functional group richness affected community stoichiometry, especially by increasing C:P and N:P ratios. Compost application to agricultural fields is an excellent natural approach to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

fighting plant pathogens. Lime products have been used to destroy *Salmonella* or other pathogens present in soil and manure, and do not require any specialized equipment to apply, so lime can be recommended for temporal use, e.g., for rapidly destroying *Salmonella* or other pathogens. Lime amendments can be beneficial to acidic soils, which, in any case, require regular lime amendments (Dowling et al., 2015). Quicklime application can improved the effects of mechanical soil aeration, which can restrain pathogenic microorganism growth by reducing soil moisture and enhancing soil permeability (Wei et al., 2015a).

Microorganisms respond quickly to modifications in the vegetation or soil properties. Understanding the relationships between soil conditioners and microbial community structure and composition may significantly contribute to our knowledge regarding the development of better fertilizer regimes for extensive vegetable cropping systems (Glaser et al., 2015). When compared with traditional techniques for determination of the biological diversity, such as Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) , fluorescence *in situ* hybridization (FISH), and terminal restriction fragment length poly-morphisms (T-RFLP), high-throughput sequencing technologies can significantly expand investigations into species and functional diversity of microbial populations (Diaz et al., 2012).

As stated above, it appears that both soil acidity and nutrient levels can be manipulated to control fungal pathogens and improve the bacterial diversity and biomass in agricultural fields. In the agricultural site studied here, a soil-borne fungal parasite (*Plasmodiophora brassicae Woron*) often causes 50-60% seasonal yield losses for the autumn cabbage crop in 35-year revaccination. We tested the hypothesis that: Soil conditioners effective in controlling pathogens may favor the development of bacteria with potential for biocontrol. We assessed the potential of these alternative control methods in soil-borne parasites and looked for significant insights into species and functional diversity of microbial populations.

Materials and methods

Experimental site and crop management

This experiment was carried out on a field site located in Baicheng County of Acheng City in Heilongjiang Province, China (45°32′N, 126°58′E and altitude 151 m). The site soil is classified as fine Mollisols Albolls Argialbolls Argiaquic. The experimental site is located within a temperate continental monsoon climate with mean annual precipitation of 600 mm and mean temperature of 4°C. For at least 35 years before the experiment (see *Table 1*), the field site crop rotation had involved early garlic (*Allium sativum L*, April to July), late Chinese cabbage (*Brassica campestris L. spp*, July to October) and winter fallow (October to April). Continuous cropping changed the soil physicochemical properties and microbial community structure and has resulted in the establishment of a fungal pathogen.

Field experiment design

This two-year experimental manipulation of soil conditioners controlling (lime, Effective Microorganisms EM, bio-organic fertilizer) soil remediation under a continuous cropping system started in 2014, and involved six soil treatments. The six treatments were located on adjacent plots and included: (1) untilled soil (NTS) within an

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

undeveloped field, means healthy soil; (2) continuously tilled soil (CTS) within a field with continuous crop production growing garlic and cabbage with raw chicken manure application (*Table 1*); In order to discuss the effects of soil conditioners on soil restoration under a 35 years old cabbage plantation under garlic revaccination. (3)-(6) treatments were set. It is forbidden to use raw chicken manure during the whole growth period. Chemical fertilizers are normally applied in accordance with conventional planting practices. (3) organic fertilizer treatment (OFT), based on a field with continuous crop production growing garlic and cabbage with base fertilizer and 1500 kg/ha bio-organic fertilizer application; (4) quicklime and organic fertilizer treatment (LOF), within a field with continuous crop production growing garlic and cabbage with 4000 kg/ha quicklime and 1500 kg/ha bio-organic fertilizer application; (5) quicklime treatment (LIM), within a field with continuous crop production growing garlic and cabbage with 4000 kg/ha quicklime (equivalent to CaO 2026 kg/ha) application; (6) EM treatment (EMT) within a field with continuous crop production growing garlic and cabbage with 5000 ml/ha EMT diluted 100 times and sprayed onto the soil after garlic and Chinese cabbage harvest. The EMT treatment also involved a supplemental application at the bulbil differentiation stage of garlic and the rosette stage of Chinese cabbage. Bio-organic fertilizer (decomposed biological organic fertilizer with microbial fungicide) included moisture content 30.0%, organic matter 25.5%, N% 2.69%, P_2O_5% 0.96%, K_2O 0.49%, bacillus bacillus and bacillus granulus ≥0.2 billions/gram. Besides the untilled soil treatment, others treatments applied the same concentration chemical fertilizer (667 m^2 Garlic, N 15 kg, P_2 O_5 20 kg, K_2 O 15 kg; 667 m^2 Chinese cabbage, N 15 kg, P_2 O_5 7 kg, K_2 O 12 kg;). The experiment was laid out as a randomized design of the six treatments with three replicates. Each plot was 60 m^2 in area and surrounded by a 30 cm buffer to prevent the water and nutrient exchange between adjacent plots.

Table 1. *Cropping model and manure input before experimental field*

Schedule	Cropping method and manure input	Planting method and density
Late April	Grown early garlic, The proper 42% compound fertilizer (N-P_2O_5-K_20=20:8:14) application and dressing ratios for garlic(Allium Sativum L.) were 600kg /ha and 2:1.	Chinese cabbage was planting by plot, with the width of 2.2 meter x length 100 meter. It is generally advisable to plant 3,800 plants/667 m^2.
Early July	Harvested early garlic	
Middle July	Applied 37500 kg/ha raw chicken manure	Garlic Seeding density using traditional narrow ridge system
Late July	Grown late Chinese cabbage, Fertilizer applied N 124 kg/ha, P_2O_5 90 kg/ha, K_2O 150 kg/ha. Chemical fertilizers are 46 % urea, 46 % triple-superphodate, 50 % potassium sulfate	45 cm x 6 cm, 24,700 plants/667 m^2
Early October	Harvested late Chinese cabbage	
Middle October	Applied 46875 kg/ha raw chicken manure and winter fallow until next April	

The nutrient content of raw chicken manure: Moisture content 50.5%; Organic matter 25.5%; N% 1.63%; P_2O_5 % 1.54%; K_2O 0.85%

Soil sampling

Soil samples were collected in October 2015 after the Chinese cabbage harvest. Soil samples, up to a depth of 0.60 m, were collected using an 85 mm diameter soil corer and divided into three depth layers (0–0.20, 0.20–0.40, and 0.40–0.60 m). For each treatment, three replicate samples were collected according to a completely randomized

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

design. Each replicate was composed of three subsamples, which were mixed and transported to the laboratory on the same day. Soil samples for microbiological analysis were subsequently wet sieved (4 mm mesh sieve) and stored at -80°C.

Soil chemical properties and microbial biomass

The air-dried soil sieved by (0.25 mm) was analyzed for organic C by vitriol acid potassium dichromate oxidation method (SOC), total N by the Kjeldahl method (TN) and total P by the $HClO_4$-H_2SO_4 method (TP), and soil pH was measured in a 1:2.5 (soil:water) mixture using the potentiometric method. Other parameters and specific analysis methods were determined as described by Forster (1995). Microbial biomass C (C_{mic}) and biomass N (N_{mic}) contents were determined on the 4°C component using the chloroform-fumigation–extraction method (Vance et al., 1987). Extracts were filtered through Whatman no.42 filter paper and analyzed for organic C content with an elemental analyzer (TOC-VCPH/CPN, Shimadzu, Kyoto, Japan).

Soil Enzyme activity measurements

In order to investigate changes to soil biological functions, activities of key enzymes were determined immediately after air-drying soil (<1 mm). Soil urease (EC 3.5.1.5) activity (URE) was measured by incubating 5 g of soil in 0.5 M urea as substrate in 0.1 M phosphate buffer at pH 7.1 under 37°C for 2 h. The formation of ammonium was determined spectrophotometrically at 578 nm and URE activity was expressed as μg NH_4–N g^{-1} soil h^{-1} (Nannipieri et al., 1974). Soil sucrase (EC 3.2.1.26) activity (SUC) was measured by incubating 3 g soil (d. w.) according to Kandeler et al. (1999). The activity of SUC was expressed as mg glucose g^{-1} soil h^{-1} (Kandeler et al., 1999). Soil catalase (EC 1.11.1.6) (CAT) was measured by incubating 5 g soil in 5 ml of a 0.3% H_2O_2 solution for 30 min at 30°C. The suspension was titrated with a 0.1 mol L^{-1} $KMnO_4$ solution. The activity of CAT was expressed as 0.1 mol L^{-1} $KMnO_4$ ml g^{-1} soil 30 min^{-1} (Author, 1964). Polyphenol oxidase (EC 1.10.3.1) activity (PPO) was determined as described by Peruccia (2000), and expressed as mg purpurogallin g^{-1} soil 2 h^{-1}. Determinations for all soil samples were performed in triplicate, and all values were reported as averages of the three determinations. All rates were standardized to oven-dried soil mass.

Soil DNA extraction and PCR amplification

Microbial DNA was extracted from soil samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer' s suggested protocols. For each sample three-four subsamples were independently taken and went through the extraction. Then the extracts from one sample were pooled together and the extracted DNA was stored at -20°C until the subsequent sequencing procedure. The V4-V5 region of the bacteria 16S ribosomal RNA gene was amplified by PCR (95°C for 3 min, followed by 27 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min) using primers (515F 5'-barcode-GTGCCAGCMGCCGCGG)-3' and 907R 5'-CCGTCAATTCMTTTRAGTTT-3', where 'barcode' is an eight-base sequence unique to each sample. PCR reactions were performed from triplicate 20 μL mixtures containing 4 μL of 5 ×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA GelExtraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer' s instructions. After extraction, amplicons were quantified using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar quantities and paired-end sequenced (2×250) on an Illumina MiSeq platform according to standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP******).

Sequence data processing

Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17) according to the following criteria: Operational Units (OTUs) were clustered using UPARSE (version 7.1 http://drive5.com/uparse/) and a 97% similarity cutoff. Chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was determined through comparisons with the Silva (SSU115) 16S rRNA database using RDP Classifier (http://rdp.cme.msu.edu/) and a confidence threshold of 70% (Amato et al., 2013).

Data analysis

Treatment effects were tested for through analysis of variance (ANOVA) using SPSA17.0, and differences among treatments were compared using Duncan' s multiple range test ($P < 0.05$). The community richness index and community diversity index were calculated as well as data preprocessing, operational taxonomic unit-based analysis and hypothesis tests were performed using mothur (http://www.mothur.org/). The histogram was created using OriginPro 10.1. Variation in community composition across the different treatments as analyzed using a principal coordinate analysis (PCoA) performed using CANOCO 4.5. Multivariate analyses investigating the relationships between environmental variables (OM, TN, available P, NH_4, and pH) and community composition were performed through a redundancy analysis (RDA) using CANOCO 4.5. The RDA invoked a manual forward-selection procedure to identify the significance of environmental variables ($P<0.05$) using a Monte Carlo test.

Results

Continuous cropping with two crops in a monoculture system caused Chinese cabbage root disease and decline of yield

Plasmodiophora brassicae Woron is a slime fungus. The dormant sporangia forms in the host cell, spherical or ovate, thin, colorless, single cell, size 4.6 to 6.0×1.6 to 4.6 (μm), germination produces swimming spores. The Spore is pear-shaped or spherical, 2.5 to 3.5 μm in diameter. From the root hair of cabbage to the host cell, after a series of evolution and expansion, it enters the formation layer from the root cortex, stimulates the division and expansion of the host parenchyma cells, causing the root system to form a tumor, and finally the pathogen forms a large number of dormant sporangia in the host cell. After the tumor decays, dormant sporangia enter the soil for wintering. Bacteria hibernate sporangia in the soil or adhere to the seeds over the winter, and can survive in the soil for 10 to 15 years. The sporangia spreads through rainwater, irrigation

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water, pests, and agricultural operations, and the germination produces swimming spores that invade the host. After about 10 days, the roots grow tumors. Bacteria can develop at 9 to 30°C, with a suitable temperature of 23°C, a suitable relative humidity of 50% to 98%, and a suitable pH 6.2. Soil water content is less than 45% and pH is greater than 7.2 or more, then pathogenic bacteria die. The disease first appears scattered in fields, but in successive seasons it will infect the entire field, reducing the yield significantly of garlic and resulting in no yield at all of cabbage in the CTS treatment (*Table 2*). The addition of agricultural lime and nutrition management is the common method for its control. But the mechanic of reducing the occurrence of cabbage clubroot in already-infected fields is not clear and this can be discussed in the next sections.

Table 2. *Soil properties in different tillage and fertilization treatments*

Treatments	pH (1:2.5H_2O)	Total N (mg/kg)	Total P (mg/kg)	Available N(mg/kg)	Available P(mg/kg)	Organic Matter (g/kg)	Garlic yield (t/ha)	Chinese Cabbage yield (t/ha)
CTS	5.90±0.08e	1.61±0.02c	1.47±0.01b	189.06±5.32a	54.4±0.98ab	23.58±0.27c	9.17±0.05e	null
NTS	6.98±0.06a	1.90±0.03a	1.52±0.03b	200.16±7.65a	53.1±1.23ab	37.16±0.70a	Null	null
EMT	6.14±0.07d	1.68±0.09bc	1.48±0.05b	197.23±2.31a	55.1±2.69ab	25.12±0.46c	12.53±0.02d	62.5±4.3a
LIM	6.57±0.10bc	1.75±0.06bc	1.59±0.05ab	194.43±6.15a	51.3±1.31b	24.66±0.30c	14.06±0.12b	69.8±5.5a
LOF	6.72±0.07b	1.78±0.03ab	1.69±0.06a	196.15±3.98a	52.2±2.32ab	25.23±0.37c	15.11±0.01a	74.3±4.3a
OFT	6.39±0.04c	1.81±0.06ab	1.69±0.09a	197.45±4.32a	56.4±1.69a	28.69±1.19b	13.28±0.19c	66.7±4.1a
ANOVA P-Values	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

NTS represents untilled soil, CTS represents continuously tilled soil, OFT represents organic fertilizer treatment, LOF represents quicklime and organic fertilizer treatment, LIM represents quicklime treatment, EMT represents effective microorganisms treatment. Values are mean±standard deviation (n=3). Values within the same column followed by the different letters indicate significant difference (Duncan’ s multiple range test (P＜0.05))

Effects of continuous ecological factors controlling on soil chemical properties

In this content, the soil conditioners controlling (Lime, EM, bio-organic fertilizer) significantly influenced ($P<0.05$) soil properties including soil pH, the concentration of TN, OM and so on (*Table 2*). Soil pH of the CTS treatment was significantly lower than for all other treatments, with the greatest difference (18.3%) occurring between the CTS treatment and NTS treatment. Compared with the CTS, all the fertilizer and regulators treatments significantly increased the garlic yield and total N, whereas TP, available P, available N and OM did not significantly differ among CTS, EMT, and LIM. There was no significant difference observed among all treatments for the Chinese cabbage yield. Changes to soil properties may affect soil enzyme activities and the composition and abundance of bacterial communities, which were further examined using qPCR techniques and the Illumina MiSeq platform.

Soil enzyme activities

Soil enzyme activities were significantly influenced by soil depth (*Figure 1*). The EMT treatment had a particularly strong stimulatory effects on PPO, URE, and SUC activities, achieving 89.7%, 15.4%, and 11.4% increases in activity respectively in shallow soil layer (0-20 cm) when compared with activities in the same soil layer for the

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CTS treatment. Likewise, Lime and organic fertilizer boosted URE, SUC, and PPO activities. The NTS treatment increased URE, SUC, and PPO activities, resulting in a 53.0%, 54.7%, and 183.4% increase in activity, respectively, in the shallow soil layer (0-20 cm), when compared with activities in the same soil layer for the CTS treatment. On the other hand, the CTS treatment stimulated CAT activity by 49.0% compared to under the NTS treatment. Among all treatments, URE and PPO activities were highest under the LOF treatment, where they were 3.84% and 22.5% greater, respectively than under the OFT. The URE and PPO activities under the LOF treatment were also 6.96% and 33.7% higher, respectively than under the LIM treatment. Under the LIM treatment, SUC activity was 2.90% and 6.46% greater than under the LOF and OFT, respectively. Under the OFT, SUC activity was 9.22% higher than under the EMT. CAT activity was highest under the EMT, followed by LIM, OFT, and then LOF.

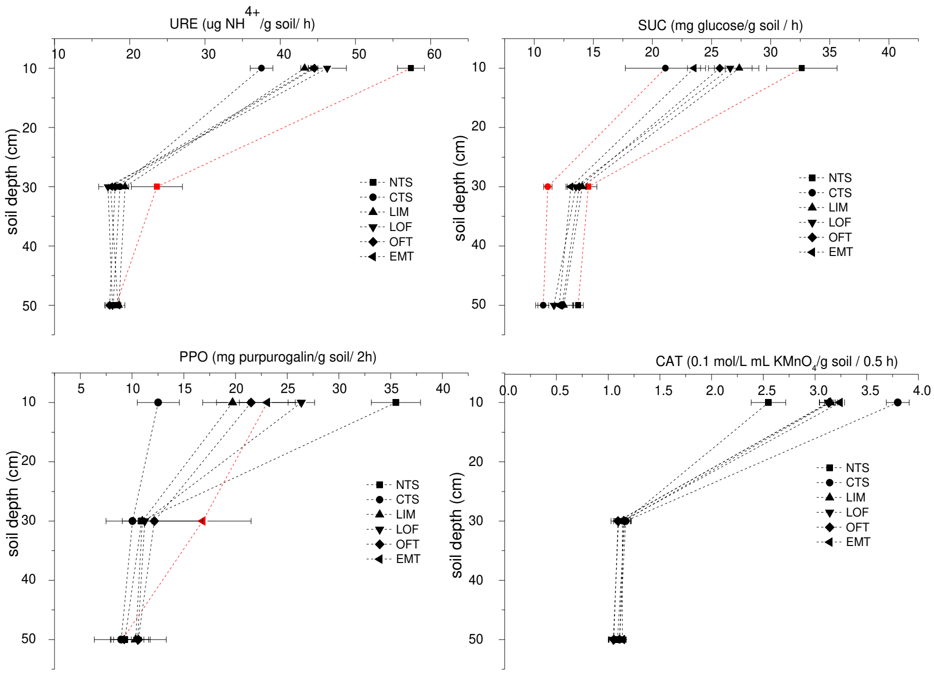

Figure 1. *Soil URE, SUC, PPO, CAT activities depending on soil depth and different treatments. Errors bars represent standard error(n=3)*

The activities of URE, SUC and PPO were consistently higher in the shallow soil layer (0-20 cm) than in the middle (20-40 cm) or deep layer (40-60 cm). SUC activity in 20-40 cm soil layer was 29.9% higher than in the 40-60 cm soil layer. Within the middle soil layer (20-40 cm), SUC activity was highest under the NTS treatment, followed, in order of decreasing SUC activity by LIM, OFT, LOF, EMT, and then CTS. The activities of SUC, PPO, and CAT did not significantly differ between the middle and deep soil layers.

In the shallow soil layer, the soil enzyme activities significant correlated with the soil chemical properties (see *Table A1*). There was a positive correlation between the activities of hydrolytic enzymes and pH and total N. We also found significant reductions in catalase activity with increasing levels of organic matter and total N.

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Microbial C and N

Different treatments caused a shift of the soil microbial biomass carbon and nitrogen in soil samples (*Figure 2*). The organic fertilizer and EM inoculant addition increased C_{mic} and N_{mic} contents relative to the CTS, EM inoculant addition resulted in the highest soil C_{mic} and N_{mic} contents. The addition of the organic fertilizer did increase C_{mic}, but N_{mic} in the LOF treatment was higher than the OFT treatment under the same input of organic fertilizer. In this study, the C_{mic}/N_{mic} ratio was the highest in the treatments of CTS and LIM followed by the OFT and EMT; the other two treatments were significantly lower (*Figure 2*). Fertilizer input type and soil pH showed significant impacts on C_{mic}, N_{mic} and C_{mic}/N_{mic} ratio. The higher rate of input significantly increased both C_{mic} and N_{mic} and decreased the C_{mic}/N_{mic} ratio. There was a significant interaction between input type and level for C_{mic} and C_{mic}/N_{mic}.

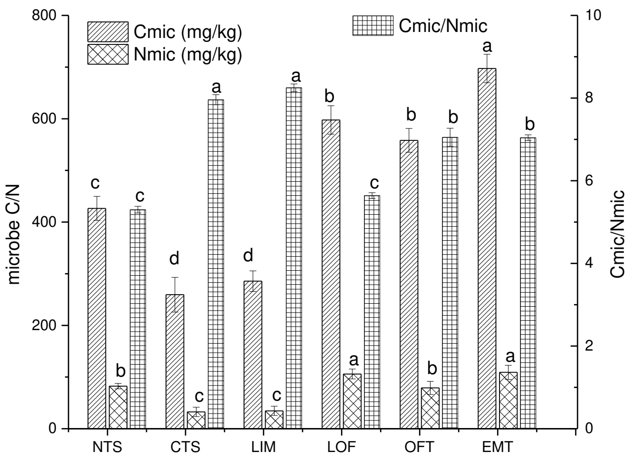

Figure 2. *Soil microbial biomass (Cmic and Nmic) in different treatments determined by the chloroform-fumigation–extraction method. Error bars indicate the standard deviation of relative biomass between three duplicate samples. The same column followed by the different letters indicate significant difference (LSD, P<0.05)*

Bacterial community composition and diversity

After two years of soil nutrition management 16S rRNA high-throughput sequencing showed bacterial community profiles to markedly differ among treatments. The Illumina-based analysis of the V3 region of the 16S rRNA gene resulted in recovery of 118, 101 high quality sequences from the six soil treatments (*Table 3*). More than 17,000 valid reads were obtained for each replicate through a sequence optimization process, and the bacterial community richness index was calculated. After quality control filtering, median sequence length of each read was 396 bp (*Table A2*). The Good' s coverage values (*Table 3*) exceeded 98% at a 97% similarity cutoff, indicating that sequence reads were sufficient to capture the bacterial diversity within these soils. Based on Mothur clustering, the number of OTUs within soil samples ranged from 919-1364, depending on treatments. The lowest number of total OTUs was observed under the NTS treatment. The highest Shannon and Chao 1 indices occurred under the EMT and OFT treatments, which suggested that bio-organic fertilizer facilitated bacterial community diversity. Rank-abundance curves can be used to explain species abundance

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and evenness of species distribution. The Rank-abundance curves for the six soil samples were shown in *Figure 3*, and it can be seen that these six curves were not flat at the end, which reflected the uneven distribution of species. The asymptote of the OFT, EMT, and LIM treatment curves appeared to occur at higher x-axis values than that of the NTS treatment curve, which indicated that bacterial community abundance in the treatments of OFT, EMT, and LIM treatments were significantly higher than the treatment of NTS. The highest bacterial abundance occurred in the treatment of OFT. Thus, two-year continuous controlling cropping increased the bacteria community diversity.

Table 3. *Summary of the pyrosequencing sequencing data and diversity estimates*

Sample	Number of reads	97% sequence identity			
		Number of OTUS	Good's coverage ratio C(%)	Shannon index (H')	CHAO1 estimator
CTS	17450	1179	98.6	5.92	1393
EMT	17209	1287	98.5	6.12	1445
LIM	19993	1285	98.9	5.95	1427
LOF	19048	1199	98.6	5.43	1397
NTS	26386	919	99.4	4.82	1032
OFT	18015	1364	98.5	6.19	1558

Chao: community richness, Coverage: sampling coverage, Shannon: community diversity

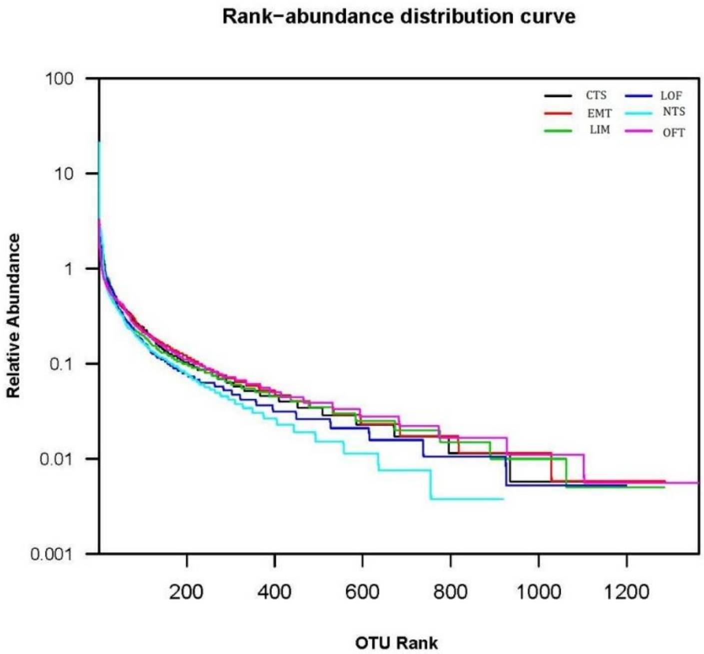

Figure 3. *OTU Rank-abundance curves for the OTUs in the soil samples of different treatments*

All sequences were classified into 51 phyla or groups using Mothur program. The overall bacterial composition was relatively consistent across treatments, whereas the distribution of each phylum or group varied among treatments (*Figure 4*). Under all treatments, *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Actinobacteria* were the five most dominant phyla, accounting for > 70% of the total

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reads. Significantly more unclassified species were detected under the CTS, EMT, LIM, LOF, and OFT treatments, which was in accordance with the higher diversity indices for these treatments. Compared with the other treatments, soils from the EMT had significantly higher percentages of *Actinobacteria* (1.3–6.5-fold), *Armatimonadetes* (1.2–7.3-fold), *WA3* (1.0–4.8-fold), and *Chlorobi* (1.6–34.5-fold), and lower percentages of *Planctomycetes* (CTS: 5.6%, EMT: 5.1%, LIM: 5.8%, NTS: 6.8%, LOF: 3.8%, and OFT: 6.1%). The percentages of *Actinobacteria* and *Firmicutes* were lowest in soils from the LOF treatment. More *Cyanobacteria* were detected in soils from the CTS treatment than from the LIM or LOF treatments, and the lowest number of *Cyanobacteria* was found in soil from the NTS treatment. A higher percentage of *Lysobacter* was found in NTS than it in other treatments. All fertilization treatments significantly impacted bacterial community structure. The abundance of *Lysobacter*, *RB41*, *Steroidobacter*, and *Planctomycetaceae* was lower in the NTS than in the LOF treatment soil. Compared with the CTS, *Xanthomonas*, *Oxalobacteraceae*, *Lineage_IIb*, and *Nitrospira* relative abundances increased in the EMT and OFT treatment soils, whereas *Haliangium* and *Sphingomonas* relative abundances decreased under these two treatments. The relative abundance of all 50 detected genera is listed in *Table A3*. *Lysobacter*, *Rhodospirillaceae*, *Comamonadaceae*, *KCM-B-112*, *Xanthobacteraceae*, and *Sporocytophaga* abundances were significantly greater in NTS treatment soil compared with CTS treatment soil, whereas *Acidobacteriaceae*, *Arenimonas*, *Sorangium*, *Rhizobium*, *Lineage_IIb,* and *Flavobacterium* abundances were higher in CTS than in NTS.

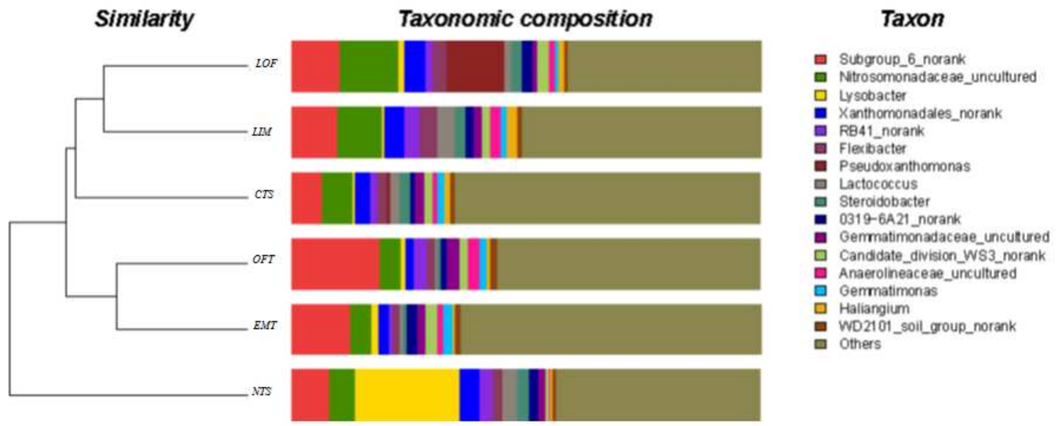

Figure 4. *Comparison of the bacterial communities at the gene level. The branch structure is used to describe and compare similarities and differences between multiple treatments. The hierarchical clustering according to beta diversity distance matrix (Hierarchical clustering) analysis. A tree structure was constructed by using the unweighted pair group method with arithmetic mean (UPGMA) algorithm*

On a genus level, all 412 detected genera were observed in soils from all treatments, except for *Xanthomonas,* which was not detected in NTS or LIM treatment soils, and *Sporocytophaga,* which was not detected in EMT or LOF treatment soils. Results of the cluster analysis for the 100 most abundant genera (assessed through counts of OTUs) within soil samples from the six treatments are displayed on a heatmap (*Figure A3*). Soils from the six treatments were classified into two groups (continuous cropping and untilled soil) according to their bacteria genera composition (*Figure 4*). The continuous

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cropping group (EMT, OFT, CTS, LIM, and LOF) subdivided into a bio-fertilizer and non-bio-fertilizer group. The untilled soil group was made up of only the NTS treatment. The taxonomic information for the OTUs was used to develop the phylogeny. The phylogenetic classification was used to analyze the relationships between microbial ancestry and abundance within soil samples from the different treatments.

In order to further investigate bacterial community composition among the different treatments, we performed a PCoA on the relative abundance of bacterial genera (*Figure 5*). Data are presented as a 2D plot to better illustrate the relationships. The PCoA clearly separated CTS from NTS. Values for LIM, LOF, EMT, and OFT grouped together along the PC1 axis. Although EMT and OFT were relatively similar, the different bio-organic fertilizer significantly affected the soil microbial community. NTS had a significantly higher PC1 value, and EMT had a higher PC2 value than other treatments.

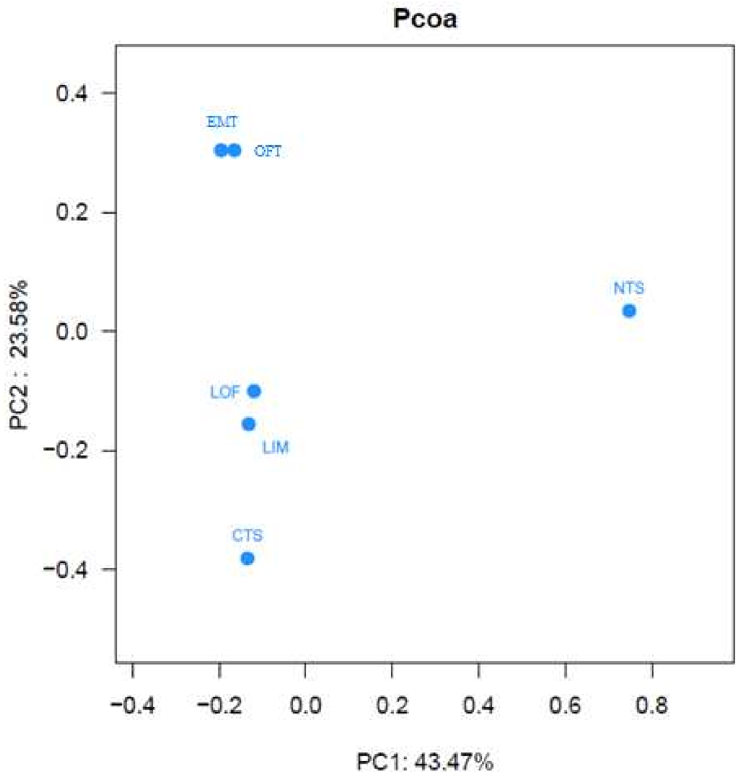

Figure 5. *Principal co-ordinates analysis (Pcoa) on the relative abundance of bacterial genera. Principal components (PCs) 1 and 2 explained 43.47% and 23.5% of the variance, respectively*

Relationships between bacterial community composition and environmental variables

According to the forward-selection CANOCO, soil organic matter and total N concentration, which accounted for 27.4% and 1.75% of the variation in bacteria community composition, were the two most important contributors to the variation among bacterial communities (*Figure 6*). All environmental variables together explained 79.3% of the variation in microbial communities composition among treatments. The soil properties, in order of decreasing influence on the bacterial community composition were: OM, TN, GY, TP, pH, CCY, available N, available P.

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The correlation (or covariance, depending on the standardization option used in the analysis) between phyla and environmental variables can be approximated through projections of two perpendicular vectors, representing each of the phyla and environmental variable. Most of the abundant phyla were significantly correlated with at least one environmental factor. However, *Sphingomonas*, *Nitrospira*, *Haliangium*, *Myxococcales*, *Flexibacter*, and *Nitrosomonadaceae*were did not significantly correlated with any of the environmental factors. The abundances of *Chitinophagaceae*, *Aquicella*, *Xanthomonadales*, *Solimona*, *Bacillus*, *Gaiella*, *Piscinibacter*, *Gemmatimonadaceae* were positively correlated with soil TN, OM, pH, and available N, whereas *Planctomycetaceae*, *Comamonadaceae*, *Lysobacter*, *Xanthobacteraceae*, and *Sporocytophaga* were positively correlated with TP. The phylum was correlate with garlic yield and Chinese cabbage yield, for some orders, such as *Nitrosomonadaceae*, *Haliangium*, *Flexibacter*, etc.

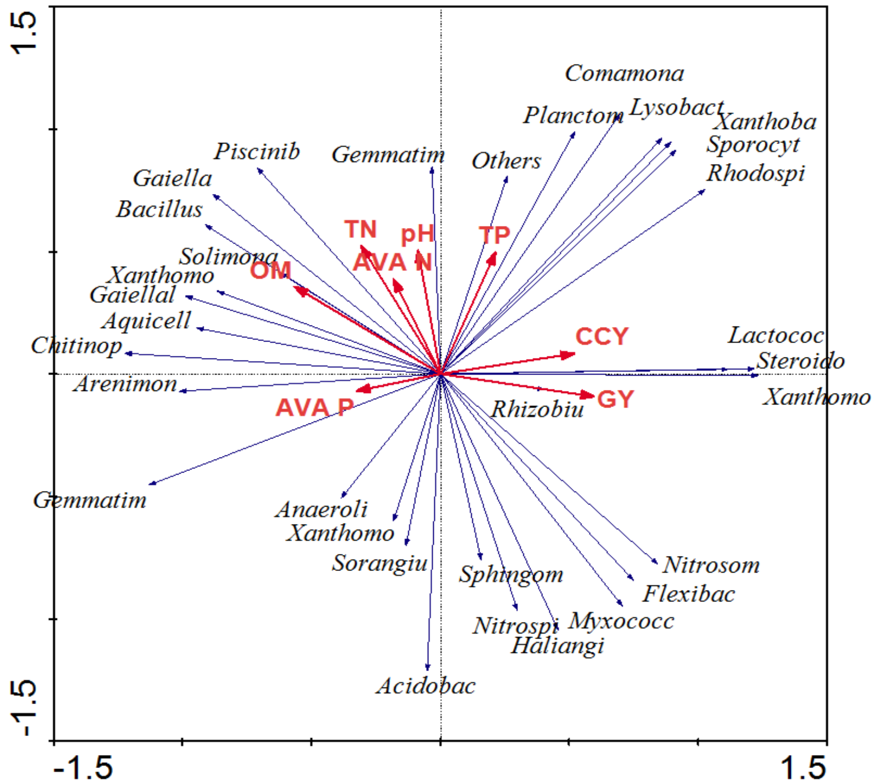

Figure 6. *RDA of soil bacterial communities and soil characteristics for each treatment. Soil factors indicated in red line include AVA P (available phosphorus), AVA N (available N), OM (organic matter), TN (total nitrogen), TP (total phosphorus), GY (garlic yield), CCY (Chinese cabbage yield), pH*

Discussion

This research approach provides insight into how soil chemical and biological properties, total bacterial community structure and soil extracellular enzyme activities respond to the contrasting lime, bioorganic fertilizer and EM to control and suppress the clubroot. Short -term soil conditioners significantly impacted soil nutrient availability, and in turn affected soil microbial community structure and function. Bacterial

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community diversity, biomass and enzyme activities involved in nutrient transformations were enhanced in treatments receiving annual applications of soil conditioners and chemical fertilizers.

Continuous cropping with two crops in a monoculture system caused root disease and decreased soil nutrient levels and pH

This Clubroot is a major issue when brassica crops are grown in short rotations (McGrann et al., 2016). Clubroot has spread rapidly across the major Chinese brassica growing regions and become established as a serious threat to production (Shakeel et al., 2016).

Many indicators of soil quality can be manipulated through long-term raw manure and mineral fertilization under monoculture continuous cropping systems. Continuous cultivation over 35 years can cause reductions in soil pH, TN, and OM compared with NTS treatment soil. Long-term raw manure fertilizer application and monoculture cropping can decrease soil pH (Wang et al., 2015). Previous studies have found the magnitude of raw manure fertilization effects on microbial biomass to be pH-dependent, which agrees well with the results reported in this study. This was also observed in our trial where microbial biomass increased with amendments application rate, regardless of the input type (*Figure 2*). The mechanism underlying declines in pH and nutrient levels with long-term monoculture cropping may be an accumulation of organic acids caused by repeated raw manure application, and leading to that in an accumulation of organic acids which decrease the ability to keep water and fertilizer deteriorated soil structure. This is consistent with the previous reports (Gaind and Singh, 2016). In this case, the similar effects on available nutrients can be concluded from the data. These findings suggest that soil pH influenced microbial activity resulting in different rates of N and P mineralized, which is supported by the variation in activities of enzymes involved in N and P cycling (e.g. urease and phosphatase) observed in this study.

Effects of soil amendments on soil pH and nutrients

It is difficult to control clubroot because the pathogen survives long periods in the soil for a long time as resting spores. Previous research has provided evidence that soil pH can influence clubroot, and liming arose as a conventional technique to control the clubroot through its influences on soil pH (Chai et al., 2014).

Niwa et al. (2007) found neutralization of soil using $Ca(OH)_2$ and $CaCO_3$ to suppress clubroot infections. Clubroot is suppressed effectively under soil of neutral pH in the presence of calcium, which has led to the hypothesis that either spore germination or root-hair infection, each part of the primary phase of the clubroot life cycle, is inhibited under such conditions (Niwa et al., 2008). In addition to these abiotic factors, it has been suggested that the microbial community can contribute to clubroot suppression. Ruaro et al found a negative correlation between severity of clubroot infection and foliage N, Ca, Mg, and B concentrations and a reduction in infection when soil was adjusted to pH 6.5 and treated with calcium nitrate (Ruaro et al., 2009). Therefore, in soils with abundant nutrients and nearly neutral pH levels, the infection of host plants with the clubroot fungus is suppressed. In this case, pH and total N and SOM content increased markedly contrasted in LOF ,OFT and EMT. Available nutrient and carbon concentrations affected the physiology of the host plant and subsequent disease development.

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

Soil amendment strategies increased bacterial richness and enzyme activities

Otherwise the untilled soil(NTS), contrasted with infected soil(CTS, LIM, LOF, EMT and OFT), is healthy and retained higher biomass. In terms of microbial activity, functional diversity was suppressed in the diseased soil samples. Activities of soil enzymes, including urease, phosphatase, and sucrose, were also reduced in infected soils. Soil urease, phosphatase, and catalase activities were determined in order to assess the potential turnover rates of soil carbon and nitrogen (Zhang et al., 2016). Enzymatic activities in diseased soils were inhibited by 45.1 to 169.2% in the LOF, EMT, OFT, LIM and CTS. In our study, the higher soil microbial biomass under the EM and organic fertilizer treatments may have been due to the addition of microbial population in the treatment and the additional C, which activates the soil indigenous microbiota. The lime was added into the infected soil that resulted in the increased pH and induced the bacterial growth. Our results corroborated findings of previous researchers. They have suggested lime and bio regulators can stimulate enzyme activities (Shrestha et al., 2015). We also found soil amendments to infected soil altered soil pH and available nutrients in favor of increasing top soil enzymatic activities and bacterial richness. It was interesting to note that we observed greater increases to soil pH through organic fertilizer application than previous studies. This suggests a threshold effect of the influences of ecological factors on enzyme activities and bacterial biomass in diseased soils (Lagerlof et al., 2015). The activity of Polyphenol oxidase was significantly higher than other treatments in the top soil. Polyphenol oxidase (PPO) is an extremely effective soil acidity and nutrient modifier. Studies have shown that PPO can contribute to pathogen resistance, especially resistance to opportunistic pathogens. Another potential effect of PPO is elevated oxidative stress in the soil (Yakushev et al., 2014). Previous researchers have suggested that potential enzyme synthesis rates were determined by microbial community composition. Although further research on the relationships between individual microorganism species, functional activities, and soil-borne pathogens is required, our results provide strong evidence of links between the microbial community composition and functional activities.

Soil amendment strategies changed bacterial community compositions

Many factors may affect the structure and function of bacterial communities within the rhizosphere soil (Simmons et al., 2016). In our study, the Chao 1 index, which is representative of bacterial phylotype richness levels, showed healthy soil (NTS) to have the highest bacterial diversity among the six treatments (*Table 3*). Diseased soil had lower bacterial richness than healthy soil. The phylum assignment results identified 99.99% of the 16S rRNA sequence reads as bacteria belonging to 16 phyla; *Lysobacter* was the dominant phylum in the healthy soil samples (NTS) (*Figure 4*). The *Xanthomonadale*and *Bacillus* phyla were significantly more abundant in healthy rhizosphere soil than in diseased soil ($P<0.05$). Otherwise, the abundances of the *Gemmatimonas*, *Acidobacteriaceae*, *Sorangium,* and *Rhizobium* phyla were higher in diseased samples (CTS) (*Table A3*). These results suggest that that the bacterial community structures were similar in diseased and healthy soils. *Lysobacter* and *Xanthomonadale*, two mainly species within the Stenotrophomonas genus were multidrug-resistant opportunistic pathogens (Dhawan et al., 2016). This was interesting because it was generally accepted that these phyla are sensitive to changes in nutrient levels. Our results provided strong evidence that these phyla respond to soil-borne

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

pathogens when the rhizosphere soil was amended, although mechanisms underlying this process were not addressed in this study. Kobayaschi et al. (2011) have also shown *Lysobacter*to possess antifungal properties, specifically against *Phytophthoracapsici*, a destructive pathogen of pepper plants (Ko et al., 2009). Previous studies have concluded that shifts in soil microbial community abundance and composition can significantly influence nutrient cycle dynamics and improve resistance to pathogens (Chavez-Romero et al., 2016). Therefore manipulations of soil acidity to a suitable pH and addition of organic manure can effectively improve soil resistance to various pathogens.

Relationships between soil properties and bacterial community composition

We hypothesized that populations of bio-control bacteria will be stimulated by adjusting the content of different substrate in soil so as to reduce the number of pathogens microbial and achieve the goal of preventing risk of cabbage infected by pathogen. Our hypothesis could be proved by the results of recent two years trial experiment (see *Figure 4*). The observed changes to bacterial community composition can be explained by the strong influences of soil amendments on soil chemistry, such as nutrients bioavailability. The higher TN, pH and OM in the surface layer soil significantly increased these bacterial phylotype richness compared with untilled soil. Similar effects were seen for total phosphorus and plant yield (*Figure 6*). These results may be explained by effects of different C substrates on host plants (Bergkemper et al., 2016). Another mechanism through which soil bacterial communities can be modified is resource availability. Large amounts of antibiotics were introduced to the soil environment with manure amendment, and a previous study showed cow manure to shift the bacterial community structure in clay soil (Fernandez et al., 2016). The relative abundance of antibiotic bacteria (*Xanthomonadales*, *Lysobacter*, *Pseudomonas*, and *Bacillus*) present within the soils treated with the bio-organic fertilizer was greater than for the soils treated with lime because of the greater organic matter and total nitrogen content of the bio-organic fertilizer. These results were similar to the observation of a previous study (Wang et al., 2016). The cause of antibiotic resistance is multifaceted, including over use, inappropriate use, and regulatory incompetence, and results in a deficiency of useful antibiotics for clinical use. The results of this study suggest that bio-organic fertilizer can be used to suppress crop pathogens.

Conclusions

Severity of brassica crop infection with clubroot is known to be linked to pH and soil nutrient content. Soil amendments, which raise pH and/or soil nutrients content, can be applied to control clubroot infection of vegetable brassica crops and manipulate bacterial community abundance and composition. The results of this study provide important insights into the influences of soil amendment strategies on the microbial community structure within monoculture agricultural systems and identify the major factors shaping microbial communities. Therefore, further research focuses on revealing the relationship between soil microbial diversity and function, and establishing a direct and quantitative relationship between soil microbial diversity and function. And the relationship between soil microbial diversity and pathogenic function of pathogenic bacteria could been revealed furtherly.

Acknowledgements. The authors thank the many farmers and advisors who sent samples for tests in this project as well as the many staff members at NEAU who assisted. This project was funded by a research grant from the National Key Technology R&D Program (2017YFD0300503), and the Applied Basic Research Programs of Science and Technology Commission Foundation of Heilongjiang Province (No. GC13B111).

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APPENDIX

I Correlation of soil enzyme activities and soil characteristics

Table A1. *Pearson correlations between URE, SUC, PPO, CAT activities and soil characteristics under different treatments for soil shallow layer samples (0-20 cm)*

		pH	Total N	Total P	Available N	Available P	Organic Matter
URE	Pearson Corr.	0.816	0.874	0.111	0.569	-0.156	0.899
	Sig.	3.68E-5	2.15E-6	0.662	0.014	0.538	3.92E-7
SUC	Pearson Corr.	0.860	0.870	0.240	0.511	-0.241	0.750
	Sig.	4.79E-6	2.65E-6	0.339	0.030	0.336	3.47E-4
PPO	Pearson Corr.	0.786	0.808	0.102	0.618	-0.155	0.8127
	Sig.	1.10E-4	4.90E-5	0.688	0.006	0.540	4.267E-5
CAT	Pearson Corr.	-0.864	-0.887	-0.238	-0.659	0.096	-0.825
	Sig.	3.91E-6	9.45E-7	0.340	0.003	0.703	2.53E-5

2-tailed test significance is used

II Soil bacterial community structure and diversity

Table A2. *Sample statistics*

Sample	Sequences	Bases (bp)	Average Length (bp)
CTS	23680	9383057	396.24
EMT	20453	8102610	396.16
NTS	32315	12808208	396.35
LIM	23773	9422177	396.34
LOT	23616	9360053	396.34
OFT	25554	10131604	396.34

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Table A3. *Percent content of all bacteria detected under the six treatments*

Taxon	CTS	EMT	LIM	LOF	NTS	OFT
Subgroup_6_norank	6.395	12.517	9.723	10.295	7.803	18.618
Nitrosomonadaceae_uncultured	6.590	4.486	9.563	12.531	5.579	4.635
Lysobacter	0.407	1.476	0.390	1.066	22.319	0.921
Xanthomonadales_norank	3.221	2.179	4.437	4.520	4.427	1.765
RB41_norank	1.628	0.831	3.096	1.711	2.823	2.859
Flexibacter	2.029	1.400	3.886	2.824	1.561	1.638
Pseudoxanthomonas	0.648	0.116	0.010	12.416	0.337	0.022
Lactococcus	2.132	0.633	3.741	1.402	3.157	0.661
Steroidobacter	2.149	0.819	2.191	2.446	2.744	0.594
0319-6A21_norank	1.095	2.226	1.846	2.200	1.899	1.371
Gemmatimonadaceae_uncultured	1.966	1.761	1.591	1.018	1.622	2.748
Candidate_division_WA3_norank	1.662	2.615	1.886	2.389	0.542	1.782
Anaerolineaceae_uncultured	1.129	1.203	2.251	1.276	0.334	2.565
Gemmatimonas	1.467	1.987	1.280	0.824	0.019	1.460
Haliangium	1.192	0.569	2.201	1.197	0.485	0.672
WD2101_soil_group_norank	1.112	1.284	0.955	0.887	0.640	1.532
Xanthomonadaceae_unclassified	0.923	3.213	0.350	0.919	0.315	0.544
OM190_norank	0.762	0.575	1.461	0.866	0.997	0.855
Rhodospirillaceae_uncultured	0.699	0.378	0.730	0.614	2.194	0.389
Acidobacteriaceae_Subgroup_1_uncultured	2.556	0.506	1.541	0.987	0.027	0.278
GR-WP33-30_norank	0.756	0.552	0.965	0.619	1.114	1.205
Aquicella	0.946	2.167	0.560	0.882	0.322	0.694
Comamonadaceae_unclassified	0.395	1.185	0.490	0.362	1.876	0.350
TRA3-20_norank	0.630	0.471	1.706	1.155	0.478	0.572
Myxococcales_norank	1.054	0.581	1.425	0.945	0.584	0.339
Bacillus	0.321	1.650	0.375	0.199	0.186	2.559
Subgroup_7_norank	0.350	0.947	0.720	0.961	0.345	1.732
Planctomycetaceae_uncultured	0.470	1.075	0.945	0.509	0.963	0.788
Nitrospira	0.854	0.378	1.676	0.751	0.299	0.766
Gaiella	0.848	1.540	0.630	0.215	0.504	0.999
SC-I-84_norank	0.739	1.127	0.560	0.772	0.307	1.143
KCM-B-112_norank	0.000	0.012	0.005	0.000	2.797	0.000
Subgroup_25_norank	0.132	1.017	0.510	0.724	0.572	0.805
Arenimonas	1.060	1.005	0.480	0.378	0.277	0.727
Chitinophagaceae_uncultured	0.648	1.517	0.415	0.541	0.011	0.927
Sorangium	3.444	0.017	0.325	0.189	0.030	0.061
Sphingomonas	0.946	0.110	0.035	2.730	0.004	0.061
Gaiellales_norank	0.848	1.290	0.550	0.142	0.273	0.705
Rhizobium	2.160	0.064	0.040	0.262	0.917	0.044
Piscinibacter	0.390	1.644	0.215	0.304	0.440	0.505
Xanthobacteraceae_uncultured	0.344	0.314	0.465	0.268	1.042	0.661
Solimonadaceae_uncultured	0.080	0.442	0.140	0.278	0.068	1.898
Xanthomonas	2.923	0.017	0.000	0.037	0.000	0.006
Sporocytophaga	0.052	0.000	0.030	0.000	1.899	0.006
Oxalobacteraceae_unclassified	0.659	0.395	0.015	1.228	0.011	0.505
Lineage_IIb_norank	1.192	0.192	0.680	0.367	0.011	0.344
Gammaproteobacteria_unclassified	0.453	1.029	0.055	0.247	0.368	0.488
Flavobacterium	1.186	1.005	0.065	0.068	0.152	0.194
Massilia	0.241	1.011	0.045	0.026	0.004	0.427
Others	36.121	36.559	32.746	22.467	24.326	34.582

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

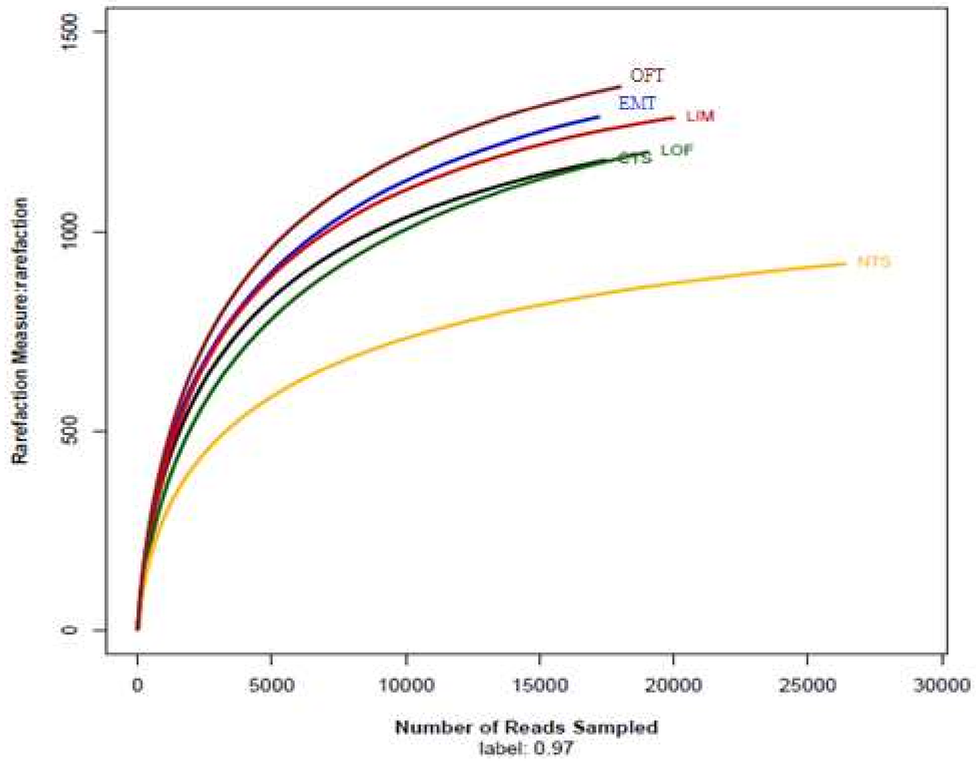

Figure A1. *Rarefaction on species-abundance data. Using 97% similarity OTUs and mothur to analyze rarefaction. This graph was made by R language tool*

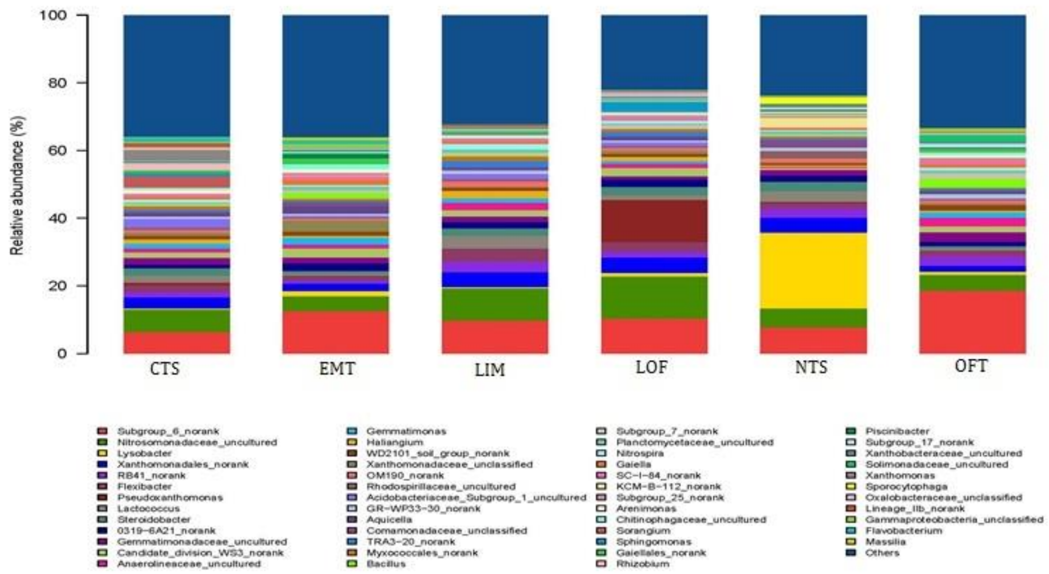

Figure A2. *The relative abundance of different bacteria communities at the genus level in the soil samples from six treatments. The different bacteria genera are distinguished with different colors. Bacteria were defined as No Rank or unclassified if there was no match in the taxonomic database, the taxonomic information was under the threshold of reliability, or there was no taxonomic information*

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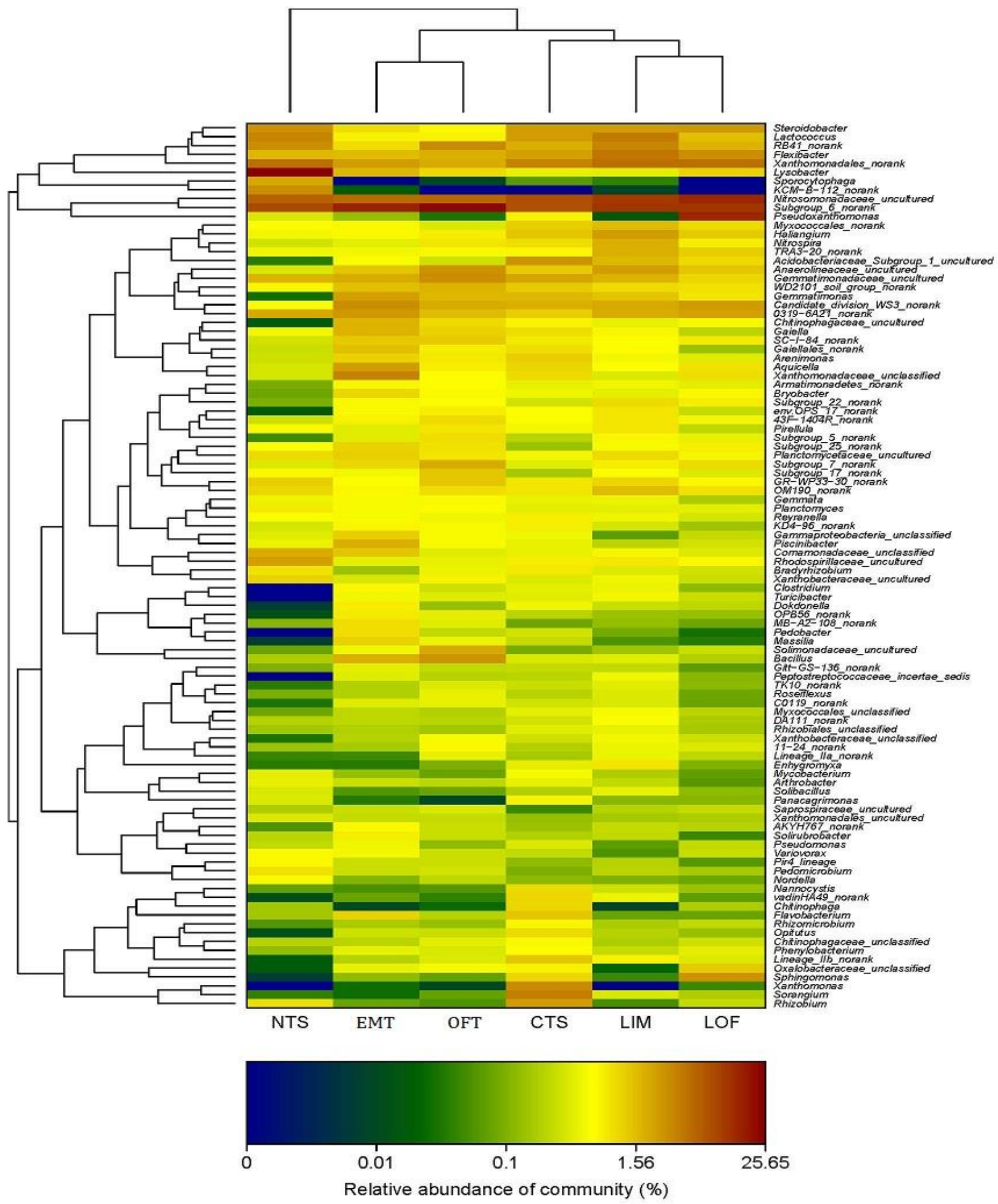

Figure A3. *Double hierarchical dendrogram of the distribution on the 100 most abundant bacteria genera among the six treatment soils. The phylogenetic tree was calculated using the Neighbor-Joining method, and relationships among the treatments were determined using Bray distance and the complete clustering method. The heatmap plot depicts the relative percentage of each bacteria genus (variables clustering on the Y-axis) with in each treatment (X-axis clustering). The relative values for bacteria genera are depicted by color intensity with the legend indicated at the bottom of the figure. Clusters based on the distance of the six samples along the X-axis and the bacteria genus along the Y-axis are indicated at the top and left of the figure, respectively*

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

NTS LOF OFT EMT LIM CTS

Figure A4. *Photo of the experiment*

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DOI: http://dx.doi.org/10.15666/aeer/1702_46554676

ANALYZING THE IMPACT OF CLIMATE CHANGE ON NATURAL VEGETATION GREENNESS USING IMPROVED STATISTICAL VEGETATION INDEX SIMULATION MODEL

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(Received 20th Dec 2018; accepted 20th Feb 2019)

Abstract. In arid regions, vegetation growth relies on water availability. Climate change can alter water cycling with subsequent effects on vegetation growth. This study examined the effects of projected climate change on vegetation greenness in the middle-reaches region of the Heihe River Basin, the second largest inland basin in China. The relationship between the Normalized Difference Vegetation Index (NDVI), an indicator of vegetation greenness derived from remote sensing data, and meteorological factors of precipitation and temperature was simulated using a modified statistical vegetation index simulation model. The model predicted that along with increases in precipitation and temperature, spring and autumn NDVI will rise about 3.8% and 7.9% by the 2050s, respectively. NDVI in summer increases about 3.9%, as increased precipitation may be superior to higher rates of soil water evaporation due to rising temperature. Hydrological processes in the Heihe River Basin are highly influenced by both climate change and human activities. The findings of this study are valuable for the development of water resource management policies that balance the water demands of both human society and the natural ecosystem.
Keywords: *NDVI, remote sensing, arid region, vegetation greenness, climate change*

Introduction

Drylands occupy more than 40% of terrestrial Earth (Slaymaker et al., 1998). The land area desertified or prone to desertification has been estimated as 57–65% of the total area of dryland ecosystems (UNEP, 1992). Land degradation in drylands has negative influences on global food security (MEA, 2005; Tscharntke et al., 2012; Weston et al., 2015). Natural vegetation plays an important role in maintaining dryland ecosystem functions (Lal, 2004; Zethof et al., 2019). As a result of the tight coupling among water, energy and biogeochemical cycles in drylands, vegetation dynamics strongly influences water cycling and responses to climate change and human activity in these systems (Wang et al., 2012; Ott et al., 2016). Natural vegetation in arid regions is particularly affected by temperature and precipitation at different temporal and spatial scales (Stellmes et al., 2010; Gessner et al., 2013; Li et al., 2015). Better understanding

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DOI: http://dx.doi.org/10.15666/aeer/1702_46774691

of plant growth responses to climatic changes will provide important insight into hydrological processes in drylands because plant growth relies on water availability.

Determination of the influence of climate change on vegetation requires long-term observation. Field observations of vegetation growth, however, are time- and labor-consuming. Remote sensing is an effective alternative way to monitor temporal trends in vegetation growth and changes in ecosystem function (Pouliot et al., 2009; Li et al., 2013; Wu et al., 2014; Liu et al., 2016). Among numerous vegetation indexes derived from remote sensing, the Normalized Difference Vegetation Index (NDVI) is widely regarded as a good proxy for terrestrial vegetation growth. NDVI, the ratio of the difference in near-infrared and red reflectance and the sum of these two variables, estimates the photosynthetically active radiation absorbed by photosynthesizing tissue (Fensholt et al., 2004). Trend analysis of NDVI data has generally been used to detect changes in vegetation dynamics at continental or regional scales. Many studies (e.g., Piao et al., 2006; Fensholt et al., 2012; Krishnaswamy et al., 2014; Wu et al., 2014) have reported strong correlations between NDVI and meteorological factors including temperature and precipitation. Los et al. (2006) built a statistical vegetation index simulation (SVIS) model to estimate the coupling strength of NDVI with temperature and precipitation using a satellite-derived monthly vegetation index and meteorological data. The model is potentially useful for predicting the response of natural vegetation to climate change in regions where field data are sparse.

The Heihe River Basin in arid northwest China is the second largest inland basin in China. The basin is adjacent to the north edge of the Qinghai-Tibetan Plateau and is characterized by diverse geomorphology. As the transition zone between the upper-reaches region of the Heihe River Basin in the southern Qilian Mountains and the lower-reach region in the northern Alxa Desert, the middle-reaches region is ecologically unique (Lu et al., 2003). It is well known for its irrigated agriculture using river water and pumped groundwater, which alters natural water cycling. Thus, natural vegetation in this region is vulnerable to both climate change and human activities (Qi and Luo, 2007; Sun et al., 2015). The objective of this study is to explore how the greenness of the Salsola passerina, which is an annual C4 plant and one representative plant in the middle-reaches region, responses to future climate changes by using a modified version of the SVIS model forced with a future climate change scenario. The prediction made by the model is important to develop water resource management policies that balance the water demand of human society and the natural ecosystem. A high-resolution meteorological forcing dataset was used as model input to account for the high spatial heterogeneity in dryland precipitation and temperature. The future climate change scenario was built by using the downscaled ensemble output of 16 general circulation models (GCMs).

Materials and methods

Study area

The Heihe River Basin (*Fig. 1*) originates in the Qilian Mountains in the northern edge of the Qinghai-Tibetan Plateau, covering an area of about 142,900 km^2. The elevation of the basin gradually decreases from the high mountain area in the south to the northern high-plain area. The Heihe River Basin is not influenced by the East Asian monsoon and has a very dry climate, with an average annual precipitation of 100–450 mm. Differences in geomorphology divide the basin into three regions: the upper-

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reaches region is in the Qilian Mountains; the middle-reaches region is part of the Hexi Corridor Plain; and the lower-reaches region is on the Alxa Plateau. The middle-reaches region is the most populated. River water and pumped groundwater are the main water resources for irrigation and domestic use. Gobi and cropland are the dominant types of land cover. Cropland is mainly distributed in the oases near the main stream, and Gobi mainly occurs between the northern edge of the Qilian Mountains and the river. The Salsola passerina is one representative natural plant in the Gobi region.

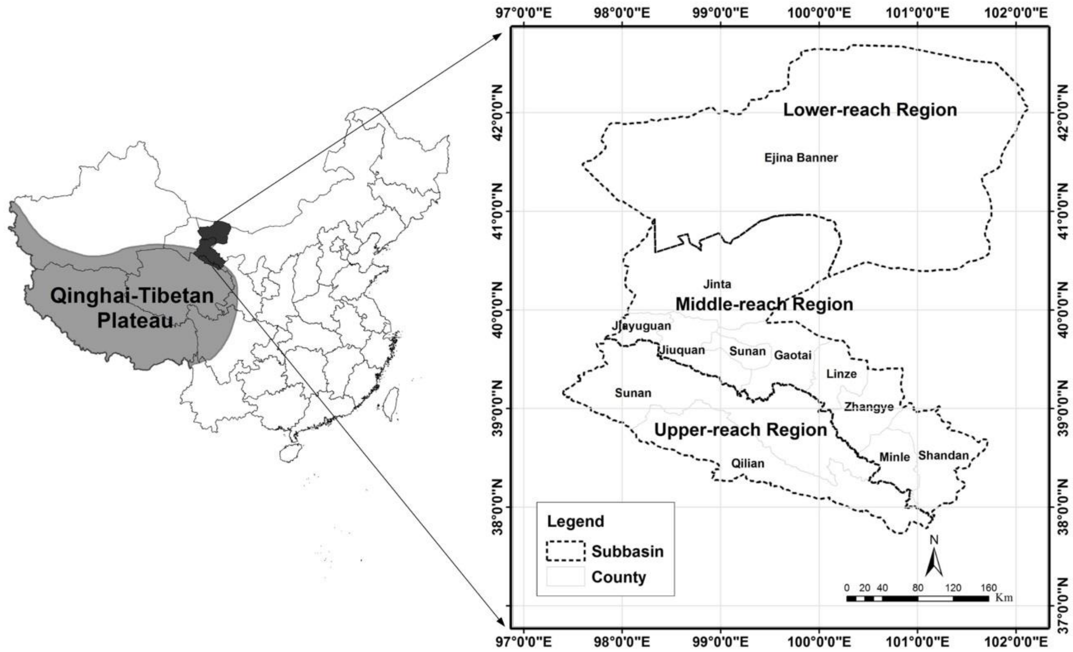

Figure 1. *Heihe River Basin and its location in China*

Dataset and preprocessing

The third generation of Global Inventory Modeling and Mapping Studies (GIMMS) NDVI dataset used in the study extends from 1982 to 2010 with an 8-km resolution and was obtained from NASA Ames Ecological Forecasting Laboratory (http://ecocast.arc.nasa.gov/data/pub/gimms/). The GIMMS data are derived from imagery taken from National Oceanic and Atmospheric Administration (NOAA, USA) satellites. Based on a field survey in 2014, a pixel of the NDVI dataset in which Salsola passerina was the dominant vegetation was selected for simulation. The location of the pixel and the characteristic landscape in this area are shown in *Figures 2* and *3*, respectively. To reduce the noise in the data, the maximum value composite method was used to generate 8-km resolution monthly NDVI data from the two images of each month. The averages of monthly NDVI and its standard deviation for 1982–2010 are shown in *Figure 4*. A sharp increment is found between March and April. And the NDVI in November is much lower than that in October. Following the definitions of Piao et al.(2011), NDVI averages from April to October represented the annual growing season, while April and May were considered spring, June to August were considered summer and September and October were considered as autumn. *Figure 5* shows the time series of growing season NDVI for the period of 1982–2010. No statistical trend has been detected at temporal domain.

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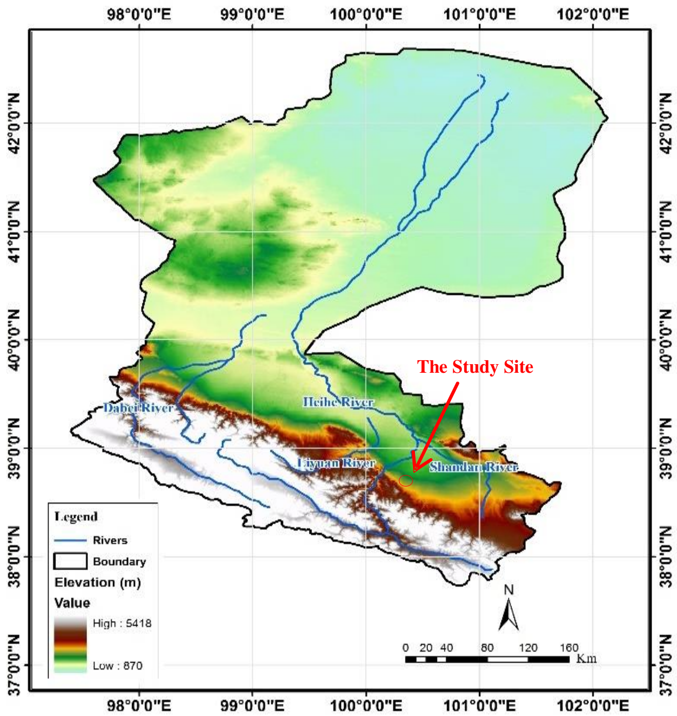

Figure 2. Location of the study site in the Heihe Basin

Figure 3. Landscape at the study site during a field survey in June 2014

The meteorological dataset used in the study covers 53 years (1958 to 2010) with spatial and temporal resolutions of 5 km and 3 h, respectively, and was developed by the Land-Atmosphere Interaction Research Group at Beijing Normal University (http://globalchange.bnu.edu.cn/research/forcing). Seven meteorological variables (i.e., air temperature, air pressure, relative humility, wind speed, precipitation, shortwave radiation and longwave radiation) were produced by combining gauge observations with reanalysis data or remote sensing data (Li et al., 2014; Huang et al., 2014). The air temperature and precipitation data from the above mentioned meteorological dataset are used in this study. Within the spatial extent of the selected NDVI pixel, air temperature and precipitation data for 1982 to 2010 were resampled to produce corresponding meteorological data. The monthly total precipitation and mean air temperature were given as the respective sum and average of the data within each month.

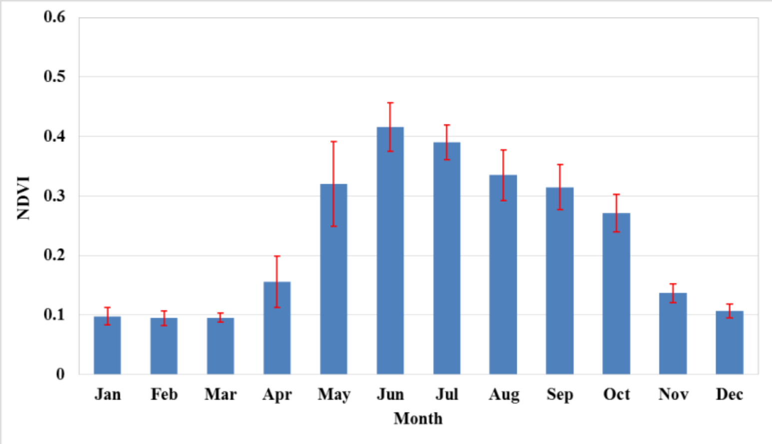

Figure 4. *Mean monthly NDVI (blue bars) and its standard deviation (red lines) at the study site for 1982–2010*

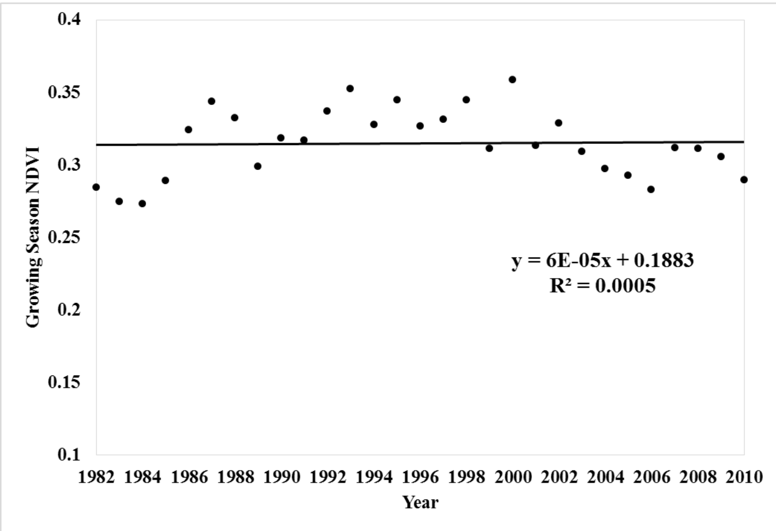

Figure 5. *Time series of growing season NDVI (black dots) and its trend line (black line) for 1982–2010*

Statistical vegetation index simulation model

The SVIS model is a conceptual model that investigates the relationship between vegetation greenness derived from remote sensing data (NDVI) and meteorological variables using multiple linear regression analysis. SVIS can predict monthly NDVI using precipitation and temperature data from the current month and the two previous months to account for the time-lag effect of meteorological factors on vegetation growth. The feasibility of the model was verified by Los et al. (2006). The model is defined as *Equation 1:*

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$$V_t = a + \sum_{i=-2}^{0} b_{t,2+i} p_{t+i} + \sum_{i=-2}^{0} c_{t,2+i} T_{t+i} + \sum_{m=-2}^{-1} d_{t,2+m} V_{t+m} \quad \text{(Eq.1)}$$

where P and T are the precipitation and temperature of the current month (t) and two previous months (t-2) and (t-1); V_t, V_{t-1} and V_{t-2}, are the NDVI of the current month t and two previous months (t-2) and (t-1); and a, b, c and d are parameters describing relationship between NDVI and the two meteorological variables.

In this study, rather than using multiple linear regression analysis, an Artificial Neural Network (ANN) was used to describe the relationship between NDVI and the meteorological variables. The development of ANN was inspired by the functionality of biological neural networks such as the human brain. Biological neural networks learn quickly from experiences and interactions between the internal and external environment to establish pattern recognition. ANN is recursive and can continually learn from the given dataset, which has proven to be efficient and time saving. ANN can also be used for the simulation of complex and nonlinear processes. The ANN model used in this study is of the form (*Eq. 2*):

$$V_\mathrm{i} = f\left(P_i, P_{\mathrm{i}-1}, P_{\mathrm{i}-2}, T_i, T_{\mathrm{i}-1}, T_{\mathrm{i}-2}\right) \quad \text{(Eq.2)}$$

where V_i, P_i and T_i are the NDVI, precipitation, and temperature of the ith month in a year; and P_{i-1}, P_{i-2}, T_{i-1} and T_{i-2} are the precipitation and temperature of two previous months *(t-1)* and *(t-2)*, respectively. f represents the nonlinear relationship between the meteorological input and NDVI output derived from the ANN. The model performance indicators were the squared correlation coefficient (R^2) and the mean absolute difference (*MAD*) as *Equations 3–4:*

$$\mathrm{R}^2 = \frac{\left[\sum_{i=1}^{n}\left(V_{obs,i} - V_{obs,a}\right)(V_{sim,i} - V_{sim,a})\right]^2}{\sum_{i=1}^{n}\left(V_{obs,i} - V_{obs,a}\right)^2 \sum_{i=1}^{n}\left(V_{sim,i} - V_{sim,a}\right)^2} \quad \text{(Eq.3)}$$

$$MAD = \frac{1}{n}\sum_{i=1}^{n}\left|V_{obs,i} - V_{sim,i}\right| \quad \text{(Eq.4)}$$

where $V_{obs,i}$ and $V_{sim,i}$ are the observed (GIMMS dataset) and simulated NDVI at time step i, and $V_{obs,a}$ and $V_{sim,a}$ are the average observed and simulated NDVI over the whole training or validation period. The simulation is made for the period of 1982 to 2010. In order to make the training period cover various climatic condition of the study site, the data of even-numbered years in this period were used to train the model. And data of odd-numbered years were used for model validation.

Future scenarios under climate change

After development, the model was applied to predict vegetation greenness under future climate change scenarios. The GCMs need to be downscaled because their outputs are too coarse to describe climatic characteristics at the regional scale. Moreover, uncertainties in climate projections from GCMs require consideration. To make a more reliable future regional climate change scenario, usually an ensemble

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analysis combing multiple GCM analysis and quantifying the probability of future climate is conducted. The Climate Wizard dataset (available at: http://www.climatewizard.org/index.html), projections of future temperature and precipitation derived from ensemble average of 16 Coupled Model Intercomparison Project phase 3 (CMIP3) GCMs, is adopted in this study to reduce possible uncertainties of GCMs. The monthly precipitation and mean temperature was downscaled to the resolution of 50 km using an empirical statistical method (details are available in Palmer et al., 2004). The data of the 2050s (2040–2069) for A1B scenario (medium CO_2 emission) were used to build regional climate change scenario of the mid-21st century at the study site. According to the intergovernmental panel on climate change special report on emission scenario, the A1B scenario corresponds to rapid growths in economy and global population, which reaches peaks in the mid-21st century and declines following a slowing growth, and is a moderate scenario for greenhouse gas emission. This study focus on how the vegetation growth responses to intermediate climate change scenario and discussions about extreme scenario are out the scope of this study. Therefore, the climate projection under A1B scenario is used in our research. It is widely used for examining potential vegetation response in the future to climate changes in previous studies (e.g., Alo and Wang, 2008; Smith et al., 2011; Waha et al., 2013). Percentage change in monthly precipitation between projections for the 2050s and the past 50 years was used to scale monthly precipitation data for 1982–2010. The absolute difference in monthly temperature between the projected (2050s) and historical (past 50 years) climate was added directly to monthly temperature data for 1982–2010. Using the future climate change scenario as input, the NDVI prediction made by the ANN model represents the status of vegetation growth in the 2050s. NDVI simulated using meteorological data from 1982–2010 was considered the baseline. The differences between the two simulations are considered as the impact of climate change on vegetation growth. Model prediction excluded the winter period and was conducted only for months during the growing season (April to October).

Results and discussion

Seasonal fluctuation in vegetation greenness and meteorological factors

Time series of monthly precipitation, temperature, and NDVI in the Heihe River Basin from 1982 to 2010 revealed unimodal seasonal patterns (*Figures 6* and *7*). Intra-annual variation patterns in the two meteorological variables were consistent with that of NDVI. All three variables showed periodic variation with maximum values in summer or fall and minimum values in winter. Average summertime NDVI was 0.38. Average wintertime NDVI was 0.11, indicating the existence of a winter dormant period. Analysis of the multi-year averaged NDVI data from 1982 to 2010 indicated that from the beginning of spring (April) to the beginning of summer (June), NDVI increases with an average magnitude of 0.32 (*Table 1*). NDVI remained high during the two subsequent summer months. *Table 1* also shows that NDVI decreases with an average magnitude of 0.20 from the end of summer (August) to the beginning of winter (November).

For both temperature and precipitation, absolute values of the increases from the beginning of spring to the beginning of summer were smaller than that for the decrease from the end of summer to the beginning of winter (*Table 1*). In contrast, the magnitude of increase in NDVI was greater than the magnitude of decrease in the same periods, indicating a time lag in NDVI response to changes in precipitation and temperature. This time lag was the justification for the use of both the current and the previous two months' meteorological data for monthly simulation of NDVI.

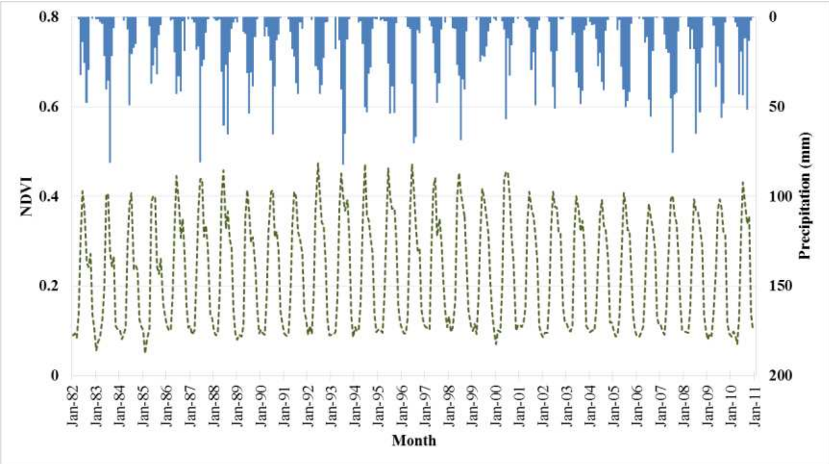

Figure 6. *Time series of monthly NDVI (green dashed line) and precipitation (blue bars) for 1982–2010*

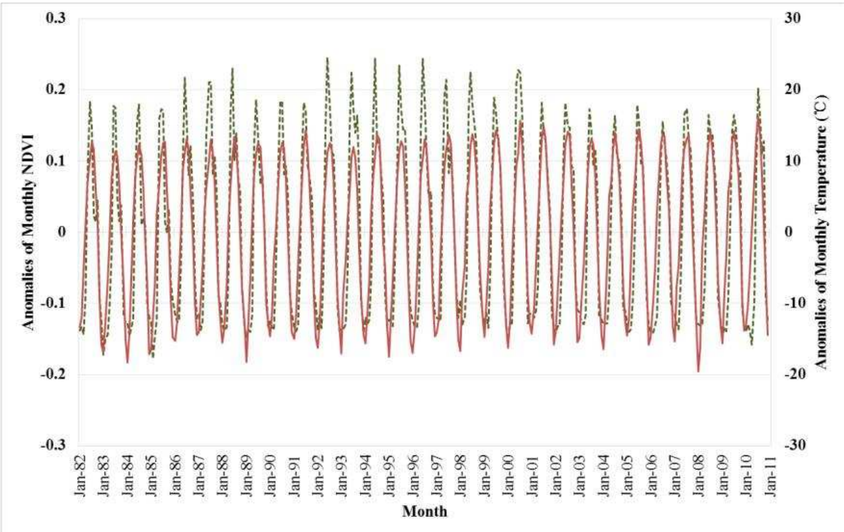

Figure 7. *Anomalies of monthly NDVI (green dashed line) and temperature (red line) for 1982–2010*

Table 1. *Differences in monthly NDVI, mean temperature and precipitation between the beginning of spring (April) and the beginning of summer (June), and between the end of summer (August) and the beginning of winter (November)*

Periods	**NDVI differences**	**Temperature differences (°C)**	**Precipitation differences (mm)**
The beginning of spring (April) to the beginning of summer (June)	0.26	16.5	30
The end of summer (August) to the beginning of winter (November)	-0.20	-20.0	-39

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Influence of future climate change on vegetation greenness

The monthly observed and simulated NDVI values from 1982 to 2010 are shown in *Figure 8*. Simulations reproduced the timing and range of variation in NDVI reasonably well. The scatter plot of observed to simulated NDVI values demonstrated a strong correlation from the low to high ranges of NDVI (*Fig. 9*). Although R^2 and *MAD* values were lower for model validation than for model training period (*Table 2*), model performance was high enough for both to justify the use of the model for prediction.

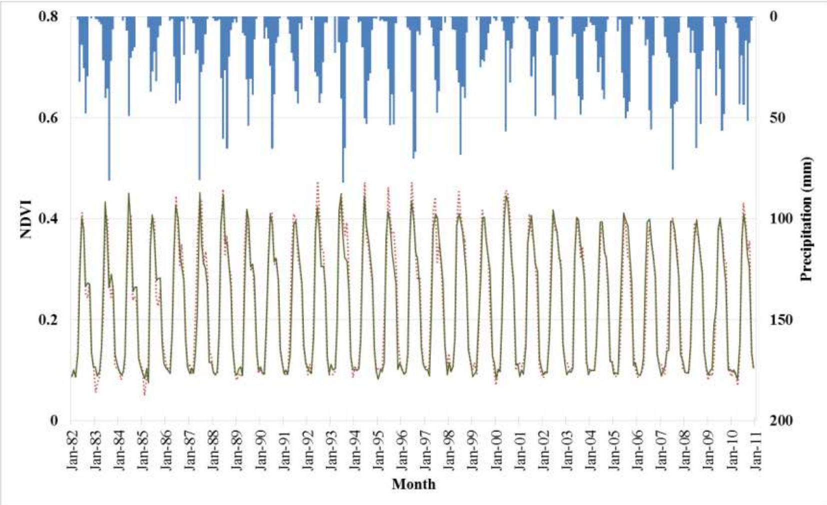

Figure 8. *NDVI simulations from 1982 to 2010 in the study area (precipitation: blue bars; observed NDVI: red dashed line; simulated NDVI: green line)*

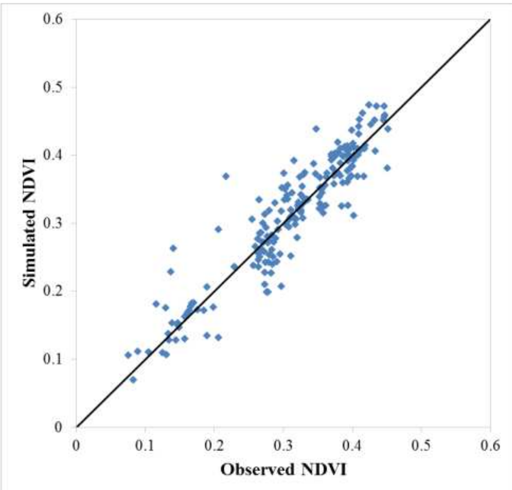

Figure 9. *Scatter plot of observed vs. simulated monthly NDVI*

Table 2. *Model performance for training and validation periods*

Performance indicators	Training period (even-numbered years for the period of 1982 to 2010)	Validation period (odd-numbered years for the period of 1982 to 2010)
R^2	0.979	0.922
MAD	0.0117	0.0243

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Projected monthly meteorological data for the 2050s under the A1B scenario were used as input for the modified SVIS model to simulate future variation in vegetation greenness. *Figures 10* and *11* depict the changes in monthly precipitation and mean temperature in the Heihe River Basin between the baseline and future climate scenario for the 2050s. Simulations predicted increases in mean monthly precipitation in all months except July, with particularly high increases (>30%) from November to March. Monthly mean temperature was predicted to increase in all months in the 2050s scenario, with an average increase of 2.77 °C. Both precipitation and rainfall were predicted to increase in spring, summer and winter (*Fig. 12*).

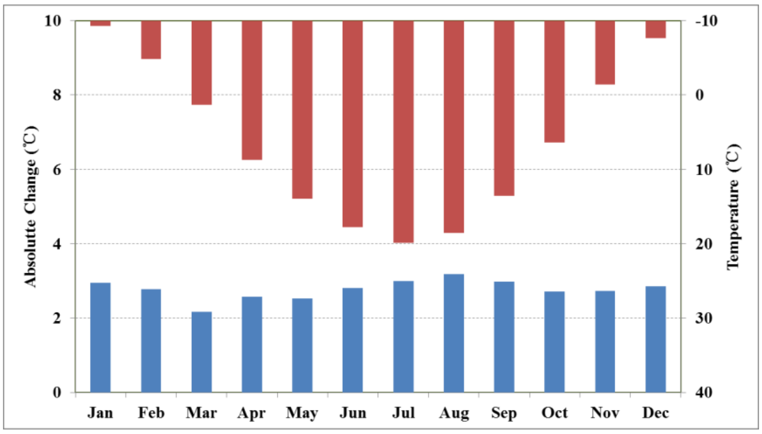

Figure 10. *Monthly temperature in the baseline period (red bars) and absolute changes of monthly temperature between baseline period and the projected climate scenario for the 2050s (blue bars)*

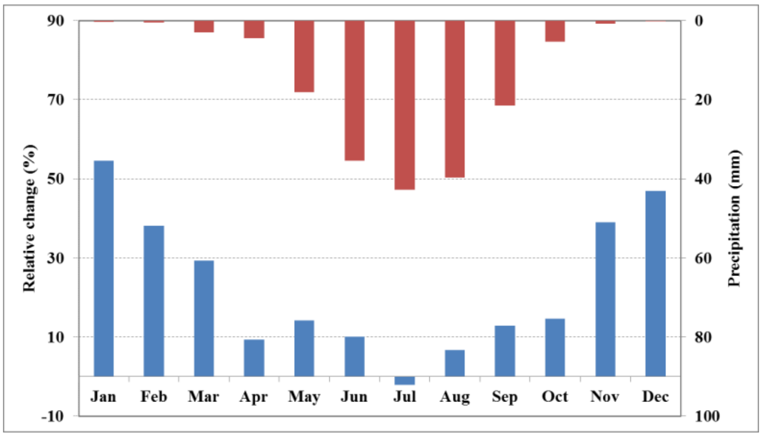

Figure 11. *Monthly precipitation in the baseline period (red bars) and absolute changes of monthly precipitation between baseline period and the projected climate scenario for the 2050s (blue bars)*

Future climatic scenario and predicted seasonal changes in NDVI are shown in *Figure 12*. Compared with the baseline period, spring and autumn NDVI were predicted to increase 3.8% and 7.9%. Increased precipitation would raise soil water content, benefitting vegetation growth. Carl et al. (2013) demonstrated that global warming may cause temporal shifts in phenology. More specifically, Piao et al. (2008) and Richardson

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et al. (2010) indicated that increasing spring and autumn temperature extend the length of growing season and correspondingly the days for biomass growth becomes more available. Yang et al. (2012) indicates that, among different climate factors, temperature and precipitations have closest relations with NDVI. Together, for the study site, increased temperature and precipitation may explain projected increases in NDVI with climate change of spring and autumn. In summer, there is an increase of 3.9% for future NDVI. Both temperature and precipitation rises in summer of the 2050s. Rising temperature would increase the energy available for soil water evaporation and thus reducing the soil water content available for vegetation growth. However, the reduction in soil moisture may be compensated by the increases in precipitation and these increases even finally lead to a rising in soil moisture, which may be the reason that NDVI become higher in summer of the 2050s.

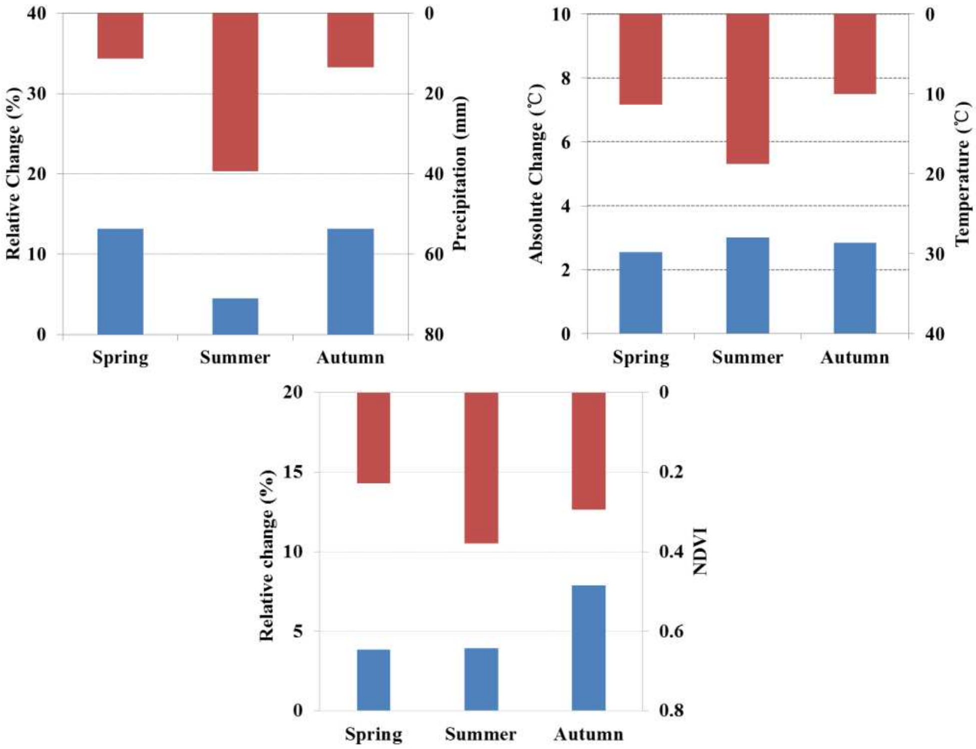

***Figure 12.** Average changes (blue bars) in seasonal mean precipitation, temperature and simulated NDVI between baseline (red bars) and the projected climate scenario for the 2050s*

Our prediction shows that for this dominant shrub in the study area, i.e. Salsola passerina, the vegetation greenness become better with the increases in temperature and precipitation. By using a dynamic global vegetation model forced with future climate change scenario, similar results are obtained by Gao et al. (2016) in the Qinghai-Tibetan Plateau, which is adjacent to the Heihe River Basin. Different results are revealed in the study of Gremer et al. (2015), which found that C4 grassland cover negatively responded to mean annual precipitation in the dryland across the south western United States. It is indicated that how natural vegetation responses to climate changes also depends on vegetation type and geophysical conditions. Similarly, Zhang et al. (2016) demonstrated that different vegetation types had very different sensitivities to climate factors in arid Central Asia. The results from model simulation in this study show that

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the increased vegetation greenness is caused by the increases of temperature and precipitation. Tang et al. (2017) demonstrated that the sensitivity of vegetation growth to the temperature and precipitation may decrease if these two climatic variables keep increasing in the Shiyang River Basin, which is also located in Northwest China and close to the Heihe River Basin. To further improve our understanding about influences of climate change on natural vegetation in dryland, regional studies based on long-term physiological and phenological observations are necessary.

Conclusions

This study focused on predicting the climate change response of Salsola passerina, a representative vegetation type in the middle Heihe Basin, China, using a modified SVIS model. The relationship between satellite-observed NDVI, an indicator of vegetation greenness, and meteorological forcing (temperature and precipitation) were evaluated with the model using 29-year datasets. The performance of both model training and validation justified use of the model for prediction. Climate change scenario data for the 2050s were constructed using the ensemble average of downscaled output from sixteen GCMs for a medium CO_2 emission scenario (A1B). NDVI was projected to increase 3.8% and 7.9% in the spring and autumn, likely because of greater soil water content with increasing precipitation and extension of growing season due to higher temperatures. Projected NDVI rises about 3.9% in the summer seasons, suggesting that increase of soil moisture due to greater precipitation is superior to loss of soil moisture caused by higher temperature. This study focused on the influence of climate change on the growth of Salsola passerina. Further understanding of how natural vegetation in the middle Heihe River Basin will react to a changing environment requires the direct incorporation of soil moisture into the SVIS model. Reliable estimation of soil moisture in this region needs a coupled model of both surface water and groundwater to account for human activity such as the use of river water and pumped groundwater for irrigation.

Acknowledgements. This study was supported by the National Key Research and Development Program of China (Grant Nos. 2018YFC0406502, 2016YFC0401308), and the National Natural Science Foundation of China (Grant No. 41671018).

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DOI: http://dx.doi.org/10.15666/aeer/1702_46774691

EFFECTS OF IBA AND PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) ON ROOTING OF RAMSEY AMERICAN GRAPEVINE ROOTSTOCK

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(Received 30th Dec 2018; accepted 27th Feb 2019)

Abstract. This experiment was conducted to reveal the effects of PGPR and IBA on rooting characteristics of Ramsey "Salt Creek" (*Vitis champini*) under the nursery. Hard wood cuttings of Ramsey were subjected to PGPR (EMA1, EMA6, EMA24, EMA MEDIA, EMA CALLUS, EMA QUICK DIP) with dipping times of 1h, 6h, 24 h. IBA concentration was 25, 2000, 4000 ppm with dipping time of 24 h in the case of long-soak method, and of 4 seconds in quick dip method. Data were recorded on specific rooting and shooting characteristic. Results obtained from treatments showed significant differences at $p \leq 0.01$ for specific rooting characteristic, like rooting rate (%), rooting degree (0-4), root length (cm), root fresh and dry weight (g) under the interaction between PGPR and IBA concentration and dipping time. The rooting rate of EMA1 was 56.7%, and was better than in IBA treatments. Rooting degree (0-4) was 3.5 with EMA1 and 3.3 with EMA MEDIA. The highest root lenght (cm) were obtained in IBA25 (37.6) EMA MEDIA (37.2) and EMA1 (34.1). The best result on fresh root weight (g) was determined with EMA1 (9.8) and EMA6 (9.5). As a conclusion, inspected rooting characteristics generally increased with the EMA1 and EMA6 treatments.
Keywords: *Vitis vinifera*, *nursery, cutting, dipping, rooting*

Introduction

One of the most cultivated fruit species is grape (*Vitis vinifera* L.) which is originated from temperate zones of Asia minor in the regions between the south of the Black and the Capsian seas and subsequently spreading towards tropical areas to the west and to the east (Vaddar, 2007; Galavi et al., 2013).

Phylloxera, an insect from North America, was detected in France in 1868. The spreading of Phylloxera in most of the French and European vineyards was extremely rapid. *Vinifera* is susceptible to the attack of phylloxera. Phylloxera attacked vineyards worldwide from the European grapevine cultivars and spreaded from vine to vine. It caused great damage all over the world. Grape plants died rapidly and in huge numbers after the roots of vinifera grapevines were attacked by phylloxera. While the phylloxera damages the roots of European vine species, *V.vinifera*, it cannot harm the roots of the American vine species. Phylloxera resistant rootstocks play an important part in viticulture. Grape rootstocks have been bred to provide resistance to phylloxera (*Daktulosphaira vitifoliae* Fitch). This situation makes the use of American vine rootstock in the production of suspended seedlings inevitably (Carew et al., 2004). Today, vinegrowers have to graft *V.vinifera* cultivars onto resistant rootstocks such as North American species (Baydar and Ece, 2005; Lowe and Walker, 2006; Sabır, 2011; Teker et al., 2014).

Grape is one of the most important horticulture crops of Turkey. Traditionally, grapevines have grown on their own roots, this is still a common practice in Turkey, but establishing vineyard with phylloxera resistant rootstocks is necessary for modern Turkish viticulture. In recent years, vineyards have established the usage of rootstock. Most vinegrower prefer rooted american grapevine cutting for establishing vineyard because it is

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DOI: http://dx.doi.org/10.15666/aeer/1702_46934705

more economical than grafted seedlings. They plant rooted american rootstock, then they make cleft grafting in the vineyard. For this reason, well rooted and full grown American rootstock cutting is very significant.

The rootstock ‘Ramsey’ (*Vitis champini*) which has great potential to be used as grape rootstock owing to its resistance to phylloxera and nematode is one of the most important rootstocks used in Turkey, especially drought climatic conditions, calcareous and high salinity soils. Ramsey cuttings are difficult to root, for this reason the root stocks cutting are dipped in 1500 to 2000 ppm of indolebutyric acid (IBA) solution in nursery.

Plant growth-promoting rhizobacteria (PGPR) are free-living soil bacteria that can affect plant growth by the synthesis of phytohormones and vitamins, inhibiting plant ethylene synthesis, enhancing stress resistance, improving nutrient uptake and mineralising organic phosphate (Barnaval et al., 2014; Daler and Çetin, 2017). PGPR can influence the expression of root traits, such as root branching intensity, average root diameter, but mainly research has been conducted to reveal the effects of PGPR on plant growth, graft success and vegetative growth (Sabır, 2013; Karaca and Sabır, 2018). Grapevines form mutualistic symbioses with PGPR that have shown enhanced plant growth and nutrition. In the field, PGPR may be low or nonexistent (in fumigated soils), suggesting the need for PGPR inoculation of grapevine plants at the nursery. Addition of PGPR inoculum to roots could be an effective strategy for increasing nursery production (Aguin et al., 2004). Using PGPR is important in nursery. Hence, future studies will focus on enhancing nursery methods to improve successful rooting rate. PGPR can have effects on initiating easy rooting of rootstock plants like Ramsey. This study was performed in nursery, in order to identify the effects of different doses of PGPR and IBA concentrations on american rootstock scion. The effectiveness of root zone inoculation with PGPR and IBA to improve successful rooting rate on Ramsey scion were studied.

Materials and Methods

This study was carried out at Tekbağ Nursery Co. Ltd. in Manisa, Turkey, in 2017-2018.

Plant Material

Hardwood cuttings of Ramsey rootstocks were used as plant propagation materials in this study. Ramsey has high vigour, nematode (Meloidogyne) resistance and salt tolerance, moderate to high phylloxera resistance, moderate tolerance of drought and lime, but can suffer from zinc deficiencies in high pH soils. However, it has the lowest success of rooting and affinity ratio when it is compared to other rootstocks (Lowe and Walker, 2006).

The cuttings were collected from dormant vines. All cuttings were taken from the same parts (between 3^{rd}–10^{th} nodium) of mother vines. They were placed in cold storage at 1–5°C and high relative humidity until the experiment. The plant materials were cut into 40 cm sections with an average of four nodes each. A total of 1800 cuttings were collected for two years.

Plant Growth-Promoting Rhizobacteria (PGPR) and IBA Applications

Plant Growth-Promoting Rhizobacteria (PGPR) and indolebutyric acid (IBA) have been applied to increase development of cuttings.

Plant Growth-Promoting Rhizobacteria: PGPR used in the research is EM•A that was obtained from the “EM AGRITON” company in the form of ready-to-use solution. It

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provides all the useful properties of active microorganisms technology. This is a one hundred percent organic product containing lactic acid bacteria (Lactobacillus Fermentum, L. Plantarum, L. Rhamnous, L. Delbrueckii), yeast (Saccharomyces Cerevisiae), phototrophic bacteria (Rhodopseudomonas Palustris) and Bacillus Subtilis. It has a 1.9×10^{8} ** total microorganism count ($^{**}6.1\times10^{7}$ aerobic, mesophilic bacteria; 1.3×10^{8}, anaerobic microarofil bacteria; 6.0×10^{5}; yeast; < 100 mildew) and a 3.89 pH.

Indolebutyric acid: IBA is a plant hormone in the auxin family and is an ingredient in many commercial horticultural plant rooting products. Generally, IBA is used to initiate root formation in nursery.

Methods

In the experiment, IBA was applied to Ramsey cuttings with different methods at different time and doses (Alley, 1980; Kroin, 2008). **EM•A** was applied to cuttings with different methods at different time and doses. All applications of **EM•A** and IBA used in the research are given in *Table 1*.

After treatments, cuttings were planted in plastic pots containing uniform mixture of peat (1.034% N, 0.94% P_2O_5, 0.64% K_2O, pH 5.88, Klassman®) and inert perlite (0–3 mm in diameter) in equal volume. Before planting, 5 cm top parts of the cuttings were covered with red colored paraffin which melts at 70°C. They were placed in a greenhouse at 28°C and 60% relative humidity for 6 weeks after preparation.

Table 1. *Introduction of applications*

EMA1	10 cm bottom parts of cuttings were immersed in EM•A solution at a dose of 100 mg/L for 1 hour and then planted in pots.
EMA6	10 cm bottom parts of cuttings were immersed in EM•A solution at a dose of 100 mg/L for 6 hour and then planted in pots.
EMA24	10 cm bottom parts of cuttings were immersed in EM•A solution at a dose of 100 mg/L for 24 hour and then planted in pots.
EMA MEDIA	EM•A at a dose of 100 mg/L was injected into the rooting media shortly after planting.
EMA CALLUS	Callus tissue was formed at the bottom of the rootstocks in the stratification room with 28 ° C temperature and 85% humidity in 21 days. The callus tissue at basal ends of cuttings were immersed in EM•A solution at a dose of 200 mg/L using quick dip method (4 second). After, cuttings were planted in pots.
EMA QUICK DIP	The basal ends of cuttings were immersed in EM•A solution at a dose of 200 mg/L using quick dip method (4 second) and then cuttings were planted in pots.
IBA25	10 cm bottom parts of cuttings were immersed in IBA solution at a dose of 25 mg/L for 24 hour (basal long-soak method) and then planted in pots.
IBA2000	The basal ends of cuttings were immersed in IBA solution at a dose of 2000 mg/L using quick dip method (4 second) and then cuttings were planted in pots.
IBA4000	The basal ends of cuttings were immersed in IBA solution at a dose of 4000 mg/L using quick dip method (4 second) and then cuttings were planted in pots.
CONTROL	Cuttings are directly planted in pots without any application.

Evaluation of Growth Parameters for Ramsey

For the analyses of root distrubution of the cuttings to PGPR and IBA plant materials were visually evaluated 6 weeks after operation for root distrubition. The growth of the cuttings was assessed by rooting rate (%), rooting degree (0-4), root number, root length (cm), fresh root weight (g), dry root weight (g), stoma number, shoot length (cm), shoot weight (g), number of internodes on main shoot, lenght of lignified summer shoot (cm),

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leaf area (LA, cm^2), leaf weight (gr), correlation between leaf area and leaf weight following the PGPR and IBA applications.

Statistical Analysis

A completely randomized simple factorial design with three replications (30 cuttings in each replication) was used in the experiment. The data were subjected to analysis of variance by SPSS (SSPS Inc. 10.0, USA, 1999) software, and differences between means were determined by Fischer's Least Significant Difference (*LSD*) test.

Results

All data examined in the research are given in *Table 2, Table 3* and *Table 4* for experimental years.

Applications were determined to have a statistically significant effect (P=0.001) on the rooting rate (%) for Ramsey grapevine rootstock in 2017 and 2018 experimental years. The EMA1 and EMA6 applications in the first experimental year were included in the same statistical group for rooting rate (%) 56.7 (a), 50.0 (ab), respectively (*Table 2, Fig.1)*, on the other hand in the second year EMA1 and EMA6 applications were in the same statistics group, 56.7 (a), 56.7 (a), respectively (*Table 3*, *Fig.1*).

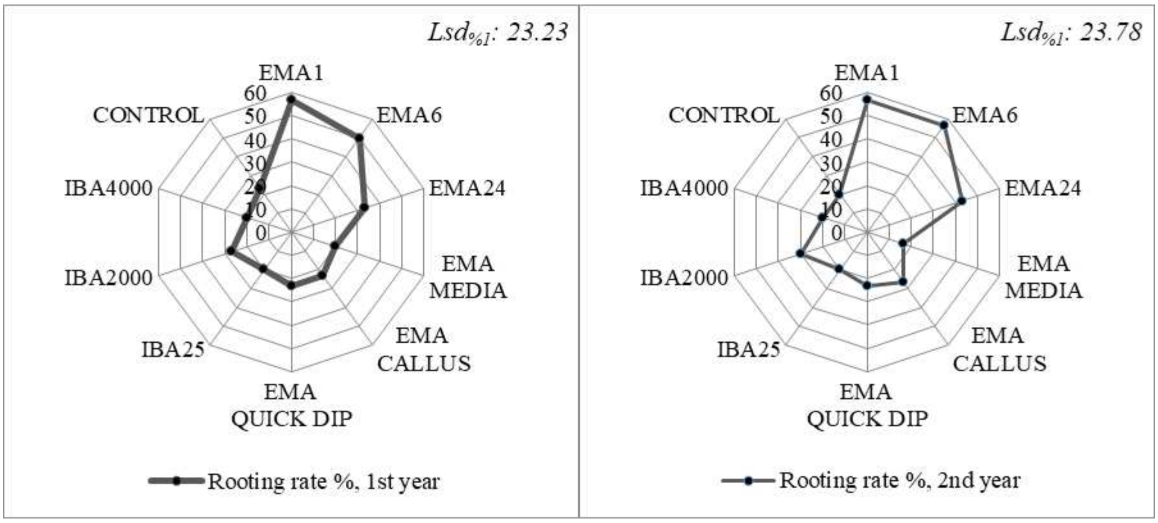

Figure 1. *Rooting rate (%) for 2017 and 2018 application years*

When we extend the statistical evaluation by considering the year as a factor, EMA1 and EMA6 applications were included in the same statistical group for rooting rate (%) performances in both experimental year, 56.7 (a), 53.3 (a), respectively (*Table 4*).

The fresh root weight (g) was measured with a 0.0001 g sensitive analytical balance and indicated in grams (g). Applications were determined to have a statistically significant effect on fresh root weight (g) in the first year, the best results were determined with EMA6 (10.6 a) and EMA1 (9.4 ab) (*Table 2*, *Fig. 2*). On the other hand there was no difference between applications in the second year (*Table 3*, *Fig. 2*). When both application years were evaluated together, EMA1 (9.8 a) and EMA6 (9.5 ab) were determined to be in the same statistical group like in the first year (*Table 4*).

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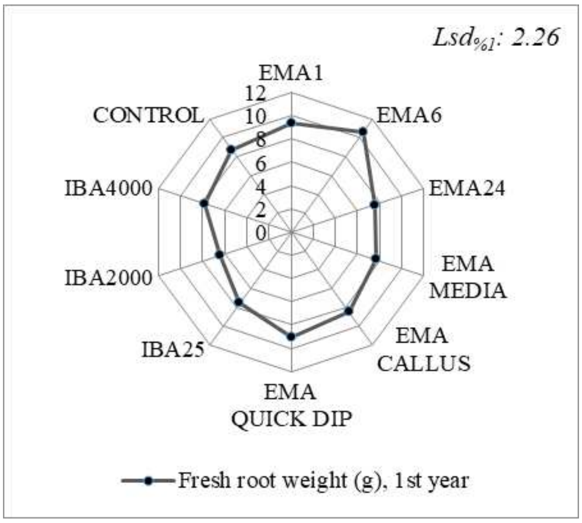

Figure 2. *Fresh root weights (g) for 2017 application year*

While the best result with dry root weight was determined with EMA6 (6.7 a) and CONTROL (6.2 a) in the first year (*Table 2*), in the second year, CONTROL (6.2 a), EMA1 (5.9 ab) and EMA24 (5.9 ab) applications were obtained in the same statistical group (*Table 3*, *Fig. 3*). In both year, best results were obtained form EMA1 (9.8 a) and EMA6 (9.5 ab) (*Table 4*).

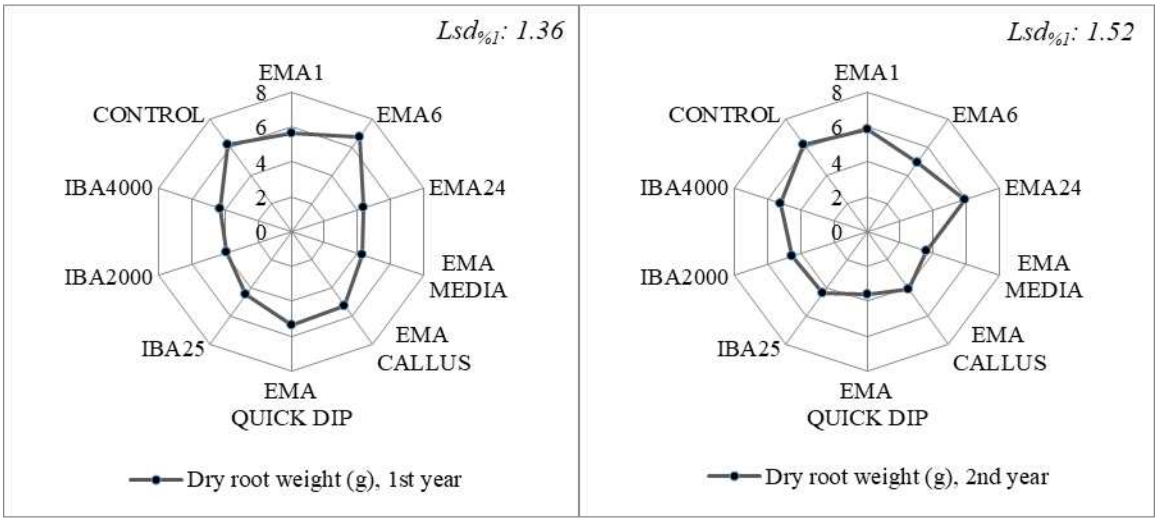

Figure 3. *Dry root weights (g) for 2017 and 2018 application years*

Stoma number was found 38.3 (a) with EMA CALLUS for the first year (*Table 2*). In the second year, EMA1 and EMA QUICK DIP application were in the same statistical group, 30.7 (a) and 29.7 (a), respectively (*Table 3*, *Fig. 4*).

When cuttings analyzed for shoot length (cm), number of internodes on main shoot, lenght of lignified summer shoot (cm), shoot weight (g) and root number, there were not significantly influenced by PGPR and IBA treatment for 2017 and 2018 treatment years (*Table 2*, *Table 3*). When we extended the statistical evaluation by considering the year as a factor, same results were obtained for all these values (*Table 4*).

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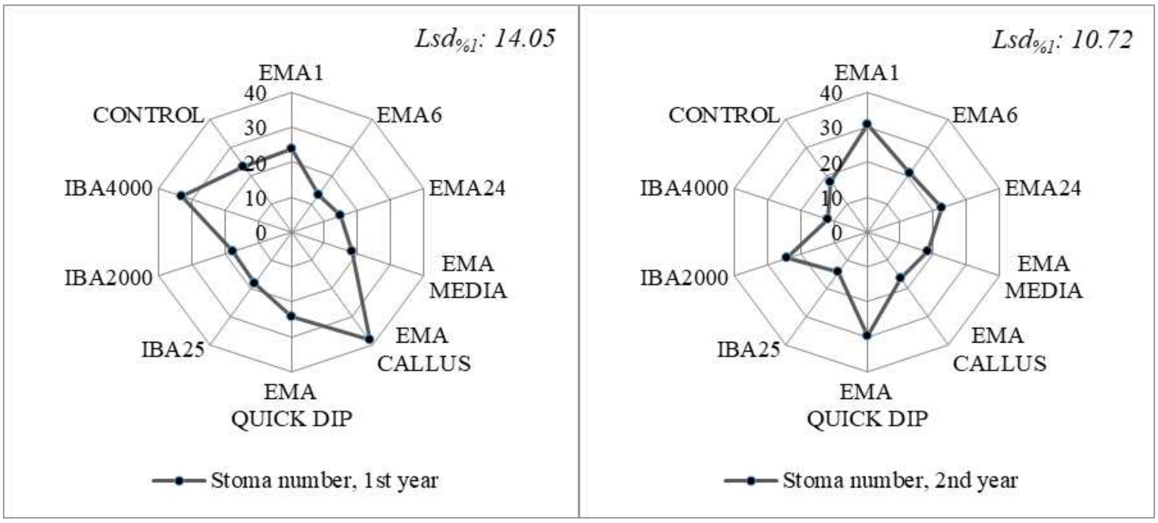

Figure 4. *Stoma number for 2017 and 2018 application years*

There was no significant difference in the success of root lenght (cm) PGPR and IBA applications in the first year of application (*Table 2*). The highest positive effect of PGPR application on root lenght (cm) of Ramsey was observed in the second year in EMA MEDIA (41.2 a) and EMA1 (35.0 ab), they were in the same statistical group (*Table 3*, *Fig. 5*).

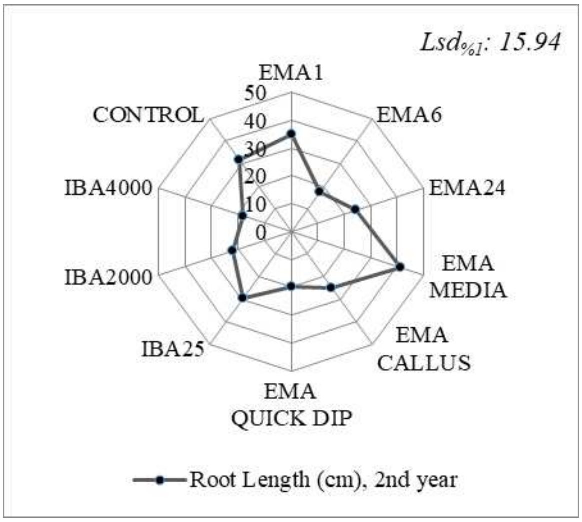

Figure 5. *Root length (cm) for 2018 application year*

The effect of EMA MEDIA (37.2 a), IBA25 (37.6 a) and EMA1 (34.1 ab) for root lenght (cm) were observed in the same statistical class in both year (*Table 4*).

The root number was not affected by PGPR and IBA applications. No statistical difference was found (*Table 2*, *Table 3*, *Table 4*).

When it comes to the effect of PGPR and IBA application on Ramsey rooting degree (0-4), it is observed that PGPR inoculated plants have the highest level of rooting degree at EMA MEDIA (3.7 a), IBA4000 (3.7 a) and EMA1 (3.3 ab) and these were in the same statistics group for the first year (*Table 2*). There was no statistical difference between the second year applications, but the EMA1 had the highest 3.7 value (*Table 3*, *Fig. 6*).

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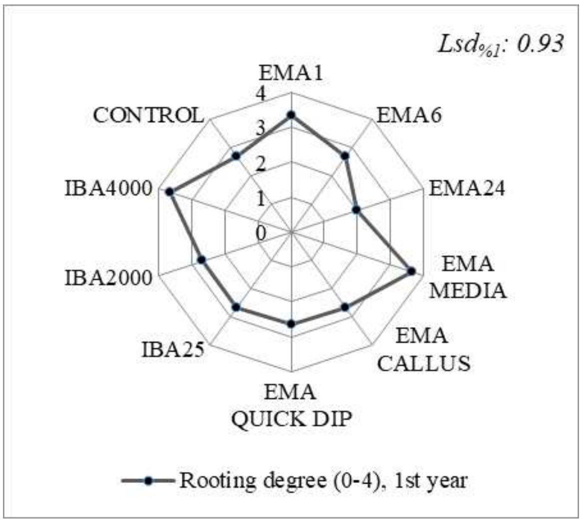

***Figure 6**. Rooting degree (0-4) for 2017 application year*

An overall evaluation of the data demonstrated that there were statistically important increases in the rooting rate (%) of Ramsey by EMA1 (3.5 a) and EMA MEDIA (3.3 ab) of PGPR when the two-year data is evaluated (*Table 4*).

In the first year, EMA1 (53.5 a), EMA QUICK DIP (52.5 a), CONTROL (52.0 a) and EMA24 (50.9 a) gave the best results for leaf area (cm^2) (*Table 2*), statistics groups changed in the second year, but EMA1 (51.6 a) application gave the best result again. EMA MEDIA (51.7 a), IBA25 (50.1 a) and IBA2000 (49.9 a) were found to be of the same importance (*Table 3*). Eventually, the leaf area was affected positively by EMA1 (52.6 a) application in both year (*Table 4*).

The leaf weight (g) value was not affected by PGPR and IBA applications, no statistical difference was found between applications in year 2017 and 2018 (*Table 2*, *Table 3*). When the two experimental years were evaluated together, the leaf weight value was increased by EMA1 (1.35 a) (*Table 4*).

As a result of the measurement of leaf area and leaf weight (g) values, it was determined that these two criteria were directly proportional to each other. It was determined that leaf weight increased as leaf area value increased (*Fig. 7*).

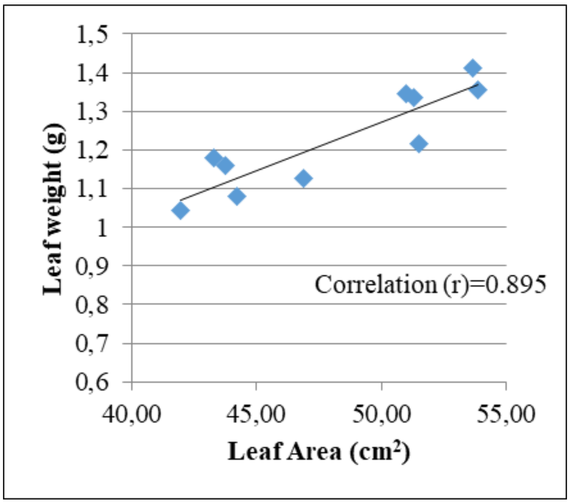

***Figure 7.** Correlation between leaf area and weight for both experimental years*

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Table 2. *First year (2017) results of PGPR and IBA applications to Ramsey rootstock cuttings.*

Applications / Criteria	EMA1	EMA6	EMA24	EMA MEDIA	EMA CALLUS	EMA QUICK DIP	IBA25	IBA2000	IBA4000	CONTROL	*LSD*
Rooting rate (%)	56.7 a	50.0 ab	33.3 bc	20.0 c	23.3 c	23.3 c	20.0 c	26.7 c	20.0 c	23.3 c	*%1*=23.23
Fresh root weight (g)	9.4 ab	10.6 a	7.6 bc	7.7 bc	8.5 abc	9.0 ab	7.6 bc	6.4 c	7.9 bc	8.7 ab	*%1* =2.26
Dry root weight (g)	5.7 abc	6.7 a	4.4 cde	4.3 d	5.2 bcd	5.4 abcd	4.5 cde	3.9 e	4.3 cde	6.2 ab	*%1* =1.36
Stoma Number	24.0 bc	13.3 c	15.0 c	18.3 c	38.3 a	24.3 abc	18.0 c	17.7 c	33.0 ab	23.3 bc	*%5*=14.05
Shoot Length (cm)	58.8	57.6	73.2	66.0	66.8	56.1	70.3	54.0	76.0	48.0	*ns*
Number of internodes on main shoot	16.3	14.3	18.3	16.2	12.4	15.0	19.6	14.0	19.1	14.8	*ns*
Length of lignified summer shoot (cm)	4.9	4.0	2.8	4.0	4.1	1.6	9.0	3.3	5.8	2.1	*ns*
Shoot weight (g)	14.3	13.4	16.8	21.1	17.9	8.6	20.7	16.0	17.3	17.0	*ns*
Root length (cm)	33.3	29.3	22.7	33.1	25.4	17.8	45.6	20.7	26.8	34.7	*ns*
Root number	6.0	6.0	5.3	7.7	3.7	7.0	7.3	6.0	8.3	5.7	*ns*
Rooting degree (0-4)	3.3 ab	2.7 bc	2.0 c	3.7 a	2.7 bc	2.7 bc	2.7 bc	2.7 bc	3.7 a	2.7 bc	*%5*=0.93
Leaf area (cm^2)	53.5 a	50.7 ab	50.9 a	46.5 abc	40.8 c	52.5 a	42.8 c	42.0 c	43.1 bc	52.0 a	*%1* =1.36
Leaf weight (g)	1.38	1.27	1.16	1.26	1.07	1.17	1.17	1.41	1.22	1.25	*ns*

Columns means followed by the same letter are not significantly different at 0.01 probability

Table 3. *Second year (2018) results of PGPR and IBA applications to Ramsey rootstock cuttings.*

Applications / Criteria	EMA1	EMA6	EMA24	EMA MEDIA	EMA CALLUS	EMA QUICK DIP	IBA25	IBA2000	IBA4000	CONTROL	*LSD*
Rooting rate (%)	56.7 a	56.7 a	43.3 ab	16.7 c	26.7 bc	23.3 bc	20.0 c	30.0 bc	20.0 c	20.0 c	*%1*=20.78
Fresh root weight (g)	10.2	8.4	9.3	6.7	7.5	6.7	7.6	7.8	8.5	8.2	*ns*
Dry root weight (g)	5.9 ab	4.9 abcd	5.9 ab	3.6 d	4.1 cd	3.6 d	4.4 cd	4.6 bcd	5.2 abc	6.2 a	*%1* =1.52
Stoma Number	30.7 a	20.7 abc	22.7 ab	18.3 bc	16.3 bc	29.7 a	14.3 bc	24.3 ab	11.7 c	17.7 bc	*%5* =10.72
Shoot Length (cm)	54.1	62.4	68.6	57.7	56.4	61.2	59.7	60.1	55.9	70.3	*ns*
Number of internodes on main shoot	16.5	14.6	16.6	15.5	14.7	16.6	16.9	15.5	14.7	17.8	*ns*
Length of lignified summer shoot (cm)	1.8	3.3	0.8	7.3	2.0	6.7	4.0	0.0	1.0	4.4	*ns*
Shoot weight (g)	11.4	16.9	16.5	16.7	9.5	13.2	13.2	9.6	25.2	16.2	*ns*
Root length (cm)	35.0 ab	17.3 c	24.5 bc	41.2 a	25.0 bc	19.8 bc	29.7 abc	22.1 bc	18.0 c	31.6 abc	*%1*=15.94
Root number	7.3	6.3	8.3	8.7	6.3	6.0	8.3	5.3	5.0	5.7	*ns*
Rooting degree (0-4)	3.7	2.3	2.3	3.0	3.0	2.0	3.3	3.0	2.3	2.7	*ns*
Leaf area (cm^2)	51.6 a	46.5 ab	42.1 b	51.7 a	46.0 ab	44.2 ab	50.1 a	49.9 a	46.5 ab	46.2 ab	*%1*=7.63
Leaf weight (g)	1.32	1.18	1.14	1.31	1.19	1.21	1.29	1.23	1.12	1.37	*ns*

Columns means followed by the same letter are not significantly different at 0.01 probability

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46934705

Table 4. *Both experimental years (2017-2018) results of PGPR and IBA applications to Ramsey rootstock cuttings.*

Applications / Criteria	**EMA1**	**EMA6**	**EMA24**	**EMA MEDIA**	**EMA CALLUS**	**EMA QUICK DIP**	**IBA25**	**IBA2000**	**IBA4000**	**CONTROL**	***LSD***
Rooting rate (%)	56.7 a	53.3 a	38.3 b	18.3 c	25.0 bc	23.3 c	20.0 c	28.3 bc	20.0 c	21.7 c	*%1*=13.63
Fresh root weight (g)	9.8 a	9.5 ab	8.4 abc	7.2 c	8.0 abc	7.9 abc	7.6 c	7.1 c	8.2 abc	8.4 abc	*%1*= 1.97
Dry root weight (g)	5.8 ab	5.8 ab	5.1 abc	3.9 c	4.65 bc	4.5 c	4.4 c	4.2 c	4.7 bc	6.2 a	*%1* =1.25
Stoma Number	27.3	17.0	18.8	18.3	27.3	27.0	16.2	21.0	22.3	20.5	*ns*
Shoot Length (cm)	56.4	60.0	70.9	61.8	61.6	58.6	65.0	57.0	65.9	59.1	*ns*
Number of internodes on main shoot	16.4	14.4	17.4	15.8	13.5	15.8	18.3	14.7	16.9	16.3	*ns*
Length of lignified summer shoot (cm)	3.36	3.6	1.8	5.3	3.2	4.6	6.5	3.3	3.8	3.3	*ns*
Shoot weight (g)	12.9	15.1	16.7	18.9	13.7	10.9	17.0	12.8	21.3	16.6	*ns*
Root length (cm)	34.1ab	23.3 bc	23.6 bc	37.2 a	25.2 abc	18.8 c	37.6 a	21.4 bc	22.4 bc	33.1 ab	*%5*=13.42
Root number	6.7	6.2	6.8	8.2	5.0	6.5	7.8	5.7	6.7	5.7	*ns*
Rooting degree (0-4)	3.5 a	2.5 cd	2.2 d	3.3 ab	2.8 abcd	2.3 cd	3.0 abc	2.8 abcd	3.0 abc	2.7 bc	*%5*=0.80
Leaf area (cm^2)	52.6 a	48.6 abc	46.5 bc	49.1 ab	43.4 c	48.3 abc	46.5 bc	45.9 bc	44.8 bc	49.1 ab	*%1*=5.39
Leaf weight (g)	1.35 a	1.22abcd	1.15 cd	1.29 abc	1.13 d	1.19 bcd	1.23abcd	1.32 ab	1.17 bcd	1.31 ab	*%5*= 0.15

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APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):4693-4705.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46934705

Discussion

Intensive nursery practice in viticulture still relies on the use of plant hormones like auxin to provide dense root formation and healty root development. It is very well known that IBA can stimulate root formation of cuttings. The essential role of IBA has been documented on induction of rooting and root formation (Galavi et al., 2013). Hartmann et al. (1997) reported that IBA application have impressive results in rooting the semi-hardwood cuttings in apple, plum and olive. Therefore, use of IBA has been recommended for rooting of grapevine rootstocks cuttings on the basis of the evidence reported in the literature by a great number of researchers (Yağcı, 2015). On the other hand, excessive use of hormone causes problems not only in terms of financal cost but also in terms of the cost to the environment. Therefore, the development and application of sustainable agricultural techniques is vital to decrease environmental pollution (Sabır, 2013). Many research on viticulture using PGPR resulted in enhanced plant growth, disease resistance, improved nutrient fixation or solution and graft success. Using different hardwood grafting type and varieties, Köse et al. (2003) and Köse et al. (2005) demonstrated that there were statistically important increase in the survival of grafts in relation with PGPR applications. In the current studies, PGPR inoculation alone or in combination markedly helped the young plants to survive and success in rooting.

This report provides the first data to demonstrate that PGPR applications can increase rooting rate (%) and full rooting characteristics in hardwood cuttings of Ramsey grapevine rootstocks. In order to determine rooting criteria of hardwood cuttings applied in the seedling production process different concentration and dipping times were tested with PGPR together with IBA applications. In contrast to the results at IBA and control, applications with PGPR increased rooting rate (%), rooting degree (0-4), root length (cm), fresh and dry root weight (g). All of these characteristics for cuttings are of the same importance in nursery practice.

PGPR and IBA applications did not have a specific influence on the root number at the base of Ramsey hardwood cuttings. However, the PGPR and IBA applications manifested their specific characteristics and showed significant variation in the number of roots, in the increasing variation of IBA4000 (8.3), EMA MEDIA (7.7), IBA25 (7.3) in the first year, and of IBA25 (8.3), EMA24 (8.3) and EMA1 (7.3) in the second year. Root number similarly responded to the change in both year in EMA MEDIA (8.2), IBA25 (7.8), EMA24 (6.8) and EMA1 (6.7).

Application of treatments, resulted in rooting rate (%) with EMA1 and EMA6 to be 56.7 a, 53.3 a, respectively (*Table 4*). Interesting thing is, IBA4000 applications have reduced rooting rate (20.0 c) in our researche. It is very well known that the hormones can stimulate root formation of cutting (Köse et al., 2003). Furthermore, Yağcı, (2015) reported the effects of IBA doses (1000, 2000, 4000 mg/L) on grafted sapling (different grafting combinations; Hamburg Misketi, Tekirdağ Çekirdeksizi, Italia and Trakya İlkeren cuttings/Ramsey rootstock). According to the researcher, the effect of IBA applications on rooting rate (%) was statistically significant. On the other hand, 4000 mg/L of IBA application adversely affected rooting rate (%), the best effect of different levels of IBA on rooting rate (%) was obtained at 2000 mg/L IBA. Some studies have reported that higher concentrations of IBA can adversely affect the rooting of cuttings (Cerveny and Gibson, 2005; Galavi et al., 2013). These results support our research. As for rooting rate (%) around hardwood cuttings of Ramsey rootstocks, had also remarkably positive influences with the highest degrees being promoted by EMA1 and EMA6 for both experimental years (*Table 4*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46934705

Even if the values of fresh root weight (g) were in positive order with EMA1 (9.8 a), EMA6 (9.5 ab), dry root weight (g) have reduced in CONTROL (6.2 a), EMA1 (5.8 ab) and EMA6 (5.8 ab) applications (*Table 4*).

As reported in some studies, IBA2000 and IBA4000 applications have reduced fresh and dry root weight (g). These results support our research. Researchers believe that high concentrations of IBA can cause damage to the cutting base (Kelen and Demirtaş, 2001; Galavi et al., 2013; Yağcı, 2015).

Correlation analyses were carried out on the parameters indicating leaf area (cm^2) and leaf weight (g). It was determined that leaf weight increased as leaf area value increased (*Fig. 7*).

When the effect of PGPR and IBA application of Ramsey hardwood cuttings is examined stoma number of the cuttings was affected by EMA CALLUS (38.3 a) in the first year (*Table 2)*. It is observed that the inoculated plants have the highest amount of stoma number with EMA1 (30.7 a) in the second year. EMA1 and EMA QUICK DIP application were in the same statistical group, 30.7 a and 29.7 a, respectively (*Table 3*).

On the other hand, an overall evaluation of the data demonstrated that there were statistically important increases in the rooting rate (%) and rooting degree (0-4) of Ramsey by EMA1 application. It is important to determine whether there is a positive relationship between these characteristics.

Comparing root lenght (cm) among the experimental groups for the two years showed a significantly ($P<0.05$) positive effect in IBA25 (37.6 a), EMA MEDIA (37.2 a) EMA1 (34.1 ab) (*Table 4*).

In *Table 4*, where the values obtained in terms of rooting degree, it is seen that plants formed root development level on a scala of 0-4 when they are inoculated with EMA1 and EMA MEDIA. There were found statistical differencies for root development level on a scala of 0-4 with EMA1 (3.5 a) and EMA MEDIA (3.3 ab), these were in the same statistical class. Köse et al., (2003) reported that positive effects of bacterial applications on rooting of rootstock having low rooting rate may be explained by auxin and/or auxin like plant growth promoting substance production of bacterial strains. They found that the bacterial combination decreased the rooting rate and rooting degree.

Conclusion

In conclusion, Ramsey is resistant to phylloxera and salt tolerance but its use is very limited due to the difficulties in rooting ability of the cuttings. Some treatments including plant growth regulators, especially IBA and other chemical substances have been applied on the cuttings for increasing the rooting percentage. The aim of this study was to investigate the effects of the PGPR, it was used with different application time on the rooting of Ramsey rootstock, having different features (with callus and without callus). These applications were compared with the most common IBA treatment and control group plants. Our results suggest that PGPR applications may have a great potential to stimulate the rooting of hardwood cuttings of grapevine rootstocks, with low rooting capability. In our study, it seems that PGPR application has a positive effect on rooting rate (%), fresh and dry root weight (g), root length (cm), rooting degree (0-4) and leaf area (LA, cm^2) of rootstocks with EMA1 and EMA6 applications. The EMA1 and EMA6 applications may be of benefit for Ramsey rootstocks, particularly for organic farming.

Acknowledgements. The author would like to thank EM AGRITON Co. Ltd. for their help in obtaining PGPR sources and Tekbağ Nursery Co. Ltd. in Turkey for plants materials.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_46934705

A STUDY ON THE PRODUCTION AND MARKETING OF BEE PRODUCTS PROVIDING BIODIVERSITY: CASE STUDY FROM TURKEY

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(Received 3rd Jan 2019; accepted 28th Feb 2019)

Abstract. This research aims to find out the marketing problems of the beekeepers in Muğla province, Milas district of Turkey and offer potential solutions. The main material of the research is the data gained via surveys, performed as face-to-face interviews with beekeeping farmers in Milas district. According to the data of Milas District Agriculture and Forestry Directorate, 721 beekeepers constitute the main part of the research. The proportional sample size has been determined as 62 people. The most significant problems that farmers face during the production are diseases and pests by 53.2%, while the most significant problem during marketing is the low selling price of honey by 80.6%.
Keywords: *beekeeping, beekeepers, honey, economy, price*

Introduction

Beekeeping is seen as a strategically important sector that should be supported in order to protect biodiversity and transfer it to next generations, provide food security, increase diversity, support domestic economy by increasing employment, and prevent erosion threat (Yılmaz, 2015).

Global climate change is one of the most important problems facing today's societies. The agricultural sector, which meets the food, nutrition and fiber needs of the society, is one of the most vulnerable sectors against climate change (Arbuckle et al., 2013). Honey bee is the most valuable pollinator of agricultural products worldwide economically. Approximately 73% of the plants cultured in the world are pollinated by bees (Reddy et al., 2012). One of the main factors affecting honey production is the climate (Malisa and Yanda, 2015). That is, beekeeping sector is dramatically affected by drought and climate change. Sustainability of soil and water resources is of vital importance for beekeeping. Sustainability of soil and water resources is of vital importance for beekeeping because the bee needs healthy vegetation and flora. On the other hand, seasonal effects of global warming negatively affect bees. The effect of climate change on bees may cause the bees not to fulfill the function expected of them in pollination. This reduces the quality and quantity of crop production and can lead to economic loss of beekeepers.

Beekeeping is not a soil-based production field, for that reason it can be an only income source for the farmers who have no or limited land. Running with a limited budget, establishing and running the business with low expenses, generating income in short terms, marketing bee products easily and providing bee and bee breeds domestically are the most significant reasons that make beekeeping attractive (OGM, 2013).

Beekeeping activities which is an important sub-sector of livestock provides a prominent added value. As a sub-sector of livestock and agriculture, beekeeping activity creates a significant added value for the economy. Sustainability of the added value is directly related to developing marketing opportunities for the products and analyzing marketing problems. Besides of having great geographical features and rich flora, Turkey has a significant place in global beekeeping sector with its colony assets (Köseman et al., 2016). Beekeeping in Turkey is an agricultural occupation done to produce honey only. Most of the beekeeping businesses are family-run businesses (Şahinler and Gül, 2003).

By the year of 2016, there are 90 564 654 bee hives in the world and they produce 1 786 996 ton honey approximately (FAO, 2018). By the same year in Turkey, these numbers are respectively 7 900 364 and 105 727.435 tons (TSI, 2018). Namely, Turkey produces 8.72% of the hive assets and 5.92% of honey production in the world. By the year of 2017, there are 83 210 beekeeping businesses in Turkey. By the same year, the number of total hives is 7 991 072. In Muğla province where the research is conducted, there are 6000 families that are bekeeping farmers in 334 villages. In Muğla province, the main product of beekeeping is pine honey; and Muğla provides 70-75% of the production in Turkey. In Muğla 35000-40000 people are employed in beekeeping sector (Şahin, 2015). By 2017, Muğla supplies 11.99% of the hive assets and 13.86% of honey production in Turkey (TSI, 2018).

Being the capital of beekeeping production, Muğla province Milas district has a significant potential in terms of beekeeping activities in Turkey. Beekeeping activities which is no longer a side income started to be basic living source for most of the families in rural areas in Milas district. By 2017, 15.39% of the beekeeping businesses of Muğla province is located in Milas district. By 2017, 18.04% of the natural honey produced in Muğla province and 2.06% of the beewax have been produced in Milas district. On the other hand, 17.74% of the total hive assets of Muğla province (old and new) is located in Milas district (TSI, 2018).

Main aim of the study is to indicate the structure of beekeeping production and marketing in Muğla province Milas district. Within this scope, firstly socio- economic structure of the beekeepers, the production and marketing structure of beekeeping goods produced in businesses and problems of the farmers that they face during production and marketing phase have determined; possible solution offers for the problems have suggested.

Materials and methods

Study area

The geographical position of Milas is 37° 18′ 59″ North and 27° 47′ 2″ East. Milas is located in the southwest of Turkey within the borders of Aegean Region in the Menteşe Mountainous area. Administratively, it is a central district located within the borders of Muğla province (*Fig. 1*). It is bordered by the city of Muğla and Yatağan district in the east, Yenihisar district of the city of Aydın in the west, Kocarlı, Karpuzlu and Çine districts in the north, Gökova Bay in the south and Bodrum district in the southwest (Çakar, et al., 2011).

It is possible to examine agriculture in Milas as fruit and vegetable, grain and industrial plant production. Because of its climate and geographical structure, Milas is one of the rare place where many products can be grown. Its primary agricultural products are olive, forage plants, wheat, barley, grain corn, cotton, tobacco, vegetables and citrus fruits. Milas is the most important olive and olive oil production area of the Aegean region. Industrialization related to olive cultivation has been the primary key industry of Milas for a very long time. Animal husbandry is also important in the district. Beekeeping and honey production as well as fish farming have a significant for district (Anonymous, 2015).

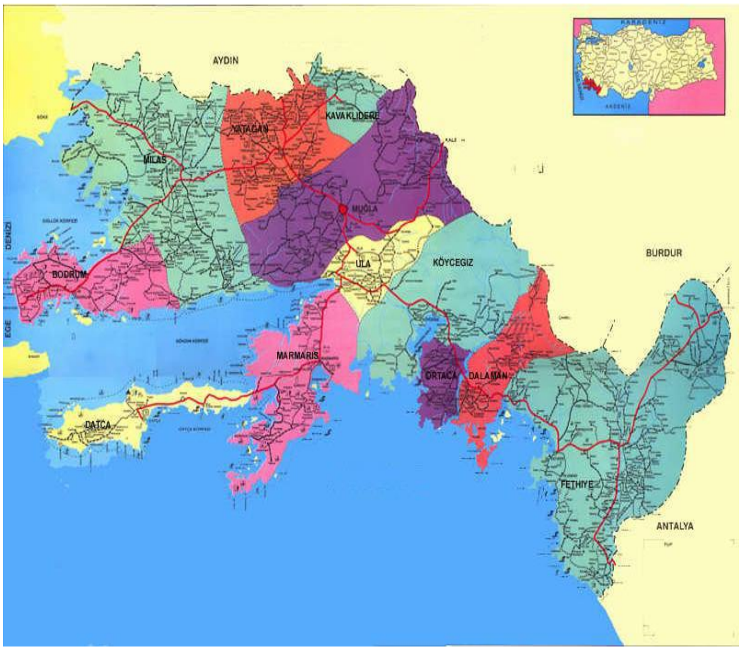

Figure 1. *Map of Muğla Province. (Source: Anonymous, 2004)*

Method applied when collecting data

The main material of the research is data gained via surveys with face-to-face interviews done among the beekeeping farmers in Milas district of Muğla province in Aegean Region. According to the records of Agriculture and Forestry District Directorate of Milas, 721 beekeepers constitutes the main part of the research. Having specified by proportional sample volume (Newbold, 1995), interviews were done with the farmers. 90% confidence interval and 10% error margin is taken as a basis in the research. The data of the study were obtained from the face-to-face surveys conducted with beekeepers in Milas district in April-May 2017. Nine neighbourhood (village) of the district is examined as the scope of the research.

$$n = \frac{Np(1-p)}{(N-1)\sigma_P^2 + p(1-p)} \quad \text{(Eq.1)}$$

In *Equation 1:* N = total number of farmers as interview; N = population; P is taken as 0.50 to be able to reach maximum sample volume; (1 - p) = 0.5; σ_{px}^2 = variance. In the calculation, total number of the farmers that is to be interviewed is figured as 62.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47074724

Method applied when analyzing data

After acquiring the data for the research, assessment phase has been started. The data from the surveys has been coded and recorded. Definitive statistical analysis is used in order to assess the data.

Results

General features of farms and farmers

The ages of the farmers range from 27 to 74, and average age of the farmers is 48. When it comes to adaptation of new techniques and technologies, education level of the beekeepers has an important role (Ogunjimi et al., 2016). The education level of the beekeepers participating in the current study was found to be low. In the research, average education year of the famers is figured as 5.9. Average household population of beekeepers is 3.6. It has been calculated that average agricultural experience of the farmers is 26.3 years, average experience as beekeepers is 19.5 years. It is found that 64.5% of the farmers who participated in the research took out loan. In the research, it is specified that only 9.7% of the farmers have business records regularly.

88.7% of the farmers (55 farmers) who participated in the research have other agricultural activities than beekeeping. Average business width of the farmers is figured as 23.1 decares. It is seen in the research that five farmers have no agricultural land. It is determined that 98.2% of the beekeeping businesses continue their agricultural activities on property land, 1.8% of them continue agricultural activities on rented land. 61.8% of the farmers make mixed production (crop+animal production), 32.7% make only crop production, 5.5% make only animal production.

Farmers who participated in the study stated that 38.1% of their agricultural income came from beekeeping activities. 25.8% of the farmers participating in the survey have non-agricultural income. Farmers are engaged in professions such as local headman, coffeehouse owner, fisherman, labourer apart from agriculture. There are farmers who are retired and traders, as well.

Findings related to production and marketing

6.5% of the farmers participating this research stated that they started beekeeping with old type hive, 88.7% of them started with buying colonies with modern hives and 8.1% of them started with buying swarm.

The reasons for farmers' to do beekeeping are examined and for this purpose *Table 1* is formed. The first reason of farmers' to do beekeeping is that there is no other source of income (37.1%). This is followed by the habit inherited from family (33.9%) and ability to access to the product in short time (29%).

Honey can be defined as the natural product in which the plant nectars, the secretions of the alive parts of the plants or the secretions of plant-absorbing insects that live on the living parts of the plants, are collected by the honeybee (Apis mellifera) and combined with their own specific substances, decreasing the water content and storing it in the honeycomb (Anonymous, 2005). Beekeeping activities can be considered as an important rural development tool, which provides a certain rate of income to a large number of farmers, in rural areas. Undoubtedly, the first condition for farmers to earn income from beekeeping activities is that they produce clean, hygienic and good quality honey. Determination of product properties, honey additives and residue levels of honey produced by farmers can be measured by honey analysis. For this reason, it is very important for farmers to make honey analysis for honey they produced. In the study, it was determined that the farmers' honey analysis rate is quite low. It was determined that only two producers had honey analysis.

Table 1. *Reasons for farmers to do beekeeping*

Reasons to do beekeeping	n	%
Ease of maintenance	10	16.1
Short production period	18	29.0
Habit inherited from family	21	33.9
Curiosity, hobby	15	24.2
There is no other source of income	23	37.1
Because the product is suitable the region	2	3. 2

*Total passes 100% since there are more than one answer

The presence of colony in the production of bee products is of great importance. Naturally, as the number of colonies increases, the production amounts of bee products are expected to increase. For this reason, in this study, farmers' colony assets are investigated. In the study, the percentage of farmers indicating the increase in the colony assets in the last three years was 17.7% and the percentage of farmers indicating the decrease in it was 48.4% (*Table 2*).

Table 2. *Change in the colony assets in the last three years*

	n	%
Increased	11	17.7
No change	21	33.9
Decreased	30	48.4
Total	62	100.0

The migratory beekeeping is based on the principle of transporting colonies from one region to another in order to benefit from different flowering periods in plants and to protect the bees from adverse winter conditions. 77.4% of the farmers participating in the research is doing migratory beekeeping, 22.6% is doing fixed beekeeping.

It was determined that the farmers moved their hives to various provinces such as Kayseri, Ankara, Eskişehir, Sivas, Konya, Nevşehir, Tekirdağ, Niğde, Kütahya, Afyonkarahisar, Kars, Sakarya, Edirne and Burdur. A farmer who stated that he was a fixed beekeeper stated that he had moved his hives to the neighbouring villages. It is specified that the farmers usually go in May and return in August. When the farmers' honey harvesting frequency was examined, it was concluded that half of the farmers participating in the study do harvesting twice a year. 30.6% of the farmers harvest three times a year (*Table 3*).

In the study, the average number of hives owned by farmers was found as 162 units. Today, beekeeping business is one of the means of living of many families in rural areas. The products obtained from beekeeping business, such as pollen, propolis, beeswax and primarily honey, provide a significant added value in the income of the

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producers engaged in beekeeping in rural areas (Çukur et al., 2016). 98.4% of the farmers interviewed in this study were identified to produce honey, 46.8% of them produce beeswax and 29% of them produce queen bees (*Table 4*).

Table 3. *Farmers' honey harvesting frequency*

	n	**%**
Once in every three weeks	2	3.2
Once in a month	6	9.7
Twice a year	31	50.0
Three times in a year	19	30.6
Four times in a year	1	1.7
Once in a year	3	4.8
Total	62	100.0

Table 4. *Number of farmers producing bee products*

	n	**%**
Number of farmers producing extracted honey	61	98.4
Number of farmers producing comb honey	17	27.4
Number of farmers producing beeswax	29	46.8
Number of farmers producing swarm	23	37.1
Number of farmers producing queen bee	18	29.0
Number of farmers producing pollen	7	11.3

The average production quantities of bee products are shown in *Table 5*. From the farmers participated in this research, it is determined that the average amount of extracted honey production was 2334.5 kg, the average comb honey production amount was 81 kg and the average beeswax production amount was 48.9 kg. In the study, average extracted honey production per hive was calculated as 14.41 kg.

Table 5. *Average production amount of bee products*

	Production amount
The amount of extracted honey (kg)	2334.5
The amount of comb honey (kg)	81.0
The amount of beeswax (kg)	48.9
The amount of swarm (unit)	10.2
The amount of queen bee (unit)	16.3
The amount of pollen (kg)	3.8

It was specified that 87.1% of farmers have problems with honey production. Farmers who have problems with honey production are asked about their problems and *Table 6* is formed according to the answers. Diseases and pests (53.2%) and negative effects of global warming (41.9%) are among the most important problems of beekeepers.

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Table 6. *Farmers' problems related to honey production*

	n	%
Diseases and pests	33	53.2
Accommodation and safety issues in migratory beekeeping	25	40.3
Negative effects of global warming	26	41.9
High transportation costs	25	40.3
High production costs	18	29.0
Lack of information about beekeeping	9	14.5
Decrease of crop production	8	12.9
Non-diversification of bee products	7	11.3
Lack of tools and equipment	5	8.1
Problems related to breeding bees	4	6.5
Difficulties in obtaining loans	4	6.5
Honey analysis results do not appear	2	3.2
Quality problems	1	1.6

*Total passes 100% since there are more than one answers

When the sales status of bee products is examined, it was specified that 91.9% of the farmers sold extracted honey and 16.1% sold comb honey and beeswax (*Table 7*).

Table 7. *Number of farmers selling bee products*

	n	%
Number of farmers selling extracted honey	57	91.9
Number of farmers selling comb honey	10	16.1
Number of farmers selling beeswax	10	16.1
Number of farmers selling swarm	2	3.2
Number of farmers selling pollen	2	3.2

When the average sales amount of bee products were examined in the study, it was identified that the average amount of extracted honey sales of the farmers is 2323.7 kg, the average comb honey sales amount is 75.7 kg and the average beeswax sales amount is 12.4 kg (*Table 8*).

The farmers' sales prices of bee products are shown in *Table 9*. As shown in the table, the sales price of comb honey is 5.96 €, extracted honey is 2.16 €, beeswax is 7.09 €, pollen is 7.70 € and swarm is 77.03 €.

Table 8. *The average sales amount of bee products*

	Sales amount
The sales amount of extracted honey (kg)	2323.7
The sales amount of comb honey (kg)	75.7
The sales amount of beeswax (kg)	12.4
The sales amount of swarm (unit)	1.6
The sales amount of pollen (kg)	3.1

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DOI: http://dx.doi.org/10.15666/aeer/1702_47074724

Table 9. *Actual and expected average sales prices of bee products. (Source: TCMB, 2018)*

	Sales price (TL)	Sales price (€)	Expected sales price (TL)	Expected sales price (€)
Comb honey	23.2	5.96	45.5	11.68
Extracted honey	8.4	2.16	14.4	3.70
Beeswax	27.6	7.09	41.9	10.76
Pollen	30	7.70	100	25.68
Swarm	300	77.03	400	102.71

*As of 15 May 2017 1 Euro = 3.8946 TL

Sales prices of bee products and sales prices of bee products according to farmers are shown in *Table 9*. As it is seen from the table, sales prices of bee products are well below the sales prices expected by farmers. The price differences were calculated as 5.72 € in comb honey, 1.54 € in extracted honey, 3.67 € in beeswax, 17.89 € in pollen and 25.68 € in swarm.

Farmers' extracted honey selling areas are presented in *Table 10*. As it can be seen from the table, the majority of the farmers sell extracted honey to the merchant (75.8%).

Table 10. *Farmers' selling places of extracted honey*

	n	%
Directly to the customer	6	9.7
Merchant	47	75.8
Wholesaler	3	4.8
Retailer	7	11.3

*Total passes 100% since there are more than one answers

In this study, it is determined that 99% of the produced honey is sold while 1% is preserved for home consumption.

Reaching agricultural information at the right time has a vital importance for the success of farmers. The access of farmers to reliable, timely and necessary information will reduce the risks and uncertainties of farmers and help them to make the right decision (Mbagwu, et al., 2018). Access to agricultural marketing information of farmers and other agricultural stakeholders can be improved through accessible agricultural marketing information systems (Amer et al., 2018). Agricultural information is a critical component in increasing small-scale agricultural production and in reaching farmers' profitable markets. In the case of reaching market information by farmers, yield increase will be achieved (Wawire et al., 2017). Access to the market is one of the most important factors affecting the performance of agriculture and plays an important role in developing and diversifying the means of living of small farmers in developing countries. Inadequate access to market information leads to moral damage and high transportation costs and hence prevents some farmers from accessing the market (Okello et al., 2014). By means of reaching agricultural markets, small-scale farmers are able to increase agricultural production, realize economic growth and reduce hunger and poverty (Magesa et al., 2014). Market information flow to small-scale farmers is poor. This complicates farmers' access to the market (Matto, 2018; Wawire

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et al., 2017). In this study, when farmers' status of access to information related to domestic (market price, production, demand) and foreign (market price, production, demand) markets is examined, it was determined that 48.4% of the farmers have access to the information related to domestic market and 17.7% of the farmers had access to the information related to the foreign market.

When the extracted honey sales of the farmers is examined, it is determined that in general they sell the extracted honey in cash (69.4%) (*Table 11*). Examining the months of extracted honey sales of the farmers, it draws attention that honey is sold intensively in September, October and November (*Table 12*).

Agricultural marketing, especially in developing countries, plays an important role in reducing poverty sustainably and ensuring household food safety (Katengeza, 2012). 83.9% of farmers were found to have problems with honey marketing when this problem was examined. Low sales price of honey (80.6%), unfair competition (38.7%), fluctuations in market prices (27.4%), and inability to access market related information (21%) are amongst the most important problems faced by farmers in honey marketing (*Table 13*).

Discussion

This study was carried out in order to reveal the production and marketing structures of bee products of beekeepers in Milas district of Muğla province. Farmers participating in the research can be said to be middle-aged. In a research done by Adedeji et al. (2016), it is specified that 90% of the beekeepers is in 40- 49 age range.

Table 11. *Farmers' form of sale of extracted honey*

	n	%
Cash	43	69.4
Dated	6	9.7
Cash+dated	8	12.9
Does not sale	5	8.0
Total	62	100.0

Table 12. *Farmers' sales months of extracted honey*

	n	%
January	6	9.7
February	3	4.8
March	1	1.6
April	1	1.6
May	1	1.6
June	-	-
July	8	12.9
August	11	17.7
September	26	41.9
October	34	54.8
November	25	40.3
December	15	24.2

*More than one answers received

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47074724

Table 13. *Farmers' problems related to honey marketing*

	n	%
Low sales price of honey	50	80.6
Unfair competition	24	38.7
Fluctuations in market prices	17	27.4
Inability to access information about the market	13	21.0
Lack of financial power	11	17.7
Distrust to the trader	10	16.1
Inadequate honey consumption of consumers	9	14.5
Non-diversification of bee products	5	8.1
Insufficient storage conditions	4	6.5
Inability to receive the sales price in time	4	6.5
Changes in consumer demand	3	4.8
No payment in advance	3	4.8
The farmers cannot see the honey analysis reports due to the fact that the analysis made by the companies	2	3.2
Inability to obtain products with the same quality	1	1.6
Insufficient support	1	1.6

*Total passes 100% since there are more than one answers

It was determined that the farmers participating in the study were highly experienced in beekeeping (19.5 years). In a research done by Belie (2009) in Ethiopia, average experience of the beekeepers is figured as 14.5 years. In a research done by Ceyhan and Canan (2017) average age of beekeepers is figured as 49, average experience is figured as 21 years.

In the study, it was determined that most of the farmers received loans (64.5%). In a research done by Belie (2009), it is determined that 85% of the beekeepers take out loan.

It was determined that very few of the farmers participating in the study kept their business records regularly (9.7%). In a research done by Mujuni et al. (2012) in Uganda, it is found that 10% of the beekeepers keep a record of hive numbers. In a research done by Şeker et al. (2017), it is pointed out that 69.1% of the beekeepers do not have a record.

It was determined that a significant number of farmers participating in the research also carried out other agricultural activities besides beekeeping. In a research done by Burucu and Bal (2018) in Kastamonu province, it is seen that 66.7% of the farmers conduct other agricultural activities than beekeeping. In a research done by Tullu (2014) in Ethiopia, it is found that the income of the farmers is supplied from beekeeping by 40%, other animal activities by 33% and crop production by 27%. In a research done by Abazinab et al. (2016), it is indicated that 63.4% of the income of the farmers is supplied by beekeeping activities. 8.82% of the farmers who participated in the research done by Kekeçoğlu et al. (2013) perform only beekeeping, the ones who does other agricultural activities and beekeeping activities are found to be 49.26%. In a research being conducted by Kalayu et al. (2017), 27.7% of farmer income is determined from crop production, 23.8% from animal production, 16.9% from beekeeping activity and 15.4% from irrigation. In the study conducted by Chauhan and Sharma (2000), it was

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determined that 69% of household income of farmers came from beekeeping. In a research done by John (2014) in Tanzania, 52.50% of the farmers' basic income source was determined to be beekeeping.

High majority of the beekeepers (88.7%) participating in the current study started the business of beekeeping with colonies in modern hives. Guyo and Legesse (2015) found that 92% of the farmers started beekeeping by trapping swarms while 7% of them started with the gift hives received from their family. In the research conducted by Tesfaye et al. (2017), it was determined that the 98.3% the farmers start beekeeping by trapping swarms and 1.7% of the farmers start with inherited hives from family. Abazinab et al. (2016) specified that 71.7% of the farmers started beekeeping via trapping swarms, while 28.3% of them started beekeeping through the gifted hives from family. In the research conducted by Gebreyohans and Gebremariam (2017), it is determined that 38% of the farmers started beekeeping through the gifted hives from family, 35% of them started via purchasing the hives and 15% of them started beekeeping using the hives retrieved from governmental and non-governmental organizations.

In the research, the most important reason for farmers to start beekeeping was determined to be obtaining yields in a short time. In the research done by Burucu and Bal (2018) in Kastamonu province, the first reason of farmers' to start beekeeping is the habit inherited from family by 67.90%. In the study conducted by Tunca and Çimrin (2012), it was determined that beekeeping activities is the only source of income for 17% of the farmers and additional source of income for %57 of them. In a research conducted by Borum (2017) in South Marmara Region, it is determined that 83.75% of the farmers do beekeeping as side job and hobby. In a research conducted by Şahinler and Gül (2003) in Hatay province, it is specified that 38.95% of the beekeepers do beekeeping to make a living, 42.11% of them do it with the aim of side income and 18.95% of them pursue it as a hobby.

The colonies possessed by nearly half of the beekeepers participating in the current research have decreased in the last three years. In a research conducted by Potts et al. (2010) that involves 18 European countries, it was determined that the number of hives decreased in central European countries while it increased in some Mediterranean countries. Demir et al. (2017) found that 8.8% of the farmers were considering to leave the beekeeping in the future. In the study conducted by Çevrimli and Sakarya (2018), the rate of farmers who is considering to increase the number of bee hives in the next years was found to be 57.5%.

It was determined that most of the beekeepers (77.4%) participating in the current research perform migratory beekeeping. In a study conducted in Croatia by Barlovic et al. (2009), it was determined that one third of the farmers were migratory beekeepers. In a study conducted in India by Sharma et al. (2013), it was found that the farmers were carrying 250-300 hives to 300-800 km away. Another study by Adgaba et al. (2014) found that 93% of the farmers were migratory beekeepers.

Environment, climate and flora have important effects on the number of harvests made. Therefore, the number of harvests may vary from region to region. It was found that half of the participating beekeepers have two honey harvests a year. In a study conducted by Gebremeskel et al. (2014), the rate of farmers who harvested once a year is specified as 61.5%, twice a year is 36.5% and three times a year is 1.9%. In their study, Kiros and Tsegay (2017) shows that more than 70% of the farmers harvested honey once a year while 25% harvest three times a year. In a study conducted by

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Abejew and Zeleke (2017), it is specified that 34% of farmers harvest more than twice per year. In a study conducted by Abazinab et al. (2017), it is showed that the rate of farmers who harvested once a year is 37.8%, twice a year is 52.8% and three times a year is 9.4%. In the study conducted by Gebreyohans and Gebremariam (2017), it was found that 75% of the farmers harvested in September and October and 34.16% in July and August.

When the literature is reviewed, it is seen that there are significant differences between the numbers of hives possessed by beekeepers because many factors can be effective on the number of hives possessed. The participating beekeepers were found to have 162 hives on average. In a study conducted by Sarab et al. (2018) in Iran the average number of hives was found as 176. In another study conducted by Kadirhanoğulları (2016) in Iğdır province, it was determined that each business has 67 units of bee hives on average. In another study conducted by Tarekegn et al. (2017), it is determined that the families doing beekeeping business has 19 hive assets on average. In the research done by Moniruzzaman and Rahman (2009), the average number of hives owned by farmers was found as 28. In the research conducted by Kezic et al. (2008), farmers with up to 60 hives were referred as hobby beekeepers, farmers who has between 61 to 150 hives were referred as part-time beekeepers and farmers with more than 151 hives were referred as professional beekeepers. In that research, it was determined that 56% of farmers were doing beekeeping as hobby, 31% of them were part time beekeepers and 13% of them were professional beekeepers. In the study conducted by Kutlu (2014) in Gaziantep province, it is determined that the rate of farmers who consider beekeeping as the main source of income is 54%, as a side income is 37% and as a hobby is 9%.

Almost all of the participating farmers (98.4%) were determined to produce honey. In the study conducted by Öztürk (2017), it was determined that 82.5% of the farmers produce only honey and 17.5% produce pollen and royal jelly besides honey production. In the study conducted by Grgic et al. (2018) in Croatia, the yield of honey per hive was found to be 18.33 kg. In the study conducted by Yıldırım and Ağar (2008) the yield of honey per hive was found to be 10.72 kg. In the study conducted by Onurlubaş and Demirkıran (2017), it was determined that on average the farmers produced 2555.75 kg of honey annually. In the study conducted by Peter (2015), the average honey yield per hive in South Africa during the 2007/08 period is reported to be 12.77 kg. In a study conducted by Vural and Karaman (2009) in Bursa province, the average hive asset of farmers was found to be 168.40 and the average honey production was 4527.33 kg.

It was determined that the most important problem faced by the beekeepers during the production phase was diseases and pests. Effective and proper fight against bee diseases and pests is seen as very important in terms of the sustainability of beekeeping in the district. In other studies on the subject, diseases and pests have been found to be an important problem. In the study conducted by Ahikiriza (2016), it was concluded that limited information about production, diseases and pests, low colony assets and fires are the most important problems related to production. In a survey conducted by Yemane and Taye (2013) in Ethiopia, it was determined that the problems of beekeeping were insufficiency of beekeeping equipment, inadequate colony assets, high prices of modern hives, pests and beekeepers' lack of knowledge. In a study conducted by Ogunjimi et al. (2016) in southwest Nigeria, it was determined that the most important problems of the beekeepers regarding the production were the lack of information about beekeeping, the

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lack of loan opportunities and the existence of insufficient processing technologies.. In the study conducted by Aksoy et al. (2017) in Erzurum province, the most important factors affecting honey production are found to be unsuitable climate conditions and winter loss. In the study conducted by Gebreyohans and Gebremariam (2017), 83.3% of the farmers stated that they see bee pastures as the most important problem especially in the dry season. In the study conducted by Ogunjimi et al. (2016), it is determined that the most important problems related to beekeeping is inadequate training related to beekeeping practices, insufficient loan opportunities and lack of beekeeping equipments.

In the current study, the most important bee product sold was found to be honey. In their study, Adedeji et al. (2016) stated that 74% of farmers produce honey for commercial purposes. In the study conducted by Burucu and Bal (2018) in Kastamonu province, it is determined that 82.72% of the farmers sell their honey.

In the research, it was determined that there were significant differences between the actual sales prices of bee products and the sales prices expected by the farmers for their products. In a research done by Kezic et al. (2008), it was found that the average selling price of honey for beekeeping professionals was 2.07 €/kg. In a study conducted by Cejvanovic et al. (2011) in Bosnia and Herzegovina the wholesale price of honey was found to be 2.11 €/kg. In a research conducted by Saner et al. (2004) in İzmir and Muğla provinces, the farmers' wholesales price of the extracted honey is calculated to be 1.65 €/kg. In a research conducted by Demir et al. (2017), it is specified that only 37.5% of the farmers were satisfied with the current honey prices. In a research conducted by Köseman et al. (2016) it is found that the rate of the farmers stating that in the last five years their beekeeping income is decreased is 38.3% while the farmers stating that their income has increased and decreased in this period is 39.6%.

In the study, it was determined that high majority of the beekeepers were found to be selling their products to traders. It was specified, by Onurlubaş and Demirkıran (2017) in their study in Edirne province, that 37.5% of the honey produced by the farmers was sold directly to the consumers.

It the current study, it was also found that the amount of honey allocated to the consumption of the beekeepers' their own families is very low (1%). In their study, Shibru et al. (2016) found that 95.8% of the farmers sold the honey immediately after the harvest while 4.2% of them stocked the honey for home use. In a study conducted by Girma et al. (2008) it is determined that 90% of the honey is sold and 10% is consumed at home. In their study Lemita (2010) found that farmers sold 97% of the produced honey in local markets for cash and 3% of them consumed at home. In a study conducted by Kinati et al. (2013) in Ethiopia, it is determined that 75% of the honey sold after between one and six months, while 1.7% of it is stocked more than two years by the farmers. In the study conducted by Ambaw and Teklehaimanot (2018), it was determined that 39.7% of the farmers did not stock honey while 41.7% of them stocked from one to six months.

In this study, it was also determined that honey was sold in almost all months. In the research conducted by Abebe (2009) in Ethiopia it was found that farmers sold 27% of the honey in December, 25% of it in January and 18% of it in February.

The low sales price of honey was determined as the most important problem faced by beekeepers in marketing. In the study conducted by Kadirhanoğulları (2016), it was determined that the lack of effective marketing cooperatives to market the produced honey was stated as the biggest problem of all the farmers. In a study conducted by

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Kumar (2013) in India, low honey sales prices, high cost of transporting hives from one place to another and financial difficulties experienced during the purchase of raw materials have been identified as problems by farmers.

Conclusion

Bees are vital for the continuity of the ecosystem as they ensure the pollination of plants. Beekeeping is a very important agricultural activity in terms of diversifying agricultural income and agricultural activities in rural areas and increasing agricultural income. Beekeeping has an important place both in Muğla and Milas.

The average honey production per hive in Turkey is about 14.63 kilograms (Şahin, 2015). In the research, the average extracted honey production per hive was found to be 14.41 kg, this figure appears to be slightly less when it is compared to average production of Turkey. It is thought that productivity will increase due to the modern beekeeping techniques of the farmers. Therefore, relevant agricultural extension programs should be organized. It was determined that 87.1% of farmers had problems with honey production. Diseases and pests (53.2%) and negative effects of global warming (41.9%) are among the most important problems of beekeepers. Beekeeping courses should be organized in order to solve the problems of farmers related to production, to increase their technical knowledge on bee farming and diseases and pests. Farmers should be educated with practical trainings.

It is stated that 83.9% of farmers have problems with honey marketing. The most important problem farmers face with honey marketing is the low price of honey (80.6). There are no beekeeping cooperatives in the research area. This reduces the bargaining power of beekeepers and beekeepers sell bee products at low prices. In the research, it was determined that the sales prices of bee products were significantly lower than the sales prices expected by farmers. On the other hand, it has been determined that the farmers' rate of accessing information about market is low. In addition to the beekeeping techniques, farmers have to know and follow supply, demand, domestic and foreign markets, sales, price formation etc. closely. Agricultural marketing extension can be defined as a training program that provides information that farmers need to solve marketing problems. Therefore, an agricultural marketing extension program should be applied to meet farmers' needs, and farmers' problems about the market should be solved.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47074724

ETHNOBOTANICAL STUDY OF MEDICINAL PLANTS OF NAMAL VALLEY, SALT RANGE, PAKISTAN

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(Received 5th Jan 2019; accepted 26th Feb 2019)

Abstract. This paper presents the first quantitative ethnobotanical knowledge and practices of using native plants for different ailments from Namal Valley of Pakistan. Data was gathered by interviewing 350 informants through semi-structured questionnaires. A total of 217 taxa belonging to 166 genera and 70 families were documented. Fabaceae and Asteraceae families were found to be the most cited families (with 19 and 18 species receptively). Herbs represent the most cited life form (71%) and flower was the most widely used part (34.8%) with decoction as main mode of the utilization (41.5%). On the basis of use values, the most commonly used ethnobotanical taxa in the Valley were reported to be *Euphorbia heterophylla* (0.7) and *Merremia dissecta* (0.6). The highest RFC value was noted for *Aloe vera* (0.14) while highest ICF value was estimated for dental problems category (0.7). Maximum similarity index was found in the studies with Bana Valley (JI 23.9). Similarity percentage of plants uses ranges from 0% to 15.7 (Bana Valley), while dissimilarity percentage varies up to 32.5% (Soon Valley). This study highlights the medicinal flora of study area that will serve as baseline for conservation and sustainable utilization through future research on bio prospecting of potential species to develop novel drugs.
Keywords: *medicinal flora; conservation; Fabaceae; novel drugs; informant consensus factor*

Introduction

Ethnobotanical studies on medicinal flora are crucial for developing novel drugs based on traditional knowledge of the local people (Heinrich and Gibbons, 2001; Mesfin et al., 2009; Vitalini et al., 2013). The usage of medicinal plants for medicinal and therapeutic purposes has been studied and documented globally (Kunwar et al., 2015; Bulut et al., 2017; Fortini et al., 2016; Menale et al., 2016; Shah and Rahim, 2017). The role of herbal treatment in curing various diseases is vital because of the fact that a large number of ailments are cured by plant based medications (Rehecho et al., 2011). In this context ethnobotanical researches demonstrate high significance and prominence of medicinal plants in cognitive pluralistic perspective. Such studies not only play an important role in upgrading the social status and economic values of an area but also preserve the aboriginal medico-ethnobotanical data of the indigenous communities that consequently preserve the global heritage (Sanz-Biset et al., 2009). Medicinal plants are considered very important among the rural communities due to their potential to cure health related problems for which several of remote communities are unreachable of modern health care facilities (Heinrich, 2000; Tabuti et al., 2003; Verma and Singh, 2008). Out of approximately 6000 plants species in Pakistan 600 to 700 are reported to have medicinal value. Among them 456 plants species are used in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

the formulation of approximately 350 synthetic drugs (Ahmad and Husain, 2008). Pakistan has variations on climatic conditions, ecological zones and topography where diverse flora of medical importance flourishes in dry to temperate habitats. Limited number of ethnobotanical expeditions have been conducted in well-known valleys of Pakistan (Ahmad et al., 2014; Amjad, 2015; Bano et al., 2014; Haidar and Qaiser, 2009; Haq, 2012; Hazrat et al., 2011; Khan et al., 2010; Khan et al., 2013; Khan and Khatoon, 2008; Shah and Rahim, 2017; Zabihullah et al., 2006) but most of the remote valleys are still unexplored There could be hundreds of plant species growing in these valleys having medicinal, industrial and economic potential. Local communities of various regions use these species in different ways on the basis of their cultural beliefs and inherited experience. However, little attention is being paid to the conservation and sustainable utilization of several of potential species. Salt Range of Pakistan is one of such region which is least explored regarding the documentation of medicinal wealth present in several of its valleys. Namal Valley is among the most fascinating area awaiting systematic study of its ethnobotanical knowledge associated with native flora of the region. Recently (Shah et al., 2018) has documented sixty eight (68) medicinal plants used in snakebite and scorpion sting used by the inhabitants of this valley who are predominantly pastoralists, peasants and farmers. Namal Valley is naturally gifted with diverse flora with social, economic and environmental importance. The objectives of this study were to collect record and document information regarding the plants used ethnobotanically by the aboriginal people especially with respect to the medicinal wealth of the plants. This extensive study carried out throughout the Valley revealed an exhaustive list profile of medicinal plants, based on quantitative evaluations adopting several ethnobotanical indices like use value (UV), frequency of citation (FC), relative frequency citation (RFC), family importance value (FIV), informant consensus factor (ICF) and the Jaccard index (JI).

Material and Methods

Study area

Geographically, the study area was located in the northwest of Punjab Province geographically ranging at 71 °48°45° E longitude and 32°40°10° N latitude, spreading over an area of 5.5 square kilometers. The valley is bounded by Salt Range Mountains and touches western border of Mianwali district (*Fig. 1*). Historical and scenic places of the Valley include Namal College Mianwali, Namal Dam, Namal Lake, Sulphur spring and shrines of Hafiz Jee and Khaki Shah. Namal valley is included among the oldest civilization in Pakistan. Raring of cattle and goats provides a livelihood for the local people Most part of the Valley is covered by forests and pastures The climate of the Valley is characterized by cold, dry weather at high altitudes and humid, warm in low altitude-lying areas. Several native tribes with a rich historical background and that use the native medicinal flora and have their own traditional healers known as tabeeb or hakeem are in the Valley. These tribes are awan, malik, niazi, shah, mian etc. Climate variation and complex topography gives way to diverse flora and fauna in the Valley. Valley is carpeted with *Dodonaea viscosa*, *Prosopis glandulosa*, *P. juliflora*, *Tamarix aphylla*, *T. dioica*, *Tephrosia pupurea*, *Withania coagulans*, *Pluchea arabica*, *Pulicaria glutinosa* and *Rhazya stricta* in the low altitudes while *Acacia modesta* mixed with *Salvadora oleoides* are commonly seen in high altitudes. *Viola cinerea* and *Pseudogaillonia hymnostephana* are among the rare taxa of the Valley that have also rare occurrence not only from the Pakistan but also globally (Shah et al., 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

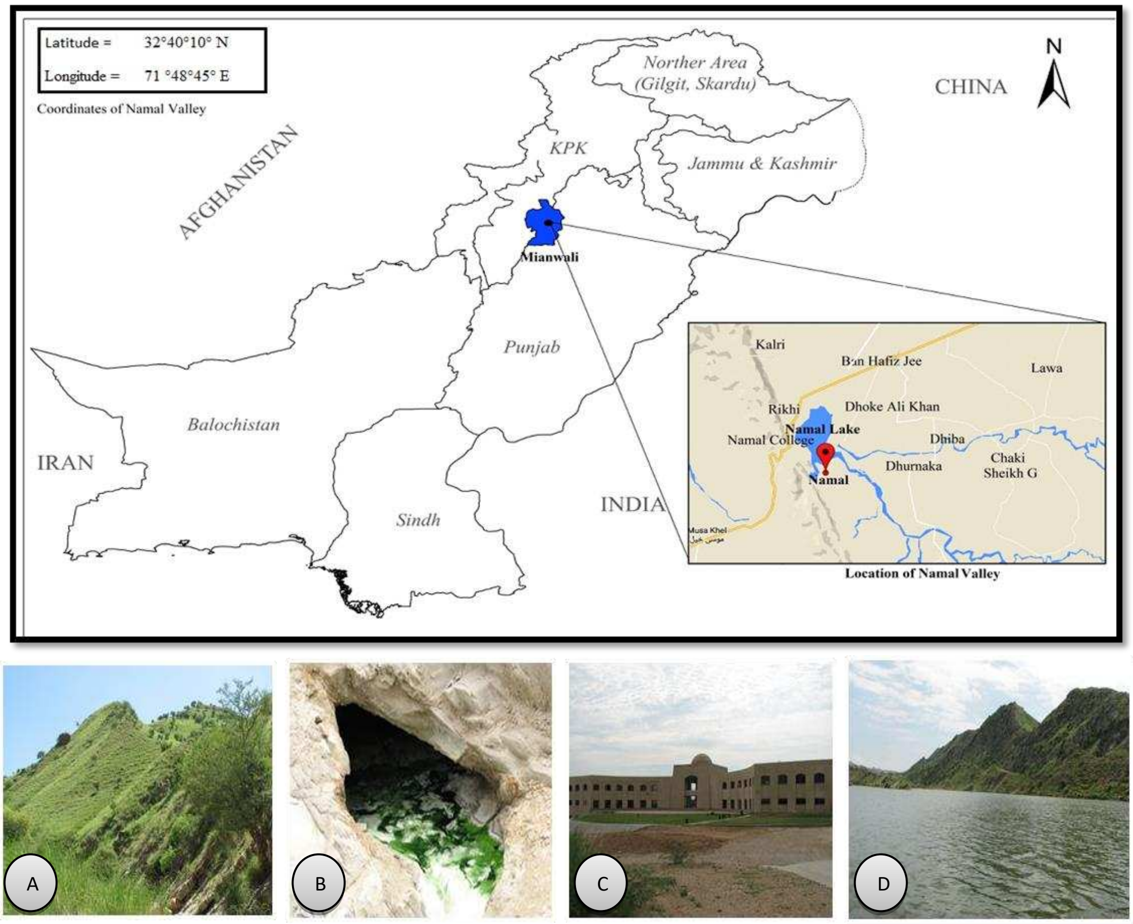

Figure 1. *Map and Panoramic views of the Namal Valley*

(A- Lush green scenic Valley hills; B- Sulphur Spring; C- Namal college; D- Namal Lake)

Ethnobotanical data collection

Data were collected from all the villages of the Valley from 2013-2016 representing all the seasons following the method of (Martin, 1985; Heinrich et al., 2009). A total of 16 field trips (representing all seasons) were carried out to collect plants and to document ethnobotanical information. Keeping in view the aim of quantitative approach to record ethnobotanical information, the participant observation method was used along with open-ended interviews and questionnaires. Knowledgeable people of the Valley including 12 traditional healers, shepherds, herbal medicinal venders, were consulted. The people in the local area speak Saraiki language and therefore, interviews were conducted in Saraiki language. All the documented data was later translated into English. A total of 350 informants (240 males and 110 females) of all group ages (25 to ≥ 65) were interviewed. Information regarding vernacular names, ethnobotanical uses etc. was recorded and the details are presented in *Table 1.*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

Table 1. *Ethnobotanical uses of plants in Namal Valley, Salt Range, Pakistan*

Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
1	*Abutilon indicum* (L.) Sweet/ Malvaceae SAN-SR-06	Shrub / W	Flower, leaves/ Decoction	Respiratory disorders, menstrual disorders	2	0.2	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
2	*Acacia modesta* Wall./ Fabaceae Phulahi SAN-SR-55	Tree/ W	Twigs, pods/ Raw, decoction	Gummosis, miswak sticks, toothache, diarrhea, Expectorant, Gynaecological problems	6	0.17	35	0.1	1*, 2▲, 3•, 4*, 5▲, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
3	*Acacia nilotica* (L.) Delile./ Fabaceae Kikar, Babool SAN-SR-64	Tree/ W	Twigs, pods / Raw, decoction	Gummosis, miswak sticks, toothache, Expectorant, Gynaecological problems	5	0.2	25	0.07	1•, 2*, 3•, 4*, 5▲, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13•, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
4	*Achyranthes aspera* L./ Amaranthaceae Puthkanda SAN-SR-45	Herb/ W	Whole plant/ Decoction	Cough, cold, febrifuge, menstrual problems, kidney pain	5	0.42	12	0.03	1*, 2*, 3*, 4▲, 5*, 6*, 7*, 8•, 9*, 10*, 11•, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23▲	
5	*Adiantum capillus-veneris* L./ Pteridaceae Paersichhayon SAN-SR-33	Herb/ W	Whole plant/ Decoction	Hair tonic, febrifuge, respiratory problems	3	0.43	7	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
6	*Aerva javanica* (Burm.f.) Juss. ex Schult./ Amaranthaceae Bui SAN-SR-91	Herb/ W	Leaves/ Decoction	Skin problems, joint pain, vomiting, eye infection	4	0.2	20	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23▲	
7	*Ageratum conyzoides* (L.) L. / Asteraceae Osarri SAN-SR-109	Herb/ W	Whole plant/ Decoction, juice	Skin problems, fever, cough	3	0.2	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9▲, 10*, 11*, 12*, 13*, 14*, 15*, 16•, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
8	*Ajuga bracteosa* Wall. ex Benth./ Lamiaceae SAN-SR-75	Herb/ W	Whole plant/ Decoction	Malaria fever, vomiting, headache	3	0.43	7	0.02	1*, 2▲, 3*, 4*, 5▲, 6▲, 7*, 8▲, 9*, 10*, 11•, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19•, 20*, 21*, 22•, 23*	
9	*Albizia lebbeck* (L.) Benth../ Fabaceae Kala Shrin SAN-SR-145	Tree/ W,C	Leaves/ Decoction	Cardio tonic, diarrhea. Aphrodisiac, skin problems,	4	0.16	25	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
10	*Albizia procera* (Roxb.) Benth./ Fabaceae Safed shrin SAN-SR-139	Tree W,C	Leaves/ Decoction	Inflammation, menstrual problems, Hepatic pain , epilepsy	4	0.11	35	0.1	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
11	*Alhagi maurorum* Medik./ Fabaceae Oont katara SAN-SR-128	Shrub/ W	Pods, leaves	Piles, laxative, obesity	3	0.5	6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
12	*Aloe vera* (L.) Burm.f. Syn. *Aloe barbadensis* Mill./ Asphodelaceae Kowar gandal SAN-SR-100	Herb / W,C	Leaves/ Latex	Skin problems, febrifuge, malaria, inflammation, obesity, digestive problems	6	0.12	50	0.14	1•, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	
13	*Alternanthera pungens* Kunth/ Amaranthaceae Khaki booti SAN-SR-68	Herb/ W	Leaves/ Decoction	Menstrual disorders, febrifuge, body pain	3	0.23	13	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
14	*Amaranthus graecizans* L./ Amaranthaceae Marierri SAN-SR-112	Herb/ W	Leaves/ Cooked	Laxative, gastrointestinal problems, galactagogue	3	0.15	20	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	
15	*Amaranthus viridis* L./ Amaranthaceae Bathu SAN-SR-01	Herb/ W	Leaves/ Cooked	Laxative, gastrointestinal problems, respiratory disorders	3	0.15	20	0.06	1*, 2*, 3▲, 4*, 5*, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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16	*Anagallis arvensis* L./ Primulaceae Bili booti SAN-SR-118	Herb/ W	Whole plant/ Infusion	Depurative, skin problems, inflammation	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5▲, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
17	*Anisomeles indica* (L.) Kuntze/ Lamiaceae Bili poodina SAN-SR-85	Herb/ W	Aerial parts/ Decoction	Malaria, gastrointestinal disorders, hypertension	3	0.17	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
18	*Argemone mexicana* L./ Papaveraceae Satia nasi SAN-SR-124	Herb/ W	Flower/ Infusion	Post-partum, menstrual problems, obesity,	3	0.27	11	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
19	*Arisaema tortuosum* var. *curvatum* (Roxb.) Engler/ Araceae Zahr mora SAN-SR-217	Herb/ W	Rhizome/ Raw, decoction	Snake bite, scorpion stings, antidote	3	0.23	13	0.04	1*, 2*, 3*, 4▲, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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20	*Arundo donax* L./ Poaceae Narrki SAN-SR-189	Herb/ W	Aerial parts/ Decoction	Fever, bloating, menstrual problems	3	0.42	7	0.02	1*, 2*, 3▲,4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
21	*Asparagus capitatus* Baker/ Asparagaceae SAN-SR-210	Herb/ W	Whole plant/ Decoction	Body pain, fever, gastrointestinal problems	3	0.16	19	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
22	*Asphodelus tenuifolius* Cav./ Asphodelaceae Wassli SAN-SR-54	Herb/ W	Seeds/ Raw	Piles, febrifuge, anthelmintic, ring worm	4	0.18	22	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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23	*Azadirachta indica* A. Juss. / Meliaceae Neem SAN-SR-93	Tree/ W	Seeds stem Leaves, fruit/ Raw, smoke, infusion, decoction	Anthelmintic, cleaning teeth, Antidiabetic, chronic malaria, piles, chicken pox, skin problems	7	0.24	29	0.08	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	
24	*Azolla pinnata* R. Br./ Saviniaceae SAN-SR-09	Fern/ W	Whole plant/ Poultice	Inflammation, wounds, skin burn	3	0.17	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
25	*Bacopa monnieri* (L.) Wettst./ Plantaginaceae Barhami booti SAN-SR-44	Herb/ W	Leaves, flowers/ Decoction	Mental disorders, anxiety, depression, tonic	4	0.25	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
26	*Barleria cristata* L. / Acanthaceae SAN-SR-161	Herb/ W	Aerial parts/ Decoction	Respiratory disorders, blood purifier	2	0.25	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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27	*Boerhavia procumbens* Banks ex. Roxb. /Nyctaginaceae It-sit SAN-SR-61	Herb/ W	Leaves/ Decoction	Poor appetite, febrifuge, snake bite, piles, diuretic	5	0.31	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
28	*Broussonetia papyrifera* (L.) Vent./ Moraceae Kagazi toot SAN-SR-214	Tree/ W	Leaves/ Infusion	Dysentery	1	0.5	2	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
29	*Calligonum comosum* L'Hér./ Polygonaceae Khippi SAN-SR-200	Shrub/ W	Leaves/ Decoction	Digestive problems, body pain, headache,	3	0.17	17	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
30	*Calotropis procera* (Aiton) Dryand/ Apocynaceae Akrra SAN-SR-16	Shrub/ W	Leaves, flower/ Latex	Snake bite, rheumatism, wound healing, febrifuge, mumps, toothache	6	0.13	45	0.13	1*, 2▲, 3*, 4*, 5•, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	

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31	*Cannabis sativa* L./ Cannabaceae/ Bhang SAN-SR-22	Herb/ W	Leaves/ Raw, Decoction	Insomnia, hypertension, abdominal pain, toothache	4	0.16	25	0.07	1*, 2•, 3*, 4▲, 5*, 6*, 7▲, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16▲, 17*, 18*, 19*, 20*, 21*, 22•, 23*	
32	*Capparis decidua* (Forssk.) Edgew./ Capparaceae Karein SAN-SR-03	Tree/ W	Fruit, flower/ Raw, juice	Digestive problems, tonic, obesity	3	0.1	30	0.08	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
33	*Capparis spinosa* L./ Capparaceae SAN-SR-47	Shrub/ W	Stem, flower/ Juice	Toothache, abortifacient	2	0.25	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10▲, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
34	*Capsella bursa-pastoris* (L.) Medik./ Brassicaceae SAN-SR-102	Herb/ W	Leaves/ Paste, decoction	Skin problems, postpartum, cardiotonic	3	0.16	19	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12▲, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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35	*Caralluma tuberculata* N.E.Br./ Apocynaceae Chungan SAN-SR-134	Herb/ W	Whole plant/ Cooked, raw	Diabetes, blood purifier, skin problems,	3	0.15	20	0.06	1*, 2*, 3*, 4*, 5•, 6*, 7▲, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
36	*Cardamine hirsuta* L./ Brassicaceae SAN-SR-27	Herb/ W	Aerial parts/ Decoction	Intestinal worms	1	0.33	3	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
37	*Cardaria draba* (L.) Desv./ Brassicaceae SAN-SR-190	Herb/ W	Aerial parts/ Infusion	Abdominal pain, vomiting,	2	0.33	6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
38	*Carthamus oxyacantha* M. Bieb./ Asteraceae Poli SAN-SR-212	Herb/ W	Leaves/ Decoction	Skin problems, febrifuge, wound healing	3	0.12	25	0.07	1, 2, 3, 4, 5▲, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	

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39	*Cenchrus echinatus* L./ Poaceae SAN-SR-24	Herb/ W	-----	-----	0		6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
40	*Centaurium pulchellum* (Sw.) Druce/ Gentianaceae SAN-SR-66	Herb/ W	Leaves/ Decoction	Fever, kidney pain, diuretic	3	0.18	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
41	*Ceratonia siliqua* L./ Fabaceae SAN-SR-125	Tree/ W	Seeds/ Decoction	Gastrointestinal problems, obesity	2	0.08	25	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
42	*Chenopodium murale* L./ Chenopodiaceae Dosaga SAN-SR-43	Herb/ W	Leaves/ Cooked	Digestive problems, menstrual problems	2	0.12	17	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19•, 20*, 21*, 22*, 23*	

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43	*Chenopodium album* L./ Chenopodiaceae Chulaii SAN-SR-122	Herb/ W	Leaves/ Cooked	Digestive problems, menstrual problems	2	0.12	17	0.05	1*, 2▲, 3▲, 4*, 5▲, 6▲, 7•, 8•, 9*, 10▲, 11*, 12▲, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
44	*Chloris gayana* Kunth/ Poaceae Pankha Ghaas SAN-SR-148	Herb/ W	-----	-----	0		12	0.03	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
45	*Chrozophora tinctoria* (L.) A. Juss./ Euphorbiaceae Hathi sundi SAN-SR-81	Herb/ W	Flowers/ Infusion, decoction	Fever, stomach ache, vomiting, wound healing	4	0.22	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
46	*Cichorium intybus* L./ Asteraceae Kasni SAN-SR-101	Herb/ W	Aerial parts/ Decoction	Kidney pain, gallbladder stone, stomach ache	3	0.25	12	0.03	1*, 2•, 3▲, 4*, 5*, 6*, 7•, 8•, 9*, 10•, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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47	*Cirsium vulgare* (Savi) Ten./ Asteraceae Laih SAN-SR-133	Herb/ W	Seeds/ Raw	Tonic, obesity	2	0.28	7	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
48	*Cistanche tubulosa* (Schenk) Wight/ Orobanchaceae Khar ghainrr SAN-SR-56	Herb/ W	Whole plant/ Decoction	Aphrodisiac	1	0.2	5	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
49	*Citrullus colocynthis* (L.) Schrad./ Cucurbitaceae Tuma SAN-SR-147	Herb/ W	Seeds/ Raw	Diabetes, malaria, stomach problems	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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50	*Clematis grata* Wall./ Ranunculaceae SAN-SR-203	Shrub/ W	Leaves/ Paste, infusion	Skin problems, vomiting, body pain	3	0.33	9	0.02	1*, 2▲, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
51	*Clerodendrum phlomidis* L.f./ Lamiaceae SAN-SR-211	Shrub/ W	Leaves/ Decoction	Sore throat, respiratory problems	2	0.5	4	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
52	*Cocculus hirsutus* (L.) Diels/ Menispermaceae SAN-SR-174	Shrub/ W	Fruit/ Decoction	Malaria, body pain, obesity	3	0.43	7	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18▲, 19*, 20*, 21*, 22*, 23*	
53	*Cocculus pendulus* (J.R.Forst. & G.Forst.) Diels/ Menispermaceae SAN-SR-215	Shrub/ W	Fruit/ Decoction	Malaria, body pain, obesity	3	0.37	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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54	*Coix lacryma-jobi* L./ Poaceae SAN-SR-79	Herb/ W	-----	-----	0	0	10	0.02	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
55	*Convolvulus arvensis* L./ Convolvulaceae Verri SAN-SR-10	Herb/ W	Whole plant/ Decoction	Wound healing, stomach problems	2	0.12	16	0.04	1*, 2•, 3*, 4*, 5▲, 6*, 7▲, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19▲, 20*, 21*, 22*, 23*	
56	*Convolvulus prostratus* Forssk./ Convolvulaceae SAN-SR-13	Herb/ W	Whole plant/ Decoction	Wound healing, stomach problems	2	0.12	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
57	*Conyza canadensis* (L.) Cronquist./ Asteraceae Paleet SAN-SR-126	Herb/ W	Aerial parts/ Decoction	Dysentery, diarrhea	2	0.06	32	0.09	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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58	*Croton bonplandianum* Baill./ Euphorbiaceae SAN-SR-25	Herb/ W	Leaf, seeds/ Infusion, raw	Blood purifier, cardiotonic, constipation	3	0.2	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
59	*Cucumis melo* var. *agrestis* Naudin/ Cucurbitaceae Chibbarr SAN-SR-80	Herb/ W	Fruit, seeds/ Raw,	Purgative, cooling effect, bloating, tonic	4	0.21	19	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
60	*Cuscuta reflexa* Roxb./ Convolvulaceae Akash-bail SAN-SR-07	Herb/ W	Whole plant/ Decoction	Gastrointestinal problems	1	0.5	2	0.01	1*, 2*, 3*, 4▲, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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61	*Cymbopogon commutatus* (Steud.) Stapf/ Poaceae Jangli lemon grass SAN-SR-157	Herb/ W	Aerial parts/ Decoction	Respiratory disorders, obesity, digestive problems, menstrual pain	4	0.17	24	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
62	*Cynodon dactylon* (L.) Pers./ Poaceae Tulla SAN-SR-166	Herb/ W	Leaves/ Decoction	Dysmenorrhea, vomiting, piles,	3	0.15	20	0.06	1•, 2•, 3▲, 4▲, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15▲, 16•, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
63	*Dactyloctenium aegyptium* (L.) Willd. / Poaceae Pankha Khabbal SAN-SR-153	Herb/ W	-----	-----	0	0	17	0.05	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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64	*Dalbergia sissoo* DC./ Fabaceae Shisham, Tahli SAN-SR-23	Tree/ W,C	Leaves, stem/ Juice, bark	Eye ailments, scabies, dysmenorrhea	3	0.10	29	0.08	1*, 2*, 3▲, 4*, 5▲, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*,18▲, 19*, 20*, 21*, 22*, 23*	
65	*Datura metel* L./ Solanaceae Dhatura SAN-SR-106	Herb/ W	Seeds/ Powder	Body pain, wound healing	2	0.08	25	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
66	*Desmostachya bipinnata* (L) Stapf./ Poaceae Dabb Ghaas SAN-SR-168	Herb/ W	-----	-----	0	0	22	0.06	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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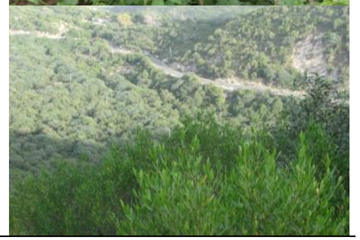
67	*Dichanthium annulatum* (Forssk.) Stapf/ Poaceae SAN-SR-196	Herb/ W	-----	-----	0	0	4	0.01	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
68	*Dicliptera bupleuroides* Nees/ Acanthaceae SAN-SR-213	Herb/ W	Leaves/ Decoction	Stomach problems, constipation	2	0.33	6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22▲, 23*	
69	*Digera muricata* (L.)Mart./ Amaranthaceae Tandla SAN-SR-198	Herb/ W	Whole plant/ Cooked, juice	Urinary problems, constipation, inflammation	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	
70	*Dodonaea viscosa* (L.) Jacq./ Sapindaceae Sanatha SAN-SR-204	Shrub/ W	Twigs, Leaves/ Infusion, raw	Skin problems, toothache, oral problems	3	0.25	12	0.03	1*, 2*, 3•, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

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71	*Echinops echinatus* Roxb./ Asteraceae SAN-SR-130	Herb/ W	Whole plant/ Decoction	Hepatitis, vomiting, nausea	3	0.2	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
72	*Eclipta prostrata* (L.)L./ Asteraceae Bhangra SAN-SR-132	Herb/ W	Aerial parts/ Decoction	Jaundice, cooling effect, constipation	3	0.33	9	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18▲, 19*, 20*, 21*, 22*, 23*	
73	*Ehretia obtusifolia* Hochst. ex A. DC./ Boraginaceae SAN-SR-36	Shrub/ W	Seeds, leaves/ Infusion, decoction	Fever, liver problems, body pain	3	0.2	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	
74	*Eichhornia crassipes* (Mart.) Solms/ Pontederiaceae Gul-e-rana SAN-SR-74	Herb/ W	Flowers/ Infusion	Malaria, fever, body pain	3	0.3	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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75	*Ephedra foliata* Boiss. ex C.A.Mey. *sy, Ephedra ciliata* Fisch. & C.A.May. Ephedraceae SAN-SR-92	Shrub/ W	Aerial parts/ Decoction	Asthma, cough, bronchitis, flu, headache, sore throat	6	0.26	23	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
76	*Epilobium hirsutum* L./ Onagraceae SAN-SR-120	Herb/ W	Leaves/ Infusion	Eczema, skin allergy, wound healing	3	0.33	9	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
77	*Equisetum arvense* L./ Equisetaceae Naarri SAN-SR-34	Herb/ W	Whole plant/ Paste, infusion	Epistaxis, diabetes, skin problems	3	0.33	9	0.02	1*, 2▲, 3*, 4*, 5*, 6*, 7▲, 8*, 9*, 10*, 11*, 12▲, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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78	*Eruca sativa* Mill./ Brassicaceae Jhamayon SAN-SR-152	Herb/ W,C	Seeds/ Oil	Anti-lice, hair tonic, anti-allergic, inflammation, dandruff , itching	6	0.17	35	0.1	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
79	*Eucalyptus camaldulensis* Dehnh./ Myrtaceae Sufeda SAN-SR-15	Tree/ C	Leaves/ Decoction	Respiratory disorders, febrifuge, malaria, insect repellent, vomiting, nausea,	6	0.21	29	0.08	1*, 2*, 3*, 4*, 5▲, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	
80	*Euphorbia helioscopia* L./ Euphorbiaceae Chhatri dodak SAN-SR-04	Herb/ W	Leaves/ Latex	Warts	1	0.33	3	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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81	*Euphorbia heterophylla* L./ Euphorbiaceae SAN-SR-65	Herb/ W	Whole plant/ Infusion, paste	Scabies , warts,	2	0.66	3	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21•, 22*, 23*	
82	*Euphorbia hirta* L./ Euphorbiaceae SAN-SR-154	Herb/ W	Whole plant/ Decoction	Cough, obesity, constipation, blood purifier	4	0.36	11	0.03	1•, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
83	*Euphorbia peplus* L./ Euphorbiaceae SAN-SR-177	Herb/ W	Leaves/ Latex, infusion	Skin problems, wound healing, blood purifier, anti-allergic	4	0.25	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
84	*Euphorbia prostrata* Aiton/ Euphorbiaceae SAN-SR-206	Herb/ W	Leaves/ Decoction	Piles, constipation, gynecological problems	3	0.5	6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19▲, 20*, 21*, 22*, 23*	

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85	*Euphorbia serpens* Euphorbiaceae SAN-SR-171	Herb/ W	Whole plant/ Decoction	Skin problems, warts, asthma, cough,	4	0.33	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
86	*Evolvulus alsinoides* (L.) L./ Convolvulaceae SAN-SR-52	Herb/ W	Whole plant/ Decoction	Obesity, fever, hypertension	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
87	*Fagonia indica* Burm.f./ Zygophyllaceae/ Dhamasa SAN-SR-108	Herb/ W	Aerial parts/Decoction	Cooling effect, vomiting, bloating	3	0.3	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
88	*Ficus benghalensis* L./ Moraceae/ Borrh SAN-SR-140	Tree/ W,C	Leaves/ Latex	Chicken pox, small pox, piles	3	0.14	22	0.06	1▲, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	

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89	*Ficus racemosa* L./ Moraceae Gularr SAN-SR-73	Tree/ W,C	Fruit, leaves/ Raw, decoction	Gynecological disorders, diabetes, cough, fever	4	0.27	15	0.04	1▲, 2•, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	
90	*Ficus religiosa* L./ Moraceae Peepal SAN-SR-110	Tree/ W,C	Leaves/ Decoction	Sexual diseases, obesity, diabetes	3	0.17	17	0.05	1▲, 2*, 3*, 4*, 5▲, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16▲, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
91	*Ficus virgata* Reinw. ex Blume/ Moraceae Jangli Injeer SAN-SR-26	Tree/ W	Fruit, leaves/ Raw	Stomach ache, constipation, menstrual problems	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
92	*Filago hurdwarica* (Wall. ex DC.) Wagenitz/ Asteraceae SAN-SR-58	Herb/ W	Leaves/ Decoction	Sore throat, cough, fever, fatigue	4	0.26	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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93	*Forsskaolea tenacissima* L./ Urticaceae Nettle SAN-SR-82	Herb/ W	Whole plant/ Decoction	Hypertension, ulcer, stomach ache	3	0.18	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
94	*Fumaria indica* (Hausskn.) Pugsley/ Papaveraceae Paaprra SAN-SR-135	Herb/ W	Flowers/ Infusion	Depurative, vomiting, jaundice, stomach ache	4	0.44	9	0.02	1*, 2▲, 3*, 4*, 5•, 6*, 7*, 8▲, 9*, 10*, 11•, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
95	*Galium aparine* L./ Rubiaceae SAN-SR-08	Herb/ W	Whole plant/ Tea, infusion decoction	Obesity, hypertension, skin problems, body pain	4	0.26	15	0.04	1*, 2*, 3*, 4▲, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12▲, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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96	*Gastrocotyle hispida* (Forssk.) Bunge/ Boraginaceae Khatol SAN-SR-113	Herb/ W	Leaves/ Decoction	Purgative, constipation, vomiting	3	0.37	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
97	*Geranium mascatense* Boiss./ Geraniaceae SAN-SR-129	Herb/ W	Aerial parts/ Decoction	Epilepsy, back ache, joint pain,	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
98	*Glandularia pulchella* (Sweet) Tronc. (Syn. *Verbena tenisecta* Briq.)/ Verbenaceae SAN-SR-136	Herb/ W	Whole plant/ Infusion	Hypertension, anxiety, fever	3	0.43	7	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
99	*Grewia optiva* J.R.Drumm. ex Burret/ Malvaceae SAN-SR-143	Tree/ W	Fruit/ Raw	Jaundice, digestive problems, tonic	3	0.15	20	0.06	1*, 2▲, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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100	*Grewia tenax* (Forssk.) Fiori/ Malvaceae Gungair SAN-SR-150	Shrub/ W	Fruit/ Raw	Jaundice, digestive problems, tonic	3	0.15	20	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
101	*Grewia villosa* Willd./ Tiliaceae SAN-SR-156	Shrub/ W	Fruit/ Raw	Jaundice, digestive problems, tonic	3	0.15	20	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14▲, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23▲	
102	*Gymnosporia spinosa* (Blanco) Merr. & Rolfe/ Celastraceae Pattassi SAN-SR-162	Shrub/ W	Seeds/ Ash	Toothache, oral infections	2	0.33	6	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
103	*Gynandropsis gynandra* (L.) Briq./ Cleomaceae Badal banga SAN-SR-11	Herb/ W	Aerial parts/ Juice	Epilepsy, menstrual pain, vomiting	3	0.33	9	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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104	*Heliotropium currasavicum* L. Boraginaceae SAN-SR-21	Herb/ W	Leaves/ Juice, decoction	Wasp bite, skin problems, cough	3	0.3	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
105	*Heliotropium europaeum* L./ Boraginaceae Hathi sundi SAN-SR-51	Herb/ W	Leaves/ Paste	Wound healing	1	0.33	3	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
106	*Hibiscus mutabilis* L./ Malvaceae Gul-e-ajaib SAN-SR-69	Shrub/ W	Flowers, leaves/ Decoction	Cough, sore throat, body pain,	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
107	*Ipomoea carnea* Jacq./ Convolvulaceae Morning glory SAN-SR-97	Herb/ W	Leaves, flowers/ Decoction	Hypertension, asthma, anxiety	3	0.18	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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108	*Juncus elegans* Royle ex Sam./ Juncaceae Water Dila SAN-SR-78	Herb/ W	-----	-----	0	0	15	0.04	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
109	*Justicia adhatoda* L./ Acanthaceae Bhaikarr SAN-SR-20	Herb/ W	Flower/ Decoction	Cold, cough, body pain, headache	4	0.2	20	0.06	1*, 2•, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11•, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
110	*Kickxia ramosissima* (Wall.) Janch./ Plantaginaceae SAN-SR-40	Herb/ W	Whole plant/ Decoction, paste	Febrifuge, body pain, inflammation	3	0.37	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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111	*Lactuca serriola* L./ Asteraceae SAN-SR-60	Herb/ W	-----	-----	0	0	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
112	*Lantana camara* L. /Verbenaceae Bhang SAN-SR-86	Shrub/ W	Leaves/ Infusion	Carminative, febrifuge, malaria, cough, cold	5	0.26	19	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16▲, 17▲, 18▲, 19*, 20▲, 21*, 22*, 23*	
113	*Lathyrus aphaca* L./ Fabaceae SAN-SR-121	Herb/ W	Leaves, seeds/ Infusion, raw	Hypertension, insomnia, fever	3	0.23	13	0.04	1*, 2•, 3*, 4*, 5▲, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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114	*Launaea capitata* (Spreng.) Dandy/ Asteraceae SAN-SR-142	Herb/ W	-----	-----	0	0	12	0.03	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
115	*Leptadenia pyrotechnica* (Forssk.) Decne/ Apocynaceae Khip SAN-SR-77	Shrub/ W	Leaves/ Decoction	Blood purifier, pimples, wound healing	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
116	*Leucaena leucocephala* (Lam.) de Wit./ Fabaceae Angrezi shareen SAN-SR-96	Tree/ W	Pods/ Decoction, raw	Skin problems, laxative, vomiting, colic	4	0.22	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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117	*Lindenbergia abyssinica* Hochst. ex Benth./ Scrophulariaceae SAN-SR-175	Herb/ W	Whole plant/ Decoction, infusion, juice	Stomach problems, epilepsy, body pain, headache	4	0.26	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
118	*Lindenbergia indica* Vatke/ Scrophulariaceae SAN-SR-207	Herb/ W	Whole plant/ Decoction, infusion, juice	Stomach problems, epilepsy, body pain, headache	4	0.26	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
119	*Linum strictum* L./ Linaceae Alsi SAN-SR-182	Herb/ W	Leaves/ Paste	Poultice, inflammation, skin irritation	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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120	*Maerua arenaria* Hook. f. & Thomson/ Capparaceae SAN-SR-201	Shrub/ W	Leaves, bark/ Decoction	Gynecological problems, blood purifier, pimples	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
121	*Malvastrum coromandelianum* (L.) Garcke/ Malvaceae SAN-SR-195	Herb/ W	Leaves/ Infusion	Diarrhea, vomiting, heart burn	3	0.16	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
122	*Marsilea quadrifolia* L./ Marsileaceae SAN-SR-188	Herb/ W	Whole plant/ Paste, decoction	Antidote, stomach problems, skin allergy	3	0.37	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
123	*Medicago sativa* L. Fabaceae Maina SAN-SR-202	Herb/ W	Flower/ Infusion	Hair tonic,	1	0.33	3	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7▲, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
124	*Melilotus indicus* (L.) All./ Fabaceae Maina SAN-SR-216	Herb/ W	Whole plant/ Juice	Wasp bite, inflammation	2	0.12	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
125	*Mentha longifolia* (L.) L./ Lamiaceae Senji SAN-SR-89	Herb/ W	Whole plant/ Infusion, decoction	Gastrointestinal disorders, obesity, aphrodisiac, febrifuge,	4	0.09	45	0.13	1*, 2•, 3*, 4*, 5*, 6•, 7•, 8•, 9*, 10*, 11•, 12•, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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126	*Merremia dissecta* (Jacq.) Hallier f./ Convolvulaceae SAN-SR-107	Herb/ W	Whole plant/ Decoction	Cough, fever, headache	3	0.6	5	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
127	*Millettia pinnata* (L.) Panigrahi/ Fabaceae Sukh chain SAN-SR-63	Tree/ W,C	Leaves/ Decoction	Febrifuge, respiratory disorders,	2	0.16	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
128	*Minuartia hybrida* (Vill.) Schischk./ Caryophyllaceae SAN-SR-41	Herb/ W	-----	-----	0	0	8	0.02	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
129	*Misopates orontium* (L.) Raf./ Plantaginaceae SAN-SR-32	Herb/ W	-----	-----	0	0	6	0.02	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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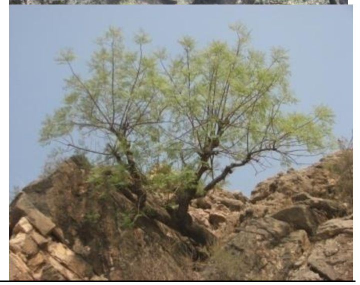
130	*Momordica balsamina* L./ Cucurbitaceae Jangli karela SAN-SR-37	Herb/ W	Fruit/ Raw	Diabetes, skin problems, blood purifier, stomach problems, throat infection, liver problems	6	0.24	25	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
131	*Monotheca buxifolia* (Falc.) A. DC./ Sapotaceae Gurgura SAN-SR-159	Tree/ W	Fruit/ Raw	Constipation, laxative, cooling effect, liver problems	4	0.2	20	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
132	*Moringa oleifera* Lam./ Moringaceae/ Sohanjna SAN-SR-103	Tree/ W,C	Pods, leaves/ Raw, powder	Skin problems, wounds, boils, depurative, febrifuge, jaundice, stomach problems	7	0.23	30	0.08	1▲, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*,20▲, 21*, 22*, 23*	

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133	*Morus alba* L./ Moraceae Safaid toot SAN-SR-146	Tree/ C	Fruit/ Raw	Laxative, expectorant, respiratory disorders, jaundice	4	0.12	33	0.09	1*, 2•, 3•, 4*, 5•, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
134	*Morus laevigata* Wall. ex Brandis/ Moraceae Shah toot SAN-SR-149	Tree/ C	Fruit/ Raw	Laxative, expectorant, respiratory disorders, jaundice	4	0.18	22	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
135	*Morus nigra* L./ Moraceae Kala toot SAN-SR-209	Tree/ C	Fruit/ Raw	Laxative, expectorant, respiratory disorders, jaundice,	4	0.13	29	0.08	1*, 2•, 3*, 4*, 5•, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
136	*Nannorrhops ritchieana* (Griff.) Aitch./ Arecaceae Mazri SAN-SR-186	Shrub/ W	Fruit, leaves/ Raw	Tonic	1	0.2	5	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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137	*Nelumbo nucifera* Gaertn./ Nelumbonaceae Kanwal SAN-SR-197	Herb/ W	Seeds/ Raw	Cardio tonic, cooling effect, hypertension	3	0.21	14	0.04	1▲, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
138	*Nerium oleander* L./ Apocynaceae Knair SAN-SR-181	Shrub/ W,C	Stem/ Latex	Snake, scorpion and wasp bite	3	0.07	40	0.11	1▲, 2*, 3*, 4*, 5▲, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
139	*Ocimum americanum* L./ Lamiaceae SAN-SR-28	Herb/ W	Leaves, seeds/ Decoction, raw	Cough, malaria, depression, constipation	4	0.25	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22*, 23*	
140	*Ocimum basilicum* L./ Lamiaceae Niazbo SAN-SR-57	Herb/ W,C	Leaves/ Decoction	Obesity, fever, hypertension, malaria, anxiety	5	0.15	32	0.09	1*, 2*, 3*, 4▲, 5*, 6*, 7•, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	

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141	*Olea ferruginea* Wall. ex Aitch./ Oleaceae SAN-SR-111	Tree/ W	Fruit/ Oil	Laxative, obesity, stomach ache, tonic, rheumatism	6	0.22	27	0.08	1*, 2*, 3•, 4*, 5•, 6*, 7*, 8*, 9*, 10*, 11▲, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
142	*Opuntia dillenii* (Ker Gawl.) Haw./ Cactaceae SAN-SR-115	Shrub/ W	Fruit, leaves/ Juice, extract	Cooling effect, liver problems, inflammation	3	0.16	19	0.05	1▲, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
143	*Otostegia limbata* (Benth.) Boiss/ Lamiaceae SAN-SR-205	Shrub/ W	Flowers, leaves/ Decoction, infusion	Hypertension, insomnia, cough, sore throat	4	0.33	12	0.03	1*, 2•, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11▲, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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144	*Oxalis corniculata* L./ Oxalidaceae Khatti buti SAN-SR-169	Herb/ W	Whole plant/ Decoction	Digestive problems, nausea, vomiting, bloating	4	0.23	17	0.05	1*, 2•, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11•, 12*, 13*, 14*, 15*, 16•, 17*, 18*, 19•, 20▲, 21*, 22*, 23*	
145	*Oxalis corymbosa* DC./ Oxalidaceae SAN-SR-173	Herb/ W	Whole plant/ Decoction	Digestive problems, nausea, vomiting, bloating	4	0.23	17	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
146	*Papaver dubium* L./ Papaveraceae Jangli afyun SAN-SR-14	Herb/ W	Leaves, seeds/ Infusion, juice	Laxative, fatigue, insomnia, cooling effect, respiratory problems, tonic	6	0.26	23	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
147	*Parthenium hysterophorus* L./ Asteraceae Dhania booti SAN-SR-39	Herb/ W	Whole plant/ Paste, infusion decoction	Piles, poor appetite, gynaecological disorders	3	0.18	16	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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148	*Peganum harmala* L./ Nitrariaceae Harmal SAN-SR-67	Herb/ W	Seeds/ Powder	Piles, malaria, febrifuge, measles, gastrointestinal disorders	5	0.13	36	0.10	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18*, 19▲, 20*, 21*, 22*, 23*	
149	*Pentatropis spiralis* (Forssk.) Decne./ Asclepiadaceae SAN-SR-127	Liana/ W	Leaves/ Infusion	Blood purifier, antidote,	2	0.25	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
150	*Periploca aphylla* Decne./ Apocynaceae Batta SAN-SR-83	Shrub/ W	Aerial parts/ Decoction	Antitumor	1	0.2	5	0.01	1*, 2*, 3*, 4*, 5•, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
151	*Persicaria glabra* (Willd.) M. Gomez/ Polygonaceae SAN-SR-138	Herb/ W	Leaves/ Infusion, decoction	Malaria fever, headache, body pain, fatigue	4	0.31	13	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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152	*Persicaria lapathifolia* (L.) Delabre (Syn. *Polygonum lapathifolium* L./ Polygonaceae SAN-SR-123	Herb/ W	Leaves/ Infusion, decoction	Malaria fever, headache, body pain, fatigue	4	0.28	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
153	*Phoenix sylvestris* (L.) Roxb. / Arecaceae Khajoor SAN-SR-95	Tree/ W	Fruit/ Raw, infusion	aphrodisiac, tonic, stomach problems, expectorant, gonorrhea,	5	0.22	22	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
154	*Phragmites australis* (Cav.) Trin. ex Steud./ Poaceae Narri SAN-SR-35	Herb/ W	-----	-----	0	0	15	0.04	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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155	*Phyla nodiflora* (L.) Greene/ Verbenaceae Bhukkan SAN-SR-84	Herb/ W	Whole plant/ Decoction	Constipation, respiratory problems, skin problems,	3	0.3	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
156	*Physalis minima* L./ Solanaceae Jangli cherry SAN-SR-12	Herb/ W	Whole plant/ Powder, paste, infusion	Chest infections, cough	2	0.25	8	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	
157	*Physorrhynchus chamaerapistrum* (Boiss.) Boiss./ Brassicaceae SAN-SR-19	Herb/ W	Whole plant/ Infusion, paste	Skin problems, headache	2	0.16	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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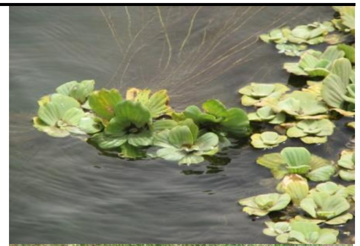
158	*Pistia stratiotes* L./ Araceae SAN-SR-208	Herb/ W	Leaves/ Decoction	Malaria, fever, headache, sore throat	4	0.30	13	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
159	*Plantago boissieri* Hausskn. & Bornm.L./ Plantaginaceae Jangli isabgol SAN-SR-179	Herb/ W	Husk	Digestive problems, cooling effect, obesity, poor appetite	4	0.33	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
160	*Pluchea arabica* (Boiss.) Qaiser & Lack/ Asteraceae SAN-SR-86	Herb/ W	Whole plant/ Decoction	Obesity, stomach ache, cough, fever	4	0.44	9	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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161	*Polygonum persicaria* L./ Polygonaceae SAN-SR-104	Herb/ W	Whole plant/ Decoction	Stomach pain, indigestion, vomiting	3	0.3	10	0.02	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
162	*Polygonum plebeium* R. Br./ Polygonaceae SAN-SR-17	Herb/ W	Whole plant/ Paste, decoction, infusion	Epilepsy, body pain, wound healing	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12▲, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
163	*Portulaca oleracea* L./ Portulacaceae Qulfa SAN-SR-53	Herb/ W	Leaves/ Cooked	Laxative, digestive problems, eczema	2	0.13	15	0.04	1*, 2•, 3*, 4*, 5*, 6*, 7*, 8▲, 9▲, 10*, 11*, 12*, 13•, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	

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164	*Prosopis glandulosa* Torr./ Fabaceae Angrezi kikri SAN-SR-90	Shrub/ W	Pods/ Decoction	Gynaecological disorders	1	0.25	4	0.01	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
165	*Prosopis cineraria* (L.) Druce/ Fabaceae Jand, Jandi SAN-SR-119	Tree/ W	Leaves/ Smoke	ENT problems, inflammation,	2	0.16	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
166	*Prosopis juliflora* (Sw.) DC./ Fabaceae Kikri SAN-SR-18	Shrub/ W	Leaves, pods/ Decoction	Poultice, gynaecological disorders	2	0.08	23	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
167	*Pseudogaillonia hymenostephana* (Jaub. & Spach) Linchevskii/ Rubiaceae SAN-SR-49	Herb/ W	Whole plant/ Decoction	Malaria fever, fatigue, body pain, cough, cold	5	0.26	19	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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168	*Pulicaria glutinosa* (Boiss.) Jaub. & Spach/ Asteraceae SAN-SR-31	Herb/ W	Whole plant/ Smoke, infusion	Evil eye, measles, skin allergy, inflammation, malaria, blood purifier	6	0.35	17	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
169	*Punica granatum* L./ Lythraceae SAN-SR-71	Shrub/ W	Fruit, peel/ Juice, paste	Malaria, pimples, anemia, cooling effect, fatigue, weakness	6	0.24	25	0.07	1▲, 2▲, 3*, 4*, 5•, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17•, 18▲, 19•, 20*, 21*, 22▲, 23*	
170	*Pupalia lappacea* (L.) Juss./ Amaranthaceae Gol Puthkanda SAN-SR-76	Herb/ W	Leaves/ Paste	Wound healing	1	0.05	21	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	

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171	*Ranunculus hispidus* Michx./ Ranunculaceae SAN-SR-176	Herb/ W	Whole plant	Vomiting, nausea, headache, fatigue	4	0.27	15	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
172	*Ranunculus muricatus* L./ Ranunculaceae SAN-SR-194	Herb/ W	Leaves/ Powder	Malaria	1	0.08	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8•, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
173	*Rhazya stricta* Decne./ Apocynaceae Weirran SAN-SR-46	Herb/ W	Leaves, stem/ Paste, miswak	Chronic wound healing, oral infection	2	0.12	17	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
174	*Ricinus communis* L./ Euphorbiaceae Harnoli SAN-SR-02	Shrub/ W	Seeds, leaves/ Oil, paste	Inflammation, abortion, hypertension, headache,	4	0.11	35	0.1	1▲, 2•, 3*, 4*, 5*, 6*, 7*, 8*, 9▲, 10*, 11▲,12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22* , 23•	
175	*Ruellia nudiflora* (Engelm. & A. Grey) Urb./ Acanthaceae Pataki SAN-SR-05	Herb/ W	Flowers, leaves/ Decoction	Cancer, pain, cough, constipation,	3	0.11	27	0.08	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
176	*Ruellia tuberosa* L./ Acanthaceae SAN-SR-42	Herb/ W	Flowers, leaves/ Decoction	Cancer, pain, cough, constipation,	3	0.11	27	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
177	*Rumex dentatus* L./ Polygonaceae Jangli palak SAN-SR-38	Herb/ W	Leaves/ Raw, cooked	Skin problems, obesity	2	0.06	32	0.09	1*, 2▲, 3*, 4*, 5▲, 6*, 7*, 8*, 9*, 10*, 11*, 12•, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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178	*Rumex vesicarius* L./ Polygonaceae SAN-SR-70	Herb/ W	Leaves/ Decoction, paste	ENT problems, scorpion bite, wasp bite, indigestion,	4	0.15	26	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
179	*Saccharum bengalense* Retz./ Poaceae Kana SAN-SR-98	Herb/ W	-----	-----	0	0	19	0.05	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
180	*Saccharum revennae* (L.) L./ Poaceae Sarkanda SAN-SR-62	Herb/ W	-----	-----	0	0	13	0.03	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
181	*Saccharum spontaneum* L./ Poaceae Kahn SAN-SR-72	Herb/ W	-----	-----	0	0	20	0.05	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	
182	*Salvadora persica* L./ Salvadoraceae Jaal, peelu SAN-SR-88	Tree/ W	Fruits, bark/ Raw, Decoction	Toothache, epilepsy, abortion	3	0.07	40	0.11	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14▲, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23▲	
183	*Salvia moorcroftiana* Wall. ex Benth./ Lamiaceae SAN-SR-99	Herb/ W	Leaves/ Poultice, decoction	Itching skin, kidney pain, colic, bloating	4	0.16	25	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12•, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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184	*Salvinia natans* (L.)All./ Salviniaceae SAN-SR-87	Herb/ W	Whole plant/ Poultice	Wound, inflammation, itching skin,	3	0.17	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
185	*Schweinfurthia papilionacea* (L.) Boiss./ Plantaginaceae SAN-SR-199	Herb/ W	Leaves/ Smoke, decoction	Skin allergy, cough, sore throat	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
186	*Senecio* sp./ Asteraceae SAN-SR-178	Herb/ W	-----	-----	0	0	4	0.01	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
187	*Sisymbrium irio* L./ Brassicaceae Khoob kalan SAN-SR-170	Herb/ W	Flowers/ Decoction	Chest infections, expectorant	2	0.08	23	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19▲, 20*, 21*, 22*, 23*	
188	*Solanum elaeagnifolium* Cav./ Solanaceae SAN-SR-192	Herb/ W	Fruits, flower/ Infusion	Analgesic, toothache, menstrual pain	3	0.14	22	0.06	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
189	*Solanum incanum* L./ Solanaceae SAN-SR-105	Herb/ W	Fruits, flower/ Infusion	Respiratory problems, gastrointestinal problems	2	0.14	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
190	*Solanum nigrum* L./ Solanaceae Mako SAN-SR-30	Herb/ W	Fruits, seeds/ Decoction	Hepatitis, respiratory problems, body pain	3	0.19	16	0.04	1•, 2•, 3•, 4*, 5•, 6*, 7•, 8▲,9*, 10*, 11*, 12*, 13*, 14▲, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	
191	*Solanum surattense* Burm. f./ Solanaceae Mahorri SAN-SR-131	Herb/ W	Fruit/ Juice, raw	Respiratory problems, febrifuge, pain	3	0.19	16	0.04	1▲, 2*, 3*, 4*, 5*, 6*, 7•, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
192	*Solanum xanthocarpum* Schrad. & H. Wendl./ Solanaceae Mahorri SAN-SR-48	Herb/ W	Fruit, leaves/ Decoction, infusion	Cough, cold, malaria, headache, febrifuge	5	0.28	18	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
193	*Sonchus asper* (L.) Hill/ Asteraceae Dhodak SAN-SR-59	Herb/ W	Whole plant/ Decoction	Stomach ache, head ache, migraine	3	0.2	15	0.04	1*, 2*, 3▲, 4*, 5▲, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
194	*Stellaria media* (L.) Vill./ Caryophyllaceae Banbatorr SAN-SR-50	Herb/ W	Aerial parts/ Paste, infusion	ENT problems, piles, jaundice	3	0.21	14	0.04	1*, 2▲, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
195	*Tamarix aphylla* (L.) H. Karst./ Tamaricaceae Khaggal SAN-SR-114	Tree/ W	Leaves/ Smoke	Measles, respiratory disorders, febrifuge, body pain	4	0.10	39	0.11	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
196	*Tamarix dioica* Roxb. Ex Roth./ Tamaricaceae Khaggal SAN-SR-94	Tree/ W	Leaves/ Smoke	Measles	4	0.09	42	0.12	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
197	*Taraxacum officinale* (L.) Weber F. H. Wigg./ Asteraceae Duddal SAN-SR-116	Herb/ W	Flower/ Infusion	Stomach problems, hepatitis,	2	0.09	22	0.06	1*, 2▲, 3*, 4*, 5*, 6*, 7*, 8•, 9*, 10*, 11*, 12▲, 13*, 14*, 15▲, 16*, 17*, 18*, 19*, 20*, 21*, 22•, 23*	
198	*Taverniera glabra* Boiss./ Fabaceae SAN-SR-117	Shrub/ W	Leaves, bark/ Decoction	Body pain, obesity, poor appetite	3	0.3	10	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
199	*Taverniera spartea* (Burm.f.) DC./ Fabaceae SAN-SR-137	Shrub/ W	Stem/ Decoction	Cancer, blood purification, pimples	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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200	*Tecomella undulata* (sm.) Seem./ Bignoniaceae Roheerra SAN-SR-141	Tree/ W	Bark/ Decoction	Urinary problems,	1	0.08	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
201	*Tephrosia apollinea* (Delile) DC./ Fabaceae SAN-SR-144	Herb/ W	Pods, flower/ Decoction	Inflammation, toothache, respiratory problems	3	0.21	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
202	*Trianthema portulacastrum* L./ Aizoaceae It sit SAN-SR-151	Herb/ W	Leaves/ Juice	Antidote, gynaecological disorders, stomach problems, eye infections	4	0.14	28	0.08	1▲, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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Sr. No.	Species/ Family Name/ Vernacular Name/ Voucher number	Habit /Type	Part(s) used/ Mode of utilization	Medicinal Value/ Disease treated	UR*	UV*	FC*	RFC*	Previous reports for comparison**	Picture
203	*Tribulus terrestris* L./ Zygophyllaceae Bhakrra SAN-SR-155	Herb/ W	Aerial parts/ Decoction	Aphrodisiac, respiratory problems, febrifuge, headache, laxative	5	0.14	36	0.10	1▲, 2▲, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
204	*Trichodesma indicum* (L.) Lehm./ Boraginaceae	Herb/ W	Leaves/ Decoction, infusion	Antidote, laxative, body pain, fever	4	0.2	20	0.05	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
205	*Typha angustata* Bory & Chaub./ Typhaceae Koondar SAN-SR-158	Herb/ W	Whole plant/ Decoction	Respiratory disorders, stomach problems, gynaecological problems	3	0.07	39	0.11	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
206	*Typha latifolia* L./ Typhaceae Koondar SAN-SR-165	Herb/ W	Whole plant/ Decoction	Respiratory disorders, stomach problems, gynaecological problems	3	0.07	38	0.11	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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207	*Urtica dioica* L./ Urticaceae Bichho bboti SAN-SR-167	Herb/ W	Flowers/ Infusion	Body pain, head ache	2	0.06	32	0.09	1*, 2▲, 3*, 4▲, 5*, 6*, 7*, 8*, 9*, 10*, 11*,12*, 13*, 14*,15▲, 16▲, 17*, 18*, 19▲, 20*, 21*, 22*, 23*	
208	*Verbascum thapsus* L./ Scrophulariaceae Kamla Tambaco SAN-SR-184	Herb/ W	Flowers/ Infusion	Headache, febrifuge, aphrodisiac, malaria, respiratory problems, gynaecological problems	6	0.26	23	0.06	1*, 2▲,3*, 4*, 5*, 6*, 7* , 8*, 9*, 10*, 11▲, 12•, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22•, 23*	
209	*Verbena officinalis* L./ Verbenaceae SAN-SR-183	Herb/ W	Leaves/ Decoction	Digestive problems, fever, menstrual problems	3	0.37	8	0.02	1*, 2▲, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
210	*Vernonia arabica* Boiss./ Asteraceae SAN-SR-163	Herb/ W	Aerial parts/ Decoction	Stomach ache, fever, colic, liver problems	4	0.28	14	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

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211	*Vicia sativa* L./ Fabaceae Rewarri SAN-SR-180	Herb/ W	Aerial parts/ Decoction	Inflammation, back ache, skin problems	3	0.25	12	0.03	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
212	*Viola cinerea* Boiss/ Violaceae SAN-SR-172	Herb/ W	Whole plant/ Infusion, decoction	Blood purifier, malaria, gynaecological disorders	3	0.12	26	0.07	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	
213	*Withania coagulans* (Stocks) Dunal /Solanaceae Khamjeera SAN-SR-29	Shrub/ W	Fruit/ Raw, infusion	Depurative, gastrointestinal disorders, menstrual disorders, obesity, respiratory disorders	5	0.14	35	0.1	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17▲, 18*, 19*, 20*, 21*, 22*, 23*	

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214	*Withania somnifera* (L). Dunal /Solanaceae Kutai lal SAN-SR-193	Herb/ W	Fruit/ Infusion	Abdominal pain, gastrointestinal problems, vomiting, skin problems, depurative, cough, cold, throat infections, insomnia	9	0.25	35	0.1	1*, 2▲, 3, 4, 5▲, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13▲, 14*, 15*, 16*, 17•, 18*, 19*, 20*, 21*, 22▲, 23•	
215	*Xanthium strumarium* L./ Asteraceae Chhota Dhatura SAN-SR-187	Herb/ W	Leaves, aerial parts/ Infusion, powder	Oral infection, dysmenorrhea, hypertension, malaria	4	0.31	13	0.04	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8▲, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19▲, 20*, 21*, 22*, 23*	

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216	*Ziziphus mauritiana* Lam./ Rhamnaceae Beri SAN-SR-185	Tree/ W	Leaves, seeds, fruits/ Raw, paste	Tonic, laxative, hair tonic, gastrointestinal problems	4	0.1	40	0.11	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20▲, 21*, 22*, 23*	
217	*Ziziphus nummularia* (Burm.f.) Whigt. & Arn./ Rhamnaceae Karkinna SAN-SR-164	Shrub/ W	Fruits, leaves, seeds/ Raw, paste	Tonic, laxative, hair tonic, stomach problems	4	0.09	42	0.12	1*, 2*, 3*, 4*, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14*, 15*, 16*, 17*, 18*, 19*, 20*, 21*, 22*, 23*	

1= Yabesh et al. (2014); 2= Haq. (2012); 3= Khan et al. (2010); 4= Phondani et al. (2010); 5= Zabihullah et al. (2006); 6= Hazrat et al. (2011); 7= Khan and Khatoon (2008); 8= Amjad. (2015); 9= Jiofack et al. (2009); 10= Bano et al. (2014); 11= Ahmad et al. (2014); 12= Khan et al. (2013); 13= Belayneh et al. (2012); 14= Teklehaymanot and Giday. (2010); 15= Pieroni and Giusti. (2009); 16= Pradhan and Badola. (2008); 17= Shah and Rahim. (2017); 18= Tetali et al. (2009); 19= Ali and Qaiser. (2009); 20= Rasingam. (2012); 21= Sanz-Biset et al. (2009); 22= Sharma et al. (2004); 23= Teklehaymanot and Giday. (2010)
UR*; Use Report, VU*; Use Value, FC*; frequency of Citation, RFC*; Relative Frequency Citation
W; Wild, C; Cultivated
** (●) – Similar use, (▲) – Dissimilar use, (*) – Use not reported

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Collection and taxonomic identification of plants

Plants were collected with the help of traditional healer and local respondents just after the formal and informal discussion on ethnobotanically important plants of the area. Local informants guided researchers to the natural habitat of the discussed species and in some cases also helped to collect voucher specimens. After recording information about plants, voucher specimen were pressed in the field and dried properly. Plants were identified following the Flora of Pakistan (Nasir and Ali, 1970-2001). The list of plant species used ethnobotanically in the area was checked and updated after verification from the online website (http://www.theplantlist.org) of the Royle Botanic Garden, Kew, assessed on April 3, 2014. Voucher specimens were preserved and deposited in the Herbarium of University of Sargodha for their taxonomic identification by comparison with specimen in the herbarium and for future reference.

Quantitative analyses of ethnobotanical data

Ethnobotanically important plant species were arranged in alphabetic order by botanical name, family, habit, parts used, modes of utilization and disease treated. Different quantitative indices including use value (UV), frequency of citation (FC), relative frequency citation (RFC), family importance value (FIV), informant consensus factor (ICF) and the Jaccard index (JI) were applied to analyze the documented data.

Use Value (UV)

The UV is a quantitative index used to determine the relative importance of an indigenous plant species. According to Phillips et al. (1994), the use value was calculated by using the formula :

$$UV = \sum U / n \qquad \text{(Eq.1)}$$

Where, "U" refers to the number of uses mentioned by the participant for a given species and "n" refers to the total number of informants interviewed that used the given species. High UV score indicates that there are many use reports for that plant and vice versa.

Frequency of Citation (FC) and Relative Frequency Citation (RFC)

The Frequency of Citation (FC) is number of informants mentioning the use of plant species.

Moreover, Relative Frequency Citation (RFC) index was evaluated by using the formula :

$$RFC = FC / N \left(0 < RFC < 1\right) \qquad \text{(Eq.2)}$$

N is the total number of informants participating in the study. The RFC index ranges from "0" when nobody referred to a plant as useful to "1" when all informants referred to as useful. (Vitalini et al., 2013).

Informant Consensus Factor (ICF)

Informant Consensus Factor (ICF) was calculated using the formula (Heinrich et al., 1998):

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$$ICF = \left(Nur - Nt\right) / \; Nur - 1 \qquad \text{(Eq.3)}$$

Where, “Nur” refers to the total number of use reports mentioned for a particular disease category and “Nt” refers to the total number of plant taxa used for particular disease category. This formula was opted to find out the homogeneity in the ethnomedicinal information documented from the traditional informants.

To calculate ICF, reported medicinal plant species were grouped into 16 categories of various diseases reported in the study area by the informants. ICF is useful to focus and to determine the consistency of the data concerning certain sorts of disease categories (Canales et al., 2005; Heinrich et al., 1998). The result of this consensus ranges from 0 to 1. A high value (close to 1) predicts that plants are used by the high percentage of the informants for a number of illness related to that comprehensive category, whereas, the low value (close to 0) shows that the plants are selected arbitrarily for a few or a single complaint or that informants did not share or exchange facts and data about the usage of plants (Abu-Irmaileh and Afifi, 2003; Akerele, 1988; Kloutsos et al., 2001). Medicinal plants for which very few diseases are referred are supposed to be pharmacologically less active and thereby have low lower ICF values (Gazzaneo et al., 2005; Sharma et al., 2012; Teklehaymanot, 2009), *Table 2*.

Jaccard Index (JI)

Jaccard index is used to compare the reported data with that of other ethnobotanical studies conducted in other areas of Pakistan and abroad (González-Tejero et al., 2008). JI is calculated by using the formula :

$$JI = c \times 100 / a + b - c \qquad \text{(Eq. 4)}$$

Where, “a” is the recorded number of species of the study area “A”(Namal Valley) and “b” is the documented number of species of the area “B”. Where “c” is the number of species common to both “A” and “B”. As far as indigenous communities are concerned, “a” is the number of species reported by indigenous community “A”, “b” is the number of species cited by the indigenous community “B” and the “c” is the number of species reported by both “A” and “B” communities, *Table 2*.

Results

A total of 350 local informants were interviewed. The age of informants ranged from 25 years to above 65. Education wise, 24.3% informants were illiterate, 20% completed their five years education, 18.6% completed their eight years education and only 13.1% informants had higher education (University level). A total of 217 plant species belonging to 166 genera and 70 families were collected from the Valley. The ethnobotanical uses of plants (vernacular name, habit/type, part used/ mode of utilization, disease treated and other information like UR, UV, FC, RFC, previous report) are included in the *Table 1*. The highest number of reported species belong to family Fabaceae (19 species) followed by Asteraceae (18 species), Poaceae (13 species), Euphorbiaceae and Solanaceae (9 species each) and Lamiaceae and Moraceae (8 species each) etc. Most of the documented species were herbs (71%), followed by shrubs (15%) and trees (14%) (*Fig. 2*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

Table 2. *Comparison of present study with previous reports at national and global level (JI*; Jaccard index)*

Sr. No.	Reference	Valley name/ Region	No. of documented plant species	Plants with similar use	Plants with dis-similar use	No. of species common in both areas	%age of species common in both areas	Species enlisted only in aligned area	Species enlisted only in study area	%age of plants with similar use	%age of dis-similar uses	JI* (*)
1	Yabesh et al., 2014	Silent Valley/ Kerala, India	102	5	12	17	16.7	85	184	4.9	11.8	6.7
2	Haq, 2012	Allai Valley/ Western Himalaya Pakistan	172	15	17	32	18.6	140	169	8.7	9.9	11.5
3	Khan et al., 2010	Poonch Valley/ Azad Kashmir (Pakistan)	169	6	7	13	7.7	156	188	3.5	4.1	3.9
4	Phondani et al., 2010	Niti Valley/ Central Himalaya, India	86		8	8	9.3	78	193	0	9.3	3.04
5	Zabihullah et al., 2006	Kot Manzaray Baba Valley/ Malakand Agency, Pakistan	82	9	15	24	29.3	58	177	10.1	18.3	11.4
6	Hazrat et al., 2011	Dir Kohistan Valley/ Khyber Pukhtunkhwa, Pakistan.	40	1	2	3	7.5	37	198	2.5	5	1.3
7	Khan and Khatoon, 2008	Haramosh and Bugrote Valleys/ Gilgit, Pakistan	98	6	5	11	11.2	87	190	6.1	5.1	4.1
8	Amjad, 2015	Bana Valley/ Kotli, Pakistan	86	12	28	40	46.5	46	161	13.9	32.5	23.9
9	Jiofack et al., 2009	Upper Nyong Valley/ Cameroon	140	0	3	3	2.1	137	198	0	2.14	0.9
10	Bano et al., 2014	Skardu Valley/ karakoram-Himalayan range, Pakistan	50	1	2	3	6	47	198	2	4	1.2
11	Ahmad et al., 2014	Chail Valley/ Swat- Pakistan	50	6	4	10	20	40	191	12	8	4.5
12	Khan et al., 2013	Naran Valley/ Western Himalaya, Pakistan	101	4	6	10	9.9	91	191	3.9	5.9	3.7
13	Belayneh et al., 2012	Erer Valley/ Eastern Ethiopia	51	2	1	3	5.9	48	198	3.9	1.9	1.23
14	Teklehaymanot and Giday, 2010	Lower Omo River Valley/ Ethiopia	38	0	3	3	7.9	35	198	0	7.9	1.30
15	Pieroni and Giusti, 2009	Upper Varaita Valley/ Piedmont	88	0	3	3	3.4	85	198	0	3.4	1.07
16	Pradhan and Badola, 2008	Dzongu Valley/ North Sikkim, India	118	3	4	7	5.9	111	194	2.5	3.4	2.34
17	Shah and Rahim, 2017	Soon Valley/ Khushab, Pakistan	70	11	17	28	40	42	173	15.7	24.3	14.9
18	Tetali et al., 2009	Parinche Valley/ Maharashtra, India	28	0	5	5	17.8	23	196	0	17.8	2.34
19	Ali and Qaiser, 2009	Chitral Valley/ Pakistan	83	4	6	10	12.04	73	191	4.8	7.2	3.9
20	Rasingam, 2012	Pillur Valley/ Tamil Nadu, India	74	0	12	12	16.2	62	189	0	16.2	5.02
21	Sanz-Biset et al., 2009	Chazuta Valley/ Peruvian Amazon	289	1	0	1	0.3	288	200	0.3	0	0.20
22	Sharma et al., 2004	Parvati Valley/ western Himalaya, India	50	4	3	7	14	43	194	8	6	3.04
23	Teklehaymanot and Giday, 2010	lower Omo River Valley/ Ethiopia	57	2	4	6	10.5	51	195	3.5	7.01	2.5

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DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

The high numbers of species recorded in the present work were wild (92.6%). In the current study, the most frequent plant part used ethnobotanically by the local people was flowers (35%), followed by leaves (18%), whole plant (12%), pods (10%), twigs (8%), aerial parts (8%), rhizome and seeds (3% each), stem (1.6%) and fruit (1%) (*Fig. 3*). A total of 261 preparations were recorded and the most preferred method of preparation was decoction (41%), followed by infusion (18%), raw form (14%), paste (8%), juice (5%), cooked form (3%), latex, smoke, poultice and powder (2% each), oil, ash and extract (1% each) (*Fig. 4*).

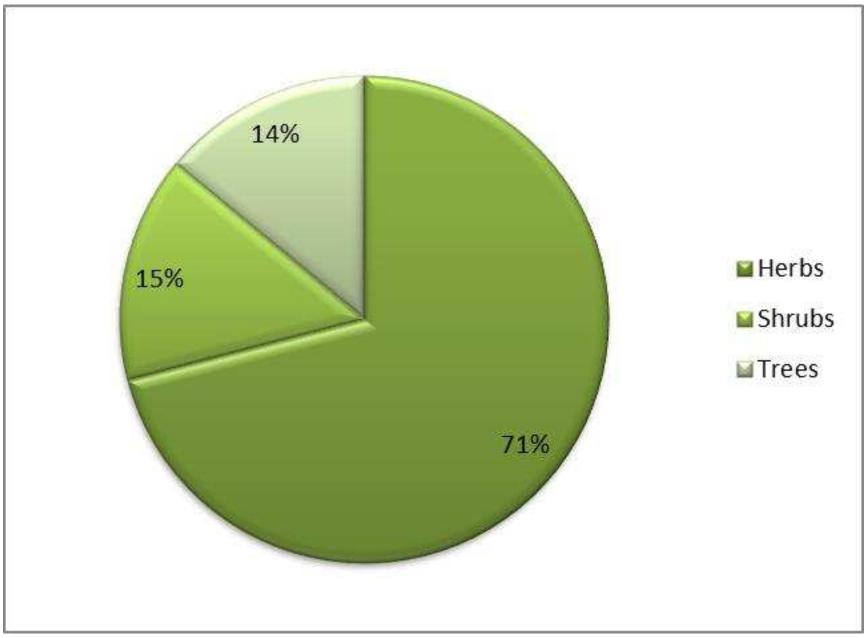

Figure 2. *Life form of reported medicinal plants*

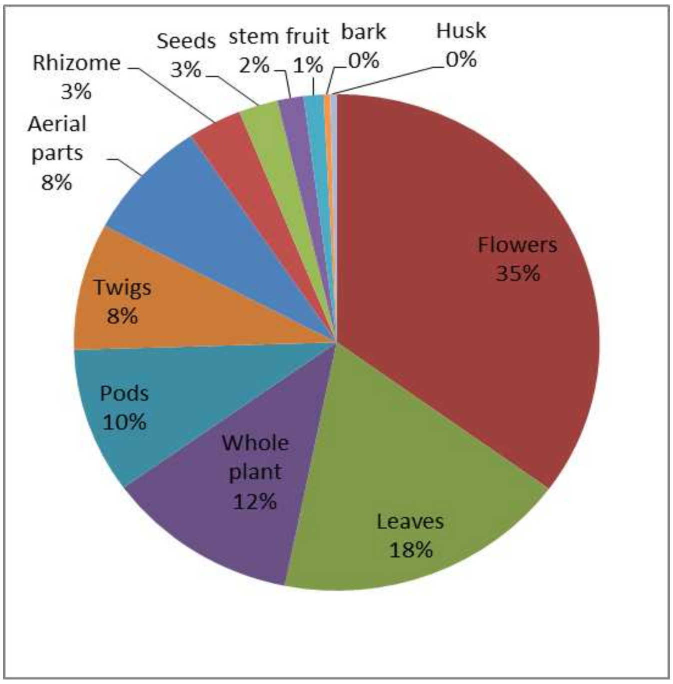

Figure 3. *Percentage of part used of plant species*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

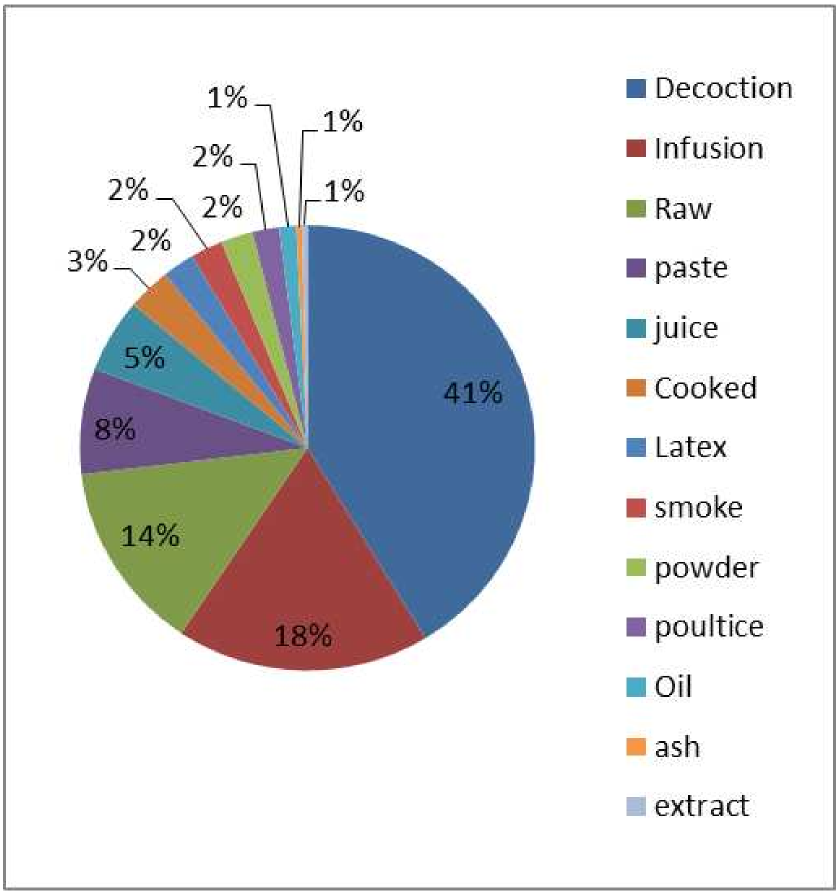

Figure 4. *Percentage of mode of utilization*

Discussion

Ethnobotanical knowledge plays an important role in exploring the human-plant relationship and medicinal uses of native flora. In this study possession of traditional knowledge about medicinal plants was found among the people with low level of education and among the age group of 46-<65. This is because inhabitants of the study area are mostly associated with raring of animals and other depends on available land for farming, they have least concern about acquiring higher education. But due to intimate relation with nature they have enough knowledge about the use of these plants. On the other hand, the informants who are educated are unaware from the uses of plants because of least interaction with the nature.

Highest use report of plants belonging to Fabaceae and Lamiaceae is due to their dominance and wider occurrence as easily available plants are likely to be used frequently by local inhabitant (Agelet and Valles, 2001; Johns et al., 1990) Similar findingis reported by Cornara et al., (2009); Giday et al., 2009; Saslis-Lagoudakis et al., 2011; Teklehaymanot, 2009). Moreover, the ascendancy of these families reveals the presence of a wide range of bioactive compounds in respective taxa belonging to these families. The diversity of bioactive compounds and secondary metabolites may be the reason for the multi-species use one or more health problems, as a single compound may not be the reason of efficacy (Verpoorte et al., 2005). The Poaceae had less aforementioned proceedings in ethno botanical investigations (Cakilcioglu and Turkoglu, 2010; Kadir et al., 2012; Rokaya et al., 2010) conducted at global level. In present study the reason of frequent use of family Poaceae is mainly the dependency of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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locals for fuel (e.g., *Saccharum bengalense*, *S. revennae*, *S. spontaneum* etc.), thatching (*Arundo donax*, *Phragmites australis* etc.) , forage (*Cymbopogon commutatus*, *Cynodon dactylon*, *Cenchrus echinatus*, *Chloris gayana*, *Coix lacryma-jobi*, *Dactyloctenium aegyptium*, *Desmostachya bipinnata*, *Dichanthium annulatum* etc.) and other ethnobotanical uses (medicinal) as compared to medicinal values, because lush herbaceous plants are abundant in study area. Such findings indicate great reliance of inhabitants on a diversity of plant species to treat ailments, and represent a good sign of the intense facts on plant based treatments (Nadembega et al., 2011). Among the reported plant species, some medicinal plants were of high indigenous priority thus are commonly cited by majority of the respondents. The highly cited medicinal plant species those having at least 32 citations or more (*Table 1*) were *Aloe vera, Calotropis procera*, *Mentha longifolia*, *Tamarix dioica*, *Ziziphus nummularia*, *Nerium oleander*, *Salvadora persica*, *Ziziphus mauritiana, Tamarix aphylla*, *Typha angustata, T. latifolia, Peganum harmala, Tribulus terrestris, Acacia modesta, Albizia procera, Eruca sativa, Ricinus communis, Withania coagulans, Withania somnifera, Morus alba, Conyza canadensis, Ocimum basilicum, Rumex dentatus* and *Urtica dioica.* These plants may reported frequently due to their abundance in the area and there is prevailing beliefs among the inhabitants to consider these plants medicinally effective, as majority of informants stated that maximum treatment were cured at domestic level because of unavailability of healthcare services and high cost of synthetic medications. The reason of frequent citation of these plants may be the presence of valuable and pharmacologically active bioactive compounds. Favorable climatic and topographic conditions of the study area might have positive contribution toward the rapid adaptation of herbaceous flora as compared to other life forms (Ayyanar and Ignacimuthu, 2011; Giday et al., 2003; Mesfin et al., 2012; Uniyal et al., 2006). According to most of the informants the fast restoration and higher potency of herbs is the main reason to have higher preferences for medicinal purposes and herbs are ephemeral in habit. Similar results were also discussed in previous documentations (Megersa et al., 2013; Upadhyay et al., 2010; Upadhyay et al., 2007). Herbs are rich in bioactive compounds (Srithi et al., 2009). The major use of flower is unique to this study when, compared with other ethnomedicinal studies (Rahman et al., 2016; Shah and Rahim, 2017) conducted in Pakistan. Local people believe that collecting a plant in flowering period is very important for the effectiveness of the formulation. This may be due to the richness of essential oils or the existence of bioactive compounds in flowers as compared to other parts. However, collection of premature flower and its excessive use may have negative impacts on plants sustainability. Furthermore, over-exploitation of seeds and roots for local uses can lead to a drastic decline in the populations of medicinal plants (Ghimire et al., 2008) and consequently an expatriation of medicinal species from the area. Therapeutic uses of leaves are defensible (Giday et al., 2003; Zheng and Xing, 2009) which is reported as the second highest part used in this study. Use of leaves in traditional medication is a common practice which is also reported in several other studies (Asase et al., 2010; Asase and Oppong-Mensah, 2009; Koudouvo et al., 2011; Nadembega et al., 2011; Nguta et al., 2010). The use of leaves may be of high preference due to its photosynthetic function and presence of secondary metabolites for defense from herbivores which could be medicinally important for curing various ailments in humans (Bhattarai et al., 2006; Ghorbani, 2005). In addition, leaf collection is ecologically sustainable and is easy to collect as compared to other plant parts (Giday et al., 2009).

Boiling the dried and stored plant parts for herbal preparation is considered more effective as compared to other methods. It is a general observation that heating process

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speed up several biological reactions ensuring the extraction of countless vigorous compound (Al-Adhroey et al., 2010; Chen et al., 2008; Han et al., 2007; Zhang et al., 2005). Second rated mode of utilization reported in the area was infusion. Fresh and healthy plant parts are used for making infusions. Local people believe that infusion is one of the highly effective recipe which is easy to prepare and several effective compounds that plays a crucial role in the medication will not degraded during the process of preparation (Dike et al., 2012; Idowu et al., 2010). A handful amount (quantity that can be held or griped in a hand) of a patient is considered as an exact dose for medication from generation to generation. In the study area this is the most common way to measure a dose of drug required for different age groups from children to adults. Doses are taken twice or thrice a day depending upon the condition of the disease treated. Water was main and common solvent used in herbal remedies (Andrade-Cetto, 2009; Lee et al., 2008; Poonam and Singh, 2009; Prashanth et al., 2001; Ssegawa and Kasenene, 2007) since most of them were prepared as decoction or infusion. This practice is highly convenient because water is easily accessible, the easiest vehicle for herbal preparation and also expected not to disturb the chemical composition of the active constituents (Nunkoo and Mahomoodally, 2012). Some other ingredients sugar, salt, honey, olive oil, mustard oil and lemon are also added to improve the taste and to have additional effective that can help to reduce nausea, vomiting and constipation.

Quantitative analysis of ethnobotanical data

Use value (UV), Frequency of citation (FC) and Relative frequency citation (RFC)

Use value implies the relative significance of plant species on the basis of number of informants (FC) who mention the uses reported for respective plant species. The plant species with high UV (*Eq.1*) are suggested for further pharmacological and phytochemical screening to develop new drugs.

Relative frequency citation (RFC) (*Eq.2*) is a quantitative index that estimates the local reputation of every plant species used by the ethnic folks (Vitalini et al., 2013). It is calculated from Frequency of Citation (FC) divided by the total number of informants (N) who participated in the survey (Tardío and Pardo-de-Santayana, 2008) (*Table 1*).

The high RFC value suggests that these plants were known to majority of the informants. The high values of RFC help to identify species for which an appropriate conservation and sustainable utilization strategies should be formulated to ensure regular supply of raw materials for the future (Asase et al., 2005).

Comparison of different indices

Informant consensus factor (ICF) (*Eq.3*) values of reported medicinal plants were calculated for the categorized ailments (*Table 1*). Fifteen primary ailment categories were identified: *Table 3*. ICF values range from 0 to 0.7 (*Table 3*). Highest ICF value (0.7) was reported for oral and dental problems group, followed by respiratory problems (0.4), gastrointestinal problems, antidote and skin problems (0.3 each). The reason of high ICF could be the common happening of these illnesses in the community due to poor sanitation practice, low economic status and lack of adequate modern health care of in the Valley (Bieski et al., 2015). High ICF values undoubtedly disclose a noteworthy number of reports on the use of these taxa for a group of health problems (Baydoun et al., 2015). The slightest agreement among the informants was detected for plants used for group of sexual disorders, ear, nose and throat problems (ENT), eye ailments and other diseases which include pain, small pox, mumps, insect repellent, antitumor and anti-allergic, all these groups containing the zero ICF value. The least

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

value of ICF for ear, nose and throat problems (ENT) and eye diseases is in accordance with other studies (Bibi et al., 2015; Jamila and Mostafa, 2014). These low ICF values could be credited to the trend of folks in native or urban societies to use conventional medicines for curing predictable diseases, even in current times (Upadhyay et al., 2011).

Table 3. *ICF Value of medicinal plants used for treatment of various diseases (ICF*=Informant Consensus Factor)*

Disease category	No. of use reports	No. of taxa	ICF*
Respiratory problems	78	49	0.4
Urinogenital diseases	9	7	0.25
Gastrointestinal problems	145	99	0.3
Musculoskeletal disorders	56	46	0.2
Sexual disorders	53	53	0
Skin problems	83	61	0.3
Glandular disorders	29	28	0.03
Antidote	22	16	0.3
Cardiovascular diseases	51	46	0.1
Neurological disorders	20	18	0.1
ENT problems	13	13	0
Eye ailments	3	3	0
Fever	68	55	0.2
Oral and Dental problems	19	7	0.7
Hair problems	6	5	0.2
Others	10	10	0

Comparative analysis of documented data with previous literature

To conclude whether the usage of plants by communities of Namal Valley were previously reported or not for medicinal value, a literature study on ethnobotanical use was done and comparative results are listed in *Table 1*. Twenty three research papers from different valleys were selected to compare the data and it was observed that various plants have novel use reports and some plant species like *Viola cinerea*, *Pulicaria glutinosa* and *Pseudogaillonia hymnostephana* from the study area are reported for the first time and previously with no or rare use reports. In this study a total of 217 plant species were documented. Literature review of described medicinal plants have shown that the similarity of uses varies from 0% to 15.7%, while dissimilar uses range up to 32.5% (*Table 2*) (*Eq.4*). In our study high degree of similarity was found with studies directed in Bana Valley in Kotli, Pakistan by Amjad. (2015) and Soon Valley in Khushab, Pakistan by Shah and Rahim. (2017) with JI 23.9 and 14.9 respectively (*Table 2*). The cause of maximum similarity index may be the resemblance in plant diversity and their multipurpose uses against numerous health related problems in the study area. Noteworthy multiethnic discrepancy may be the cause of deviation in JI that are topmost symbolic of variation in habitation and populace (Leonti et al., 2009). The resemblance and inconsistency in ethnomedicinal studies appear to point out the significance of tribal wakefulness of medicinal plants in miscellaneous regions where chronological (Moerman, 1998) environmental (Ladio et al., 2007), phytochemical and organoleptic (Leonti et al., 2003) structures add in their assortment.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Limitations of the study, insights gained and approaches for improvements

This study was primarily aimed to document indigenous ethnobotanical knowledge of native communities of Namal Valley and evaluates their reliability within Pakistan and globally. However, few limitations still exists regarding sufficient interpretation of data. For example the native people have no acquaintance of common names/ vernacular names of some plant and similarly no report about their ethnomedicinal value and their use is confined to fuel, forage, thatching etc. Another obstruction of the study was that it did not investigate into the danger of invasive species and, documentation and dispersal of the introduced species.

Side effects of medicinal and other plants

In this study the documented ethnobotanical data generally elaborate the medicinal usage of the indigenous plant species, but it was observed that informants were much careful in using some of the plants such as *Achyranthes aspera, Calotropis procera, Cannabis sativa, Croton bonplandianum, Cuscuta reflexa, Datura metel, Dodonaea viscosa, Euphorbia helioscopia, E. heterophylla, E. hirta, E. peplus, E. prostrata, E. serpens, Heliotropium currasavicum, H. europaeum, Ipomoea carnea, Lantana camara, Lathyrus aphaca, Nerium oleander, Ricinus communis, Rhazya stricta, Solanum elaeagnifolium, S. xanthocarpum, S. nigrum, S. surattense, S. incanum*. The inhabitants are fully aware about the fact that these plants can cause symptom of toxicity including abortion, restlessness, depression, skin inflammation, vomiting, abdominal pain, nausea, impotency, sterility, dizziness and hallucination. People have a general knowledge about toxic signs and their interpretation of toxicity is based on the observation from generation to generation. Foremost taxonomic classification level for assessing the efficacy of plant to native societies is family (Thomas et al., 2009). Same is factual for the toxicity of plants (Huai et al., 2010). Fabaceae, Asteraceae, Euphorbiaceae and Apocynaceae have been described as the chief families comprising toxic plants in various studies (Levetin and McMahon, 2008; Huai et al., 2010; Ozturk et al., 2008). The reason of toxicity of these families is the presence of different toxins such as alkaloids, dicoumarin, glycosides, photosensitizing compounds, saponin, selenium (Fabaceae), acrid substances, alcohol, alkaloids, glycosides, nitrogenous compounds, photosensitizing compounds, saponins, selenium, volatile oils (Asteraceae), acrid substances, croton oil, photosensitizing compounds, biterpinoids, triterpenoids, steroids, alkaloids, cyanogenic glycosides and glucosinolates (Euphorbiaceae) and resin, glycosides (Apocynaceae) (Barla et al., 2006; Madureira et al., 2004; RIZK, 1987; Yamamura et al., 1989; Zhang and Guo, 2006). Further study of herbal toxicity in human is needed to certify safety and boost patient confidence about herbal remedies.

Conclusions and recommendations

This study exposed that traditional medication, mainly comprising the practice of medicinal plants playing a substantial role in curing the basic healthcare necessities of the inhabitants of Namal Valley, Pakistan. Usage of traditional medicine as a dominant part of their beliefs, inadequate access to recent health care conveniences and the cheerful prosperity of natural assets could be deliberated as the key influences for the prolongation of these traditional practices. The instant and thoughtful hazard to the native medicinal flora in the study area appears from the growing impact of overharvesting, overgrazing and deforestation due to human activities. There is dire necessity for thoughtful exertions to create awareness in the native people so that the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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proper actions should be taken to protect the suitable surroundings/habitations mandatory to defend and conserve the medicinal plants in their natural ecosystems. Because of lack of written documents of conservation of plants and habitats, researches like this study is compulsory to protect primeval retentions, to encourage the flow of information to the younger generations, to preserve ethno-biodiversity and to deliver a beginning point for further pharmacological, biological and chemical investigations on medicinal objects.

Acknowledgements. The authors appreciatively thank all the guides the traditional healers who accepted to participate to the study. The study was supported by Higher Education Commission Pakistan (Programme No. 20-1599/ 09/3007).

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DOI: http://dx.doi.org/10.15666/aeer/1702_47254805

SPATIAL AND TEMPORAL CHANGES OF HABITAT QUALITY IN JIANGSU YANCHENG WETLAND NATIONAL NATURE RESERVE – RARE BIRDS OF CHINA

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(Received 26th Jan 2019; accepted 8th Mar 2019)

Abstract. Taking the core area of Jiangsu Yancheng Wetland National Nature Reserve as a case, spatial and temporal changes of habitat quality were analyzed from the viewpoint of *Red-crowned crane* (*Grus japonensis* (Statius Müller, 1776)), and other rare birds. The results showed that the area of mudflats and *Suaeda salsa* marshes decreased, the area of *Spartina alterniflora* marshes, *Phragmites australis* marshes, aquaculture ponds and roads increased during 1983 to 2011. The value of habitat degradation index increased from 0.006 to 0.024 during 1983 to 2011, which meant the habitat degradation was becoming more serious. The spatial distribution of habitat degradation had been a distinct longitudinal band from an obvious lateral belt. The value of habitat quality index decreased from 0.863 in 1983 to 0.742 in 2011, which meant the habitat quality became worse due to the expansion of aquaculture ponds and *Spartina alterniflora*. Based on 1983, the value of habitat scarcity index increased from -0.10 to 0.173 during 1997 to 2011, indicating that the ecosystem function was declining. The values of habitat scarcity index of *Suaeda salsa* marsh and mudflat were obviously rising and were also the highest which were 0.628 and 0.637 in 2011, respectively. It indicated that *Suaeda salsa* marsh and mudflat became more and more scarce. They needed more protection and restoration.
Keywords: *landscape change, habitat quality, spatial differentiation, InVEST, coastal wetland*

Introduction

Habitat quality is one of the important service functions of the ecosystem and the objective existence of the essential attributes of the ecological environment (Xiao, 2011). However, with the increase of human disturbance, especially the change of land use mode, intensity and pattern caused by large-scale human activities, it had a heavy impact on the quality of biological habitat, and even affected the biodiversity of the entire region, and even subsequently the planet (Zhong et al., 2017). Therefore, the study of regional habitat quality was of great significance for maintaining regional ecosystem stability and improving ecosystem functions.

On the whole, the habitat quality assessment research can be divided into two categories. First, the habitat quality assessment based on on-the-spot investigation was mostly limited to a small scale, such as small cities, rivers, nature reserves and so on. Belt transect method or quadrat method was often used to investigate animals and plants, so as to obtain parameters related to habitat quality, constructing an assessment system and using certain mathematical methods to evaluate (Wu et al., 2015), which was mainly used to predict the spatial distribution of habitat or select location of nature reserves in the future, which can analyze the spatial distribution of species and habitat conditions, and assess the suitability of the species habitat (Chen et al., 2016). For example, Guo et al. (2016) evaluated the regional habitat quality of Zagunao river valley in the upstream of Minjiang River by using Analytic Hierarchy Process (AHP) according to 9 factors such as topography, soil, water and vegetation. However, this method is time-consuming and labor-consuming, making it difficult to observe for a long time.

The second is the index system model based on landscape pattern. With the widespread use of remote sensing and Geographic Information System (GIS) technology, integrating the landscape pattern and the distribution of threat sources into the habitat quality model, establishing a structural spatial display model has become a new research focus, is suitable for large and medium-sized research on the spatial and temporal changes of habitat quality, and is the basis for the identification, definition and conservation of ecosystem health (Gong et al., 2018). Artificial Intelligence for Ecosystem Services (ARIES), Multiscale Integrated Models of Ecosystem Services (MIMES) and Integrated Valuation of Ecosystem Services and Tradeoffs (InVEST) models have done creative research in this respect, among which the InVEST model is the most mature and most widely used ecological function evaluation model at present, and the method of evaluating habitat quality based on habitat threaten is widely used (Bhagabati et al., 2014). Terrado et al. (2016) did a comparative analysis of the calculation results of habitat quality module in model with the results of biodiversity observation. Research showed that there was a significant correlation between them, proving the reliability of the InVEST model. Chu et al. (2015) studied the changes of landscape pattern and habitat quality in Liaoning coastal zone from 2000 to 2010 by using the InVEST model, pointing out that reclamation of wetlands and urbanization expansion were the main reasons for the degradation of habitat quality. Wang (2016) evaluated the changes of habitat quality in Sanjiangyuan from 2000 to 2010 based on the InVEST model, and pointed out that the improvement of habitat quality in Sanjiangyuan in the past 10 years was a manifestation of the effectiveness of ecological restoration and construction. Bai et al. (2015) studied the changes of habitat quality in Minjiang River Basin from 2000 to 2010 by using the InVEST model, and pointed out that the interference of roads, industrial land, residential areas and mining sites were the main driving factors for the changes of habitat quality.

Yancheng Coastal Wetland is located in the central coast of Jiangsu Province of China, and is one of the most typical and representative distribution areas of muddy coastal wetlands in China and even the world, integrating intertidal beaches, tides, rivers, salt marshes, *Phragmites australis* marshes and *Spartina alterniflora* marshes. Over the years, under the dual influence of nature and human activities, especially under the high-frequency and high-intensity human production activities, the natural wetland area has been reduced and biodiversity has been lost, and the problem of ecological environment quality has become increasingly prominent, which has become one of the current focus issues, especially the study of suitability of *Red-crowned Crane* habitat. Sun et al. (2011) and Ou et al. (2015) pointed out the impact of changes in wetland landscape structure and connectivity under the influence of human activities on the habitat quality, especially on the habitat suitability of *Red-crowned Cranes*. Liu et al. (2016) studied the population dynamics of Red-crowned Cranes and their selection of habitat in Yancheng coastal wetland. Wang et al. (2015) selected nine ecological factors such as land use status, plant coverage, soil environment, biomass, water quality, water supply, biodiversity, wetland threated status and annual growth rate of wetland degradation, taking GIS technology,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48074821

and the ecological suitability of nature reserves was analyzed quantitatively by using AHP and factor weighted superposition. However, the study on the spatial differentiation of habitat quality with long time series, quantification and visualization is rarely reported in Yancheng coastal wetland. Therefore, taking the core area of Jiangsu Yancheng Wetland National Nature Reserve Rare Birds as an example, this paper studied the spatial and temporal changes of the habitat quality of the core area from 1983 to 2011 by using the habitat quality module in the InVEST model, and analyzed the impact of landscape changes on the habitat quality, which can provide a reference for the construction and management of Yancheng Coastal Wetland.

Material and methods

Study area

Yancheng coastal wetland is located in the central coastal area of Jiangsu Province of China, facing the Yellow Sea in the east. The coastline is 582 km long and the wetland area is 4.53×10^5 hm^2. It is the largest tidal flat coastal wetland in the Asian continent. The nature reserve is located in the transition zone between subtropical and warm temperate zone. The monsoon is remarkable and the climate is mild. The annual precipitation is about 1000 mm. Jiangsu Yancheng Wetland National Nature Reserve • Rare Birds (32°20′N～34°37′N，119°29′E～121°16′E) was established in 1983. It was upgraded to a national nature reserve in 1992. It is an important member of World Biosphere Reserve, Northeast Asia Crane Protection Network and East Asia--Australian Wader Migration Network. It has been entered into the International List of Important Wetlands. The study selected the core area of Jiangsu Yancheng Wetland National Nature Reserve • Rare Birds (*Fig. 1*) as the case area. The core area is to Xinyanggang River in the north, to Doulonggang River in the south, to the seawall road in the west, and to edge of mudflats (-3 m isobath) in the east. It is a typical silt-type tidal flat wetland with a total area of 1.92×10^4 hm^2. The landscape types in the core area are divided into natural wetlands, constructed wetlands and non-wetlands. Natural wetlands include *Phragmites australis* marsh, *Spartina alterniflora* marsh, *Suaeda salsa* marsh, mudflat and river. Constructed wetlands are culture ponds. Non-wetlands are roads.

Data sources

We used MSS image in 1983 (M4119037_037119831114), TM image in 1997 (P119737_5t19920607) and ETM image in 2011 (L71119037_03720110924) as the data sources. At the same time, we also used 1:200,000 geomorphic map, 1:200,000 vegetation map and 1:200,000 land use map of Xinyangang River estuary in "atlas of comprehensive survey of coastal and tidal marsh resources in China - fascicule of Jiangsu" which was published by the State Oceanic Administration of China and the State Bureau of Surveying and Mapping of China in 1988.

In ENVI 4.7, we first removed the trips 0n the image in 2011 by using the stripper tool. Then, we used the FLAASH module to perform atmospheric correction on remote sensing images. After atmospheric correction, we performed geometric correction of remote sensing images through GPS positioning in the field, in which the correction accuracy was less than 0.5 pixels. We classified remote sensing images in 1983 and 1997 by using the maximum likelihood method in supervised classification. Based on the principal component analysis, we combined the multispectral band with the panchromatic band for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48074821

the image in 2011 (Serious phenomenon of foreign body with spectrum). We used a combination of unsupervised classification and decision tree classification, and used survey data of field to verify, so that computer interpretation and visual interpretation could be combined to achieve image interpretation accuracy of more than 95%. We produced a series of landscape types of the study area in 1983, 1997, and 2011 by ArcGIS.

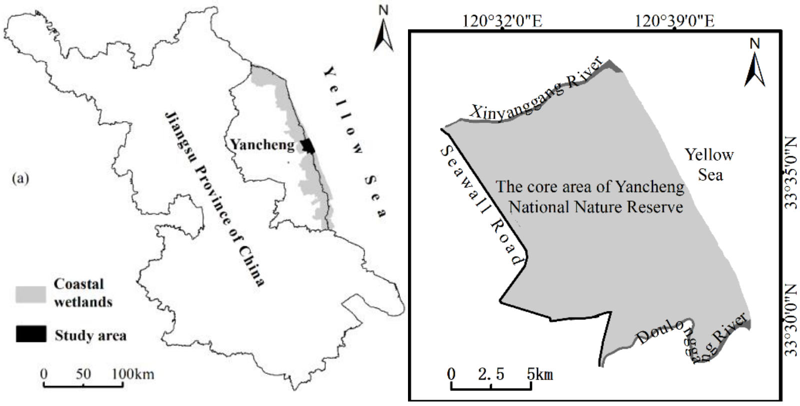

Figure 1. *Location and scope of the study area. (a) The study area is located at the core zone of Yancheng National Nature Reserve, which is situated on the center of the coastal of Jiangsu Province, China. (b) The scope of the study area*

Methods

The InVEST model was developed by Stanford university, the Nature Conservancy (TNC), the World Wildlife Fund (WWF) and other research institutions to evaluate Ecosystem service functions and their economic value, and support Ecosystem management and decision-making (Hall et al., 1997; Sharp et al., 2017; Zhang et al., 2017).

Habitat Quality Module in InVEST 3.2 was a combination of the landscape type map and the threat factors of biodiversity to generate a map of habitat quality. The Habitat Quality Module was based on the type of landscape that had different levels of sensitivity to different threat factors. It clearly characterized the response of various landscape types to threat factors and assessed the interaction between threat factors. The better the habitat quality was, the higher the degree of biodiversity conservation would be in the region. The more frequent human activities were, more threat factors there were, and more changes the landscape had, the greater impact on habitat quality it would have. Therefore, using the Habitat Quality Module could effectively analyze the relationship between landscape changes and habitat quality. The basic data required for the operation of the Habitat Quality Module included the map of landscape type, the map of threat factors, the influence distance table of threat factors and the table of sensitivity of landscape types to threat factors (Nelson et al., 2009; Goldstein et al., 2012; Mdk et al., 2013; Chaplinkramer et al., 2015; Zhang et al., 2016; Zhao et al., 2017).

Map of landscape types

Taking the series of landscape maps of the study areas in 1983, 1997 and 2011 as the basic data, and choosing the map of 1983 as the baseline. We also set the unified grid size to 30m×30m.

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Threat factors

Based on the landscape type of Yancheng coastal wetlands, we selected aquaculture ponds, rivers, roads and *Spartina alterniflora* (Invasive species) that were affected by human factors greatly as threat factors. Since there were no aquaculture ponds in the study area in 1983, the pond was not used as a threat factor in 1983. We referred to the relevant literature (Yuan et al., 2009; Sun et al., 2011; Meng et al., 2012; Yang et al., 2014), combined with the characteristics of the study area, set the maximum influence distance of the threat factors, the weight of the threat factors, and the linear correlation of the decline as shown in *Table 1*.

Table 1. *Attribute table of ecological threat factors*

Threat factors	Maximum impact distance (km)	Weight	Linear correlation of regression
Aquaculture pond	1	0.6	0
River	3	0.4	1
Road	5	0.6	1
Spartina alterniflora	3	0.8	0

A table of threat factors in the model, includes the relative impact of threat factors on habitat types and the distance at which habitat degradation occurs. There are two kinds of calculation formulas for the influence distance of threat factor, such as *Eqs. 1* and *2*.

$$i_{rxy} = 1 - \frac{d_{xy}}{d_{rxy}} \text{ if linear} \tag{Eq.1}$$

$$i_{rxy} = exp\left(-\frac{2.99}{d_{rxy}}\right) \text{ if exponential} \tag{Eq.2}$$

In *Equations 1* and *2*, i_{rxy} represents the degree of influence of the threat factors $\boldsymbol{r}$ in the grid unit $\boldsymbol{y}$ on the habitat grid unit $\boldsymbol{x}$; d_{xy} represents the distance between the two grid units $\boldsymbol{x}, \boldsymbol{y}$; and d_{rxy} represents the maximum influence distance of the threat factors (Sharp et al., 2017).

Sensitivity of landscape types to threat factors

Habitat suitability was bigger if it was closer to the original ecological system. Based on the function of maintaining the stability of the coastal wetland ecological environment, as well as the foraging and habitat environment standards for rare species of *Red-crowned crane*, we set the highest habit suitability was 1, the lowest was 0.

Different landscape types had different sensitivities to threat factors. Compared with the artificial landscape, the natural landscape had significantly weaker anti-interference ability to the threat factors and had greater sensitivity. We set the sensitivity range between 0 and 1, and the higher the sensitivity was, the larger the value would be. If the landscape type was not sensitive to the threat factors, it was represented by 0, and if it was extremely sensitive to the threat factors, it was represented by 1 (Bao et al., 2015; Li et al., 2016;

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Zhang et al., 2018). The sensitivity index of each landscape type to the threat factors in the study area was shown in *Table 2.*

Table 2. *Habitat suitability and sensitivity to threat factors of different landscape types*

Landscape types	Habitat suitability	Culture pond	River	Road	*Spartina alterniflora*
Aquaculture pond	0.5	0	0.5	0	0.5
River	0.5	0.5	0	0.8	0.5
Mudflat	0.8	0.5	0.3	0.3	0.8
Road	0	0	0	0	0
Phragmites australis marsh	1	0.3	0.6	0.6	0.5
Spartina alterniflora marsh	0.5	0.5	0.5	0.7	0
Suaeda salsa marsh	1	0.5	0.8	0.5	0.8

Habitat degradation index

The degree of habitat degradation was closely related to the sensitivity of landscape type to threat factors, the number of threat factors, the influence distance of threat factors and the weight of threat factors. The model assumed that within a certain range, the closer the habitat to the threat factor was, the higher the sensitivity and the habitat degradation index would be. The formula for calculating the habitat degradation was as follows.

$$D_{xj} = \sum_{r=1}^{R} \sum_{y=1}^{y_r} \left(\frac{w_r}{\sum_{r=1}^{R} w_r}\right) r_y i_{rxy} \beta_x s_{jr} \quad \text{(Eq.3)}$$

In *Equation 3*, D_{xy} is the habitat degradation index, which characterizes the degree of habitat degradation; R refers to the number of threat factors; y_r refers to the number of grid cells on the threat factor layer; w_r refers to the weight value of the threat factor; $r_y$refers to the number of raster unit threat factors in the layer; β_x refers to the degree of protection; s_{jr} refers to the sensitivity of the threat factor, and the value range is from 0 to 1.

Habitat quality index

Habitat quality Index depends on habitat degradation index and habitat suitability. In general, habitat quality index increases with the increase of habitat suitability and decreases with the increase of habitat degradation Index. Therefore, the more frequent the landscape type changes were and larger the scope was, the higher the degree of habitat damage would be, greater the adverse impact on species survival and reproduction it would have, and worse the habitat quality would be. The higher the habitat suitability was and the better the habitat environment of the species was, the higher the habitat quality would be. The habitat quality index was calculated as follows.

$$Q_{xj} = H_j \left(1 - \frac{D_{xj}^Z}{D_{xj}^Z + k^Z}\right) \quad \text{(Eq.4)}$$

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In *Equation 4*, Q_{xj} refers to the habitat quality index; H_j represents the habitat suitability; D_{xj} is the degree of habitat degradation; *k* is the half-saturation coefficient, we set it to 15 in the study (It is half the raster resolution). *z* is generally set to 2.5.

Habitat scarcity index

Habitat scarcity could reflect the scarcity of the overall habitat of the region and the scarcity of a certain landscape type. The more the habitat scarce was, the more protected it should be. The higher the habitat scarcity index was, the lower the scarcity degree would be. It could be calculated as follows.

$$R_j = 1 - \frac{N_j}{N_{jbase}} \quad \text{(Eq.5)}$$

$$R_x = \sum_{x=1}^{X} \sigma_{xj} R_j \quad \text{(Eq.6)}$$

In *Equation 5*, R_j is the habitat scarcity index of landscape type j. N_j is the number of grids of landscape type j in the current or future map. N_{jbase} is the number of grids of landscape type j in the baseline landscape map. If there is no landscape type j, $R_j = 0$.

In *Equation 6*, R_x is the habitat scarcity index. σ_{xj} is the discriminant coefficient of the landscape type j.When the grid is the current landscape type j, $\sigma_{xj} = 1$, otherwise $\sigma_{xj} = 0$.

Results

We loaded the resulting image of the model run (including the habitat degradation raster map and the habitat quality raster map) into ArcGIS, analyze the degree of habitat degradation and habitat quality in each year by reclassifying images furtherly. It was possible to compare the degree of habitat degradation and the changes in habitat quality through grid calculation.

Landscape changes

From the perspective of landscape composition, the landscape types of the study area were mainly aquaculture pond, *Phragmites australis* marsh, *Suaeda salsa* marsh, *Spartina alterniflora* marsh and mudflat. Comparing the changes during 1983 to 2011 (*Table 3*), it could be found that the area of aquaculture pond, *Phragmites australis* marsh, and *Spartina alterniflora* marsh in the study area increased, while the area of *Suaeda salsa* marsh first increased and then decreased. *Phragmites australis* marsh increased from 19.62% in 1983 to 31.46% in 2011, and the growth was faster and faster. *Spartina alterniflora* marsh increased from 1.69% in 1983 to 23.77% in 2011, and the expansion rate was getting faster and faster. The area percentage of *Suaeda salsa* marsh was 26.85%, 29.13% and 9.98% in 1983, 1997 and 2011, respectively, and it first increased and then decreased, and it decreased rapidly, the main reason for the increase was that *Spartina alterniflora* marsh had not formed a close interlaced zone with

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Suaeda salsa marsh, and there were original ecological evolution sequences of mudflat to *Suaeda salsa* marsh between 1983 and 1997. The sharp decrease between 1997 and 2011 was due to the two-way extrusion of *Phragmites australis* marsh and *Spartina alterniflora* marsh, and the area of *Suaeda salsa* marsh continued to shrink. Mudflat decreased from 46.93% in 1983 to 17.63% in 2011, and the rate of reduction was also increasing faster, mainly due to the expansion of *Suaeda salsa* marsh and *Spartina alterniflora* marsh. The area of the aquaculture pond had changed greatly, from 0% in 1983 to 12.11% in 2011. The road area had nearly doubled in 2011 compared with 1983. According to the two periods, it could be concluded that the effect of human activities on the study area was continuous and stable.

Table 3. *Area changes of landscape types in the study area*

Landscape types	1983		1997		2011	
	Area ($\times 10^2 hm^2$)	The percentage (%)	Area ($\times 10^2 hm^2$)	The percentage (%)	Area ($\times 10^2 hm^2$)	The percentage (%)
Aquaculture pond	0	0	11.67	6.06	23.32	12.10
River	6.41	3.33	4.16	2.16	4.11	2.13
Mudflat	90.39	46.93	63.72	33.08	33.95	17.62
Road	3.04	1.58	4.81	2.50	5.64	2.93
Phragmites australis marsh	37.79	19.62	42.65	22.14	60.6	31.46
Spartina alterniflora marsh	3.25	1.69	10.51	5.46	45.8	23.77
Suaeda salsa marsh	51.71	26.85	55.13	28.62	19.23	9.98

According to the area and number of landscape patches, it could be found that the number and area of landscape patches changed significantly from 1983 to 2011 and the number of patches increased from 53 to 291, an increase of 294.34%; the average patch area decreased from 363.47 hm^2 66.20 hm^2, a decrease of 81.79%. The landscape fragmentation trend in the study area was obvious, and the landscape segmentation phenomenon was intensified. The contagion index and aggregation index were reduced from 61.50 and 96.05 to 45.84 and 89.36, respectively. The mean shape index dropped from 1.59 to 1.30, indicating that the landscape in the study area was gradually increased by human disturbance, and the landscape patch shape tended to be regular.

Habitat degradation analysis

The degree of habitat degradation had certain reference value in predicting the possibility of habitat damage in the future. The degree of habitat degradation directly reflected the threat of threat factors to landscape types and was directly proportional to them. The higher the degree of habitat degradation was, the greater the impact of the threat factors on the region would be. The core area of the Jiangsu Yancheng Wetland National Nature Reserve • Rare birds had a high degree of ecological environmental protection and a low degree of habitat degradation generally. However, in terms of time, habitat degradation was becoming more and more serious, and the value of habitat degradation index was increasing more and more quickly. The value of habitat degradation index was 0.006, 0.009 and 0.024, in 1983, 1997 and 2011, respectively. It can be seen from *Table 4* that the value of habitat degradation index was most

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concentrated between 0 and 0.02 and the area percentage were 100%, 90.73% and 44.39% in 1983, 1997 and 2011, respectively. In 1983, the degree of habitat degradation was low, and the value of degradation index was all concentrated in the range of 0-0.02. In 1997, the value of habitat degradation was still low, but compared with the 1983, the area percentage in the range of 0.02-0.04 was 9.27%, the area with the value between 0.04-0.06 was very small. In 2011, the habitat degradation was intensified, and the area percentage of degradation ranging from 0 to 0.02 was less than half of that in 1997, the area ratio in the range of 0.02-0.04 reached 30.19%, the area ratio in the range of 0.04-0.06 reached 22.99%, the area ratio in the range of 0.06-0.08 was 2.43%, and the highest value of habitat degradation index reached 0.072.

Table 4. *Habitat degradation degree classification statistics from 1983 to 2011*

Grading ranges	1983		1997		2011	
	Area ($\times10^2hm^2$)	The percentage (%)	Area ($\times10^2hm^2$)	The percentage (%)	Area ($\times10^2hm^2$)	The percentage (%)
0-0.02	192.60	100	174.78	90.73	85.51	44.39
0.02-0.04	0	0	17.85	9.27	58.17	30.19
0.04-0.06	0	0	0.02	0	44.28	22.99
0.06-0.08	0	0	0	0	4.69	2.43

It can be seen from *Fig. 2* that what had no habitat degradation or low degree of degradation were mainly threat factors themselves, namely *Spartina alterniflora*, roads, aquaculture ponds and rivers. The region with the higher value of habitat degradation index was mainly located in the neighborhood of the threat factors. From 1983 to 2011, as the intensity of threat factors increased, the area of the region with the high value of habitat degradation index also expanded, directly causing the value of habitat degradation index to become higher. In 1983, the areas with the high value of habitat degradation were mainly located on both sides of Xinyanggang River and Doulonggang River and the spatial differentiation of habitat degradation had obvious lateral belt distribution. In 1997, the area with the high value of habitat degradation was mainly distributed around the breeding ponds, the Doulonggang River, and the large *Spartina alterniflora* plaques in the south of the study area. In 2011, the areas with the high value of habitat degradation were mainly distributed in the interlaced belts between aquaculture pond and *Phragmites australis* marsh, *Salsa* marsh and *Spartina* marsh, mudflat and *Spartina* marsh. The spatial differentiation of habitat degradation had a distinct longitudinal band distribution.

Habitat quality assessment

During 1983 to 2011, the habitat quality of the core area of Yancheng Wetland National Nature Reserve was degraded. The value of habitat quality index decreased slightly from 0.863 to 0.846 during 1983 to 1997, the habitat quality declined obviously from 1997 to 2011, it fell from 0.846 to 0.742, a decrease of 12.29%.

The value of habitat quality index was divided into four sections: 0-0.25, 0.25-0.5, 0.5-0.75, and 0.75-1, which indicated that the habitat quality grades were poor, medium, good, and excellent respectfully. It can be seen from *Table 5* that the habitat quality of the study

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area was high generally, and the grade of habitat quality were mostly excellent, and then medium and poor successively, there was no distribution at the good grade. Thus, in terms of regional differences, the habitat quality in the study area showed a "polarization" trend. According to the changes of the area ratio in the three levels, it showed "two increasing and one decreasing". The area of the excellent habitat quality was in a state of decline, and the area ratio had decreased from 93.57% in 1983 to 59.08% in 2011, the decline reached 36.86%, and the decline from 1983 to 2011 was getting faster and faster. The areas of poor and medium habitat quality were increasing, and the area of poor habitat quality increased from 1.40% in 1983 to 2.91% in 2011, which was more than doubled. The area of medium habitat quality increased from 5.03% in 1983 to 38.01% in 2011, an increase of more than 6.5 times, and the increasing speed of the areas of poor and medium habitat quality were becoming faster and faster.

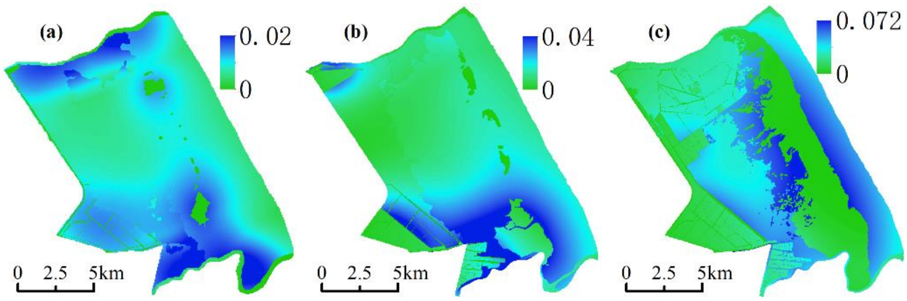

***Figure 2.** Habitat quality degradation index distribution maps. (a)It is a habitat quality degradation index distribution map in 1983. (b) It is a habitat quality degradation index distribution map in 1997. (c) It is a habitat quality degradation index distribution map in 2011*

***Table 5.** Habitat quality classification statistics from 1983 to 2011*

Grading ranges	1983		1997		2011	
	Area ($\times10^2$hm^2)	The percentage (%)	Area ($\times10^2$hm^2)	The percentage (%)	Area ($\times10^2$hm^2)	The percentage (%)
0-0.25	2.70	1.40	2.77	1.44	5.61	2.91
0.25-0.50	9.69	5.03	26.61	13.81	73.22	38.01
0.50-0.75	0	0	0	0	0	0
0.75-1.00	180.21	93.57	163.27	84.75	113.82	59.08

From the spatial differentiation maps of habitat quality (*Fig. 3*) and the habitat quality change maps (*Fig. 4*), it can be seen that the areas with relatively poor habitat quality were mainly concentrated in roads, rivers, aquaculture ponds and *Spartina alterniflora*. Especially the expansion of the aquaculture pond and *Spartina alterniflora* led to a rapid increase of the area with lower-level habitat quality. The areas with relatively good habitat quality were mainly in the original ecological vegetation distribution area, including *Phragmites australis* marsh, *Salsa* marsh and mudflat. The area where the habitat quality changes were located in the landscape interlaced belt, that was, the area where the landscape type changes, including the *Phragmites australis* marsh--aquaculture pond

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interlaced belt, *Salsa* marsh-*Spartina* marsh interlaced belt, and mudflat-*Spartina* marsh interlaced belt.

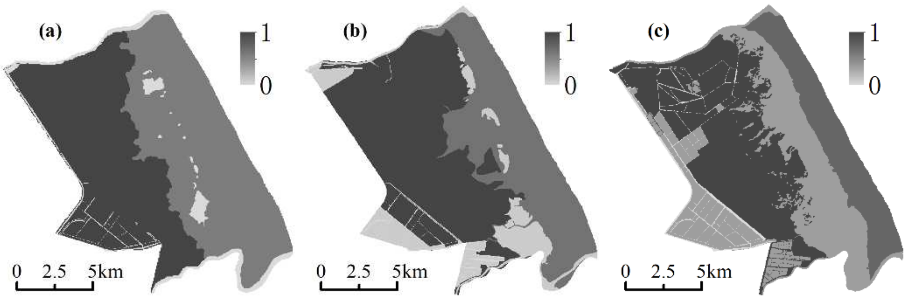

Figure 3. *Habitat quality index distribution maps. (a) It is a habitat quality index distribution map in 1983. (b) It is a habitat quality index distribution map in 1997. (c) It is a habitat quality index distribution map in 2011*

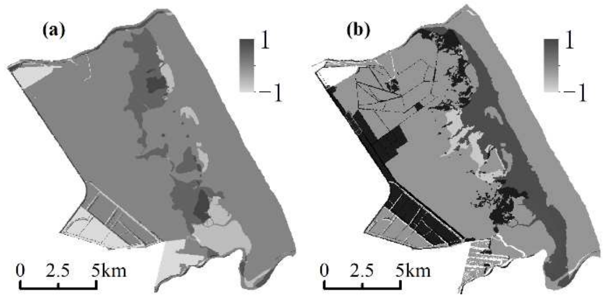

Figure 4. *Spatial variation of habitat quality index. (a) It is the spatial variation of habitat quality index from 1983 to 1997. (b) It is the spatial variation of habitat quality index from 1997 to 2011*

Habitat scarcity evaluation

The higher the scarcity index was, the more stable the overall ecosystem tended to be, and the ecosystem structure and function would decline. The lower the scarcity index was, the more fragile the ecosystem would be, but at the same time, it tended to be the more susceptible and need to strengthen protection. Based on the conditions of no introduction of *Spartina alterniflora* in the mudflat in 1983, the scarcity index was -0.10 in 1997, the scarcity index was 0.173 in 2011, and the scarcity index was rising, indicating that the ecosystem was stable and the ecosystem function was declining when the region was in human activities and invasion of alien species. The value of R_j was close to 1 and the current or future landscape type was more important to biodiversity conservation when the type of landscape was rare. The R_j value of *Salsa* marsh and the mudflat were obviously rising, and they were closer to 1. In 2011, the R_j values of salsa marsh and the mudflat were the highest, 0.628 and 0.637 respectively, indicating that these landscape types were decreasing and became more and more scarce in the study area. They needed more protection and ecological restoration.

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DOI: http://dx.doi.org/10.15666/aeer/1702_48074821

Discussion

The spatial and temporal changes of habitat quality in the core area of Yancheng Wetland National Nature Reserve • Rare birds are mainly caused by human activities and the expansion of *Spartina alterniflora*. The study area focused on protecting the wintering habitat of *Red-crowned cranes*. In order to create good artificial habitats for rare species such as *Red-crowned cranes*, it began to establish some artificial wetlands in 1993, and implemented a large area of *Phragmites australis* marsh recovery work. Driven by economic interests, some of the projects had changed the original plan, and the area of *Phragmites australis* base and aquaculture pond were mainly based on economic development, had increased significantly. As a result, the area of aquaculture ponds in the study area has increased from 0% in 1983 to 12.10% in 2011, and the increase in two periods almost the same, further illustrating the sustainability and stability of human activities. The aquaculture ponds were mainly developed from *Phragmites australis* marsh, and the original ecological landscape was transformed into a threat factor, which caused the habitat to degenerate and the habitat quality decreased. On the other hand, the artificial embankment not only generated a new threat factor, but also changed the hydrological process, blocked the intrusion of tidal water, effectively stored fresh water, further affected the soil hydrological properties, and accelerated the speed of the transformation of *Suaeda salsa* marsh to *Phragmites australis* marsh and changed the landscape structure of the wetlands.

In the 1980s, *Spartina alterniflora* was successfully introduced in the coastal of Jiangsu Province. After the formation of a large-area community in the 1990s, the area expanded rapidly, forming a coastal vegetation with a few kilometers wide and a north-south coastal wetland. Although *Spartina alterniflora* had played an active role in protecting beaches, it was a threat factor for habitats of rare species such as *Red-crowned cranes*. On the one hand, the superior silt-promoting function of *Spartina alterniflora* resulted in a significant increase in the elevation of the beach surface and the thickness of the silt reached 1.5 m on the spot, which made it difficult for the tide to pass through *Spartina alterniflora* marsh and reach *Suaeda salsa* marsh. The habitat of *Suaeda salsa* caused significant degradation. On the other hand, on the lower edge of *Suaeda salsa* marsh, the settlement of *Spartina alterniflora* made the repeat of the niche, while the ecological amplitude characteristics of *Spartina alterniflora* and the sexual and asexual dual reproduction mode made it the inter-species competition was in a dominant position, and eventually *Suaeda salsa* marsh was constantly replaced by the *Spartina alterniflora* marsh. In the landscape composition of the study area, *Suaeda salsa* marsh, which is inhabited by rare species such as *Red-crowned cranes*, has been transformed into a habitat quality threat factor, resulting in a decline in habitat quality.

Human activities and the expansion of *Spartina alterniflora* have made the core area of Yancheng Wetland National Nature Reserve • Rare birds more threatening, ecosystem function decline, ecological restoration will become an important research topic in the region. At present, positive results have been achieved. The core area had basically completed “removing aquaculture ponds and restoring marshes” project, the buffer zone “removing aquaculture ponds and restoring marshes” was also actively carried out. However, the threat of *Spartina alterniflora* expansion is still growing and will continue to threaten the habitat quality of the study area. On the one hand, it is necessary to control the expansion of *Spartina alterniflora*, maintain a moderate scale, give full play to its revetment function, and reduce the threat to landscape habitats. On the other hand, it is

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DOI: http://dx.doi.org/10.15666/aeer/1702_48074821

necessary to actively restore *Suaeda salsa* marsh, restore the evolution sequence of mudflat to *Suaeda salsa* marsh, maintain biodiversity and improve regional habitat quality.

Conclusion

The landscape composition of the core area of Yancheng Wetland National Nature Reserve• Rare birds is mainly aquaculture pond, *Phragmites australis* marsh, *Suaeda salsa* marsh, *Spartina alterniflora* marsh and mudflat. From 1983 to 2011, the change of landscape composition showed that the aquaculture pond, *Phragmites australis* marsh and *Spartina alterniflora* marsh increased continuously, and the increasing speed became faster and faster. The area of mudflat decreased, and the area of *Suaeda salsa* marsh first increased and then decreased, and the corresponding decrease was faster and quicker. The landscape changes are mainly conversions of mudflat into *Spartina alterniflora* marsh and *Suaeda salsa* marsh, *Suaeda salsa* marsh into *Phragmites australis* marsh and *Spartina alterniflora* marsh and *Phragmites australis* marsh into culture pond.

From 1983 to 2011, although the habitat quality of the core area of Yancheng Wetland National Nature Reserve • Rare birds were at the excellent level, the degradation trend was also obvious. From 1997 to 2011, the value of habitat quality index decreased from 0.846 to 0.742, a decrease of 12.29%. The areas with relatively poor habitat quality mainly concentrate on the distribution areas of threat factors such as *Spartina alterniflora* marsh, roads, aquaculture ponds, rivers, etc. The area with relatively good habitat quality mainly distributed in the original ecological vegetation distribution areas, including *Phragmites australis* marsh, *Suaeda salsa* marsh and mudflat; and the area of habitat quality change were located in the landscape ecotone. The value of habitat degradation index was increasing. The spatial heterogeneity of the habitat degradation was characterized by a transition from a transverse strip distribution to a longitudinal strip distribution. The habitat scarcity index increased significantly, indicating that the ecosystem of the study area was stable and the ecosystem function was declining.

The degradation of coastal wetland habitat seriously threatened the habitat of Red-crowned Crane and other rare species. Yancheng coastal wetland had joined the World Heritage List, and scientific protection and restoration is imminent. According to the characteristics of Yancheng coastal wetland, systematically analyzing the degradation mechanism of coastal wetland habitat quality, clarifying the mechanism of ecological restoration from the perspective of landscape structure and ecological process, reconstructing and optimizing regional habitat ecological protection network, and carrying out ecological restoration technology integration and demonstration in typical areas need to be strengthened in the future research.

Acknowledgements. This research was supported by National Natural Science Foundation of China (No.41771199 and No.41501567) and the Natural Science Foundation of Jiangsu Province, China (Grants No. BK20171277), Natural Science Research Projects of Colleges and Universities in Jiang Province (No.18KJD170001) and Qing Lan Project in Jiangsu University of China. We acknowledged Yancheng Natural Reserve Management Office for giving us the opportunity to enter the study area and carry out the field works gratefully.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48074821

AN EMPIRICAL RESEARCH ON ECO-COMPENSATION STRATEGY FOR HANDLING NON-POINT SOURCE POLLUTION OF WATER BODIES

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. This paper attempts to disclose the farmers' selection bases among different eco-compensation options for the reduction of fertilizer use, a typical non-point source (NPS) of water pollution. Firstly, the reduction methods were divided into six types. Then a survey was carried out on the farmers near two water bodies in south-eastern China's Zhejiang Province, namely, Qiaodun Reservoir and Siming Lake Reservoir. It was found that when multiple options are available, financial compensation is the most favoured one among farmers, followed by technical learning and policy compensation; the organic fertilizer has the lowest acceptance. Next, a simultaneous bivariate probit model was established to illustrate the effect of each factor on farmers' selection between intellectual and non-intellectual technical compensations, and between alternative and organic fertilizers. Meanwhile, the farmers' intentions on policy compensation were examined by the binary probit model. The modelling results show that: the farmers prefer compensation options that complement each other and wish the government could offer integrated compensation plans of these options; the age and education level of house owner, coupled with family income, farmland area, farmland fragmentation, production efficiency and farmers' willingness to reduce fertilizer application all have important impacts on the selection of compensation means.
Keywords: *chemical fertilizer, non-point source (NPS) pollution, direct eco-compensation, reservoir, farmers' participation*

Introduction

Humans freely receive ecological services from properly-functioning ecosystems like the aquatic ecosystem, i.e. the ecosystem in a body of water (Karabulut et al., 2016). Water bodies are the sources of water supply to urban and rural areas, and require strict measures on environmental safety (Ademila and Saloko, 2018; Joldeș et a., 2017; Jian, 2018; Li et al., 2015; Ganiron, 2017; Grafton et al., 2017; Tian et al., 2018). However, fresh water resources have been utilized in a non-sustainable way, as they are widely viewed as inexhaustible, free or low-cost public resources (Perry and Berry, 2016; Yihdego and Khalil, 2017). To solve the problem, China has restricted and even banned the development of many water bodies, and invested more into the construction of sewage pipes and other discharge facilities. These moves have paid off: industrial point source (PS) pollution is placed under control, and domestic wastewater can now be disposed of satisfactorily (Moges et al., 2016; Fales et al., 2016).

Despite the above efforts, the agricultural non-point source (NPS) pollution in crop farming still poses a challenge to the safety of water bodies, because the NPS pollution is highly dispersed, concealed, random, uncertain, extensive and undetectable (Álvarez et al., 2017; Smith et al., 2017; Herrera et al., 2017). This universal problem, especially the fertilizer NPS pollution, has attracted much attention from scholars around the world (Jin, 2013; Pott and Fohrer, 2017). Studies have

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

shown that dissolved fertilizer, a main pollutant to water quality, can enter water bodies through surface runoff, farmland irrigation and drainage (Kreuger et al., 2008; Kronvang et al., 2008; Neumann et al., 2011; Sun et al., 2012).

On the correlation between agriculture and water quality, irresponsible use of farmland has been widely regarded as the primary cause of agricultural NPS pollution to water bodies (Rodrigues et al., 2018; Corneil et al., 2018; Li et al., 2015). Since the "household responsibility system" was implemented in late 1970s, Chinese farmers have been officially recognized as the basic units of agricultural production, management and decision-making (Ji et al., 1999). The agricultural NPS pollution can be attributed to the irrational decisions of farmers under various factors, ranging from social environment, item property, individual preference to laws and regulations (Feng et al., 2006; Liang et al., 2015). The farmers tend to make irrational decisions when they are worried about the yield and production scale, unclear of the pollution source or the importance of pollution control, and in lack of practice instructions or convenient farming techniques (Zhang, 2015).

One of the viable options to reduce agricultural NPS pollution to water bodies lies in conservation tillage (Doreen et al., 2018). This is a tillage system that creates a suitable soil environment for growing a crop and that conserves soil, water and energy resources mainly through the reduction in the intensity of tillage, and retention of plant residues. However, the conservation tillage may bring the risk of output reduction, and make farming income uncertain. As a result, many farmers are reluctant to implement conservation tillage without proper compensation, even if they are risk takers (Klemme, 1985).

In the last two decades, many international organizations and governments have provided eco-compensations for protective actions aiding water bodies (Wunder, 2015; Ze et al., 2017). A perfect eco-compensation mechanism helps to enhance the values and benefits of ecological services, remove policy constraints on farmers' demand for individual development, and arouse the enthusiasm of farmers in pollution control activities (Xie and Li, 2016; Vogl et al., 2017; Villarroya et al., 2014; Chang et al., 2013). Out of the many eco-compensation policies, financial compensation like transfer payment has long been the most effective way to prevent agricultural NPS pollution to regional water bodies, because it is easy to quantify and measure. As farmers now expect more diverse means of compensation, it is imperative to develop various compensation mechanisms according to local conditions (Research Group on Eco-Compensation Mechanism and Policies in China, 2007).

The farmers must make prudent choices among various compensation mechanisms. After all, not every form of compensation is effective, and some may even have negative consequences. For example, over compensation may dampen the farmers' enthusiasm about sustainable farming and environmental protection, in that the subsidy to be received is negatively correlated with the environmental improvement (Aretino et al., 2001). Moreover, subsidy may distort the resource allocation, giving subsidized farmers more market power over the unsubsidized (Macintosh and Denniss, 2004). Rationality and fairness are two key attributes of a good compensation mechanism. If either of them is overlooked, the compensation will not achieve the desired goals.

Currently, the government, the dominating party of eco-compensation practices, fails to understand the wishes of farmers or their reasons to change behavior (Huber et al., 2012). With the changes in farmers' demand, the compensation effectiveness now

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DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

hinges on the rationality of the compensation means, in addition to the amount of compensation. However, there is no consensus on what makes a compensation policy rational. Some scholars held that farmers are biased towards cash compensation (Xu et al., 2013; Yang and Cai, 2012), while some others believed that farmers prefer technical compensation (Shi et al., 2014). To sum up, these discussions show that the limitations should be considered in the design of any compensation method, because the farmers' preference for compensation means varies with regions, cultures and populations. This means the choice of compensation tools should be discussed from a more microscopic perspective. Therefore, this paper explores farmers' choice between different compensation means for the abandonment of chemical fertilizer, a typical NPS of agricultural pollution to water bodies.

Selection bases of compensation means

Classification of compensation means

The compensation methods for prevention and control of agricultural NPS pollution mainly fall into four categories, namely, financial compensation, physical compensation, technical compensation and policy compensation. The details on these types of compensation are provided below.

(1) Financial compensation

As its name implies, financial compensation refers to the direct financial incentives for farmers to adopt protective measures, or the financial subsidy for the cost of taking such measures. It is commonly applied in China's "Grain for Green" program.

(2) Physical compensation

Physical compensation, e.g. seed compensation and gasoline compensation, is a collective term for any compensation means that improves the productive capacity of ecological service providers through the provision of production factors or living factors.

(3) Technical compensation

Technical compensation is either intellectual or non-intellectual. The intellectual technical compensation aims to enhance the implicit, materialized technologies, such as the farmers' ability of unified farmland management, through training, demonstration and subsidies. By contrast, the non-intellectual technical compensation mainly subsidizes farmers for the implementation of knowledge-based technologies.

(4) Policy compensation

Policy compensation generally means the preferential policies issued by the superior government to the lower government, giving the recipients various preferential treatments on agricultural structure, employment and social security.

Each of the above compensation methods has its advantages and disadvantages. In fact, the selection of compensation means is tradeoff between financial and non-financial compensations, between transfusion and hematopoietic compensations, as well as between coupling and decoupling compensations.

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DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

Typical selection bases

For the farmers who have adopted conservation tillage, eco-compensation may exist in direct forms as funds, physical objects, technologies or policies, and in indirect forms as government-funded projects on environmental protection, economic construction and social security. The direct compensation boosts the income and productivity of the farmers, while the indirect compensation benefits the farmers through the improvement of the social environment.

In this paper, the direct compensation is taken as the example to illustrate the bases of farmers' selection among different compensation means. This is because direct compensation reminds farmers of the purpose of compensation, while giving them direct benefits. Moreover, direct compensation can be investigated by closed and open questionnaire surveys, which are familiar to farmers.

Considering the important supplementary function of policy compensation, this paper makes "other jobs" an indicator of policy compensation related to agricultural activities. Here, "other jobs" refers to the jobs created by agricultural restructuring and offered by other industries, with the aim to encourage farmers to stop un-green farming practices, such as planting polluting crops.

In view of the general fertilization techniques of farmland, our questionnaire offers the farmers different combinations of compensation options targeting six conventional control methods for NPS pollution of chemical fertilizer. These control methods directly affect the farmland management (*Table 1*):

1. Financial compensation;
2. Physical compensation: Grain compensation;
3. Technical compensation: Training on "formula fertilization by soil testing" (FFST) (technical learning); provision of FFST fertilizer (alternative fertilizer); subsidies for commercial organic or green manure (organic fertilizer);
4. Policy compensation: Provisions of other job opportunities (other jobs).

The above compensation means all belong to direct compensation. The financial compensation and physical compensation are transfusion compensation, while technical compensation and policy compensation are hematopoietic compensation. Moreover, financial, physical and technical compensations fall in the category of coupling compensation, while policy compensation fits into decoupling compensation.

Table 1. *Eco-compensation means and NPS pollution control methods*

<table>
<tr><th>Option content</th><th>Financial compensation</th><th>Grain compensation</th><th>Technology learning</th><th>Alternative fertilizer</th><th>Organic manure</th><th>Other jobs</th></tr>
<tr><td rowspan="4">Division method</td><td>Financial compensation</td><td>Physical compensation</td><td colspan="3">Technical compensation</td><td>Policy compensation</td></tr>
<tr><td>Financial compensation</td><td colspan="5">Non-financial compensation</td></tr>
<tr><td colspan="2">Transfusion compensation</td><td colspan="4">Hematopoietic compensation</td></tr>
<tr><td colspan="5">Coupling compensation</td><td>Decoupling compensation</td></tr>
</table>

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DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

Materials and methods

Study area overview

Our research targets Qiaodun Reservoir in Yuyao City and Siming Lake Reservoir in Cangnan County, southeastern China's Zhejiang Province. The two medium-sized reservoirs are the major sources of water supply to the county. However, both water bodies have suffered from severe eutrophication, and face potential agricultural pollution.

Thanks to the provincial eco-compensation practices, the local farmers are now aware of conservation tillage, laying a good basis for our survey. However, the farmers mainly rely on agriculture for a living and have not acquired sufficient knowledge on scientific farming. Since rice is the dominant crop in the study area, rice growers were selected as the research objects.

Survey design description

From March 2014 to January 2015, our survey was carried out in 12 villages near Qiaodun Reservoir and 9 villages near Siming Lake Reservoir. These villages were identified by stratified random sampling, considering such factors as rice planting area, economic development and location. In each village, 30 farmers were selected for the one-on-one interview, which was designed to acquire their basic information and selection bases for compensation means.

A questionnaire was prepared for the interview. Since real-world eco-compensations often combine multiple methods, our questionnaire offers the farmers with six compensation options targeting conventional control methods for NPS pollution of chemical fertilizer, and asks them to choose three compensation methods out of the six options.

Based on the questionnaire, the interview was conducted in three steps: (1) Present the compensation options, ask the farmer to select the most desired option, and mark the selected option with the number "1"; (2) Ask the farmer to select the next desired option from the unselected options, and mark the selected option with the number "2"; (3) Ask the farmer to select the next desired option from the unselected options, and mark the selected option with the number "3".

The option selected in Step 1 is the compensation means with the maximum utility. The farmers have basically the same demand for compensation means, except a slight difference between individuals. The general principle is that the attributes and features of the selected option must satisfy the farmers' requirements of use. In other words, the compensation options are selected based on the farmers' demand and preference on the attributes of such options.

A total of 360 questionnaires were sent out to the villages near Qiaodun Reservoir and 270 to those near Siming Lake Reservoir. In the end, 323 and 218 valid questionnaires were returned from the two groups of villages, respectively.

Sample features

Table 2 sums up the features of the interviewees. It can be seen that 90% of the interviewees are males, which reflects the high labor intensity of rice production. Besides, the preference for food crop planting increases with the age of farmers, as over 80% of food crop growers are over 50.

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Table 2. *Sample features*

Variable	Option	Total	Qiaodun	Siming Lake
Gender	Female	21	8	13
	Male	520	315	205
Family population	<3	138	68	70
	3~5	245	131	114
	>5	158	124	34
Income (unit: RMB)	<10,000	13	13	0
	10,000~20,000	39	31	8
	20,000~30,000	31	13	18
	30,000~50,000	137	92	45
	50,000~70,000	114	90	24
	>70,000	178	84	94
Education level	Illiteracy	264	201	63
	Primary school has not graduated	29	12	17
	Primary school graduation	146	79	67
	Junior high school	71	29	42
	Senior high school	28	2	28
	College or above	3	0	3
Age	>40	25	10	15
	40~50	76	25	51
	50~60	149	86	63
	60~70	210	137	73
	>70	81	65	16

Methods and modelling

The farmers' willingness to select a compensation means is a binary discrete choice variable. Both financial and physical compensations belong to transfusion compensation, and directly make up for the economic losses of farmers. There might be some correlations between the two compensation options, because they tend to be selected by farmers under the same influencing factors. The same relationship may exist between intellectual and non-intellectual technical compensations.

Since financial and grain compensations are independent of each other, there are four possible choices for farmers: selecting both options, selecting financial compensation, selecting grain compensation and selecting neither option.

Let Y_1 and Y_2 be the selection of financial compensation and that of grain compensation, respectively. If the farmer being interviewed selects financial compensation, the value of Y_1 should be recorded as 1; otherwise, the value of Y_1 should be recorded as 0. Similarly, if the farmer selects grain compensation, the value of Y_2 should be recorded as 1; otherwise, the value of Y_2 should be recorded as 0. Then, the four possible choices can be expressed as (1,1), (1,0), (0,1) and (0,0), respectively.

In light of the above, a simultaneous bivariate probit model was established to illustrate the effect of each factor on farmers' selection (Greene, 2008). Extended from the probit model, the established model is suitable for simultaneous equations satisfying the following two conditions: the random perturbations of the equations are inter-

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DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

correlated, such that the equations can be estimated at the same time; the model has two output variables. The mathematical expression of the model is shown in *Equation 1.*

$$\begin{cases} Y_1^* = \beta_1 + \beta_1' X_1 + \varepsilon_1 \\ Y_2^* = \beta_2 + \beta_2' X_2 + \varepsilon_2 \\ E(\varepsilon_1) = E(\varepsilon_2) = 0 \\ \operatorname{var}(\varepsilon_1) = \operatorname{var}(\varepsilon_2) = 1 \\ \operatorname{cov}(\varepsilon_1,\ \varepsilon_2) = \rho \end{cases} \tag{Eq.1}$$

where Y_1^* and Y_2^* are unobservable potential variables depicts the farmer' evaluation of financial and grain compensation, respectively; X_1 and X_2 are influencing factors of the farmer' selection; β_1, β_1', β_2 and β_2' are estimation parameters; ε_1 and ε_2 are random perturbation terms that obey bivariate normal distribution, ρ is the correlation coefficient of ε_1 and ε_2.

If $Y_1^* > 0$, the financial compensation has a positive utility and is favored by the farmer. In this case, the output variable Y_1 equals 1. Otherwise, $Y_1 = 0$. If $Y_2^* > 0$, the grain compensation has a positive utility and is favored by the farmer. In this case, $Y_2 = 1$. Otherwise, $Y_2 = 0$.

Under the condition of a significant ρ value, ε_1 and ε_2 are irrelevant if $\rho = 0$, that is, the two equations of our model can be estimated separately, and the estimated results agree with the results of simultaneous estimation; if $\rho > 0$, Y_1 and Y_2 complement each other; if $\rho < 0$, Y_1 and Y_2 exclude each other.

The simultaneous bivariate probit model was also adopted to analyze the farmers' selection between intellectual and non-intellectual technical compensations, and between alternative and organic fertilizers; the farmers' intentions on policy compensation were examined by the binary probit model.

Variable selection

As shown in *Table 3,* the influencing factors in this research include personal information, farmer productivity, family information, farmland features, farmland management, village economy and regional variable. Specifically, personal information covers the age (age) and education level (edu) of house owner; family information refers to the annual family income (inc); farmland features include farmland area (ara) and farmland fragmentation (ldf); farmland management features refers to production efficiency (eff) and farmers' willingness to reduce fertilizer application (wtr); Regional variable (reg) refers to the investigation site the farmer belonged to, Qiaodun reservoir watershed or Siming Lake reservoir watershed.

Results

With the aid of Stata 9.0 software, the simultaneous bivariate probit model and the binary probit model were adopted for the empirical analysis on farmer's selection of eco-compensation options for chemical fertilizer reduction. The estimation results and significance test results are listed in *Table 4*.

As shown in *Table 4,* all four models have high goodness of fit, and some coefficients of the influencing factors passed the significance test and fulfilled the

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expectations. The correlation coefficient of model 1-1 was significant on the 1% level, revealing the correlation between the selection of financial compensation and that of grain compensation. Meanwhile, the positive value of the correlation coefficient (ρ = 0.4668) shows the complementary effect between the two compensation options: a farmer is more likely to choose grain compensation, if he/she has chosen financial compensation. Similarly, the estimation results of model 1-2 demonstrate that intellectual and non-intellectual technical compensations complement each other, i.e. a farmer tends to select both options at the same time. However, the correlation coefficient of model 1-3 failed the significance test, indicating that farmers' selection of alternative fertilizer is independent from that of organic fertilizer. The binary probit model estimation results of Model 1-4 demonstrate that farmers' choice of other jobs are easily influenced by age of house owner, education level, farmland fragmentation, farmers' willingness to reduce fertilizer application, and regional variable. The impacts of different influencing factors are detailed below.

(1) Age of house owner

The age of house owner has a positive impact on farmers' selection of financial compensation ($p < 1\%$). Then, it can be deduced that old farmers are willing to accept grain compensation, considering the complementary effect between financial and grain compensations. This deduction is proved by the results in the above table: the age of house owner has a positive impact on farmers' selection of grain compensation. These relationships can be explained as follows. Compared with young farmers, old farmers are concerned with collective interests and government instructions, and thus expect a limited compensation for chemical fertilizer reduction. Therefore, financial compensation, known for its simplicity, directness and flexibility, wins more recognition among old farmers than young farmers.

Table 3. *Influencing factors*

Type	Variable	Variable definition	Average value	Standard deviation
Personal information	Age of house owner	Actual years reported by farmers (years)	1.9630	0.5794
	Education level of house owner	1 = illiteracy; 2 = elementary school graduation and below; 3 = elementary school and above	1.8226	0.7441
Family information	Annual family income	Farmers reported annual household income (RMB)	5.5174	0.1420
Farmland management features	Production efficiency	Real value calculated according to input-output data	0.6143	0.0074
	Farmers' willingness to reduce fertilizer application	Farmers' willingness to report reductions (%)	0.2886	0.0110
Farmland features	Farmland area	Farmers reported farmland area (Mu)	2.3950	1.6339
	Farmland fragmentation	Number of cultivated lands separated from each other (block)	2.3272	0.8290
Regional variable	Investigation site	1 = Qiaodun reservoir watershed; 0 = Siming Lake reservoir watershed	0.5970	0.4910

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Table 4. *The estimation results and significance test results*

Variable	Simultaneous bivariate Probit model						Probit model
	Model 1-1		Model 1-2		Model 1-3		Model 1-4
	Financial compensation		Technical compensation				Other jobs
	Financial compensation	Grain compensation	Intelligence compensation	Non-intelligence compensation	Alternative fertilizer	Organic manure	
age	0.4488*** (3.0255)	0.4749*** (4.2698)	-0.1893* (-1.6679)	0.3772*** (3.1433)	-0.2055** (-1.9669)	0.3831*** (3.3316)	-0.8816*** (-7.2297)
edu	0.0151 (0.1385)	-0.2176** (-2.5559)	0.0925 (1.0485)	-0.0448 (-0.4739)	0.0271 (0.3274)	0.0766 (0.8508)	0.1668* (1.9021)
inc	-0.1701* (-1.7929)	0.0210 (0.3164)	0.0717 (1.0293)	-0.0624 (-0.8398)	0.1022 (1.5850)	-0.0183 (-0.2639)	0.0528 (0.7719)
ara	-0.0831** (-1.9845)	-0.0113 (-0.3064)	0.0421 (1.1373)	0.1093* (1.9570)	-0.0025 (-0.0675)	0.0867* (1.8170)	-0.0243 (-0.6266)
ldf	-0.0162 (-0.1529)	-0.0251 (-0.3135)	0.0831 (0.9696)	0.1185 (1.3445)	0.0440 (0.5632)	0.1191 (1.4127)	-0.1676** (-2.0207)
eff	0.2819* (1.7792)	0.1499 (1.1473)	-0.2998** (-2.2499)	-0.1372 (-0.9216)	-0.2913** (-2.2862)	0.1789 (1.2315)	0.1095 (0.8088)
wtr	0.1906 (0.6211)	0.7996*** (3.3399)	-0.7809*** (-2.8854)	-0.2463 (-0.9707)	-0.6674*** (-2.8124)	-0.3125 (-1.2817)	0.4372* (1.8231)
reg	0.5008** (2.2180)	0.4745*** (2.7598)	-0.5050*** (-2.7256)	-0.7776*** (-4.0248)	0.2300 (1.3553)	-1.2717*** (-6.6279)	1.0471*** (5.6409)
Constant	0.8519 (1.5329)	-0.8377** (-2.0067)	-0.4420 (-1.0160)	0.3865 (0.8477)	-0.2493 (-0.6208)	0.0917 (0.2113)	0.6685 (1.5673)
Log likelyhood	-506.8631		-562.0101		-655.8215		-315.3154
ρ	0.4668***		0.1895**		-0.0115		
Pseudo R^2							0.1285***

*, ** and *** mean p < 10%, p < 5% and p < 1%, respectively

The age of house owner has a negative impact on farmers' selection of intellectual compensation (p < 10%). This is because old farmers are confident about their farming experience, and find re-education unnecessary. In addition, non-intellectual compensation is positively affected by the age of house owner, due to the positive impact of the age on organic fertilizer. In fact, the age is negatively correlated with alternative fertilizer. After all, old farmers are very aware of long-term ecological benefits, as organic fertilizer is the most effective way to reduce chemical fertilizer in individual farmland management.

Furthermore, the age of house owner has a negative impact on policy compensation (other jobs) (p < 1%), i.e. old farmers do not like job opportunities other than farming. There are two possible reasons: old farmers want to maintain a stable income and consumption structure, rather than take the risk of reemployment or job-hopping; most employers only recruit people under 60.

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(2) Education level

The education level only directly affects the selection of grain compensation and policy compensation (other jobs): it is negatively correlated with the selection of grain compensation ($p < 5\%$), and positively with that of policy compensation ($p < 1\%$). A possible reason lies in the strong learning and cognitive abilities of well-educated farmers. These farmers are good at acquiring information, clear about government intentions and concerned with rights. In their eyes, compensation is often insufficient and poor in quality. Thus, many of the well-educated farmers want to have other job opportunities. It is worth mentioning that 83% of well-educated respondents are younger than 50, which confirms the negative correlation between age and policy compensation.

(3) Annual family income

The annual family income mainly has a negative impact on farmers' selection of financial compensation ($p < 10\%$). Rich families are not very dependent on farm income. Their engagement in agricultural activities is mainly a pursuit for high-quality food. Farmers from these families are discouraged by the labor-intensity and strict requirements of conservation tillage. Therefore, financial compensation is not favored by rich families. On the contrary, farmers from poor families prefer financial compensation, and often ask for excessively high compensation due to the poor awareness of scientific farming and conservation policies. Apart from this, the annual family income has a very limited impact on the selection of other compensation options.

(4) Farmland area

The farmland area has a negative impact on farmers' selection of financial compensation ($p < 5\%$). The farmers with lots of farmland prefer non-financial compensation, because of the positive correlation between farmland area and the risk of yield loss under self-management. This is partly verified by farmers' selection of non-intellectual compensation. As shown in *Table 4,* the selection of non-intellectual compensation is positively affected by the farmland area ($p < 10\%$), so does organic fertilizer ($p < 10\%$). In addition, it can be seen from farmers' selection of organic fertilizer in different farmland areas that: farmers managing a larger farmland are more willing to make long-term investment. The reason is very simple: such farmers hold a favorable opinion of farming and want to enhance crop yield with organic fertilizer.

(5) Farmland fragmentation

Farmland fragmentation has a negative impact on farmers' selection of policy compensation ($p < 5\%$), which goes against the conventional knowledge that farmers are eager to change jobs when their farmland is fragmented, as fragmentation pushes up transport cost and hinders mechanized farming. Here, farmland fragmentation has no significant impact on other coupling compensation, indicating that farmers' demand for compensation remains constant despite difference in farmland conditions. Even if the farmland is highly fragmented, the farmers will not choose other jobs, because they rely on agriculture for a living. Lacking the knowledge of other jobs, they hope to maintain the current production scale, and safeguard the basic livelihood.

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(6) Production efficiency

Production efficiency has a positive impact on farmers' selection of financial compensation ($p < 10\%$), and a negative impact on that of intellectual technical compensation and alternative fertilizer ($p < 5\%$). The higher the production efficiency, the more favorable is financial compensation among the farmers. This agrees well with the provision condition of financial compensation: farmers must know the way to allocate fund to farmland management in an efficient manner. The same explains why farmers do not want technical compensation, especially intellectual compensation like education and training, when the production efficiency is already high.

(7) Farmers' willingness to reduce fertilizer application

Grain compensation ($p < 1\%$) and policy compensation ($p < 10\%$) are positively affected by farmers' willingness to reduce fertilizer application, while intellectual technical compensation and alternative fertilizer ($p < 1\%$) are negatively affected. In other words, the farmers willing to reduce fertilizer application prefer financial compensation to technical compensation, as such farmers are optimistic about the yield loss. Note that some of these farmers are willing to reduce the amount of fertilizer as a way of conservation tillage. They are willing to bear yield loss as long as it can be eventually compensated. Thus, these farmers have a low demand for intellectual technical compensation and alternative fertilizer.

(8) Regional variable

Regional variable has a positive impact on financial compensation, grain compensation and policy compensation ($p < 1\%$), and a negative impact on intellectual and non-intellectual technical compensations ($p < 1\%$). Overall, the farmers living near Qiaodun Reservoir are more likely to accept financial and policy compensations and less likely to accept technical compensation than those near Siming Lake Reservoir. This conclusion is consistent with the actual situation. In the periphery of Qiaodun Reservoir, more than 60% of family income is from agriculture, owing to the dense population, poor traffic and lack of competitive industry. By contrast, those living near Siming Lake Reservoir have plenty of job opportunities in other industries, and farming income only accounts for 10% of family income.

Discussion

This paper explores the farmers' selection of compensation means for chemical fertilizer reduction, and divides the reduction methods into six types, namely, financial compensation, grain compensation, technical learning, alternative fertilizer; organic fertilizer, and policy compensation. Through a questionnaire survey, the farmers' acceptance of different compensation options was summed up as: Besides the popularity option of financial compensation, the farmers are willing to accept non-financial compensation means, and the options with features of transfusion and coupling compensations; the stability is the top concern in the selection process, while function and flexibility are also considered; when multiple options are available, financial compensation is the most favorite one among farmers, followed by technical learning and policy compensation; the organic fertilizer has the lowest acceptance. Next, a simultaneous bivariate probit model was established to illustrate the effect of each

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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factor on farmers' selection between intellectual and non-intellectual technical compensations, and between alternative and organic fertilizers. Meanwhile, the farmers' intentions on policy compensation were examined by the binary probit model. The following conclusions were drawn through the empirical analysis on the modelling results.

The farmers' selection process is greatly affected by their awareness of the yield loss risk incurred by the reduction of chemical fertilizer. This risk is difficult to control at least in the short term if the farmers abandon long-term fertilization. However, chemical fertilizer is more controllable than pesticide, and the reduction consequences are not hard to predict. Our research reveals that farmers prefer the combination of compensation options that complement each other, and wish the government could provide compensation in a concentrated and practical manner.

The age of house owner is the key determination of the farmers' selection process. Old farmers are more conservative about alternative options, and stick to financial compensation and grain compensation. By contrast, young farmers are willing to improve farmland management and learn new techniques through intellectual technical compensation.

The education level of house owner has a negatively impact on the selection of grain compensation and intellectual technical compensation. Well-educated farmers are interested in non-financial compensation and policy compensation (other jobs).

The farmland area is negatively correlated with the selection of financial compensation. The farmers owning a large piece of farmland tend to choose alternative options and non-intellectual technical compensation.

The farmland fragmentation suppresses farmers' demand for non-agricultural job opportunities. Even if the farmland is highly fragmented, the farmers will not choose other jobs when they rely on agriculture for a living.

The production efficiency and farmers' willingness to reduce fertilizer application have similar effects on farmers' selection process. The farmers with high production efficiency are willing to reduce the use of chemical fertilizer and receive financial compensation instead of technical compensation. The farmers living near Qiaodun Reservoir, where the social and economic environment is relatively inferior, are more likely to accept financial and policy compensations and less likely to accept technical compensation, than those living near Siming Lake Reservoir. Even so, these farmers expect the government to provide intellectual technical compensation to control the risk of yield loss. In a word, the farmers living near Qiaodun Reservoir demand more opportunities for personal development.

Conclusions

Based on the above discussions, the following measures were designed for the government to encourage farmers' participation in fertilizer reduction and protect the quality of water bodies.

(1) Integrate different compensation options into a scientific and rational plan for farmers.

Considering the complementary effect between different compensation options, the government is advised to combine several compensation options that serve the same purpose and suit the same type of farmers into an integrated compensation plan, in light

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of the local conditions. Such a plan could promote the farmers' awareness of fertilizer pollution and boost their willingness to adopt reduction measures.

(2) Highlight the guidance of financial compensation on farmers' behaviors.

The financial compensation improves the direct income of any type of farmers, and thus enjoys a high acceptance among them. Despite its obvious incentive effect, the financial compensation should not be provided in advance, due to the presence of moral hazard. Instead, this compensation option should be given after the fertilizer reduction, and used to reward the farmers' purchase of techniques.

(3) Improve the acceptance of technical compensation through various channels.

The rejection of technical compensation is mainly attributable to the myth that farming is an empirical, not technical, process, as well as the farmers' poor awareness of environmental protection and blind trust in traditional low-cost, efficient but polluting tillage methods. To solve the problem, the government should provide regular training in the field and through channels like TV, radio and the Internet, remind the farmers of the importance of water conservation and the harm of chemical fertilizer to water bodies, and subsidize the purchase of alternative techniques to fertilizer application.

(4) Create job opportunities for farmers less willing to cut fertilizer application.

For various reasons, farmers may reject any compensation for fertilizer reduction. These farmers often have a special preference for jobs in other industries. Thus, it is suggested that the government implement "agricultural to non-agricultural" plans, giving these farmers new job opportunities. In places with good agricultural conditions, efforts should be made to develop green agriculture (e.g. family farm and ecological farm) and plant crops (e.g. potatoes and corns) requiring little fertilization. In places with poor agricultural conditions, the excess labor forces should be transferred to the downstream through ecological migration or non-agricultural reemployment. The emigration will benefit the ecology of the water bodies.

On the whole, the paper focuses on discussing whether there is a substitution relationship or complementary relationship between farmers' acquisition intentions of same type of compensation tools, and proposes that various means of compensation should have corresponding optimal providing ways (providing separately or in combination). As the acquisition and adoption of a single compensation tool is not the major concern of the study, there is no further research on the hematopoietic compensation type compensation, "other jobs" as career transfer, which can thoroughly eradicate pollution and is conducive to farmers' creating income. Farmers' career transfer involves the transportation and interconnection of agricultural labor in different employment areas. There are still lots of practical problems crying out for solution such as "what kind of local career transfer mode should be established", "how to formulate farmers' career transfer compensation scheme", "how to guarantee the continuity of employment", "how to ensure the stability of farmers' incomes", and so on. Therefore, it is an area worth further research.

Acknowledgements. Support for this research was provided by Water Culture Research Program of Zhejiang University of Water Resources and Electric Power (Grant No. SWH201802), Project of the Zhejiang Soft Science Research plans (Grant No. 2018C35064), Ministry of Education Humanities and Social Sciences Research Youth Foundation (Grant No.14YJC790124), Zhijiang Young Scholar Program of Social Science Planning of Zhejiang Province (Grant No. 13ZJQN056YB), the Major Program of National Social Science Foundation of China (Grant No. 14ZDA070), National Natural Science Foundation of China (Grant No. 71773114).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48234838

ANALYSIS AND PREVENTION OF THE IMPACTS OF METRO CONSTRUCTION ON GROUNDWATER ENVIRONMENT

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. Despite its positive impacts on traffic convenience and resource delivery, metro construction tilts the balance of groundwater resources and destabilizes the groundwater environment. This paper explores the impacts of shield tunnel construction on groundwater environment from the design and construction to an operating metro project, and verifies these impacts through an analysis on the tunnel project in Jinan Metro Line R3. The research shows that the grouting in shield tunneling has a local influence on the groundwater quality and pH, which attenuates with the increase of distance to the tunnel axis; the scope of influence is constrained by the formation lithology. Then, the backpropagation (BP) neural network was adopted to predict the ejection volume of groundwater, and the main influencing factors of water environment were disclosed by analytical hierarchy process (AHP). Finally, the author discussed the key techniques and prevention systems for prevention of the impacts of shield tunnel construction on water environment.
Keywords: *shield tunnel, backpropagation (BP) neural network, prediction of spring flow, analytical hierarchy process (AHP), key to groundwater control*

Introduction and survey of literature

Metro construction makes traffic convenient and efficient, enhances the overall competitiveness of cities, and improves people's living standards. However, the construction process also has negative impacts on groundwater environment.

During the construction of metro tunnels, dewatering is a necessity for engineering excavation. The rapid discharge of water from the tunneling section undermines the groundwater regulation and the spatial distribution of water resources. With the advancement of the tunnel, an increasing part of the water environment is destroyed, which changes the conditions of water-soil conservation and aggravates the imbalance between surface water and groundwater system. Zheng et al. (2016) and other scholars classified the impact of soft soil, high water level, liquid-rich powder, sand layer and underground engineering construction on the environment and the causes of disasters. Luo et al. (2018) analyzed the characteristics of urban underground engineering, and discussed the environmental hazards of underground engineering construction.

Surface subsidence caused by instability of tunnel excavation will lead to complex environmental problems. Li (2014) analyzed the influence of excavation disturbance zone on surface and surface deformation, and made exploratory research for the selection of excavation design methods and schemes, subsidence prediction and damage evaluation. Surface subsidence changes the water supply, runoff and discharge modes of surrounding water-bearing strata, thus damaging the overlying aquifer and breaking the balance of water environment. Guo (2018) put forward the countermeasures to reduce the adverse impact of tunnel construction on the water environment and improve the stability of the water environment system. In the process of tunneling, the water quality of the water environment will be damaged. According to the target layer, criterion layer, element layer and index layer, Fu (2018) constructed the index system and factor set of the influence of underground waterproof construction on water quality in urban tunnel engineering, and analyzed the influence of main pollutants discharged in the process of underground waterproof construction on water quality in urban tunnel engineering. Engineering wastewater discharged into the water environment without treatment will cause pollution of surface water resources, environmental degradation and other ecological problems.

Materials and methods

Analysis on the impacts of metro construction on groundwater environment

Overview of metro construction

The metro tunnel usually passes through several formations with complex geological conditions and diverse geological features. These formations differ greatly in water content, compressibility, void ratio, strength, as well as surface water reserve and topography. Therefore, the tunneling technique needs to be selected in light of the specific geological environment and hydrological conditions.

The shield method is a fully mechanized “deep bore” construction approach. As the shield tunnel boring machine (TBM) advances underground, the surrounding rock is supported by the shell and segments to prevent the tunnel from collapsing, while the soil on the tunnel face is excavated by the cutting device and transported outside the tunnel by the conveyor (Li, 2018). The advancement is propelled by the pressure of jacks in the rear of the shield TBM. In addition, the precast concrete segments are pieced up to form the tunnel structure.

Analysis on the impacts during the construction

Metro construction involves the pouring of concrete and the use of other chemicals (Zheng et al., 2016). The injected grouts have a certain impact on the quality and pH of the water in the surrounding rock. During the construction, the grouts, which contain lots of additives, are injected with the advancement of the shield TBM and added again after the installation of the segments (Guo, 2018). The grouts are prepared from a single type of liquid or two types of liquids. To make the single-liquid grout, the raw material needs to be mixed into a flowing liquid in a stirrer, and then solidified on the working face through flow and plastic condensations. The two-liquid grout generally refers to chemical grouting, i.e. mixing liquid A (cement) and liquid B (water glass) into a colloidal solution, whose viscosity increases with time, and relocating the solution to flow and plastic condensation zones (Cai et al., 2018). The mix ratios of the two types of grouts are listed in the *Tables 1* and *2*.

The injection of a large amount of grouts can easily pollute groundwater resources. What is worse, epoxy resin and other compounds commonly used in the construction are highly toxic and harmful to the human body (Huang et al., 2018). During the tunneling process, the deep groundwater and shallow groundwater are mixed together. The pollutants in the shallow groundwater will diffuse into the deep groundwater, affecting the direction of the water vein and water quality.

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The impacts of tunneling on water environment are constrained by the formation lithology. The permeability and compactness of the stratum both have an impact on the stability of water environment, which is negatively correlated with the distance to the tunnel axis.

During the construction, the dewatering well directly bears on the water level in the formation, resulting in temporary reduction of water level in local areas and damages to the soil structure. When water is discharged through the well, the groundwater level dropped across the board around the well. The curve on water level variation takes the shape of a funnel. Within the scope of influence, the soil layers will witness a continuous decline in the amount of pore water and the loss of water pressure balance. Meanwhile, the sediment will flow out from broken filter tube and filter layer of the well, reducing the natural compactness, water content and stability of the soil layers.

Table 1. *Common mix ratio of single-liquid grout*

Cement (kg)	Fly ash (kg)	Bentonite (kg)	Sand (kg)	Water (kg)	Additives
58	410	33	440	335	To be determined through experiment in light of the field conditions

Table 2. *Common mix ratio of two-liquid grout*

Water-cement ratio	Liquid A : Liquid B (volume ratio)	Coagulation time (s)
1:1	1:1	20~48

Analysis on the long-term impacts after the completion

Since the metro construction tilts the original balance of the groundwater environment, the long-term impacts on water environment can be evaluated against the reserve and ejected amount of groundwater. The latter can be predicted using the backpropagation (BP) neural network below (Isah et al., 2017; Li, 2017; Neelapu et al., 2018; Sánchez-Escalona and Góngora-Leyva, 2018; Sun et al., 2017a).

Let n, m and r be the number of neurons on the input layer, output layer and hidden layer in the BP neural network, which will be trained by K samples. The corresponding K input-output pairs can be expressed as $(X_k, Y_k), k = 1,2,\cdots,k$, where $X_k = (x_1, x_2,\cdots,x_n)$ is the input vector of the k-th sample and $Y_k = (y_1, y_2,\cdots,y_n)$ is the output vector of the k-th sample. Meanwhile, the expected value vector, the input layer-to-hidden layer weight matrix, and the hidden layer-to-output layer weight matrix are denoted as$D_k = (d_1, d_2,\cdots,d_n)$, $V = (V_1, V_2,\cdots,V_n)$,$V_j = (V_{j1}, V_{j2},\cdots,V_{jn})^T, j = 1,2,\cdots,n$, and $W = (W_1, W_2,\cdots,W_m)$ $W_i = (w_{i1}, w_{i2},\cdots,w_{in})^T, j = 1,2,\cdots,r$, respectively. The threshold of each hidden layer neuron and that of each output layer neuron can be expressed as $\alpha = (\alpha_1, \alpha_2,\cdots,\alpha_r)$ and $\beta = (\beta_1, \beta_2,\cdots,\beta_m)$, respectively. The initial weights and thresholds are configured by random.

Activation function: this function describes the change between the input and output of the neuron. The signals received by the hidden layer and the output layer are excited or suppressed by the calculation of the activation function. Here, the sigmoid function is selected as the activation function *Equation 1:*

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$$f(net) = \frac{1}{(1+exp(-net))} \quad \text{(Eq.1)}$$

where net is the summation unit.

Summation unit *Equation 1* represents the weighted summation of the input signals of the hidden layer (He, 2018). Since the weighted input of the hidden layer unit j is $netb_j = x_k \cdot v_j + a_j$, the actual output of the hidden layer unit *Equation 1* can be expressed as *Equation 2:*

$$b_f = (netb_j), j = 1,2 \cdots, n \quad \text{(Eq.2)}$$

Thus, the output matrix can be obtained as $B_k = (b_1, b_2, \cdots b_r)$.

Summation unit *Equation 2* represents the weighted summation of the input signals of the output layer. Since the weighted input of the output layer unit i is $nety_j = b_k \cdot w_i + \beta_i$, the actual output of the output layer unit *Equation 2* can be expressed as *Equation 3:*

$$y_i = f(nety_i), i = 1,2 \cdots r, j = 1,2, \cdots, n \quad \text{(Eq.3)}$$

Error calculation and backpropagation: Taking the root mean square errors of the output value and the expected value as the loss functions, the error index function of learning a single sample and the global error index function of learning the entire training set can be respectively expressed as *Equations 4* and *5:*

$$E_k = \frac{1}{2}\sum_{i=1}^{m}(y_i - d_i)^2 = \frac{1}{2}\sum_{i=1}^{m}\{f\{[\sum_{j=1}^{r} f(x_k \cdot v_j + \alpha_j) \cdot w_{ij}] + \beta_i\} - d_i\}^2 \quad \text{(Eq.4)}$$

$$E = \sum_{i=1}^{k} E_k \quad \text{(Eq.5)}$$

The learning and training of the BP neural network generally rely on the negative gradient descent method to adjust weights and thresholds. Based on *Equations 1–3,* the adjusted values of the weights and thresholds can be obtained through derivation by the complex function chain law, as shown in *Equations 6–9:*

$$\Delta v_j = -\mu \frac{\partial E_k}{\partial v_j}, j = 1,2, \cdots, n \quad \text{(Eq.6)}$$

$$\Delta \alpha_j = -\mu \frac{\partial E_k}{\partial \alpha_j}, j = 1,2, \cdots, r \quad \text{(Eq.7)}$$

$$\Delta \beta_j = -\mu \frac{\partial E_k}{\partial \beta_j}, j = 1,2, \cdots, m \quad \text{(Eq.8)}$$

$$\Delta w_j = -\mu \frac{\partial E_k}{\partial w_j}, j = 1,2, \cdots, r \quad \text{(Eq.9)}$$

where $\mu \in (0,1)$ is a constant representing the learning efficiency.

The adjusted weights and thresholds are adopted for the calculation of the next training sample. The learning should be terminated when the global error reaches the pre-set value after learning all training samples. Otherwise, the weights and thresholds should be initialized again for a new round of training until the said termination condition is satisfied.

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Evaluation of the negative impacts of metro construction on water environment

Analysis on the negative impacts on water environment

The negative impacts on water environment can be determined through the judgement of water environment damages caused by human activities and their impacts (Dai and Lv, 2018). In this paper, the analytical hierarchy process (AHP) is employed to scientifically assess water environment problems induced by tunnel construction (Dai and Zhao, 2018). The assessment was carried out in the following steps.

(1) Setting up the judgement matrix: Denote the target as A, the factors as u_i and u_j (*i*, *j* = 1, 2, ⋯, n) and the importance of u_i to u_j as u_{ij} (Sun et al., 2017b). On this basis, construct the A-U judgment matrix P below:

$$p = \begin{bmatrix} u_{11} & u_{12} & \cdots & u_{1n} \\ u_{21} & u_{22} & \cdots & u_{2n} \\ \cdots & \cdots & \cdots & \cdots \\ u_{n1} & u_{n2} & \cdots & u_{nn} \end{bmatrix}$$

(2) Calculating the importance rank: Find the eigenvector w corresponding to the largest characteristic root λ_{max}, according to the judgement matrix, as shown in *Equation 10:*

$$P_w = \lambda_{max} \cdot w \qquad \text{(Eq.10)}$$

Normalize the obtained eigenvector w, that is, sort the importance of all evaluation factors, yielding the weight distribution.

(3) Consistency test: Check if the weights are reasonable through consistency test on the judgement matrix, as shown in *Equation 11:*

$$CR = \frac{CI}{RI} \qquad \text{(Eq.11)}$$

where CR is the random consistency ratio of the judgment matrix; CI is the general consistency index of the judgment matrix, as shown in *Equation 12:*

$$CI = \frac{\lambda_{max}}{n-1} \qquad \text{(Eq.12)}$$

The RI is the mean random consistency index of the judgment matrix P. If the CR of the judgment matrix P is smaller than 0.1 or $\lambda_{max} = n$ and CI = 0, then the judgment matrix has satisfactory consistency; otherwise, the elements in the matrix should be adjusted to ensure the consistency. The negative effect of water environment is evaluated by ahp to realize the health analysis of water environment (Yang, 2018).

Results

Analysis of the influence of Jinan subway construction on groundwater environment

Jinan, the capital of Shandong Province, China, is widely known as the City of Springs. It is said that “every household in the city has a willow-shaded spring at the

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DOI: http://dx.doi.org/10.15666/aeer/1702_48394854

doorstep." There are many water bodies in the city, including the Yellow River, the Yufu River, the Beisha River, the Xiaoqing River and the Daming Lake. Unfortunately, the construction of Jinan Metro Line R3 has disturbed the topography, stratum altitude, stratum lithology and geological structure, hindered the ejection of underground springs and damaged the balance of groundwater environment. Located in the east of the city, Line R3 is a 49.4 km-long north-south express line linking up transport hubs like the new East Railway Station and Yaoqiang Airport. Many water bodies are scattered along the route of the line, such as the Xiaoqing River, the Daxin River, the Zhangma River, the Longji River, the Hancang River and the Mengjiazhuang Reservoir.

The construction area of Line R3 spans northward to the west of the springs in the Baiquan Spring Area, and belongs to the discharge zone of the area. The relative location between the project site and the Baiquan Spring Area is shown in *Figures 1* and *2*.

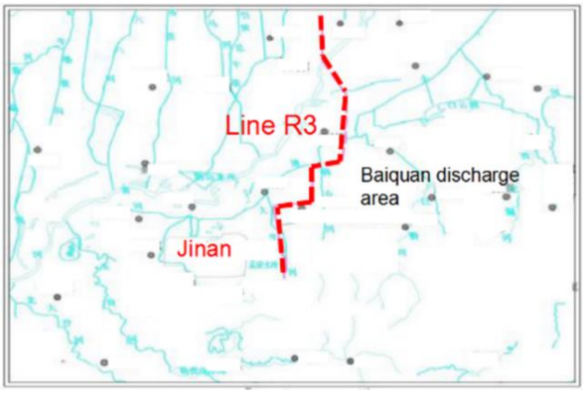

Figure 1. *Project site of Jinan Metro Line R3*

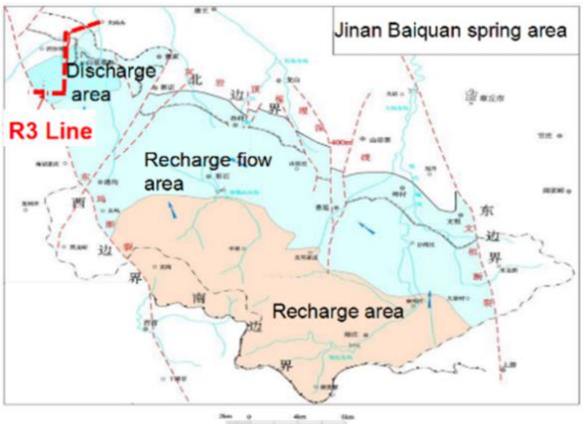

Figure 2. *Baiquan Spring Area*

The Mengjiazhuang~Long'ao section, an important passenger section of Line R3, is located in Lixia District of Jinan. Starting from the Mengjiazhuang Station, the metro line goes north along the Longding Avenue to the headquarters of the Armed Police Fire Corps, and then turns slightly towards the west, crossing the Daxin River and a mountain, and finally reaches the Long'ao Station on the Olympic West Road to the west of the Municipal Procuratorate. In this section, the crossings are mostly

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underground, along the road direction. The main environmental risk sources include the Daxin River, the DN1000 raw water pipe at the river bottom, and several roads undercrossed by the metro line, as well as the developed caverns near the side-crossing of the Daxin River Bridge. In the target section, the 1,816.811 m-long right line is designed between SK1 + 909.933 (right) and SK3 + 726.744 (right), while the left line of the same length is designed between XK1 + 909.933 (left) and XK3 + 726.744 (left). Constructed by shield tunneling, the tunnel in the section has a standard single-hole single-line circular profile. The left and right lines are both composed of straight segments, three circular curve segments with radius R = 1,000 m, and gentle curve segments. The line spacing is 13~14 m, and the covering soil is 2.3~80 m thick.

The construction area is dominated by south-north flowing pore water, which is replenished by the upstream runoff, karst water from the roof, and the atmospheric rainfall. The water is eventually discharged into the Xiaoqing River.

Since Line R3 lies in the discharge zone of Baiquan Spring Area, the first tunnel segment (north) in the Mengjiazhuang~Long'ao section passes through a 1,232.266 m-long layer of moderately weathered limestone and marlstone, while the second segment (south) through a 584 m-long gravel layer and the enrichment zone of karst groundwater in Baiquan Spring Area. Considering the dense water outlets, a huge amount of two-liquid grout was injected in actual construction. The liquid B is sodium water glass $Na_2O \cdot nSiO_2$, a popular chemical in civil engineering. The relevant chemical reaction can be described by the following formula:

$$xCa(OH)_2 + Na_20 \cdot nSi0_2 + mH_20 = xCaO \cdot Si0_2 \cdot yH_20 + 2NaOH$$

The product NaOH is alkaline. The two-liquid grout affects the pH of the water system around the grouting project and changes the pH of the local groundwater. The analysis results on local water sample are presented in *Figure 3*.

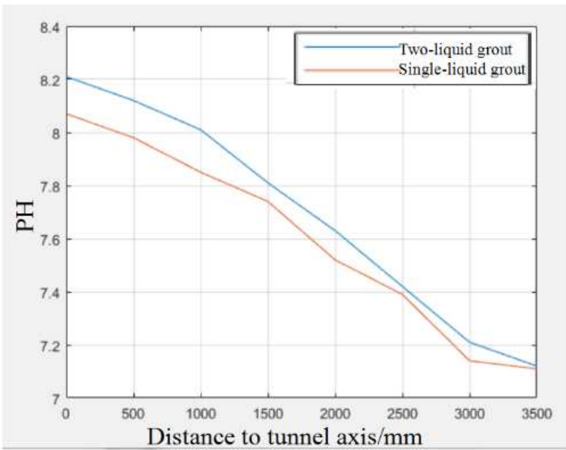

Figure 3. *The pH curve of local water sample*

As shown in *Figure 3,* the two grouts significantly pushed up the pH of the water around the tunnel, and the two-liquid grout had a greater impact on the pH than any single-liquid grout. This is because the two-liquid grout, as the main filling material, exerted an impact on the pH of the water in the secondary grouting, and the grouting process damaged the internal environment of the rock and soil, affecting the flow path

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of the water system. The author carried out an experiment on how much the tunnel blocks the water system, which shows that the grouting enhanced the water blocking effect by 20%~30% from that of cylinder volume of the tunnel.

Through investigation, it is learned that the scope of influence on water environment is maximal at the crossing through the gravelly soil layer, followed in descending order by the crossing through moderately weathered limestone and diorite, and the crossing through the silty clay layer.

As shown in *Table 3*, the impacts of the tunnel project on water environment belong to the 0~5,000 m from either side of the tunnel axis in the gravelly soil layer. Specifically, the severely affected area generally concentrates in the 0~1,500 m from either side of the tunnel axis. This area is characterized by the water seepage from Mengjiazhuang Reservoir, the depletion of surface water, wells and springs in the surroundings, the settlement, subsidence and cracking of the ground, and the complete change of the land use. The heavily affected area concentrates in the 1,500~3,000 m from either side of the tunnel axis. This area is characterized by the severe leakage of surface water, though not complete dry, obvious reduction of groundwater level, small local ground subsidence and change of local land use. The general affected area concentrates in the 3,000~5,000 m from either side of the tunnel axis. This area is characterized by the slight leakage of surface water and unobvious change of groundwater level.

Table 3. *Influence of formation lithology on water environment during metro construction*

	Gravelly soil layer	**Moderately weathered limestone layer (fragmented, with moderately developed caverns)**	**Moderately weathered limestone and diorite layer**	**Silty clay layer**
Permeability coefficient (10^{-6} cm/s)	8000	9000	6000	50
Scope of influence (relative to the tunnel axis)	0~ 5000 m	0~ 3000 m	0~ 2000 m	0~ 1000 m
Affected areas (relative to the tunnel axis)	0~ 1500 m	0~ 1000 m	0~ 800 m	0~ 500 m
Phenomena in the severely affected area (relative to the tunnel axis)	Water seepage from Mengjiazhuang Reservoir; depletion of surface water, wells and springs in the surroundings	Severe leakage of surface water, though not completely dry; obvious reduction of groundwater level	Slight leakage of surface water; unobvious change of groundwater level	Slight leakage of surface water

In the moderately weathered limestone layer (fragmented, with moderately developed caverns), the tunnel project has a moderate scope of influence on water environment, which covers the 0~3,000 m from either side of the tunnel axis. Specifically, the severely affected area generally concentrates in the 0~1,000 m from

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either side of the tunnel axis. This area is characterized by the severe leakage of surface water, though not complete dry, obvious reduction of groundwater level, small local ground subsidence and change of local land use. The general affected area concentrates in the 1,500~2,000 m from either side of the tunnel axis. This area is characterized by the slight leakage of surface water and unobvious change of groundwater level.

In the moderately weathered limestone and diorite layer, the tunnel project has a small scope of influence on water environment, which covers the 0~2,000 m from either side of the tunnel axis. Specifically, the severely affected area falls in 0~800, which is characterized by the slight leakage of surface water and unobvious change of groundwater level.

In the silty clay layer, the impacts of the tunnel project on water environment belong to the 0~1,000 m from either side of the tunnel axis. The severely affected area falls in 0~500 m, which is characterized by the slight leakage of surface water.

Near the target metro section, the large surface water systems include the Daxin River, which has a large north-south height difference, and the Mengjiazhuang Reservoir, which is rich in water resources. The groundwater beneath the riverbed is formed by the infiltration of rainwater along the gravel and bedrock fissures. The groundwater level is not uniform and varies greatly from season to season. The field survey on the reservoir confirms that there are 33 spring water outlets in the reservoir area (*Figures 4* and *5*), including 9 active springs and 24 inactive ones.

Figure 4. *Geographical map of Mengjiazhuang Reservoir*

Figure 5. *Distribution of spring water outlets near Mengjiazhuang Reservoir*

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Here, the water ejection volume of each inactive spring is estimated by the BP neural network (Fu, 2018), while the water amount of the 9 active springs are analyzed by water volume monitoring. As shown in *Table 4,* the water ejection volume of spring outlet A was reduced by 26% and that of spring outlet I was reduced by 3%, due to the tunnel blockage and geological disturbances.

Table 4. *Daily ejection volume of active springs*

Spring No.	A	B	C	D	E	F	G	H	I
Pre-excavation ejection volume (m^3/d)	2.051	0.304	4.547	0.841	0.592	2.820	1.426	0.975	0.106
Post-completion ejection volume (m^3/d)	1.518	0.274	4.092	0.774	0.539	2.679	1.369	0.678	0.103
Reduction (%)	26	15	10	8	9	5	4	11	3

The measuring points were arranged at an interval of 200 m to monitor the groundwater level near Mengjiazhuang Reservoir. Then, the variation in the ejection volume of each active spring was predicted based on the monitoring data. The deviation between the predicted value and the original value was plotted as a curve in *Figure 6*. It can be seen that the predicted value agrees well with the original value, indicating that the prediction result is close to reality.

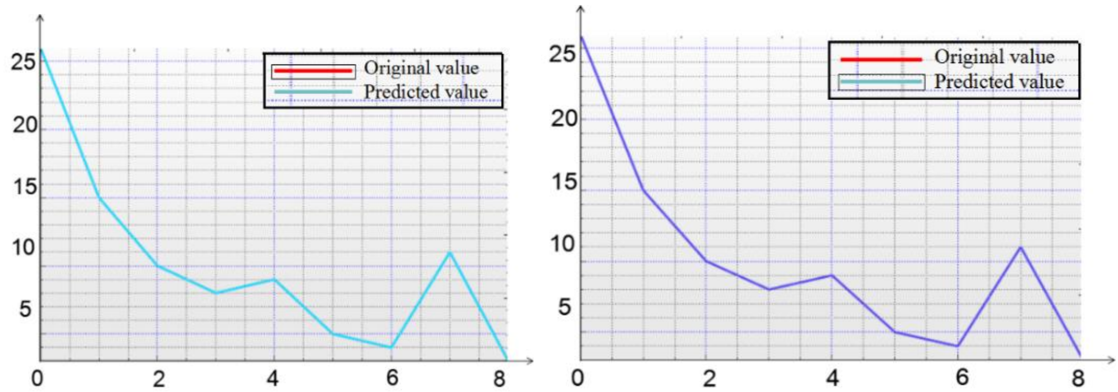

Figure 6. *Deviation between the predicted value and the original value*

The analysis on the prediction result shows that the metro tunnel construction caused an average reduction of 16% in the ejection volume of the active springs near Mengjiazhuang Reservoir. Besides, the ejection volume is correlated with the location of water outlets: the closer to the tunnel, the more the reduction in the ejection volume; the inverse is also true.

Analysis of the negative effects of subway construction on water environment

The target tunnel was divided into four parts (*Table 5*) according to the difference in geological conditions and covering soil environment of Line R3. Through the AHP, the judgment indices were allocated to three categories, namely, engineering geological conditions (Fang et al., 2018), hydrogeological conditions, and tunnel engineering

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conditions, and sub-divided into 13 special groups. Finally, 80 experts were invited to evaluate these indices. In this way, the author acquired the weights of the negative impacts of metro construction on water environment in the target metro section.

Table 5. *Division of groups and categories*

No.	Mileage	Length/m	Crossed stratum	Overlying stratum	Roof depth/m	Construction environment
A	SK3+726.7~SK3+400	326.7	Moderately weathered limestone	Plain fill, silty clay, gravelly soil	15.23-28.33	Crossing rainwater box culvert and tourist road
B	SK3+400~SK2+460	940	Moderately weathered limestone	Plain fill, moderately weathered diorite, gravelly soil	2.31-80	Crossing a mountain, karst caves, the Daxin River and DN1000 raw water pipe
C	SK2+460~SK2+280	180	Moderately weathered limestone, gravelly soil	Plain fill, silty clay	11.15-12.55	Crossing DN600 rainwater pipe; the stratum is soft on the top and hard on the bottom
D	SK2+280~SK1+909.9	370.1	Gravelly soil, silty clay	Plain fill, silty clay	10.47-13.1	Crossing the planed road and DN400 heat pipe

The weights (*Fig. 7*) were determined through selection of the evaluation method, construction of the evaluation model, the identification of the evaluation indices and the weight analysis.

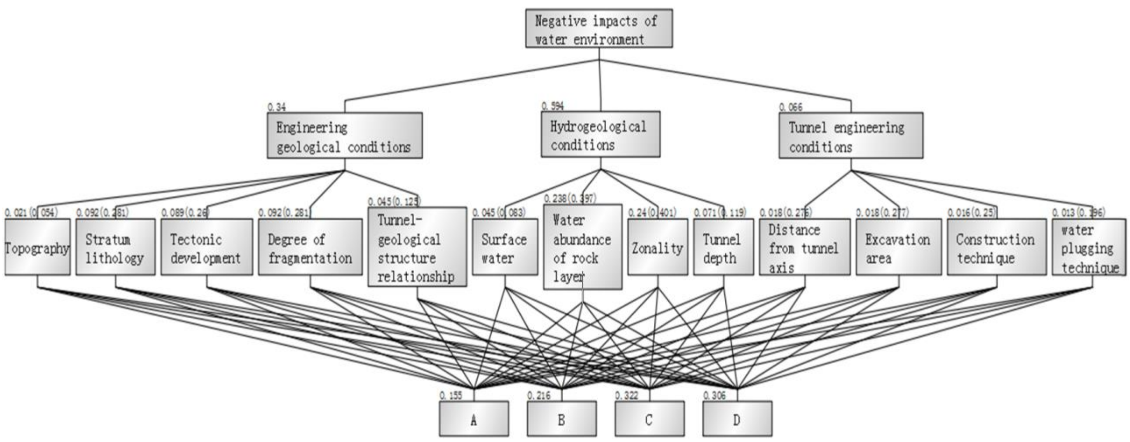

Figure 7. *AHP analysis on tunnel water environment*

The AHP weight analysis reveals that the negative impacts on water environment mainly come from the hydrogeological conditions (Tang et al., 2019). In the target tunnel, the SK2 + 460~SK2 + 280 segment had the greatest negative impacts on water environment, followed by the SK2 + 280~SK1 + 909.9 segment and the SK3 + 726.7~SK3 + 400 segment.

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Discussion

Prevention of the impacts of metro construction on groundwater environment

Key prevention technologies

To reduce the impacts of metro construction on groundwater environment, it is necessary to step up prior control, rationalize route selection and optimize the design. The design plan should be pertinent and the planned route should be optimized according to the survey report. If a section of poor geological conditions is unavoidable, the best construction plan must be prepared in light of the geological conditions and construction technologies.

As mentioned before, the shield tunnel has a certain blocking effect on the flow of groundwater. It is impossible to identify the direction of all spring waters through geological survey, for the water flow of most small springs, generated the infiltration of surface water, may be affected by the stratum and groundwater level. To minimize the impacts of formation damage on spring water, fixed-point monitoring should be carried out on the water pressure of the water and soil layer near the tunnel, and recharge technique should be adopted to replenish the spring water.

As a permanent project, the tunnel will have an impact on the groundwater environment. In the short term, the local osmotic pressure of the water will be greatly affected, which will change the flow direction of the groundwater. The sealing effect of the multi-layer grouting on the water will induce changes to the groundwater system. To reduce the damage of grouts on water environment, it is advised to adopt high-quality shield segments, enhance the water resistance of the segments, standardize the field construction process, and cut down the amount of secondary grouting.

Considering the health impacts of groundwater quality and pH, the tunneling damages on water environment must be minimized. To lower the material pollution to water environment, the possible measures include replacing toxic compounds with green materials and traditional construction techniques with advanced techniques.

Main prevention techniques

The in-process control and post-action control should also be enhanced during metro construction. The water level should be reduced to about 50 mm below the excavation face when the last 3~5 ring segments are installed, so that no water or sand will flow out at the tunnel breakthrough. To meet this goal, it is recommended to implement in-situ dewatering at the recharge well (Guo et al., 2018). The process flow of this technique is shown in *Figure 8*. It can be seen that the pumping device is used to lower the water level of the excavated soil layer to below the excavation face.

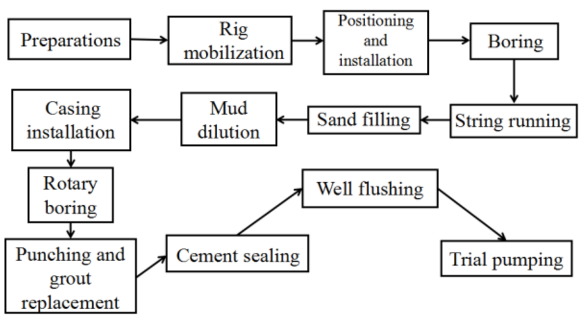

Figure 8. *In-situ dewatering at the recharge well*

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As the water is injected back into the well, the groundwater level around the well will gradually rise, and the post-injection water level is called the recharge level. The water injected into the well can seep into the aquifer, owing to the water head difference between the recharge level and the groundwater level. When the recharge well is under construction in the foundation pit, the static water level and the dewatering-induced water level variation should be observed, so as to determine the remaining construction of the well. The recharge should commence if the groundwater level changes by over 300 mm per day, and stop if the recharge amount reaches 80% or the ground heaves. The recharge water mainly comes from the groundwater in the pumping well within the foundation pit. No additional pressure is needed in the early phase of the recharge. However, a proper amount of external pressure should be applied if the groundwater level cannot meet the target or the recharge amount is difficult to increase. Note that the recharge pressure should not be too large, but controlled within 0.5 MPa. During the recharge, the water level should be monitored every 12 h in the observation wells inside and outside the foundation pit. Meanwhile, the pumping well in the foundation pit should continue to operate.

Key techniques for water environment management in Jinan

The areas with developed water systems should be avoided in the design phase of the metro tunnels in Jinan. To protect springs, the metro lines should be circumvent springs and avoid water veins. Special efforts should be paid to set up a wide network of water level monitoring systems, covering 2~3 times the area of normal water level detection systems. In addition, the shield segments must contain sealing strips and receive testing before use, aiming to minimize the risk of water leakage due to broken sealing strips.

According to the above design requirements, the in-situ recharge should be performed in time after the dewatering of the tunnel and stations in the target section. The in-situ recharge of groundwater can be controlled in an automatic and precise manner, through the coordination between pressurized recharge well, large variable diameter pressurized recharge well, surrounding water environment detection system, integrated pumping and recharge device and smart control system. The test data show that the overall recharge rate surpassed 80%, indicating that the proposed techniques can effectively protect the total amount of water resources, stabilize the groundwater pressure and maintain the balance of the groundwater environment.

Prevention system

The metro construction brings a wide range of far-reaching and long-lasting damages on water environment (Zhang et al., 2017). To protect the water resources of Jinan, the author set up a prevention system against the negative impacts on water environment, covering the entire process from design, construction, operation to maintenance. As shown in *Figure 9,* the proposed system relies on big data collection and analysis platforms (e.g. digitalization, the Internet of Things, cloud computing and collaborative management), integrates underground engineering construction techniques, and involves such systems as organizational system, capital system and institutional system. The system supports real-time warning or adjustment according to geological, hydrological, and engineering inspection data, realizes the intelligent decision-making on the prevention of negative impacts on water environment, and achieves the dynamic optimization of water environment treatment techniques and processes.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48394854

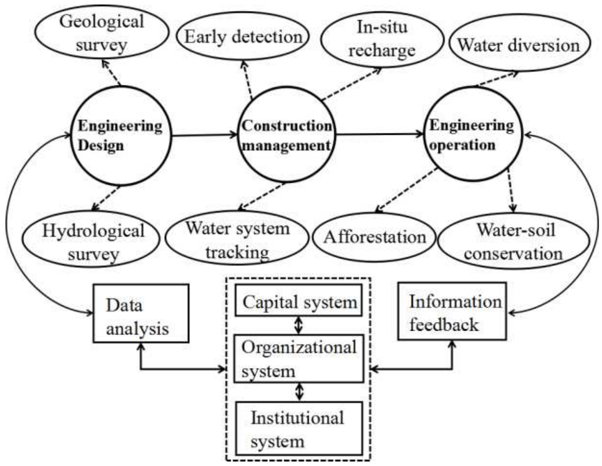

Figure 9. *The whole process prevention system against negative impacts on water environment*

During the engineering design, the survey data on water level are is analyzed by cloud computing and collaborative management system, and the results are directly applied to the construction management. The construction plan is guided by the real-time data on the field water environment, which are collected through water system tracking and early detection during the design and construction management, laying a solid basis for emergency instructions and groundwater protection.

In this paper, the in-situ recharge in Jinan uses an intelligent control system for integrated pumping and recharge. Coupled with the prevention system, the intelligent control system can promote the protection of water environment. During the project operation, water-soil conservation and afforestation work will be carried out to protect the soil and water resources in a targeted manner. The whole system forms a closed-loop control through the feedback mechanism, which can effectively reduce the negative effects of underground engineering on water environment and protect water environment.

Conclusions

During metro construction, it is imperative to maintain the original groundwater quality and flow, reduce the impacts on the surrounding water environment, and coordinate the relationship between metro construction and water environment.

(1) In this paper, the impacts of shield tunnel construction on groundwater environment are analyzed in the entire process from construction to operation. The research shows that the shield grouting can affect the quality and pH of groundwater in local areas, and the effect attenuates with the increase of the distance to the tunnel axis. These results were verified through the analysis on the construction of a section in Jinan Metro Line R3.

(2) The impacts of metro tunnel construction on water environment are constrained by stratum lithology, and abide by a certain law. This law was identified as “small in the distance and big on the contrary”, based on the data collected from the section between Mengjiazhuang Station and Long’ao Station in Jinan Metro Line R3.

(3) The BP neural network was adopted to predict the reduction of groundwater flow induced by metro construction. Meanwhile, the ejection volumes of 24 active springs

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DOI: http://dx.doi.org/10.15666/aeer/1702_48394854

near Mengjiazhang Reservoir were predicted. Through these predictions, it is concluded that the ejection volume of the local springs will be reduced by 16% on average.

(4) The main factors were acquired concerning the impacts of metro construction on water environment through the AHP. The analysis shows that the negative impacts on water environment in the target section mainly come from hydrogeological conditions, and peak in the SK3 + 726.7~SK3 + 400 segment.

(5) The author put forward the key prevention techniques for the negative impacts on water environment, and constructed a five-in-one prevention model for negative impacts on water environment, which consists of a construction system, a data analysis system, an organizational system, a capital system and an institutional system.

(6) During the construction of metro tunnels, Multi-angle analysis technology and judgment indicators can better protect the groundwater environment. In-depth exploration of the influence factors of underground engineering on the water environment and comprehensive analysis of the negative effect data of groundwater environment will be the focus of future research on the impact of underground engineering on the water environment and prevention.

Acknowledgements. This work is supported by Natural Foundation of Shandong Province, China [Grant No. ZR2017MEE069]. Thanks for the support of Natural Foundation of Shandong Province.

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APPLIED ECOLOGY AND ENVIRONMENTAL RESEARCH 17(2):4839-4854.
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48394854

POLLUTION HETEROGENEITY-BASED ANALYSIS ON INFLUENCES OF RESEARCH AND DEVELOPMENT (R&D) SUBSIDY ON ENERGY CONSUMPTION AND ENERGY STRUCTURE IN THE CHINESE INDUSTRIAL SECTOR

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. Based on the data on the Chinese industrial sector in 2009~2015, this paper empirically analyses the effect of research and development (R&D) subsidy on energy consumption and energy structure of the industry in China. The research results show that the R&D subsidy slowed down the growth of the total energy consumption in the Chinese industrial sector and promoted the optimization of the energy structure in the short term. On this basis, the author investigated how these impacts varied within so called pollution departments. The investigation reveals a significant impact of R&D subsidy in high-pollution departments: the subsidy greatly suppressed the energy consumption and optimized the energy structure of these departments; for low-pollution departments, the R&D subsidy had no major impacts on energy consumption or energy structure.
Keywords: *R&D subsidy, compensation effect, price effect, scale effect, regression analysis, heterogeneity analysis*

Introduction

Over the years, China has consumed a large amount of basic resources, especially energy, for industrial development. With abundant coal and limited oil, China's industrial development relies on the direct use of coal and the consumption of secondary energy (e.g. thermal power) produced from coal. The coal-based energy structure has brought serious pollution. In his report at the 19th National Congress of the Communist Party of China (CPC), Xi (2017) said, "To ensure harmony between humanity and nature, we must realize that lucid waters and lush mountains are invaluable assets and act on this understanding, implement our fundamental national policy of conserving resources and protecting the environment." Under his call, the government, researchers and the public began to focus on industrial development and environmental issues, striving to optimize consumption and energy structure, and achieve sustainable development (Shang and Zhu, 2018).

In addition to promoting stable economic growth, technological progress can reduce pollution and facilitate energy conservation and emission reduction in the industry. The R&D activities have significant public attributes and positive externalities. By subsidising the research and development (R&D) of manufacturers, the government can correct market failures in R&D activities, lower the R&D risks of enterprises, and improve the technical level of the industry as a whole (Xu et al., 2014). Despite the numerous studies on the relationship between R&D subsidy and technological innovation (Wu and Liu, 2014; Xiao and Lin, 2014; Zhao and Yao, 2017), there are still two unresolved questioned: Is it possible for the government to simulate technological progress of producers through R&D subsidy, thus improving the overall energy

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

conservation of the industry? Are there any differences in the energy-saving effects of government R&D subsidy for industries at different levels of pollution or technology? Answering these two questions will help China formulate future industrial subsidy policies, transform and upgrade the industry, and achieve energy conservation and emission reduction.

As mentioned before, the previous studies on R&D subsidy mainly tackle the following issues: how does R&D subsidy impact enterprise R&D inputs (Busom, 2000; Jiang and Huang, 2010), whether R&D subsidy affects innovation efficiency (Lv and Sun, 2014) and what is the crowding-out effect of R&D subsides (Lach, 2002; Wallsten, 2000). However, there is little report on the influence of R&D subsides over socioeconomic development or environmental improvement (Song, 2018). Particular attention should be paid to the positive externalities of subsidy, a common financial remedy to market failures, on a social basis. Therefore, this paper intends to expand the scope of existing research into the relationship between R&D subsidy and innovation.

Moreover, China is now faced with serious resource misallocation due to the legacy of the planned economic system. The problem is particularly severe in the field of science and technology (Han and Zheng, 2014). Against this backdrop, some inefficient industries waste R&D subsidy in blind expansion of low-efficiency production, rather than develop and upgrade technology. The resulting pollution and excess capacity hinder the industrial transformation and upgrading and harm the environmental protection in China. As a result, this research aims to help the government design and develop more flexible and effective R&D subsidy policies.

Overall, this paper attempts to disclose the effects of R&D subsidy on energy consumption and energy structure, and discover the far-reaching impact of R&D subsidy on China's environment, laying a valuable basis for the pursuit of an ecological civilization.

Materials and method

Theoretical analysis

The R&D subsidy affects energy consumption and energy structure through three channels, namely, the compensation effect, the price effect and the scale effect. Below is a detailed description of each channel.

Compensation effect

The public attributes of producers' technical R&D activities often result in high cost, high risk and a long-term investment (Danneels and Kleinschmidtb, 2010). Owing to the spill-over of producers' R&D activities, the expected returns are reduced to a level far below the generated social benefits. Hence, the producers are deprived of the resources for further R&D activities (Cohen and Levinthal, 2015). The government's R&D subsidy partially offsets the producers' loss of expected returns caused by technology spill-over, and compensates or even enhances the expected returns of the producers' R&D activities. This brings positive signals and stimuli to producers' R&D behaviour. Meanwhile, the government's R&D subsidy partially lowers the risks generated by R&D activities, suppresses the negative impact of uncertain R&D outcome on the expected returns, and arouses producers' interest in and continuous inputs to R&D (Lichtenberg, 2001). To sum up, the government's R&D subsidy can

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

bolster the R&D inputs of the producers, and promote the technological progress and innovation ability of the producers and whole industry. This, in turn, encourages the energy conservation and emission reduction in the whole industry, leading to lower energy consumption and more efficient energy use.

Price effect

The price effect is demonstrated in the indirect impacts on the efficiency and price of energy production. On climate change policies, Sinn (2008) discovered the "green paradox": the policies against climate change will lead to the growth in energy consumption and the accumulation of greenhouse gases. Sinn presented the following explanation to the transmission mechanism of "green paradox": all industries will enjoy more efficient production and better technologies thanks to technological progress; in the energy industry, the results of technological progress are demonstrated as improved efficiency of energy production and the reduced energy price; as the price drop reduces production costs, producers tend to consume more energy and release more greenhouse gases. Maria et al. (2014) analysed the coal prices in the US from 1986 to 1994, noting that the reduction of energy price caused by technological progress has increased energy consumption. Thus, the R&D subsidy, through the technological progress in the energy industry, can lower the energy supply price, and in turn affect the total energy consumption in other industries.

Scale effect

The scale effect of government R&D subsidy mainly comes from the crowding-out effect, which propels the expansion of production scale. When the government subsidises producers' R&D activities, the government funds will displace the producers' R&D inputs (Guellec and Van Pottelsberghe, 2003), allowing producers to invest the inputs to expanded reproduction. The expansion of production scale will push up energy consumption. This situation is clearly seen in high-pollution industries. In China, high-pollution industries are large in scale, high indebted, and have a low the added value of products. These industries often spend spare funds on the expansion of production scale, aiming to reduce unit cost of products and realize returns to scale. To some extent, this explains the expansion and overcapacity of some traditional industries in China, despite the continuous growth in R&D subsidy in recent years.

Samples and data sources

The relevant data on R&D subsidy and energy consumption of the energy industry from 2009 to 2015 were adopted for this study. The data in 2009 and 2010 were adjusted according to the latest classification method for industrial sectors issued by China in 2011. For instance, the plastic products industry and rubber products industry were merged into the rubber and plastic products industry, while the automobile manufacturing industry was combined with the manufacturing industries for railway, shipping, aerospace and other transportation equipment into the transportation equipment manufacturing industry. After the adjustment, the data were divided into 36 industrial sectors according to the 2011 industrial sector classification method, excluding those on the other mining industry. The data on R&D subsidy were extracted from *Statistics Yearbook on Science and Technology Activities of Industrial Enterprises* and *China Statistical Yearbook on Science and Technology*, the data on energy were

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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extracted from *China Energy Statistical Yearbook*, and the data on other indices of industrial economy and development were extracted from *China Industry Statistical Yearbook*. To eliminate the interference of price on the empirical results, the relevant economic indices were deflated against the benchmark of the 2009 price.

Definition and measurement of variables

The explained variables in this paper are the total energy consumption and energy structure.

The former describes the general trend of consumption. Here, its value is expressed as standard coal converted from the data released by the statistical department. The energy structure reflects the composition of energy consumption. It is usually expressed as the ratio of the total coal-based energy consumption to the total energy consumption. The total coal-based energy consumption equals the sum of the standard coal converted from the consumption of each type of coal-based energy.

The government's R&D subsidy is treated as an explanatory variable in this research. From the data on R&D funds, the subsidy funds allocated by the government directly to the industries were identified manually.

A total of six control variables are adopted for this research. Among them, the industry scale was described by the proxy variable of the number of employees in the industry in the current year; the fixed assets investment was extracted directly from the data released by the statistical department; the energy price index was the ex-factory price of power fuel released by the statistical department (the value was converted against the benchmark price in 1985 to eliminate the interference of price on the empirical results); the export volume, the total revenue and the R&D expenditure were directly extracted from the data released by the statistical department and subjected to deflation. The meanings of the above variables are given in *Table 1*.

Table 1. *Meanings of variables*

Variables	Description	Unit
Energy consumption	The amount of energy that one industrial sector consume in 1 year	104 tons of standard coal
Energy structure	Ratio of coal-based energy to total energy	%
R&D subsidy	Funds that the government allocates to enterprise for R&D	104 Yuan
Size	Size of one industrial sector	104 persons
Fixed asset investment	Expenses for the construction and purchase, such as capital construction, renovation and major repairs, etc	109 Yuan
Energy prices index	An index of the price of energy purchased by producers	
Export	The total revenue that a producer sell its products abroad	109 Yuan
Revenue	The total revenue that a producer sell its product or service in 1 year	109 Yuan
R&D expenditure	Total expenditure invested by producers for scientific research and development activities	104 Yuan

Construction of regression equations

The following econometric equations were established to test the impact of government R&D subsidy on energy consumption and energy structure. In *Equations 1*

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and *2,* the current year data on R&D subsidy are taken as the explanatory variables, aiming to measure the short-term impact. In *Equations 3* and *4,* the subsequent year data on R&D subsidy are taken as the explanatory variables, aiming to measure the long-term impact.

$$EC_{it} = \alpha_0 + \alpha_1 Subs_t + \alpha_2 Size_{it} + \alpha_3 FI_{it} + \alpha_4 P_{it} + \alpha_5 Exp_{it} + \alpha_6 R_{it} + \alpha_7 RD_{it} + \varepsilon_{it} \quad \text{(Eq.1)}$$

$$ES_{it} = \alpha_0 + \alpha_1 Subs_t + \alpha_2 Size_{it} + \alpha_3 FI_{it} + \alpha_4 P_{it} + \alpha_5 Exp_{it} + \alpha_6 R_{it} + \alpha_7 RD_{it} + \varepsilon_{it} \quad \text{(Eq.2)}$$

$$EC_{it} = \beta_0 + \beta_1 Subs_{it-1} + \beta_2 Size_{it} + \beta_3 FI_{it} + \beta_4 P_{it} + \beta_5 Exp_{it} + \beta_6 R_{it} + \beta_7 RD_{it} + \varepsilon_{it} \quad \text{(Eq.3)}$$

$$ES_{it} = \beta_0 + \beta_1 Subs_{it-1} + \beta_2 Size_{it} + \beta_3 FI_{it} + \beta_4 P_{it} + \beta_5 Exp_{it} + \beta_6 R_{it} + \beta_7 RD_{it} + \varepsilon_{it} \quad \text{(Eq.4)}$$

where EC is the total energy consumption; ES is the energy structure; Subs is the R&D subsidy; Size is the industrial scale; FI is the total fixed asset investment; P is the energy price index; Exp is the export volume; R is the total revenue; RD is the R&D expenditure; the subscript i refers to the industry; the subscript t refers to the year.

Results

Statistical description

According to the statistical description of data in *Table 2*, the energy consumption ranged between 69,543.27 and 110.10, revealing a huge gap in energy consumption among different industries; the minimum energy structure stood at 0.02 only, a mirror of the intensive and efficient use of non-coal-based energy in some industries, which explains the massive gap in total energy consumption among different industries; the industries also differed greatly in the amount of R&D subsidy, indicating the sharp difference in R&D level and technical level among the industries.

Table 2. *Statistical description of data*

Variables	Observations	Mean	Std. dev	Min	Max	Unit
Energy consumption	252	5085.74	11270.81	110.10	69543.27	10^4 tons of standard coal
Energy structure	252	0.28	0.19	0.02	0.81	%
R&D subsidy	252	110326.80	227060.60	194	1790916	10^4 Yuan
Size	252	262.92	208.07	18.09	909.26	10^4 persons
Fixed asset investment	252	4206.14	3878.60	208.38	20260.41	10^9 Yuan
Energy prices index	252	99.94	9.64	88.70	116.30	
Export	252	2834.67	6665.19	0.09	46165.14	10^9 Yuan
Revenue	252	24742.62	22280.99	966.33	91606.58	10^9 Yuan
R&D expenditure	252	1899258	2803182	1457	1.61e+07	10^4 Yuan

Variable correlation analysis

Table 3 records the results of correlation analysis on the variables. The analysis focuses on the correlations between government R&D subsidy and the two explained variables. As shown in Table 3, the R&D subsidy is positively correlated with the total

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

energy consumption and negatively with the energy structure. These correlations were subjected to empirical analysis in the next section.

Table 3. *Variable correlation analysis*

Variables									
Energy consumption	1.0000								
Energy structure	0.0767	1.0000							
R&D subsidy	0.0025	-0.1965	1.0000						
Size	0.2531	0.1177	0.5814	1.0000					
Fixed asset investment	0.3247	0.0537	0.4928	0.6255	1.0000				
Energy prices index	-0.0274	-0.0212	-0.0439	-0.0007	-0.1423	1.0000			
Export	-0.0316	-0.2303	0.5228	0.6508	0.2235	-0.0082	1.0000		
Revenue	0.5304	-0.0440	0.6279	0.7922	0.7959	- 0.0636	0.5094	1.0000	
R&D expenditure	0.2753	-0.2126	0.8404	0.7397	0.5883	-0.0928	0.7121	0.8256	1.0000

Analysis of empirical results

Before the regression analysis, the collinearity between the variables was tested. The results show no sign of collinearity. Next, the correlations between R&D subsidy and the two explained variables were tested and analysed on Stata 14.

Table 4 shows the effect of R&D subsidy on the total energy consumption. Under the control of time trend, Models (1) and (2) are a random effect model and a fixed effect model on the short-term effect, respectively. The results of the random effect model were adopted according to the Durbin–Wu–Hausman test. The results of Model (1) show that, in the short term, the R&D subsidy had a significant negative correlation with the total energy consumption. This means the energy consumption will decline with the increase in the R&D subsidy. The results also prove that the total energy consumption is positively correlated with the industrial scale, the total revenue and the R&D expenditure, but negatively with the fixed asset investment and the export volume.

Under the control of time trend, Models (3) and (4) are a random effect model and a fixed effect model on the long-term effect, respectively. The results of the fixed effect model were adopted according to the Durbin–Wu–Hausman test. It can be seen that the growth in total energy consumption was significantly suppressed with the increase in R&D subsidy.

Table 5 describes the effect of R&D subsidy on the energy structure. The results of Model (5) were verified by Durbin–Wu–Hausman test. According to these results, the R&D subsidy improved the energy structure and reduced the ratio of coal-based energy in the energy consumption of production in the short term. In particular, the energy price index significantly boosted the improvement of the energy structure. Hence, the R&D subsidy can elevate the efficiency of the energy industry to a certain extent and thus reduce the price of energy supply and service. In the long run, R&D subsidy did not exert a significant impact to improvement of the energy structure. According to the results of Durbin–Wu–Hausman test, the R&D subsidy in the subsequent year had no major impact on the improvement of the energy structure.

In summary, the above results indicate that R&D subsidy alleviated the growth trend of the total energy consumption and improved the energy structure in the short term.

Table 4. *Effect of R&D subsidy on the total energy consumption*

Energy consumption	**Model (1)**	**Model (2)**	**Model (3)**	**Model (4)**
Subsidy	-0.0173*** (0.00274)	-0.0166*** (0.00277)		
Subsidy (t-1)			-0.0185*** (0.00408)	-0.0167*** (0.00421)
Size	11.27** (4.833)	11.63** (5.629)	8.905 (5.875)	12.08* (7.013)
Fixed asset investment	-0.586*** (0.140)	-0.488*** (0.141)	-0.486*** (0.176)	-0.345* (0.182)
Energy price index	-18.19 (12.27)	-17.08 (12.02)	-10.04 (19.28)	-16.58 (19.18)
Export	-0.684*** (0.130)	-0.641*** (0.142)	-0.674*** (0.169)	-0.631*** (0.206)
Revenue	0.238*** (0.0425)	0.202*** (0.0433)	0.284*** (0.0592)	0.209*** (0.0636)
R&D expenditure	0.00126*** (0.000317)	0.00125*** (0.000320)	0.00103** (0.000405)	0.00103** (0.000418)
Cons_	1,974 (1,953)	2,063 (1,624)	404.3 (2,681)	1,245 (2,556)
Wald chia2/F	181.72 (0.0000)	21.57 (0.0000)	120.26 (0.0000)	12.10 (0.0000)
Time	Yes	Yes	Yes	Yes
Hausman test	1.02[0.7959]			9.56[0.0888]
Observations	252	252	216	216
R^2		0.419		0.329

Significance: ***p < 0.01, **p < 0.05, *p < 0.1

Pollution heterogeneity-based analysis

After examining the impact of R&D subsidy on energy consumption and the energy structure of the entire industrial sector in China, this paper further explores whether the impact differs with the pollution levels. According to the *Guide for Environmental Information Disclosure of Listed Companies* issued by the Chinese Ministry of Ecology and Environment, the 36 samples were divided into high-pollution departments (16) and low-pollution departments (20), and subjected to analysis separately. The results are listed in *Tables 6* and *7*.

In *Table 6*, Models (9) and (10) are about the short- and long-term impacts of R&D subsidy on energy consumption in different pollution industries, respectively. For high-pollution departments, the R&D subsidy exerted a significant negative impact on energy consumption in both short- and long-term, indicating that the subsidy can indeed curb the growth of energy consumption in high –pollution departments. As shown in Models (11) and (12), the R&D subsidy had no significant effect on energy consumption of low-pollution departments. In high-pollution industries, the R&D subsidy can promote the upgrading of industrial technology, and thus bolster the implementation of energy

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

conservation and emission reduction. The bolstering effect is particularly obvious in energy-sensitive areas like high-pollution departments. By contrast, low-pollution departments are not sensitive to energy demand, for they emphasize on with the improvement of product technology over the change of production technology.

Table 5. *Effect of R&D subsidy on energy structure*

Energy structure	Model (5)	Model (1)	Model (7)	Model (8)
Subsidy	-1.94e-07** (7.60e-08)	-1.95e-07** (7.87e-08)		
Subsidy (t-1)			-6.11e-08 (1.09e-07)	-5.01e-08 (1.16e-07)
Size	0.000764*** (0.000128)	0.000924*** (0.000160)	0.000602*** (0.000149)	0.000724*** (0.000192)
Fixed asset investment	-1.39e-05*** (3.88e-06)	-1.57e-05*** (4.01e-06)	-1.15e-05** (4.70e-06)	-1.35e-05*** (4.98e-06)
Energy price index	-0.00112*** (0.000345)	-0.00121*** (0.000341)	-0.000630 (0.000524)	-0.000453 (0.000526)
Export	-9.52e-06*** (3.50e-06)	-7.04e-06* (4.02e-06)	-8.49e-06** (4.27e-06)	-7.08e-07 (5.66e-06)
Revenue	-4.24e-07 (1.18e-06)	-3.00e-07 (1.23e-06)	1.10e-06 (1.55e-06)	1.79e-06 (1.74e-06)
R&D expenditure	1.43e-08 (8.81e-09)	1.37e-08 (9.07e-09)	-9.69e-10 (1.08e-08)	-3.84e-09 (1.15e-08)
Cons_	0.278*** (0.0505)	0.244*** (0.0461)	0.230*** (0.0688)	0.153** (0.0701)
Wald chia2/F	13.52 (0.0000)	11.29 (0.0000)	30.54 (0.0001)	5.01 (0.0000)
Time	Yes	Yes	Yes	Yes
Hausman test	7.65[0.1051]			12.95[0.0239]
Observations	252	252	216	216
R^2		0.274		0.169

Significance: ***p < 0.01, **p < 0.05, *p < 0.1

Table 7 illustrates the impacts of R&D subsidy on the energy structure in different pollution departments. As shown in models (13) and (14), the R&D subsidy exerted a significant negative impact on energy structure in both short- and long-term for high-pollution departments, indicating that the subsidy reduces the use of coal-based energy in these departments; for low-pollution departments, the R&D subsidy had no significant short- or long-term effect on energy consumption. This situation can be attributed to two factors. First, the R&D subsidy promotes the industrial transformation and upgrading, especially in high-pollution industries. These departments are less dependent on traditional coal-based energy, due to the improvement in production flow and processes. The consumption of coal-based energy is further reduced by the increase in total factor productivity induced by the R&D subsidy. Second, the R&D subsidy drives the development of new energy production, leading to lower prices of energy

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

supply and energy service. Thus, the traditional departments could use new energies at a much lower cost, which indirectly optimizes the energy structure of high-pollution departments.

Table 6. *Impact of R&D subsidy on energy consumption of each pollution industry*

Energy consumption	**Model (9)**	**Model (10)**	**Model (11)**	**Model (12)**
Subsidy	-0.0458*** (0.0103)		3.93e-06 (0.000243)	
Subsidy (t-1)		-0.0356*** (0.0126)		-6.11e-05 (0.000329)
Size	28.88*** (7.791)	33.24** (12.87)	0.670 (0.530)	-0.160 (0.615)
Fixed asset investment	-0.459* (0.237)	-0.385 (0.300)	0.0101 (0.0169)	0.0227 (0.0196)
Energy price index	-27.66 (23.60)	-37.55 (34.30)	-1.968 (1.250)	1.858 (2.014)
Export	-0.566 (0.873)	0.0922 (1.370)	-0.0147 (0.0109)	-0.00929 (0.0125)
Revenue	0.143* (0.0760)	0.117 (0.114)	0.0346*** (0.00571)	0.0474*** (0.00685)
R&D expenditure	0.00336*** (0.000699)	0.00326*** (0.000968)	-0.000138*** (3.09e-05)	-0.000183*** (3.57e-05)
Cons_	1,765 (3,314)	345.8 (4,393)	530.5*** (171.8)	95.81 (249.2)
Wald chia2/F	197.50 (0.0000)	14.53 (0.0000)	211.42 (0.0000)	194.74 (0.0000)
Time	Yes	Yes	Yes	Yes
Hausman test	6.48[0.1659]	10.85[0.0543]	2.71[0.4378]	4.30[0.2308]
Observations	112	96	140	120
R^2	0.680	0.582	0.592	0.604
	High polluting sectors	High polluting sectors	Low polluting sectors	Low polluting sectors

Significance: ***p < 0.01, **p < 0.05, *p < 0.1

Discussion

In China, the R&D subsidy has a direct and targeted effect on the total energy consumption. For one thing, the Chinese government wants to promote industrial transformation and upgrading through subsidising the R&D activities of enterprises in an industry. The subsidised enterprises are required to invest the subsidy to R&D activities. In this way, the R&D subsidy pushes up industrial R&D expenditure, and in turn the overall technical level and productivity of the industry. This is conducive to the energy conservation and emission reduction in the short- and long-term, and the efficiency of energy use, which slows down the growth of energy demand in China's industrial sector. For another, the R&D subsidy favours high and new technology enterprises. Since these enterprises are more likely to receive a huge amount of R&D

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

subsidy, the high and new industries will expand at the cost of the traditional industries. The spill-over effect of these industries will spread new technologies across different sectors, slowing down the growth of energy demand in China's industrial sector.

Table 7. *Impact of R&D subsidy on energy structure of each pollution industry*

Energy structure	**Model (13)**	**Model (14)**	**Model (15)**	**Model (16)**
Subsidy	-1.34e-07** (2.28e-07)		2.02e-07 (9.87e-08)	
Subsidy (t-1)		-5.69e-07** (2.64e-07)		-1.12e-07 (1.39e-07)
Size	0.000652*** (0.000203)	0.000281 (0.000269)	0.00142*** (0.000307)	0.00126*** (0.000362)
Fixed asset investment	-1.80e-05*** (5.40e-06)	-1.76e-05*** (6.28e-06)	-9.84e-06 (6.74e-06)	-4.22e-06 (8.05e-06)
Energy price index	-0.00145*** (0.000517)	-0.000364 (0.000717)	-0.00101** (0.000471)	2.24e-05 (0.000785)
Export	4.12e-06 (2.12e-05)	3.63e-05 (2.87e-05)	-8.78e-06* (5.13e-06)	-6.66e-07 (6.64e-06)
Revenue	-6.41e-07 (1.72e-06)	1.73e-06 (2.39e-06)	-3.27e-06 (2.50e-06)	-2.07e-06 (3.20e-06)
R&D expenditure	1.64e-08 (1.57e-08)	-1.05e-08 (2.03e-08)	1.49e-08 (1.25e-08)	8.87e-10 (1.52e-08)
Cons_	0.351*** (0.0638)	0.242** (0.0919)	0.119* (0.0708)	-0.0156 (0.109)
Wald chia2/F	5.79 (0.0000)	3.03 (0.0075)	6.86 (0.0000)	4.03 (0.0007)
Time	Yes	Yes	Yes	Yes
Hausman test	10.32[0.0354]	13.94[0.0160]	16.15[0.0028]	11.46[0.0095]
Observations	112	96	140	120
R^2	0.313	0.225	0.298	0.233
	High polluting sectors	High polluting sectors	Low polluting sectors	Low polluting sectors

Significance: ***p < 0.01, **p < 0.05, *p < 0.1

The short-term optimization of the energy structure relies on the changing scale of different industries. For one thing, low-pollution and high-tech industries expand exponentially under the R&D subsidy. These industries consume much more power and new energy than the traditional ones, which lowers the total consumption of traditional energy. For another, the traditional industries are shrinking and reorganizing. To control pollutant emissions, the Chinese government has implemented strict policies on emission control, and even shut down some small enterprises with high pollution and high energy consumption. For industrial transformation and upgrading, the government require traditional industries to merge and reorganize, in addition to issuing R&D subsidy to enterprises. To realize economies of scale and energy conservation, some traditional enterprises have implemented bankruptcy procedures and resource

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

restructuring of their subsidiaries, and directly diverted the R&D subsidy to technical progress. These measures have improved the energy structure of traditional industries to a certain extent, and affected the energy structure of the entire industrial sector in the short term.

Conclusions

This paper studies the impacts of R&D subsidy on industrial energy consumption and the energy structure, and further analyses how these impacts vary between high- and low-pollution departments. It is concluded that the R&D subsidy has indeed slowed down the growth of energy consumption in China's industrial sector, thanks to the positively impact on technology level. According to pollution heterogeneity-based analysis, the slowdown effect is attributed to the change of energy consumption in China's high-pollution departments. In other words, the change of energy consumption in high-pollution departments determines the shift in energy consumption of China's industrial sector. It is also concluded that the R&D subsidy has optimized the energy structure of high-pollution departments through the technical progress and substitution effect. The technical progress is the result of the industrial transformation and upgrading in each department, while the substitution effect means the displacement of traditional coal-based energy with low-price new energies. As a result, high-pollution departments are the main beneficiaries of the R&D subsidy, and the key to energy conservation and emission reduction in China.

The author put forward the following recommendations according to the research conclusions. First, the high-pollution departments should receive even more R&D subsidy. The subsidy should be directed at the optimization of the production flow in high-pollution departments, aiming to boost the innovation in production flow and product technologies. This will benefit industrial transformation and upgrading, as well as energy conservation and emission reduction. More R&D subsidy should be allocated to industries that have successfully upgraded and transformed their technologies, turning them into leaders in industrial transformation and upgrading.

Second, the receivers of the R&D subsidy should be further clarified. The existing distribution mode of R&D subsidy must be replaced with one that allocates special funds to key receivers. For high-pollution departments, a group of enterprise with strong technical skills and in good business conditions should be selected to enjoy special funds for technical transformation and upgrading. For enterprises and departments with weak technical skills and in poor business conditions, the R&D subsidy should be gradually reduced to prevent misappropriation. For low-pollution departments, more R&D subsidy should be allocated to some key industries to improve the scale and technology of high and new industries.

Third, the technological progress should be further shared between different departments. The spill-over and transformation of technological results are important drivers of energy conservation and emission reduction in China. The government should encourage high and new technology enterprises, especially new energy enterprises, to implement technological transformation, and promote the introduction and application of new energies and new technologies in high-pollution departments. These moves will optimize the energy structure of the entire industrial sector, curb the growth of energy consumption, and suppress pollutant emissions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

For the future studies, some research issues need to be given further discussion. On one hand, the researchers should pay more attention to the relationship between R&D subsidy and innovation. R&D subsidy provides more support for enterprises on technology development because those enterprises would invest more funds in technology development after getting R&D subsidy. And that, for the measurement of innovation, the researchers should select a new method of measuring innovation instead of patent used in previous articles. On the other hand, the researchers focus not only on innovation but also other approaches of technological progress, such as technological cooperation and technological exchange.

Acknowledgements. The research-launching funding project for introduced high-level talents.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48554867

CARBON FLOW COST CONTROL OF COAL-FIRED POWER PLANT BASED ON "ENERGY FLOW–VALUE FLOW" ANALYSIS

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. In response to the commitment that China will begin a comprehensive national carbon emissions trading system in 2017, and considering the needs of a green development strategy, in this paper we adopt a coal-fired power generation company as the research object to study its typical carbon emissions characteristics. Through theoretical analysis and modelling dominated by energy characteristics, the energy flow analysis is separated from the material flow analysis, and we construct an "energy flow—value flow" analysis model. Based on the link between energy use and carbon flow, we divide carbon flow cost into two parts: low carbon control costs and internal and external damage costs. A quality cost control idea is introduced to build a quantitative carbon flow cost control model, and we study the relationship between the total cost of carbon flow and the control of carbon pollution. The case calculation shows that coal enterprises should begin by examining their energy flow, invest reasonably in measures to control carbon, improve their energy efficiency, enhance their control of carbon flow costs and actively focus on relevant policies that control carbon flow effectively; following this process will allow them to be proactive in the new carbon market.
Keywords: *energy flow analysis, value flow analysis, carbon flow, cost control model, model application*

Introduction

Emitting greenhouse gases (primarily CO_2) cause externalities that have environmental effects on all of mankind (Cao and Zhang, 2010; Lodi et al., 2017). Since the Kyoto Protocol promulgated in 1997, the external costs of greenhouse gas emissions have been gradually internalized by market mechanisms (Liu and Huang, 2011). Thus, if today's businesses ignore the costs associated with greenhouse gas emissions, they may face difficulties in terms of environmental payments (Zhang et al., 2005). After extensive long-term economic development, China has begun to emphasize the transformation and upgrading of its economic structure. In particular, industries that have traditionally emitted high levels of carbon dioxide, such as power generation, steel, and coal among others, are now being strictly regulated to protect the environment (Capoor and Ambrosi, 2009; Zhang, 2017). The National Development and Reform Commission announced in January 2016 that progress has been made toward implementing a national carbon emissions trading market (Lu et al., 2010; Tu and Ma, 2018), and China pledged in 2017 to begin its carbon emissions trading system. In this context, clarifying the cost of carbon flow for enterprises will help them control costs, enhance their corporate image, and enable them to occupy a strong competitive position in the new carbon market.

The 13th Five-Year Plan (2016-2020) emphasized the importance of low carbon development, required enterprises to take the initiative to control carbon emissions and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48694882

strengthen control of their energy consumption. Controlling carbon emissions requires tracking the carbon flow path and calculating relevant costs. The core of studying carbon flow cost involves quantifying the flow of carbon energy inside the enterprise. From an economic standpoint, both carbon energy and value flow during an enterprise's production and management, and both flows affect the enterprise's financial costs (Wang, 2015). Therefore, we focus on the dual nature of energy and value flows, with high carbon emissions from coal-fired power generation enterprises as our research object. This paper constructs a simulation of carbon flow cost control based on "energy flow-value flow" to provide a model for the concrete implementation of carbon flow cost control.

Research methods

Energy flow, value stream analysis method

Energy flow analysis is a category of material flow analysis. During the 1980's, scholars studied the interaction between economic systems and the natural environment and developed a system known as material and energy flow accounting (MEFA) (Chang, 2012). In 2001, through an analysis of the energy flow of national economic systems, Harberl and others put forward a material and energy flow accounting framework and applied it to analyze sustainable development problems (Helmut et al., 2004). Teresa Torres applied material and energy flow accounting to analyze the status of clay in roofing tiles (Torres et al., 2008). Chinese scholar Li Xingji, after analyzing the role of logistics in urban pollution, put forward the idea of using energy flow accounting to protect the urban environment (Li, 1979). Liu et al. (2011) and other scholars wrote that energy flow accounting helps reduce environmental pollutants, because it provides a theoretical basis for enterprises' efforts to save energy and reduce emissions (Liu et al., 2017). The core of energy flow analysis is the concept of energy flow management (Bendriss et al., 2017; Makni et al., 2017). Quantitatively analyzing energy flows produced by social economic activities will help us to understand and best utilize the flow and flux of energy.

In 1997, the value stream concept was proposed by James Martin, an American management scientist, who believed that value streams exist in a group from the beginning to the end of continuous activities, and that customers were satisfied by consuming all kinds of resources as cost flows (James, 1997). Womack et al. (1997) and other scholars proposed that resources, time and costs could be saved by using value stream analysis to analyze production and maximize product values (Lalami et al., 2017). Xiao and Liu (2004) and others scholars wrote that "flow" can be used to reveal the direction, speed and strength of the interactions among different elements, and that the concept of "flow" could be used to measure the value of diverse elements dynamically. The practical application of value stream analysis requires the collection of information and the analysis of data. By managing value stream analysis, enterprises can gain a competitive advantage (Zhang and He, 2002).

Existing research has focused mostly on material flow and value stream analysis, without stripping out source analysis. This paper focuses on the costs of the carbon flow of coal-fired power enterprises, for which energy is the main input material. We argue that an energy flow and value stream analysis method is more targeted than a material flow and value stream analysis.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48694882

The relationship between energy utilization and carbon flow cost

Carbon element flows are the carrier of fossil energy. The energy input mainly consists of the combustion of coal in coal-fired power enterprises, and energy is released by the oxidation of carbon. Along the flow path of this fossil energy, carbon is transformed during the different production processes and eventually is fixed into products or becomes CO_2 emissions that are released into the external environment. Scholars such as Yin et al. (2013) wrote about a "low-carbon revolution" that would focus on energy saving and emission reduction; use less coal, oil and other carbon-containing energy sources; and ultimately reduce the negative effects on the external environment. Following the internal transport path of carbon energy and calculating the value stream may help enterprises understand the value of energy flows in their production processes and allow them to control the cost of their carbon flows. There are six strands energy flow that influence the unit process of carbon flows in coal-fired power enterprises, as shown in the following *Figure 1:*

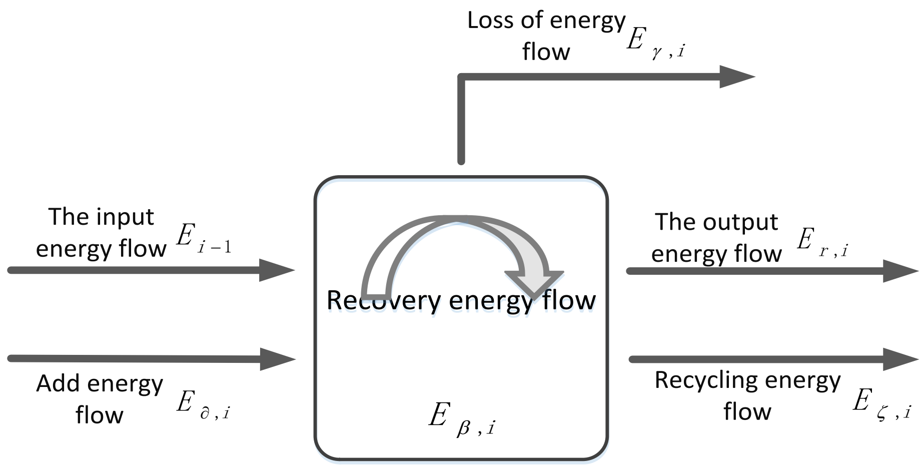

Figure 1. *Coal-fired power generation enterprise unit process flow diagram*

As *Figure 1* shows, the energy flow suggests that the process of the i-1 is the i process of energy flow, and it represents the flow of external added energy, including fuel, electricity, steam and other means. *Figure 1* also suggests that the output of energy is an energy flow loss. It shows that the recovery of energy refers to the process of recycling during the production of energy flow.

Concept and classification of carbon flow cost

Scholars of ecology, energy science and engineering have written that carbon flow draws on the resource flow cost and the concept of energy and value flow; however, they have not focused on carbon flows. Qi et al. (2010) proposed studying carbon flows from a homogeneous carbon source to carbon sequestration. In a coal-fired power generation enterprise, carbon energy is also produced in the process of the treatment.

Carbon flow cost may be evaluated by examining environmental and carbon costs. Gray and Bebbington (1993) used the concept of life cycle to argue that carbon costs include all costs associated with carbon emissions that may occur in the production process. Wang et al. (2011) wrote that carbon costs may be divided into ex ante costs,

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testing costs and loss costs. Considering the production cost, the cost of detection and prevention before and after the production of carbon flows, and the cost of these carbon flows, cost may be divided into four categories: the cost of carbon flow prevention, inspection costs, and the carbon emissions themselves (including carbon trading and carbon tax).

Model design

Content of carbon flow cost control

Carbon cost flow control is also dependent on the concepts of carbon costs and environmental cost control. USEPA introduced relevant environmental cost control tools and pointed out that government and enterprises should jointly bear the responsibility for controlling environmental costs. Gray and Bebbington (1993) wrote that comprehensive control should be based on the life cycle of carbon costs. Wang et al. (2011) proposed using the PDCA (plan, do, check, and action) method to control the environmental costs of enterprises. Zhou et al. (2016) believe in controlling the cost of carbon emissions and carbon emission reductions. He and Li (2015) proposed focusing on logistics to optimize energy flows and help companies control the cost of carbon energy.

The existing research on the cost control of carbon flows generally does not focus on the idea of “low carbon”, and little research has been done on the development of China’s carbon market and the specific characteristics of Chinese enterprises. This paper focuses on the quality cost control model and the characteristics of the carbon flow costs of coal-fired power generation enterprises to establish a carbon flow cost control model.

Classification of carbon flow cost control factors

In the middle of twentieth century, American quality management experts Feigenbaum (1961) and Juran (1988) put forward the concept of “total quality management” for the purpose of achieving high quality products with few defects. In this paper, we regard damage to the internal and external environment as a quality defect. Combined with considering the characteristics of carbon flow costs incurred by coal-fired power generation enterprises, this paper divides the carbon flow cost control model into two categories: low carbon control costs and internal and external costs, specific classification as shown in *Table 1*.

Low carbon control costs

Low carbon control costs are mainly divided into two categories: prevention costs and testing costs. The prevention cost is what the enterprise must spend in order to improve energy efficiency, reduce carbon waste emissions, and achieve a “low carbon” design. In a certain range, the cost of prevention is inversely proportional to the cost of the carbon. Detection of cost measures the cost of carbon flows, including training costs and labor costs; these element of the cost remains relatively stable.

Internal and external damage costs

This section contains two factors: the internal loss cost of carbon flow and the external environmental damage cost. The carbon flow internal loss costs include loss cost, processing cost, resource utilization cost and profit loss. Negative cost relative to the cost

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of products includes the output of the products. Processing cost refers to the use of carbon emission reduction devices and the cost of processing. Resource utilization cost and income includes enterprise measures and carbon recycling resources. Carbon flow external environmental damage cost includes 5 elements: (1) carbon waste emissions cost; (2) carbon trading costs and benefits (enterprises must buy emissions quotas issued by the government according to their actual carbon emissions); (3) costs or gains from the sale of surplus quota; (4) carbon taxes, and (5) fines.

If the penalties (costs incurred) for not controlling carbon emissions are increased, then pollution will be reduced, and the cost of internal and external loss will be reduced. However, the cost of internal and external losses will increase. In order to better express the relationship between the two, this paper introduces the quality cost control model.

Table 1. *Classification of carbon cost control model factors in coal-fired power generation enterprises*

Carbon flow cost control model factor classification		
Low carbon control cost	Preventive cost	Design labor cost Equipment purchase Surrounding greening Equipment update and maintenance
	Detection cost	Testing training fee Detection of labor costs
Internal and external damage cost	Internal loss cost of carbon	Lost cost Treatment cost Resource utilization cost and benefit
	Carbon and sulfur external environment loss cost	Carbon waste disposal costs CO_2 Emission cost Carbon trading costs and benefits Carbon tax Fine

Construction of carbon flow models of cost control

Conventional quality cost control models

Mathematical models express the quantitative relationship between quality cost based: C: Quality costs, C_1: Cost identification of prevention, C_2: Quality loss cost, q: average rate of products $(0 \leq q \leq 1)$, F: Unit cost of nonconforming, d: Unqualified product rate $[d = 1 - q(0 < d < 1, 0 < q < 1)]$, x: and thus Output.

From *Equation 1, Equation 2* is obtained.

$$Fdx = C_2qx \quad \text{(Eq.1)}$$

$$C_2 = \frac{Fd}{q} = \frac{F(1-q)}{q} \quad \text{(Eq.2)}$$

Set: K for C_1 with $\frac{d}{p}$ scale factor, then *Equations 3-4* are obtained.

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$$C_1 = \frac{Kq}{d} = \frac{Kq}{1-q} \tag{Eq.3}$$

$$C = C_1 + C_2 = \frac{Kq}{1-q} + \frac{F(1-q)}{q} \tag{Eq.4}$$

C is minimized, that is $C_1 = C_2$, when *Equations 5-6* are obtained.

$$q = \frac{1}{\sqrt{\frac{K}{F}}+1} \tag{Eq.5}$$

$$C = C_1 + C_2 = 2\sqrt{KF} \tag{Eq.6}$$

Early quality cost control models for quality and cost control and management thinking. However, in practice there are quality limitations and problems such as lack of theoretical calculations, such that the intersection curves represent only the low total cost and are contrary to the actual situation. Based on Japan’s successful zero defects theory, there has been improvement in quality cost control models.

Improving cost control model of carbon flow

Japan scholar Dr’s study points out that: “the concept of quality usually refers to the same utility function under products to users with less failure, low energy consumption, long life, high efficiency characteristics, comprehensive loss.” Thus, a product can be drawn to society brought about by the total loss of a function expression, provided with $T_{(q)}$ said. $T_{(q)} = C_{(q)} + L_{(q)}$, Which $C_{(q)}$ the cost function for the enterprise, $L_{(q)}$loss function, $q$the quality characteristics of the product vector. When quality level $q = 1 - d$ ($d$is the carbon losses and the pollution of the environment), then the carbon emissions control level is equal to the success rate of carbon emissions. Taking into account the recent coal-fired power plant investment and production decisions made for long-term economic benefit, we can establish the following mathematical model (*Eq. 7)*.

$$T_{(q)} = C_{(q)} + L_{(q)} \tag{Eq.7}$$

$T_{(q)}$ represents the total cost of the carbon flow and for low carbon costs under control, $L(q)$ is the internal and external damage costs.

Low-carbon model

We used the Cobb Douglas function to represent the relationship between preventive costs C_1 and testing costs C_2 (*Eq. 8*).

$$q = AC_1^{\alpha} C_2^{\beta} \tag{Eq.8}$$

Constraint conditions (*Eq. 9*):

$$C_1 + C_2 = C_{(q)} \tag{Eq.9}$$

Using the Lagrange multipliers method provides *Equation 10*.

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$$\varphi(C_1, C_2, q) = AC_1^{\alpha} C_2^{\beta} - \lambda[C_{(q)} - (C_1 + C_2)] \quad \text{(Eq.10)}$$

where λ is the Lagrange multiplier and *Equation 11* can be obtained.

$$C_{(q)} = q^{\frac{1}{\alpha+\beta}} / [A(\frac{\alpha}{\alpha+\beta})^{\alpha}(\frac{\beta}{\alpha+\beta})^{\beta}]^{\frac{1}{\alpha+\beta}} \quad \text{(Eq.11)}$$

Let $a_1 = [A(\frac{\alpha}{\alpha+\beta})^{\alpha}(\frac{\beta}{\alpha+\beta})^{\beta}]^{\frac{-1}{\alpha+\beta}}$, $b_1 = \frac{1}{\alpha+\beta}$, *Equation 12* can be obtained.

$$C_{(q)} = a_1 q^{b1} \quad \text{(Eq.12)}$$

Internal and external damage cost model

Businesses must pay for ecological damages caused by the pollution they emit. When the deviation $|q - 1| = 0$ shi, then internal and external losses are at a minimum, and constant $L(1) = a_2$ and $a_2 \neq 0$. In$q = 1$, we have the Cheng Tailei series, *Equation 13* can be obtained.

$$L_{(q)} = L(1) + \frac{L'(1)}{1!}(q-1) + \frac{L''(1)}{2!}(q-1)^2 + \frac{L'''(1)}{3!}(q-1)^3 + \ldots \quad \text{(Eq.13)}$$

$L_{(q)}$ in $q = 1$ obtains the extreme-one derivative $L'(1) = 0$. In addition, taking into account the deviation $|q - 1|$ value minimum, and the Taylor expansions, we find that the fourth and following are much smaller than the third, and the internal and external damage cost functions $L_{(q)}$ yield the approximate expression (*Eq. 14*):

$$L_{(q)} = L(1) + \frac{L''(1)}{2!}(q-1)^2 \quad \text{(Eq.14)}$$

Order $a_2 = L(1)$, $b_2 = \frac{L''(1)}{2!}$, *Equation 15* can be obtained:

$$L_{(q)} = a_2 + b_2(q-1)^2 \quad \text{(Eq.15)}$$

Total cost of carbon control model

As mentioned above, the total cost of carbon $T_{(q)}$ comprises low carbon, control costs and external costs, and thus *Equation 16* can be obtained from *Equations 12* and *15:*

$$T_{(q)} = C_{(q)} + L_{(q)} = a_1 q^{b1} + a_2 + b_2(q-1)^2 \quad \text{(Eq.16)}$$

where $a_1 = [A(\frac{\alpha}{\alpha+\beta})^{\alpha}(\frac{\beta}{\alpha+\beta})^{\beta}]^{\frac{-1}{\alpha+\beta}}$, $b_1 = \frac{1}{\alpha+\beta}$, $a_2 = L(1)$, $b_2 = \frac{L''(1)}{2!}$.

Equation 16 shows the total cost of carbon $T_{(q)}$. Numerically, it is low carbon cost $C_{(q)}$ with internal and external damage costs $L_{(q)}$ overlaid. $C_{(q)}$ has a relationship with q in

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the form of indices, Therefore, the logarithmic processing on both sides of *Equation 17* is obtained.

$$log(C_{(q)}) = log^{a_1} + b_1 \, log^q \quad \text{(Eq.17)}$$

$log(C_{(q)})$ with log^q constitutes a slope b_1. The intercept of log^{a_1} A is a linear relationship, yielding a different$q$. $C_{(q)}$ values can be obtained by linear regression a_1 and b_1. The values obtained $C_{(q)}$ with q have a relationship.

On the other hand, $L_{(q)}$with$q$provides a functional quadratic expression, but when $(1-q)^2 = q_1$ are the variables, we get *Equation 18*.

$$L_{(q_1)} = a_2 + b_2 q_1 \quad \text{(Eq.18)}$$

As can be seen in the $L_{(q_1)}$ with log^q formation of slope b_2, the intercept a_2 is a linear relationship, and the different values for q, $L_{(q)}$, a_2 and b_2 can be obtained by linear regression.

For the $C_{(q)}$, $L_{(q)}$ and $L_{(q_1)}$ expressions, we can discuss q and the $T_{(q)}$ impact of its extremes.

Application of carbon flow models of cost control

Data collection and processing

This paper takes a large coal-fired power plant as its study object; such plants use inputs like water, coal, oil, natural gas and other types of raw energy, convert them into electric secondary energy forms, which become inputs in the power grid and provide energy to users. A typical energy conversion of a coal-fired power plant consumes many resources. Power plants convert the chemical energy of fuels (mainly coal into electrical energy via the transformation process shown in *Figure 2*. *Table 2* provides carbon cost data for a coal-fired power plant during the period from 2010 to 2015. *Table 3* shows more data for the same time period.

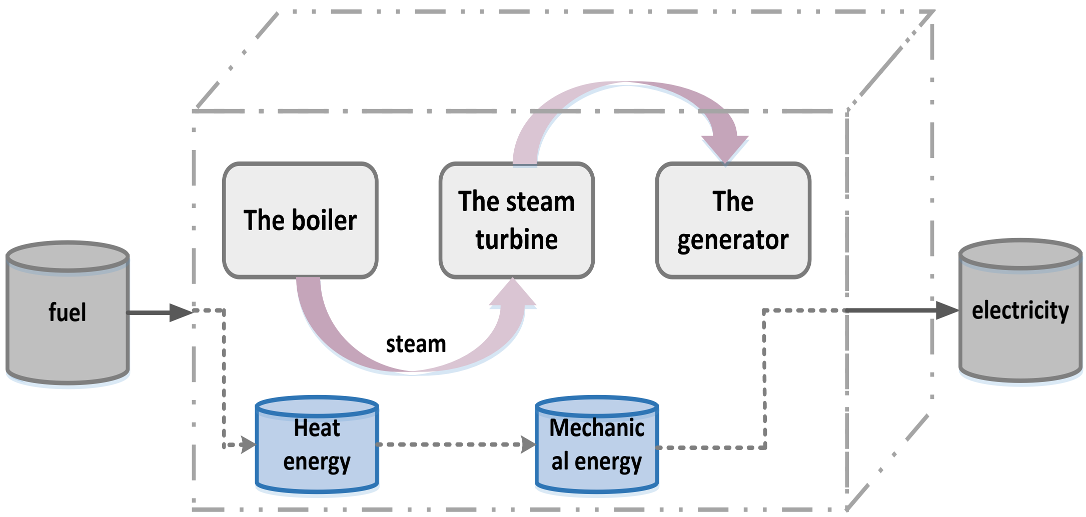

Figure 2. *Energy transformation diagram of a coal-fired power plant*

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Table 2. *Carbon costs of a coal-fired power plant from 2010~2015*

Cost of carbon flow classification	Cost items	2010	2011	2012	2013	2014	2015
Low carbon cost	The cost of prevention	94.40	109.50	129.20	140.50	186.51	259.40
	Testing costs	19.07	27.20	40.30	63.80	72.70	88.90
	Subtotal (C)	113.47	136.70	169.50	204.30	259.21	348.30
Internal and external damage costs	Carbon flow loss cost	629.80	553.01	420.76	358.90	320.80	301.60
	Carbon external environmental damage costs	361.69	326.04	252.04	179.24	125.12	102.09
	Subtotal (L)	991.49	879.05	672.80	538.14	445.92	403.69
Total (T)		1,104.96	1,015.75	842.30	742.44	705.13	751.99
Comprehensive compliance rate (%)		87.27	88.98	90.72	92.14	93.71	95.46

Table 3. *Statistics for a coal-fired power plant for the period 2010~2015*

Vintage	2010	2011	2012	2013	2014	2015	Energy-saving Total (tce)
Electricity generation (million kWh)	5,401.48	6,505.38	6,000.00	6,000.00	6,000.00	6,000.00	437.57
Power supply coal consumption (g/kWh)	310.32	309.47	307.94	305.70	303.90	302.13	
Standard coal (tce)	41.27	62.10	91.80	134.40	108.00	106.20	

From *Tables 2* and *3*, one may see the relationship among energy-saving, internal and external damage costs and the costs of low carbon control (see *Fig. 3*). The increase in energy-saving, source reduction, and low carbon control cost allows for a reduction in carbon-containing waste material and in internal and external damage costs.

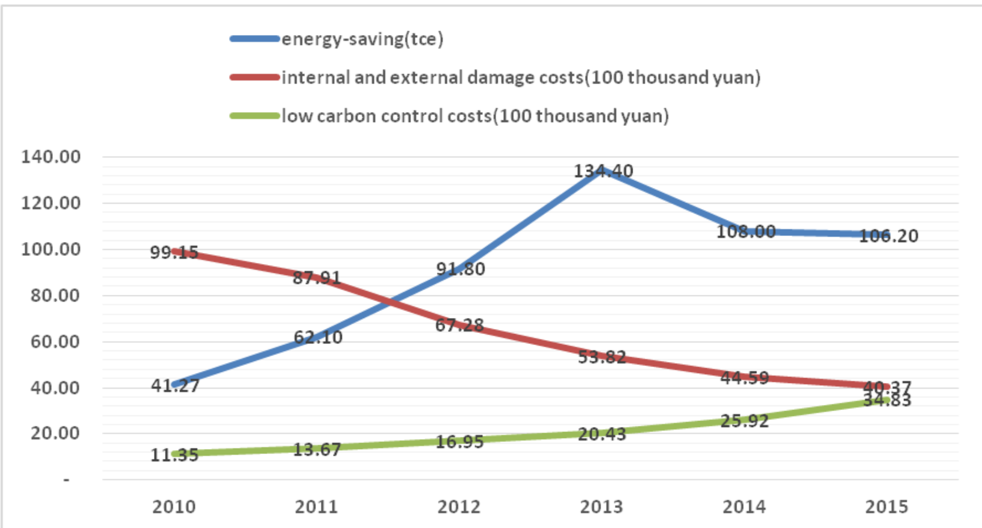

Figure 3. *The relationship among energy-saving, internal and external damage costs and low carbon control costs*

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Results and discussion

Based on the expression of functions $C_{(q)}$ and $L_{(q)}$ in combination with the data in *Table 2*, we used MATLAB software and an analysis of statistical regression models to calculate the variable parameters of our model. The results of the main performance parameters of the model are shown in *Table 4*. As calculated by *Equations 19* and *20,* the original scattered data points and the fitting result curves are shown in *Figure 4*. From *Table 1*, we can see the equivalent substitutions for $C_{(q)}$ and $L_{(q)}$ and the significant linear relationship q. The R^2values were 0.986 and 0.978, and the significance levels F were 422.26 and 261.932. The F values show that the statistical equations and the calculated P order of magnitude 10^{-6} were far less than the criterion value of 0.05. This proves that this paper constructed a regression model with high reliability.

Table 4. *Calculation results of* $C_{(q)}, L_{(q)}$ *and* $T_{(q)}$

	R^2	F	P	MSE
C(q)	0.986	422.26	8.6e-7	17.7885
L(q)	0.978	261.932	3.53e-6	29.1789
T(q)	--	--	--	46.5544

$$C_{(q)} = 669.73q^{13.74} \quad \text{(Eq.19)}$$

$$L_{(q)} = 337.5 + 40460(1\text{-q})^2 \quad \text{(Eq.20)}$$

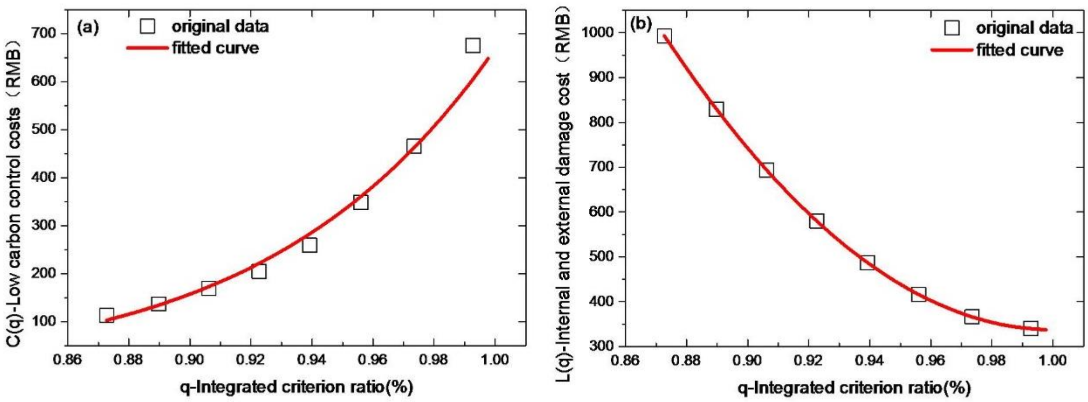

Figure 4. *Comparison of scatter plots and fitting curves*

As can be seen from *Figure 4*, *Equations 19* and *20* fit the regression of low carbon cost control, and of the internal and external damage costs, with the original plot value and the change trend of q. When the variable q is gradually increased, the corresponding function $C_{(q)}$ (low carbon control costs) gradually increased, and $L_{(q)}$ (the value of internal and external damage costs) gradually decreased.

According to the relationship between $C_{(q)}$ and $L_{(q)}$, the total carbon flow cost control function is obtained (*Eq. 21*).

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$$T_{(q)}=C_{(q)}+L_{(q)}=669.73q^{13.74}+337.5+40460(1-q)^2 \qquad \text{(Eq.21)}$$

We then combine *Equations 19* and *20* to draw $C_{(q)}$, $L_{(q)}$, $T_{(q)}$ and q with the change of the curve, as shown in *Figure 5*.

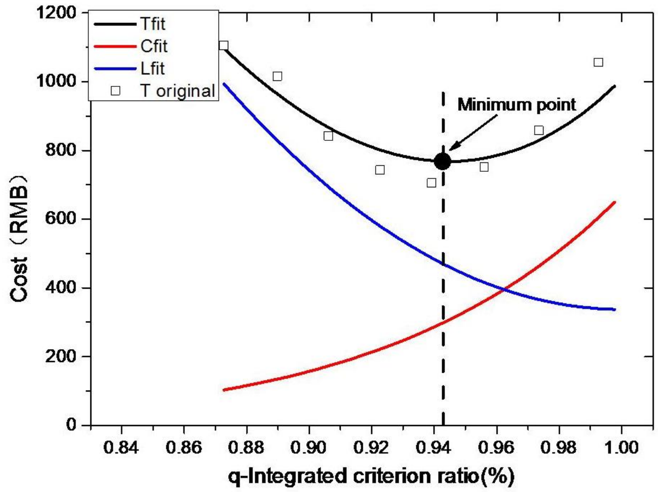

Figure 5. *Comparison of scatter plots and fitting curves*

Figure 5 shows that q has different effects on $C_{(q)}$ and $L_{(q)}$ trends, and $T_{(q)}$ decreases gradually with the increase of q, and after reaching its lowest point, it then increases gradually again. The minimum value as the black circle in the x = 0.942 graph represents the position and the corresponding gross $T_{(q)}$ is 768.0516.

Table 2 shows that in 2010, coal-fired power plants implemented certain low carbon control cost measures, and while the effect on the cost of carbon flow control was not obvious, the potential for a reduction in emissions is enormous. Beginning in 2011, power plant shave been gradually screened for small energy sources. Significant increases have been made in the research and development of low carbon technology, and energy utilization rates have improved. In 2014, the total carbon flow cost reduction was 3,998,300 yuan, compared with a 2010 decrease of about 36.18%. Low carbon control costs doubled, compared to the cost of internal and external damage, which decreased to 44.97% in 2010. In particular, the reduction of coal consumption reduced the cost of carbon flow. Improved technology, low carbon control costs, and energy conservation clearly help to reduce the total carbon flow cost. When the pollution control level is more than 94.2%, the rate of the enterprise’s low carbon cost increases more than the rate of internal and external damage costs declines, which leads to an increase in the total carbon flow cost. Therefore, in the long run, low carbon cost control is not possible for coal-fired power generation enterprises. Enterprises should avoid excessive investment in low carbon balance, and in the control of costs, because these measures result in unnecessary waste. *Figure 5* shows that the carbon flow cost curve improves, according to the specific situation of enterprises, depending on the low carbon control and damage cost functions. The lowest total cost of carbon flow is calculated on this basis, and likely the two intersection functions may also be offset, if we used an improved model data to compensate for the lack of the original model.

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Conclusion

Against the background of climate warming, the analysis and control of carbon flow cost is an important condition to understand in order for industrial enterprises to realize sustainable development. In this paper, based on the concept of “energy flow and value flow,” and using an improved model of carbon flow cost control, the following conclusions and implications are obtained:

Coal-fired power generation enterprises should take the initiative to control the cost of carbon flow, at a reasonable increase in the cost of low carbon control inputs. Low carbon control costs should reduce the cost of carbon flow, which is an important guarantee for an enterprise. However, because low carbon cost control is not possible, enterprises should aim to achieve the optimal emissions compliance rate to control their carbon flow cost, as well as to avoid losing control because of a lack of investment, and to avoid wasting resources by focusing too much on low carbon investment control.

Further increasing R&D investment and power will maximize the utilization rate of energy and reduce the coal consumption. Based on the “energy flow, value flow” concept, enterprises should pay attention to the optimization and control of carbon flow cost from the source. First, they should choose low pollution sources of energy and materials and reduce their unit energy flow. Second, they should increase low carbon technology development and invest more in carbon flow loss processing equipment to ensure the transition to becoming green, low carbon enterprises.

Coal-fired power generation enterprises should combine their own characteristics to improve the cost control system of their carbon flow. The key to improving carbon flow cost curves is determined according to the actual situation of the enterprises, as well as to their low carbon control and damage cost functions. Given this, in this paper, we used statistical analysis software to find a comprehensive compliance rate for the control of carbon flow in a coal-fired power enterprise; this rate is of practical significance for the enterprise’s cost control.

The accounting of carbon flow costs in coal-fired power generation enterprises is relatively wide-ranging. To accurately calculate and achieve carbon flow cost control, a lot of in-depth and meticulous research is needed. Future research can consider the following aspects. (1) It is relatively easy to quantify the cost of low carbon control and the internal loss of carbon flow. However, the technology that can be used to determine the external loss cost according to the degree and type of energy damage needs to be further improved. In addition, accurate accounting of carbon emissions remains a challenge. These will affect the collection of cost data, which in turn affects the model analysis. (2) We may have different classifications for various cost items of coal-fired power generation enterprises. After accurate accounting of carbon flow costs, it is necessary to further study the classification according to what standards. (3) When testing the model, the target company needs a large amount of carbon flow cost data for many years. The lack of data will affect the model rationality test. In the future, more data can be collected to test the model.

Acknowledgements. This research was supported by the Projects of National Social Science Foundation (18BJY085), the Natural Science Funds of Hunan Province (2017JJ3399), the Think Tank Special Project of Hunan Social Science (18ZWB22), the Project of Social Science Achievement Evaluation Committee of Hunan Province.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48694882

THE ROLE OF *DREB2* GENE IN DROUGHT TOLERANCE OF COMMON WHEAT (*TRITICUM AESTIVUM* L.) ASSOCIATED WITH *AZOSPIRILLUM BRASILENSE*

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(Received 31st Jul 2018; accepted 27th Feb 2019)

Abstract. Dehydration causes loss of wheat (*Triticum aestivum* L.) yield. Inoculation of wheat with *Azospirillum brasilense* improves its tolerance to drought. Although up-regulation of *DREB2* gene has increased drought tolerance of wheat, less information exists about *DREB2* expression under inoculation with *A. brasilense*. In this study, the physiological responses of different pairs of six wheat cultivars with *A. brasilense* Sp7 and Sp245 strains were evaluated to drought and the tolerant (Roshan-Sp245), sensitive (Shahpasand-Sp7), moderately tolerant (Roshan-Sp7) and moderately sensitive pairs (Shahpasand-Sp245) were selected. Afterward, in the second experiment, *DREB2* expression of the selected pairs was evaluated under inoculation and/or dehydration (ψ_w = -90 MPa) at 0, 120 and 360 minutes. At 120 min, *DREB2* expression was more in the tolerant and moderately tolerant pairs than others and also higher in inoculated and/or dehydration conditions. Root's *DREB2* was up-regulated almost 700-fold in the tolerant and moderately tolerant pairs under dehydration. In contrast, in the sensitive cv., *DREB2* expression did not change due to dehydration or inoculation. This could be the effect of compatibility or homology of *A. brasilense* strains and wheat cultivars which affected *DREB2* expression. Therefore, *DREB2* expression had a significant effect on increased drought tolerance in tolerant and moderately tolerant pairs.
Keywords: *wheat, Azospirillum brasilense, Sp7, Sp245, drought tolerance, DREB2 gene, dehydration*

Abbreviations: *DREB2* gene, DRE-binding factor gene; FC, Field capacity; PAL, Phenylalanine Ammonia lyase enzyme (EC 4.3.1.24); TAL, Tyrosine Ammonia lyase enzyme (EC 4.3.1.23); RDW, Root Dry Weight; SDW, Shoot Dry Weight; MSL, Maximum Shoot Length; ARL, Average Root Length; NMR, Number of Main Roots; NRB, Number of Root Branches; RTP, Root Total Protein; STP, Shoot Total Protein; RPAL, Root PAL activity; SPAL, Shoot PAL activity; RTAL, Root TAL activity; STAL, Shoot TAL activity

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

Introduction

Drought is one of the major or more significant stresses which adversely affect the plant water status and crop yield (Kramer and Boyer, 1995; Bray, 2000). Dehydration or loss of water causes reduction of cell turgor pressure, an increase in negative water potential and consequently less plant production (Liu et al., 2005). *Azospirillum brasilense* is one of the PGPRs (Plant Growth Promoting Rhizobacteria) that can associate with wheat and increase crop quality and quantity through suitable physiological and biochemical modification in plants (Dobbelaere et al., 2003; El-Samad and Hetta, 2005; Pereyra et al., 2006; Hungria et al., 2010; Veresoglou and Menexes, 2010). The effect of PGPRs on dehydration responsive genes in plants has been evaluated but not well studied specifically about the *DREB2* gene expression during inoculation of wheat with *A. brasilense*. Therefore, the objective of this study is to evaluate the effect of *A. brasilense* on *DREB2* gene expression in wheat cultivars under dehydration condition.

Review of Literature

The ability of *Azospirillum* to establish an association system with plants such as wheat through which it promotes better growth and development (Dobereiner and Day, 1976; Bashan and Holguin, 1997) is due to its capacity for N_2 fixation capacity (Bermner et al., 1995) and production of some bacterial compounds having plant phytohormone properties such as nitric oxide, IAA (indole-3-acetic acid) polyamines and amino acids (Hartmann et al., 1994; El-Khawas and Adachi, 1999; Pakdaman et al., 2014). These bacterial compounds produced in wheat rhizosphere caused structural changes in wheat roots, stimulated root growth and increased water and minerals absorption by the plant (Perrig et al., 2007; Amooaghaie et al., 2004; Casimiro et al., 2003; Correa-Aragunde et al., 2004). These advantages for plants are also reported at dehydration condition for different pairs of plant and bacteria especially at the early stage of growth (Bashan and Holguin, 1997; Hartmann and Baldani, 2006; Yang et al., 2009). For instance, promotion of dehydration tolerance is reported in different associated pairs obtained from some *A. brasilense* strains and wheat (*Triticum aestivum* L.) cultivars (Díaz-Zorita and Fernández-Canigia, 2009).

PGPRs can affect the expression of stress-responsive genes and thus modulate plant responses to stress (Jakab et al., 2005; Gachomo et al., 2014; Barnawal et al., 2017). For example, up-regulation of *RD29A* gene in *Arabidopsis* plants inoculated with a PGPR strain (*Bradyrhizobium japonicum* IRAT FA3) was observed when compared to non-inoculated plants (Jakab et al., 2005; Gachomo et al., 2014). *DRE/CRT* (Dehydration responsive element/C-repeat), a cis-acting element in the promoter region of *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 2005; Jia et al., 2012; Gachomo et al., 2014) interacts with a series of transcription factors known as DREBs proteins (DREB/CBF; DRE-binding factor/C-repeat binding factor) inducing a set of downstream dehydration responsive genes that improve dehydration tolerance of plants (Baker et al., 1994; Sakuma et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). *DREBs* genes family which encode DREBs proteins has a main role in the gene expression and regulation of plant’s response to dehydration stress (Sakuma et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005; Agarwal et al., 2006, 2010). A rapid and transient increase in *DREB2* expression causes an increase in the regulatory and/or functional proteins which shows the upstream role of *DREB2* in a signaling pathway and improves plant tolerance to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

short-time dehydration. Recently, the effect of some PGPRs such as *Arthrobacter protophormiae* (SA3), *Dietzia natronolimnaea* (STR1) and *Bacillus subtilis* (LDR2) on *TaCTR1/TaDREB2* expression (Barnawal et al., 2017) and improvement of wheat tolerance to abiotic stress has been reported but, the effect of *A. brasilense* on *DREB2* expression is not well studied in inoculated wheat (*T. aestivum* L.) cultivars under dehydration condition.

Materials and methods

Primary experiment

A primary experiment (unpublished data) was conducted to choose the most tolerant and sensitive cultivars to drought as well as drought tolerant and sensitive associated pairs among the combination of six wheat cultivars of common wheat (*T. aestivum* L.) named Roshan, Sardari, Tabasi, Shahpasand, Omid and Shoaleh inoculated with two strains of *A. brasilense* called Sp7 (as standard strain) and Sp245 (reported to produce significant ABA). The seeds were prepared from Agriculture and Natural Resources Research Center, Isfahan, Iran. The strains of *A. brasilense* strains were obtained from Research Association of AMP Research Unit Microbe-Plant Interactions, Germany.

The growth and biochemical parameters of six wheat cultivars as well as their combinations with two strains of *A. brasilense* (Sp7 and Sp245) including roots and shoots dry weight (RDW, SDW), average roots length (ARL), number of main roots (NMR) and the number of root branches (NRB), maximum shoot length (MSL) as well as total protein of roots and shoots (RTP, STP), PAL enzyme activity in roots and shoots (RPAL, SPAL) and activity of TAL enzyme in roots and shoots (RTAL, STAL) were measured. Adopted method of Bradford (1976) was used for protein assay and the method of Beaudoin-Egan and Thorpe (1985) was used for TAL and PAL enzymes activity analysis. The experiment was conducted under drought condition (40% of field capacity) and compared to 80% moisture content (data were not shown). Then, the data was ranked for the best and the worst responses to drought (*Appendix 1*). Afterward, according to the measured parameters, the two cultivars of Roshan and Shahpasand were selected as drought tolerant and sensitive cultivars, respectively. Simultaneously, the associated pairs of cultivar-strain including Roshan-Sp245, Roshan-Sp7, Shahpasand-Sp7 and, Shahpasand-Sp245 were selected as tolerant, moderately tolerant, sensitive and moderately sensitive to drought within all possible combinations of wheat cultivars and *A. brasilense* strains (*Appendix 1*). Then, in the second experiment, the selected cultivars and associated pairs were evaluated for *DREB2* gene expression under optimum (ψ_w = 0 MPa) and dehydration (ψ_w = -90 MPa) conditions.

Second experiment

A. brasilense strains were cultured on solid NFB (Nitrogen free basal) medium and incubated at 32°C. Then the bacteria were cultured in liquid NFB medium enriched with ammonium chloride (0.1% w/v, 1 g L^{-1}) and incubated at 30°C for 48 h. Bacterial concentration of 10^8 CFU mL^{-1} was adjusted by reading optical density at 600 nm using McFarland table (Krieg and Dobereiner, 1984; McFarland, 1907).

Seeds were sterilized using the method adopted by Sweet and Bolton (1979). Then the sterilized seeds were incubated in sterile distilled water at room temperature for 3 h. Wheat seeds were shaken in high phosphate Nfb (HPNfb) liquid medium enriched with

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

0.1% ammonium chloride contain 10^8 CFU mL^{-1} of *A. brasilense* stain and shake in 100 rpm for 3 h (Shaker Model INFORS AG, BOTTMINGEN) (Okon et al., 1977). Under sterile laminar airflow, the seeds were left to reduce the seeds moisture down to almost 14%.

Sterilized wheat seeds (Shahpasand as sensitive and Roshan as tolerant cultivar) were planted on sterile water agar (8% w/v) and kept in dark at room temperature for 48 h for germination. The uniform small seedlings were transferred into test tube (3 × 25 cm) containing 40 ml of autoclaved MS medium (30 g sucrose, 7 g agar, pH ~ 6.5) (Murashige and Skoog, 1962) and then located in growth chamber at 22 ±2°C, 60% RH, and photoperiod of 16 L/8 D (210 photons m^{-2} s^{-1}) for 3 more days. Half of the seedlings (5-day-old) were inoculated with specific *A. brasilense* strains.

To inoculate seedling, Nfb liquid medium enriched with 0.1% ammonium chloride containing 10^7 CFU ml^{-1} concentration of *A. brasilense* Sp245 and Sp7 was prepared. Then under sterile laminar airflow, 1 mL of Nfb liquid medium containing Sp245 or Sp7 was added separately into each test tube, containing three 5-day-old seedlings and kept for 2 more days. After 2 days, seedling's roots were checked for the number of bacteria. To do so, the samples of seedling's roots were stained with Iodonitrotetrazolium chloride (INT) dye (0.25% w/v aqueous solution) and then many cross-sections of root were provided manually (Sriskandarajah et al., 1993). Afterward, the presence of the bacteria within root cross-sections was checked using a light microscope. Meanwhile, the number of bacteria colonies (CFU mL^{-1}) within the roots was counted. To do so, the root samples were placed in 95% (v/v) ethanol for 3 sec, then in 0.01% (w/v) $HgCl_2$ for 15 sec and washed several times with sterile water for surface-sterilization (Sriskandarajah et al., 1993). Then, 1 g of root samples were grounded using 5 mL of 1X phosphate buffered saline (PBS) (pH ~ 7.4) at the sterile condition and serially diluted (10^{-1} up to 10^{-9}) (Stets et al., 2015). 5 μl of the final dilution (10^{-9}) were cultured on solid medium (8% w/v agar) of HPNfb enriched with ammonium chloride (0.1% w/w) and incubated at 32°C for 72 h. At last, the number of colonies formed on the plates was counted and considered as bacterial population in 1 g of tissue fresh weight. The number of bacteria in root was almost 10^8 CFU mL^{-1} for Sp245 and 10^7 CFU mL^{-1} for Sp7.

One series of 7-day-old seedlings (including non-inoculated and inoculated with *A. brasilense* strains) were transferred into sterile test tubes (3 × 25 cm) which had been saturated with water vapors (RH ≈ 100%) at 25°C (Thomson, 1872). According to Kelvin equation and after equilibrium of water vapor pressure, this was equal to maximum water potential (ψ_w = 0 MPa) and was considered as optimum water condition (Butt et al., 2006). The second series of 7-day-old seedlings were transferred into sterile test tubes with RH equal to 50% (ψ_w = -90 MPa) at 25°C as dehydration treatment. After implementation and equilibrium of treatments, plant samples were collected at 0, 120 and 360 minutes. Samples were separated immediately into shoot and root and frozen in liquid nitrogen to store at -80°C for RNA isolation.

Total RNA extraction and quantitative Real-Time PCR (qRT-PCR) analysis

Total RNA of the shoot and root samples of wheat cultivars were extracted using Irizol kit (Irizol RNA Biotech, Iran) at 4°C. To remove the possible DNA contamination, the RNA samples was treated with RNase-free DNase I (TaKaRa, Japan).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

About 1 µg of total RNA was used to synthesize first-strand cDNA primed with oligo (dT) in a 10 µl reaction mix using reverse transcriptase (TaKaRa, Japan) according to the instructions of the manufacturer. QRT-PCR was performed using SYBER-Green dye (TaKaRa, Japan) on real-time PCR machine (Bio-Rad, USA) in triplicate. The *ARF* (ADP-ribosylation factor) gene (AB050957) was used as the reference gene. The primers (*ARF*, F: 5′-CTGACGCCGAGGATATCCA-3′; R: 5′-GCCTTGACCATACCAGTTCCA-3′) used for Real-Time PCR analysis were designed using AlleleID, version 7. The amount of transcript accumulated for the *DREB2* as the target gene (AY781345) (*DREB2,* F: 5′-AAGAAAACAGGCGACAAGAT-3′; and R: 5′- GTTGTTGGTTCACTTCTTTC -3′) (Egawa et al., 2006) normalized to ARF gene as internal control. The value of *DREB2* transcript level was reported relative to the ARF gene ($-\Delta C_T$) which is the negative difference between the threshold cycle (C_T) of the target gene and C_T of the reference gene. Then, the relative expression of *DREB2* in treated conditions (inoculated and/or dehydrated) was calculated using the $2^{-\Delta\Delta C_T}$ method relative to control condition (non-inoculation and optimum water condition) (Livak and Schmittgen, 2001). The real-time reaction efficiencies of the target and reference genes (E = 0.93 and 0.96, respectively) were assumed to be equal, nearly 1. The PCR cycling conditions including an optimum concentration of primers and cDNA as well as optimum temperature for *ARF* and *DREB2* genes were set up. So, real-time PCR program was: incubation at 95°C for 10 min, 95°C for 10 s, 58°C for 25 s for 40 cycles, 72°C for 30 s and 72°C for 7 min.

Statistical analysis

The primary experiment was conducted in a split plot layout with 3 replicates. The main plot was wheat cultivars, sub-plot was bacterial strains and sub-sub-plot was water condition. The second experiment also carried out as split-plot with 3 replicates. The main plot was sensitivity to drought, inoculation as sub-plot and water condition as sub-sub-plot.

The ANOVA were obtained using SPSS and the mean values were compared using Duncan῾s multiple range tests at 95% confidence interval.

Results

According to the primary experiment, the associated pairs (wheat cultivars-*A. brasilense* strains) including tolerant (Roshan-Sp245), moderately tolerant (Roshan-Sp7), sensitive (Shahpasand-Sp7) and moderately sensitive (Shahpasand-Sp245) to drought were selected (*Appendix 1*). Physiological responses in drought condition are presented in *Tables 1 and 2*.

Physiological responses of sensitive and tolerant associated pairs to drought

Cross-inoculation of Roshan and Shahpasand cultivars with Sp245 or Sp7 strains formed different associated pairs with different physiological responses in optimum water condition ($P \leq 0.05$) (*Tables 1-4*). In optimum water condition, most physiological parameters increased (except for NMR and SPAL) ($P \leq 0.05$) when inoculated tolerant cultivar (Roshan- Sp245) compared to non-inoculated one (Roshan). However, in Roshan-Sp7, ARL, RDW and, RTP were the only parameters positively affected by inoculation in optimum water condition (*Tables 3 and 4*). Although,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

inoculation of the sensitive cultivar (Shahpasand) with Sp7 did not significantly affect any of the physiological parameters at optimum water condition in Shahpasand-Sp245, NRB, SDW, RTP and, RPAL increased due to inoculation ($P \leq 0.05$) (*Tables 1-4*). It seems that Sp245 has more homology or compatibility potential to associate with different wheat cultivars than Sp7.

Table 1. *Average values of growth parameters including maximum shoot length (MSL), average root length (ARL), number of main roots (NMR), number of root branches (NRB) and shoot and root dry weight (SDW and RDW, respectively), in non-inoculated cultivars (Roshan and Shahpasand as tolerant and sensitive to drought, respectively) as well as different pairs of wheat cultivars and A. brasilense strains (Sp245 and Sp7) including Roshan-Sp245 (tolerant), Roshan-Sp7 (moderately tolerant), Shahpasand-Sp7 (sensitive), Shahpasand-Sp245 (moderately sensitive) under drought (40% FC) and optimum water conditions (80% FC). Data represent the mean values ± SE (n = 3). Different letters in the mean values indicated the difference between the mean values (Duncan's multiple range tests, P≤0.05). MSL and ARL are in cm plant^{-1}; SDW and RDW are in mg plant^{-1}. FC, stand for moisture content at field capacity*

FC	Cultivar, Strain	MSL	ARL	NMR	NRB	SDW	RDW
80%	Roshan cv.	25.3 ± 0.2^{b}	5.7 ± 0.2^{c}	5.0 ± 0.01^{a}	0.7 ± 0.6^{c}	25.0 ± 0.01^{b}	9.0 ± 0.01^{c}
	Roshan-Sp245	31.2 ± 0.8^{a}	10. 6 ± 0.1^{a}	5.3 ± 0.6^{a}	2.3 ± 0.6^{b}	29.3 ± 0.6^{a}	17.3 ± 0.6^{a}
	Roshan-Sp7	26.3 ± 0.3^{b}	7.4 ± 0.2^{b}	5.0 ± 0.01^{a}	1.3 ± 0.6^{c}	26.0 ± 1.0^{b}	12.7 ± 0.6^{b}
	Shahpasand cv.	17.8 ± 0.3^{c}	7.1 ± 0.1^{b}	4.0 ± 1.0^{b}	0.0 ± 0.01^{d}	20.7 ± 0.6^{c}	7.0 ± 0.01^{c}
	Shahpasand-Sp245	19.7 ± 0.6^{c}	7.6 ± 0.1^{b}	4.0 ± 0.01^{b}	1.0 ± 0.01^{c}	24.0 ± 1.0^{b}	7.0 ± 0.01^{c}
	Shahpasand-Sp7	18.8 ± 0.3^{c}	7.4 ± 0.1^{b}	3.7 ± 0.6^{b}	0.7 ± 0.6^{c}	22.0 ± 0.01^{c}	7.0 ± 0.01^{c}
40%	Roshan cv.	24.0 ± 0.2^{b}	5.4 ± 0.1^{c}	5.0 ± 0.01^{a}	2.0 ± 0.01^{b}	24.0 ± 0.01^{b}	8.0 ± 0.01^{c}
	Roshan-Sp245	26.4 ± 0.5^{b}	7.4 ± 0.4^{b}	5.0 ± 0.01^{a}	3.7 ± 0.6^{a}	27.7 ± 0.6^{a}	12.0 ± 0.01^{b}
	Roshan-Sp7	24.3 ± 0.2^{b}	6.2 ± 0.1^{b}	5.0 ± 0.01^{a}	2.3 ± 0.6^{b}	25.0 ± 0.01^{b}	9.0 ± 0.01^{c}
	Shahpasand cv.	13.8 ± 0.5^{d}	5.4 ± 0.1^{c}	4.3 ± 0.6^{b}	2.0 ± 0.01^{b}	16.0 ± 1.0^{d}	5.0 ± 0.01^{d}
	Shahpasand-Sp245	13.5 ± 0.5^{d}	5.4 ± 0.1^{c}	4.7 ± 0.6^{ab}	1.3 ± 0.6^{c}	16.0 ± 1.0^{d}	5.0 ± 0.01^{d}
	Shahpasand-Sp7	9.4 ± 0.5^{e}	3.8 ± 0.1^{d}	3.0 ± 0.01^{c}	1.7 ± 0.6^{b}	11.0 ± 1.7^{e}	2.0 ± 0.01^{e}

Table 2. *Average values of total protein in shoots (STP) and roots (RTP), activity of PAL enzyme in shoots (SPAL) and roots (RPAL), activity of TAL enzyme in shoot (STAL) and root (RTAL) in non-inoculated cultivars (Roshan and Shahpasand as tolerant and sensitive to drought, respectively) as well as different pairs of wheat cultivars and A. brasilense strains (Sp245 and Sp7) including Roshan-Sp245 (tolerant), Roshan-Sp7 (moderately tolerant), Shahpasand-Sp7 (sensitive), Shahpasand-Sp245 (moderately sensitive) under drought (40% FC) and optimum water conditions (80% FC). Data represent the mean values ± SE (n = 3). Different letters in the mean values indicated the difference between the means (Duncan's multiple range tests, P ≤ 0.05). STP and RTP are is mg gFW^{-1}; SPAL, RPAL, STAL and RTAL are in U mg protein^{-1}. FC, stand for moisture content at field capacity*

FC	Cultivar, Strain	STP	RTP	SPAL	RPAL	STAL	RTAL
80%	Roshan cv.	44.0 ± 0.5^{b}	37.4 ± 1.1^{c}	17.4 ± 0.01^{c}	11.7 ± 0.2^{b}	20.7 ± 0.1^{c}	15.3 ± 0.01^{d}
	Roshan-Sp245	86.2 ± 0.5^{a}	79.3 ± 1.0^{a}	20.6 ± 0.1^{b}	14.5 ± 0.3^{a}	23.6 ± 0.1^{b}	19.3 ± 0.6^{c}
	Roshan-Sp7	45.0 ± 0.2^{b}	47.3 ± 0.7^{bc}	18.0 ± 0.1^{c}	12.4 ± 0.1^{b}	21.9 ± 0.1^{c}	16.0 ± 0.4^{d}
	Shahpasand cv.	32.7 ± 0.3^{c}	26.0 ± 1.3^{d}	10.8 ± 0.1^{d}	9.0 ± 0.01^{c}	19.4 ± 0.1^{c}	12.1 ± 0.3^{e}
	Shahpasand-Sp245	37.6 ± 0.5^{c}	35.0 ± 1.5^{c}	12.3 ± 0.2^{d}	11.5 ± 0.3^{b}	21.0 ± 0.1^{c}	13.3 ± 0.3^{e}
	Shahpasand-Sp7	36.1 ± 1.2^{c}	29.2 ± 1.2^{d}	11.1 ± 0.3^{d}	9.9 ± 0.1^{c}	19.7 ± 0.1^{c}	12.5 ± 0.3^{e}
40%	Roshan cv.	50.9 ± 0.9^{b}	56.1 ± 4.6^{b}	23.6 ± 0.6^{a}	12.9 ± 0.1^{b}	24.8 ± 0.2^{b}	18.3 ± 0.6^{c}
	Roshan-Sp245	55.7 ± 0.7^{b}	79.3 ± 0.0^{a}	26.2 ± 0.1^{a}	15.4 ± 0.01^{a}	30.3 ± 0.1^{a}	25.0 ± 0.7^{a}
	Roshan-Sp7	52.0 ± 0.4^{b}	70.3 ± 1.8^{a}	24.2 ± 0.1^{a}	13.5± 0.3^{b}	26.0 ± 0.1^{b}	22.3 ± 1.2^{b}
	Shahpasand cv.	30.3 ± 0.3^{c}	21.5 ± 1.1^{d}	9.7 ± 0.1^{d}	8.1 ± 0.1^{ce}	17.9 ± 0.1^{c}	10.2 ± 0.3^{e}
	Shahpasand-Sp245	32.2 ± 0.7^{c}	21.2 ± 0.2^{d}	8.6 ± 0.1^{d}	7.2 ± 0.3^{e}	16.6 ± 0.1^{c}	9.20± 0.3^{ef}
	Shahpasand-Sp7	32.3 ± 0.3^{c}	19.4 ± 0.9^{d}	8.3 ± 0.01^{d}	7.0 ± 0.2^{e}	15.2 ± 0.1^{d}	6.5 ± 0.6^{f}

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

Table 3. *Based on the values of Table 1, the percent of changes in each parameter of different associated pairs due to inoculation and/or drought conditions. Optimum water condition defined as 80% FC and drought as 40% FC (moisture content at field capacity). Abbreviation: maximum shoot length (MSL), average roots length (ARL), number of main roots (NMR), number of roots branches (NRB) and shoots and roots dry weight (SDW and RDW, respectively)*

Growth parameters	Sensitivity of associated pairs to drought	At 80% FC Inoculated versus Non-inoculated	For non-inoculated 40% versus 80%	For Inoculated 40% versus 80%	Inoculated 40% versus Non-inoculated 80%
		Inoculation effect	Drought effect	Drought effect under inoculation	Dual effect
MSL	Tolerant	23	0	-15	0
	Moderately tolerant	0	-	0	0
	Sensitive	0	-25	-50	-47
	Moderately sensitive	0	-	-31	-24
ARL	Tolerant	85	0	-30	29
	Moderately tolerant	30	-	0	9
	Sensitive	0	-25	-50	-46
	Moderate sensitive	0	-	-29	-24
NMR	Tolerant	0	0	0	0
	Moderately tolerant	0	-	0	0
	Sensitive	0	0	-18	-25
	Moderately sensitive	0	-	0	0
NRB	Tolerant	250	198	61	448
	Moderately tolerant	0	-	77	229
	Sensitive	0	2*	143	1.67*
	Moderately sensitive	1*	-	0	1.3*
SDW	Tolerant	16	0	0	12
	Moderately tolerant	0	-	0	0
	Sensitive	0	-25	-50	-47
	Moderately sensitive	16	-	-33	-23
RDW	Tolerant	89	0	-31	33
	Moderately tolerant	41	-	-29	0
	Sensitive	0	-25	-71	-71
	Moderately sensitive	0	-	-28	-28

*: Indication increased NRB from 0

The selected cultivars or associated pairs showed different physiological responses to drought ($P \leq 0.05$) *(Tables 1-4)*. At drought condition, MSL, ARL and, RDW in Roshan cv. (drought tolerant) did not decrease as compared to optimum water condition, but these parameters were decreased in Shahpasand cv. (*Tables 1 and 3*). In drought condition, RDW decreased in all pairs with the highest in Shahpasand-Sp7 and the lowest in Roshan-Sp245. SDW of Roshan cv. in non-inoculated and inoculated status did not change in drought condition but SDW of Shahpasand cv. and inoculated Shahpasand cv. with any of two strains were decreased. The drought condition had a negative effect on NMR of Shahpasand with Sp7. However, NRB increased in all cultivars and pairs except for Shahpasand-Sp245 under drought condition ($P \leq 0.05$) (*Tables 1-4*).

STAL and RTAL were increased in inoculated Roshan cv. with Sp245 or Sp7 but they decreased in Shahpasand cv. with both strains. In contrast, SPAL in Roshan cv. and Roshan inoculated with Sp245 or Sp7 were increased in drought condition but they did

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

not change in Shahpasand cv. or Shahpasand with Sp7 or Sp245. STP did not change due to drought in all cultivars and pairs except for Roshan-Sp245. Meanwhile, RTP did not change in Roshan-Sp245 and Shahpasand-Sp7 but it was increased in Roshan-Sp7 and decreased in Shahpasand-Sp245 under drought condition (*Tables 2 and 4*).

Table 4. *Based on the values of Table 2, the percent of changes in each parameter of different associated pairs due to inoculation and/or drought conditions. Optimum water condition defined as 80% FC and drought as 40% FC (moisture content at field capacity). Abbreviation: total protein of roots (RTP) and shoots (STP, the activity of PAL enzyme in shoots (SPAL) and roots (RPAL), the activity of TAL enzyme in shoots (STAL) and roots (RTAL)*

Growth parameters	Sensitivity of associated pairs to drought	At 80% FC Inoculated versus Non-inoculated	For non-inoculated 40% versus 80%	For Inoculated 40% versus 80%	Inoculated 40% versus Non-inoculated 80%
		Inoculation effect	**Drought effect**	**Drought effect under inoculation**	**Dual effect**
STP	Tolerant	96	0	-35	0
	Moderately tolerant	0	-	0	0
	Sensitive	0	0	0	0
	Moderately sensitive	0	-	0	0
RTP	Tolerant	110	50	0	112
	Moderately tolerant	26	-	49	88
	Sensitive	0	0	0	0
	Moderately sensitive	35	-	-39	0
SPAL	Tolerant	0	36	27	50
	Moderately tolerant	0	-	34	39
	Sensitive	0	0	0	0
	Moderately sensitive	0	-	0	0
RPAL	Tolerant	24	0	0	31
	Moderately tolerant	0	-	0	0
	Sensitive	0	0	-29	-29
	Moderately sensitive	28	-	-37	-20
STAL	Tolerant	14	20	29	47
	Moderately tolerant	0	-	19	26
	Sensitive	0	0	-23	-22
	Moderately sensitive	0	-	-37	0
RTAL	Tolerant	27	20	29	37
	Moderately tolerant	0	-	39	46
	Sensitive	0	0	-48	-47
	Moderately sensitive	0	-	-31	-24

The dual effects of inoculation and drought were evaluated by comparing an inoculated cultivar which was grown under drought condition with the respective non-inoculated cultivar at optimum water condition (named control). When inoculated Roshan cv. with Sp245 or Sp7 under drought condition were compared with control, ARL, NRB, RTP, SPAL, RTAL and, STAL were higher in inoculated one. In contrast, RDW, SDW, ARL, MSL, RPAL, RTAL in Shahpasand cv. with Sp7 or Sp245 under drought condition were lower than control. Simultaneously, MSL, NMR and STP in inoculated Roshan cv. and RTP, STP and, SPAL of inoculated Shahpasand cv. did not show any changes. However, NRB increased in inoculated Shahpasand cv. as compared to control (*Tables 1-4*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

In brief, the most beneficial changes in the physiological parameters were observed in the tolerant pair (Roshan-Sp245) followed by moderately tolerant (Roshan-Sp7), moderately sensitive (Shahpasand- Sp245) and sensitive pairs (Shahpasand-Sp7) at either drought or optimum water conditions (*Tables 1-4*).

DREB2 expression in selected pairs under different water potentials

At optimum water potential (ψ_w = 0 MPa), *DREB2* expression in the roots of inoculated seedlings (*Tables 5, 6*) was less relative to non-inoculated cultivars (ranged from 0.05 up to 0.57- fold for tolerant to sensitive pairs ($P \leq 0.05$)). However, their expressions were similar at 0, 120 and 360 min after dehydration imposed (*Table 5*). *DREB2* in the shoots of all selected pairs were up-regulated as compared to non-inoculated cultivars. *DREB2* up-regulation was more in the shoots of tolerant and moderately tolerant pairs than the sensitive and moderately sensitive pairs ($P \leq 0.05$) (*Table 6*). Therefore, at optimum water condition, *DREB2* down-regulated in the roots (*Table 5*) but up-regulated in the shoots of tolerant and sensitive pairs relative to non-inoculated cultivars.

Table 5. *DREB2 expression (fold) in different times (0, 120 and 360 min) after dehydration (ψw = -90 MPa) imposed in the roots of different associated pairs of wheat cultivars and A. brasilense strains (Sp245 and Sp7) as responded to drought including: tolerant (Roshan-Sp245), moderately tolerant (Roshan-Sp7), sensitive (Shahpasand-Sp7), moderately sensitive (Shahpasand-Sp245). The result was compared to optimum water condition (ψw = 0 MPa). Data represent the mean values ± SE (n = 3). Different letters in the mean values indicated the difference between the means (Duncan's multiple range tests, P ≤ 0.05)*

Time (min)	Ψw (MPa)	Sensitivity of associated pairs to drought	Non-inoculated Dehydration versus optimum	Inoculated Dehydration versus optimum	Inoculated versus Non-inoculated
0	0 (optimum)	Tolerant	-	-	0.06 ± 0.02[c]
		Moderately tolerant	-	-	0.05 ± 0.01[c]
		Sensitive	-	-	0.15 ± 0.04[c]
		Moderately sensitive	-	-	0.15 ± 0.02[c]
120	0	Tolerant	-	-	0.07 ± 0.02[c]
		Moderately tolerant	-	-	0.06 ± 0.02[c]
		Sensitive	-	-	0.14 ± 0.02[c]
		Moderately sensitive	-	-	0.16 ± 0.01[c]
	-90 (dehydrated)	Tolerant	2.65 ± 0.70[a]	777.8 ± 100.76[a]	19.20 ± 4.83[a]
		moderately tolerant	-	710.8 ± 84.44[a]	17.19 ± 2.12[b]
		Sensitive	1.60 ± 0.02[b]	20.9 ± 1.16[b]	1.87 ± 0.23[c]
		Moderately sensitive	-	19.1 ± 2.72[b]	1.86 ± 0.42[c]
360	0	Tolerant	-	-	0.07 ± 0.01[c]
		Moderately tolerant	-	-	0.06 ± 0.02[c]
		Sensitive	-	-	0.20 ± 0.52[c]
		moderately sensitive	-	-	0.20 ± 0.11[c]
	-90	Tolerant	0.51 ± 0.14[c]	3.6 ± 0.68[b]	0.51 ± 0.06[c]
		Moderately tolerant	-	3.4 ± 0.43[b]	0.43 ± 0.10[c]
		Sensitive	1.93 ± 0.48[b]	5.1 ± 0.56[b]	0.51 ± 0.09[c]
		Moderately sensitive	-	5.5 ± 5.06[b]	0.57 ± 0.02[c]

Dehydration had a significant effect on *DREB2* expression of roots and shoots in inoculated and non-inoculated cultivars ($P \leq 0.05$) (*Tables 5, 6*). The result showed that in the tolerant cultivar (Roshan cv.) at 120 min after dehydration, *DREB2* ($2^{-\Delta\Delta C_T}$) up-regulated 2.7 and 3.4- fold in the roots and shoots, respectively as compared to optimum

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

water condition. In the sensitive cultivar (Shahpasand cv.), *DREB2* did not change in the roots or shoots due to dehydration ($P \leq 0.05$) (*Table 5*). At 360 min, *DREB2* expression acts vice versa under dehydration, means, down-regulated in the roots of Roshan (0.5-fold) and up-regulated (2.6- fold) in the shoots, but in Shahpasand, *DREB2* up-regulated in the roots (1.9- fold) and down-regulated in the shoots (0.6- fold).

The change in *DREB2* expression due to dehydration in non-inoculated cultivars, and selected association pairs (the inoculated cultivars) had different sensitivity to drought (*Table 5*). For example, at 120 min after dehydration, *DREB2* up-regulated in the roots of tolerant, moderately tolerant, sensitive and moderately sensitive pairs (778, 711, 21 and 19- fold, respectively) while, *DREB2* up-regulated (1-fold) in the shoots of tolerant and moderately tolerant pairs and down-regulated (almost 0.4- fold) in the sensitive and moderately sensitive pairs (*Tables 5, 6*). However, at 360 min, *DREB2* up-regulation in the roots of all pairs were so less (ranged from 3.4 to 5.5- fold). In contrast, *DREB2* down-regulated due to dehydration in the shoots of the tolerant, moderately tolerant (0.2- fold), sensitive and moderately sensitive pairs (0.4- fold) (*Tables 5, 6*).

Table 6. *DREB2 expression (fold) in different times (0, 120 and 360 min) after dehydration (ψ_w = -90 MPa) imposed in the shoots of different associated pairs of wheat cultivars and A. brasilense strains (Sp245 and Sp7) as responded to drought including: tolerant (Roshan-Sp245), moderately tolerant (Roshan-Sp7), sensitive (Shahpasand-Sp7), moderately sensitive (Shahpasand-Sp245). The result was compared to optimum water condition (ψ_w = 0 MPa). Data represent the mean values ± SE (n = 3). Different letters in the mean values indicated the difference between the means (Duncan's multiple range tests, $P \leq 0.05$)*

Time (min)	Ψw (MPa)	Sensitivity of associated pairs to drought	Non-inoculated	Inoculated	Inoculated versus Non-inoculated
			Dehydration versus optimum	Dehydration versus optimum	
0	0 (control)	Tolerant	-	-	3.43 ± 0.47^a
		Moderately tolerant	-	-	3.27 ± 0.70^a
		Sensitive	-	-	1.39 ± 0.32^b
		Moderately sensitive	-	-	1.40 ± 0.12^b
120	0	Tolerant	-	-	3.35 ± 0.64^a
		Moderately tolerant	-	-	3.32 ± 0.19^a
		Sensitive	-	-	1.27 ± 0.01^b
		Moderately sensitive	-	-	1.28 ± 0.03^b
	-90 (dehydrated)	Tolerant	3.35 ± 0.33^a	1.22 ± 0.29^a	1.22 ± 0.32^b
		moderately tolerant	-	1.12 ± 0.17^a	1.11 ± 0.19^b
		Sensitive	1.02 ± 0.31^b	0.38 ± 0.14^b	0.48 ± 0.18^d
		Moderately sensitive	-	0.40 ± 0.09^b	0.50 ± 0.06^d
360	0	Tolerant	-	-	3.29 ± 1.21^a
		Moderately tolerant	-	-	3.08 ± 0.85^a
		Sensitive	-	-	1.17 ± 0.02^b
		moderately sensitive	-	-	1.18 ± 0.34^b
	-90	Tolerant	2.64 ± 0.47^a	0.16 ± 0.04^c	0.20 ± 0.05^e
		Moderately tolerant	-	0.16 ± 0.08^c	0.19 ± 0.05^e
		Sensitive	0.59 ± 0.09^b	0.43 ± 0.14^b	0.85 ± 0.18^c
		Moderately sensitive	-	0.45 ± 0.09^b	0.90 ± 0.26^c

To evaluate dual effects of inoculation and dehydration in each pair, the inoculated seedlings which exposed to dehydration condition were compared to the respective non-inoculated seedlings grown at optimum water condition. The result showed that at 120 min after dehydration, transcripts level of *DREB2* (-ΔCT) in the tolerant and moderately

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

tolerant pairs was more than tolerant cultivar grown at optimum water condition (11.4- fold for roots and 2.3- fold for shoots). Meanwhile, in dehydrated roots of sensitive and moderately sensitive pairs, transcripts level of *DREB2* was more than non-inoculated and non-dehydrated roots (1.5- fold) but in their shoot *DREB2* was less than non-inoculated ones at optimum water potential (2.6 and 2.4- fold, respectively) ($P \leq 0.05$) ($t = 120$).

In the last measurement after dehydration (360 min), the transcripts level of *DREB2* in the tolerant and moderately tolerant pairs decreased (almost 5 and 6-fold for roots and almost 1-fold for shoot, respectively) as compared to non-inoculated cultivars grown under optimum water potential while in the sensitive and moderately sensitive pairs, there was no change in the roots but it was lower in the shoots (3.7 and 3.4-fold, respectively) ($P \leq 0.05$).

Discussion

The primary experiment was necessary to find out the most sensitive and tolerant cultivars to drought among the usual cultivated wheat cultivars (Roshan, Sardari, Tabasi, Shahpasand, Omid and Shoaleh). Then finding the compatible (or homologous) and incompatible (heterogonous) pairs among association pair of these six cultivars with two strains of *A. brasilense* (Sp7 and Sp245) under drought condition (Baldani et al., 1983; Jain and Patriquin, 1984; Amooaghaie et al., 2004). This is important due to the complexity of interaction between bacteria and host plant which can affect the successful infection, the establishment of bacterial in host plant and the following appropriate physiological responses (Jain and Patriquin, 1984; Sumner, 1990; Baldani et al., 1986, 1987). According to the physiological parameters such as growth and defensive parameters as well as total protein content, the two cultivars of Roshan and Shahpasand were determined as the most tolerant and sensitive cultivars to drought, respectively (Baldani et al., 1983; Estabrook and Sengupta-Gopalan, 1991; Vasse et al., 1993; Santos et al., 2001). Simultaneously, the best partner of wheat cv. and *A. brasilense* strains as tolerant pair to drought was Roshan-Sp245 (homologous) and the worst as the most sensitive pair was Shahpasand-Sp7 (heterologous). Also, other pairs from cross-inoculation of Roshan and Shahpasand cultivars with Sp245 and Sp7 strains indicated that Roshan-Sp7 as moderately tolerant and Shahpasand-Sp245 as moderately sensitive pairs.

Physiological responses of the selected pairs to drought

Cross-inoculation of Roshan and Shahpasand cultivars (as tolerant and sensitive to drought, respectively) with *A. brasilense* Sp245 and Sp7 strains make different associated pairs with different physiological responses in drought and optimum water conditions. Inoculation of Roshan cv. with *A. brasilense* Sp245 improved the growth of wheat seedlings except for NMR and SPAL. Similar results have been reported that Sp245 caused higher shoot length, root length and the number of roots (Baldani et al., 1987; Hamdia et al., 2004; Akbari et al., 2007; Cohen et al., 2008; Spaepen et al., 2009). Also, in the moderately tolerant (Roshan-Sp7) pair, ARL, RDW and RTP increased due to inoculation at optimum water condition (*Tables 3 and 4*). The positive effect of *A. brasilense* to promote wheat growth could be partly related to the production of more roots and consequently uptake more water and nutrient from the soil (Casimiro et al., 2003; Correa-Aragunde et al., 2004). However, inoculation of Shahpasand cv. (sensitive

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DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

to drought) with Sp7 (sensitive association pair) did not show any positive effect on the growth parameters but in Shahpasand-Sp245 (moderately sensitive), NRB, SDW, RTP and, RPAL increased due to inoculation. Regarding to previous works, it can be concluded that any strain of bacteria should be homologous to specific wheat cultivar to expect stimulation effect on growth parameter (Baldani et al., 1983; Amooaghaie et al., 2004). In this experiment, not only Sp245 was in favor of tolerant cultivar, but also did a good job with Shahpasand cultivar. In contrast, Sp7 act in favor of Roshan cv. but has no effect on Shahpasand cultivar.

The effect of any bacterial strain on growth and development of wheat cultivars is neither the same nor ended to the growth parameters because in some cases there are no changes in growth indexes but biochemical parameters were affected. For example, total protein of roots (RTP) did not change in Roshan and Shahpasand with Sp7 at optimum water condition, meanwhile, other parameters behave differently (*Tables 2 and 4*). In contrast, RTP in the tolerant and moderately sensitive pairs of Roshan and Shahpasand with Sp245 were increased due to inoculation. An increase in the protein of plants have been reported in an association of different plant species and bacterial strains ((Vasse et al., 1993; Gamas et al., 1998). For example, different strains of *A. brasilense* have been caused an addition of total protein in wheat and tomatoes (Saubidet et al., 2002; Mangmang et al., 2015).

PAL and TAL activity in the roots and shoots of Roshan cv. inoculated with Sp245 were increased compare to non-inoculated cultivars at optimum water condition but there was no change in other association pairs except for RPAL in the moderately sensitive pair (Shahpasand-Sp245) which increased by 28%. *A. brasilense* Sp245 may enhance the defensive mechanism of wheat cultivars via changing the activities of these enzymes same as other PGPRs, then less reduction in growth parameters was observed in Rhoshan-Sp245 compare to other pairs (Maksimov et al., 2015). There is an evidence that an establishment of a proper relation between plant and bacteria can stimulate the biosynthesis pathway of phenolic compounds as a plant defense mechanism (Vasse et al., 1993). TAL and PAL enzymes are involved in the biosynthesis of phenolic compounds via biosynthesis pathway of phenylpropanoids (Lawton et al., 1983; Nemat Alla et al., 2002). Therefore, increase in the activity of TAL and PAL can cause more phenolic compounds in inoculated plants with a homologous bacterial strain which is an efficient mechanism to deal with the adverse effects of reactive oxygen species during the infection process (Estabrook and Sengupta-Gopalan, 1991). This may be a reason for the less damage to the growth rate in this experiment in inoculated as well as drought conditions.

All growth parameters in non-inoculated tolerant cultivar did not change due to drought condition (except more number of root branches, NRB). However, the most growth parameters in sensitive cultivar were severely decreased. Other reports also show that the reduction in growth parameters in the sensitive cultivars of wheat is more than the tolerant cultivars (Liu et al., 2004). Significant reduction in dry weight and other growth parameters of the sensitive cultivars under drought condition may be due to reduced plant water potential and impairment of mineral absorption and less photosynthesis, protein synthesis and carbohydrate metabolism (Tekle and Alemu, 2016). Among the growth parameters, the number of root branches was increased due to drought in both sensitive and tolerant cultivars with the different rate. Development of an extensive root system in drought condition is an avoidance mechanism to obtain more water from the rhizosphere to protect themselves against drought stress (Liu et al.,

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2004). The root and shoot elongation not only was observed in drought condition but also osmotic stress can cause higher root length and also higher activity of PAL and TAL enzymes in wheat seedlings inoculated with *A. brasilense* strains (Nabti et al., 2010; Hamdia et al., 2004).

Drought condition caused the addition of total protein in root in non-inoculated tolerant cultivar but no change in the sensitive one as compare to optimum water condition. Addition of total protein due to drought stress indicated the role of organic osmolytes in osmotic adjustment for adaptation of plant to drought stress (Serraj and Sinclair, 2002).

Dual effect of inoculation and drought condition simultaneously caused mostly higher growth parameters in Roshan with Sp245 (except for NMR and MSL) as well as with Sp7 (except ARL and NRB), while the growth parameters in Shahpasand cv. (except for NRB) were significantly decreased as compared to non-inoculated cultivars at optimum water condition. Similar studies showed that inoculation of wheat with Sp245 strain increased growth parameters more than Sp7 due to the capability of Sp245 to colonize interior parts of wheat root unlike the Sp7 (Michiels et al., 1991). The Sp245 strain could improve drought tolerance of wheat by the production of ABA, a key phytohormone in plant response to drought stress (Michiels et al., 1991; Cohen et al., 2008).

Dual effect of inoculation and drought condition show that Roshan cv. with Sp245 caused an increase in PAL and TAL activities compared to non-inoculated cultivars at drought or optimum water condition. It has been suggested that inoculated of wheat cultivars with Sp245 has a positive effect on the activity of these enzymes either at drought or optimum water condition. Hence, *A. brasilense* Sp245 can enhance the defensive mechanism of wheat at the drought condition (Maksimov et al., 2015). PAL and TAL have a main role in drought tolerance in wheat plants through phenylpropanoids biosynthesis pathway and accumulation of more phenolic compounds in plants (Redmann, 1974; Kaur and Zhawar, 2015).

Therefore, it can be concluded that positive effects of inoculation on biochemical properties of the wheat cultivars are mostly related to homology properties of wheat cultivar and bacterial strain not to the sensitivity of cultivar to drought. This is the reason that Roshan (tolerant) and Shahpasand (sensitive) cultivars with Sp245 showed the better results than Sp7. However, the dual effect of inoculation and drought condition represents the interaction effect on the association of wheat cultivar with the strain of bacteria when compared to separate effect.

DREB2 expression of the selected association pairs in different water potentials

At optimum water condition (ψ_w = 0 MPa), *DREB2* of inoculated cultivars was down-regulated in the root but up-regulated in the shoot of all selected pairs relative to the respective non-inoculated cultivars. It seems that over-expression of *DREB2* in the shoot of inoculated seedlings may be due to the basic effect of *A. brasilense* strains on the wheat seedling. However, the positive effect in the shoot of tolerant and moderately tolerant pairs is more than sensitive and moderately sensitive pairs. The effect of some other PGPRs such as *Arthrobacter protophormiae* (SA3), *Dietzia natronolimnaea* (STR1) and *Bacillus subtilis* (LDR2) on *TaCTR1*/*TaDREB2* expression in wheat has been shown the enhancement of Ta*DREB2* gene expression encoding for a transcription factor and improving the tolerance of plants to abiotic stress conditions (Barnawal et al., 2017).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

DREB2 expression in non-inoculated cultivars and the cross-inoculated with *A. brasilense* strains was also varied under dehydration condition. *DREB2* up-regulated in root and shoot of non-inoculated tolerant cv. but did not change in the sensitive cv. due to dehydration. *DREB2* up-regulation has been reported in dehydration condition in the tolerant cv. of wheat. For example, Shen and colleagues (2003) reported that drought stress increased *TaDREB2* gene expression in tolerant cultivars of wheat. Meanwhile, transgenic *RD29A-GmDREB* wheat plants had a better tolerance compared to wild type plants which shows the contribution of these two genes in wheat drought tolerance (Shiqing et al., 2005). At 360 minutes after dehydration imposed, *DREB2* down-regulated in the root of Roshan and up-regulated in the shoot. As an early gene, *DREB2* expression is rapid and transient (peaking at 2 or 3 hours) and then their mRNA levels will decrease (Yamaguchi-Shinozaki and Shinozaki, 2005; Agarwal et al., 2006, 2010). *DREB2* down-regulation at longer time of dehydration (360 min) in the tolerant cv. may be due to the transient role of this transcription factor. In contrast, in the sensitive cv., *DREB2* up-regulated at this time in the root and down-regulated in the shoot. It seems that in the sensitive cv. at the shorter time (120 min), dehydration could not make *DREB2* up-regulation unless dehydration duration would be longer. Longer dehydration (360 min) means more loss of water potential and more osmotic signals which were produces by the root cells (Taylor et al., 2000; Stein et al., 2000; Rock, 2000; Liu et al., 2005). Likely, the production of signals for stimulation of *DREB2* expression was enough only for the roots of sensitive cv. but not for its shoots. This is the reason for *DREB2* down-regulation even at 360 min after dehydration applied.

In this experiment, up-regulation of *DREB2* in the tolerant and moderately tolerant pairs and its down-regulation in the sensitive and moderately sensitive pairs at dehydration supported the role of *DREB2* gene as an essential early stress-responsive gene in plant tolerance to dehydration (Yamaguchi-Shinozaki and Shinozaki, 2005; Fujita et al., 2011). The early genes encode regulatory proteins such as transcription factors, signal transduction related proteins, osmolytes biosynthesis (Agarwal et al., 2010). There is evidence for better growth of recombinant *E. coli* cells expressing *SbDREB2A* (obtained from *Salicornia brachiata*) in the culture mediums enriched by NaCl, PEG, and mannitol due to the role of plant's *SbDREB2A* in the regulation of other stress-regulated genes. This function represents certain interactions between the plant's *SbDREB2A* with a transcriptional network of the bacterial cells (Gupta et al., 2010). According to our results, it seems that there is probably proper crosstalk between the transcriptional network of *A. brasilense* Sp245 (the most homologous strain) with Roshan (the most proper cultivar of wheat) which causes highest *DREB2* up-regulation in the root of the tolerant pair. *DREB2* up-regulation in the root of tolerant pair was more than the shoot which might be due to the localization of *A. brasilense* Sp245 in the root of Roshan cv. and supports this crosstalk between the transcriptional network of bacteria and plant. *DREB2* up-regulation in the root (778-fold) of tolerant pair was more than shoot (1.2-fold) but in the tolerant cv. *DREB2* expression was less in the root than the shoot under dehydration condition. This result supports the role of Sp245 to change *DREB2* expression in the root of Roshan cultivar. In the sensitive cv., unlike the tolerant cv., *DREB2* expression did not change.

Dual effects of dehydration and inoculation on *DREB2* expression in the tolerant and sensitive pair were different. This effect was positive in the tolerant and moderately tolerant pairs but it was mostly negative or ineffective in the sensitive and moderately sensitive pairs. Meanwhile, the quantity of dual effect was dependent on dehydration

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

course. According to our result, it is suggested that inoculation of wheat with *A. brasilense* Sp245 can provide a proper tool to improve tolerance of wheat to dehydration via regulation of *DREB* expression.

Conclusion

In conclusion, the effect of inoculation on physiological (growth and biochemical) parameters of wheat seedlings in the sensitive and moderately sensitive pairs was less than the tolerant and moderately tolerant pairs at drought or optimum water condition. The dual effects of dehydration and inoculation on *DREB2* expression were positive in the tolerant and moderately tolerant pairs and mostly negative or ineffective in the sensitive and moderately sensitive pairs. However, these effects were depending on the duration of dehydration. It seems that dehydration alone could not cause up-regulation of *DREB2* in non-inoculated wheat cultivars unless the time of stress would be enough meanwhile over-expression of *DREB2* in inoculated wheat cultivars with *A. brasilense* after dehydration may be more due to the effect of the bacteria on wheat seedlings. The authors feel further research is needed to find out the details of the effect of *A. brasilense* on *DREB2* gene expression in wheat including signal transduction pathways and their components using wheat and or *A. brasilense* mutants.

Acknowledgements. Authors wish to thank Dr. Michael Rothballer from AMP Research Unit Microbe-Plant Interactions for providing the bacterial strains. Financial support by the University of Isfahan is gratefully acknowledged.

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DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

APPENDIX

Appendix 1. *The averages of ranking numbers in the associated pairs between wheat (Triticum aestivum L.) cultivars (Omid, Roshan, Sardari, Shahpasand, Shoaleh and Tabasi) and two strains of A. brasilense (Sp7, Sp245) at optimum water content (80% FC) and drought condition (40% FC) based on growth and biochemical parameters. At 80% FC, the minimum and maximum numbers show the most compatible (homologous) and most non-compatible (heterologous) association pairs, respectively; At 40% FC, the minimum and maximum numbers show the most tolerant and most sensitive association pairs to drought, respectively; At 40% FC, Tabasi cultivar is not included due to lack of its successful association with both bacterial strains under optimum water condition) (NI: non-inoculated)*

Pairs		**Ranking number**	
Wheat cultivars	***A.brasilense* strains**	**80% FC**	**40% FC**
Omid	Non-inoculated	8.2	8.5
	Sp7	6.3	7.2
	Sp245	4.5	5
Sardari	Non-inoculated	12.2	10
	Sp7	9	7.3
	Sp245	7.7	5.5
Roshan	Non-inoculated	6.5	4
	Sp7	3.3	2.7
	Sp245	**3** (Minimum)	**1** (Minimum)
Sloaleh	Non-inoculated	13.8	15.0
	Sp7	10.3	14
	Sp245	7.5	11.2
Tabasi	Non-inoculated	12.5	-
	Sp7	10.5	-
	Sp245	10.5	-
Shahpasand	Non-inoculated	12.2	10.8
	Sp7	**11** (Maximum)	**16** (Maximum)
	Sp245	8	11.7

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_48834902

COMPARISON OF HABITAT SUITABILITY INDEX MODELS FOR PURPLEBACK FLYING SQUID (*Sthenoteuthis oualaniensis*) IN THE OPEN SOUTH CHINA SEA

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(Received 29th Aug 2018; accepted 7th Nov 2018)

Abstract. Fisheries resources in inshore regions of the South China Sea (SCS) have been depleted by overfishing. Purpleback flying squid (*Sthenoteuthis oualaniensis*) fishery is considered as a potential resource in the open SCS. However, little is known about their optimal habitat. Here, habitat suitability index (HSI) models were constructed using the external envelope and arithmetic mean methods to identify optimal habitats of purpleback flying squid. The matched fishery and environmental data (sea surface temperature, sea surface height anomaly, and ocean net primary productivity data) were collected from 2010 to 2014 in the open SCS. We calculated catch per unit effort (CPUE) and fishing effort to estimate the suitability index. The estimated suitable ranges for sea surface temperature, sea surface height anomaly, and net primary productivity were 27.1–30.4 °C, 0–0.15 cm, and 236.2–492.8 mg C/m^2 d based on the HSI model incorporating fishing effort. The corresponding suitable ranges estimated by the HSI model incorporating CPUE were 27.4–30.7 °C, 0.03–0.18 cm, and 238.4–475.5 mg C/m^2 d, respectively. When HSI values for 2014 were >0.6, the HSI model based on fishing effort explained 72.6% of the fishing effort and the average CPUE was 4.29 t/d. The HSI model based on CPUE explained 66.39% of the fishing effort for 2014 and the average CPUE was 4.23 t/d. The accuracy of the forecasting model based on fishing effort was higher than that based on CPUE. This HSI model can be used to aid in the management and conservation of purpleback flying squid in the SCS.
Keywords: *ocean remote sensing, SCS, ecological model, fishery resources, catch per unit effort*

Introduction

Commercial fisheries in the South China Sea (SCS) contribute to the tota China's marine fishery by 27.4% (Qiu et al., 2008). However, fisheries resources have depleted because of overfishing in coastal regions of the SCS. More fishing activities spread to the open SCS, capturing cephalopods as main targets (Chen et al., 2013). Specifically, purpleback flying squid (*Sthenoteuthis oualaniensis*), a cephalopod species inhabiting warm waters, is widely distributed in the open SCS (Siriraksophon et al., 2001a, 2011b; Zhang et al., 2015b). As a potential commercial fishery resources for sustainable exploitation, the available annual capture is estimated between 1.3 and 2.0 million tons in the SCS, while the actual capture was 0.05 million tons in 2011 (Siriraksophon et al., 2001a; Ji et al., 2015). Thus, purpleback flying squid fishery will play a crucial role in the SCS's fishery. Although it has been considered to support potentially a more robust fishery in the open SCS because of its abundance, short life span, fast growth rate, high

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

fecundity, and high nutritional value (Chen et al., 2013; Zhang et al., 2015b; Yu et al., 2012), there is no complete and scientific research about the relationship between purpleback flying squid and environmental factors in the open SCS. It is crucial to sustain and protect the fishery in the SCS under environmental changes in future (Ji et al., 2015).

Commercial fisheries data at large-scale are available as an important index to understand status of fishery resource (Hilborn and Walters, 1992). Catch and effort from commercial fishery were commonly used to develop models to predict distribution of fish species (Morris and Ball, 2006; Tian, et al., 2009). The catch per unit effort (CPUE) is commonly used to analyze the relationship between environmental variables and fish habitat, but commercial fisheries CPUE is influenced by other variables. Thus CPUE must be standardized to be a reliable abundance index (Zhang et al., 2015a). In addition, comparison of fishing effort and CPUE as an abundance index are be necessary especial for purpleback flying squid with high swimming speed (Tian et al., 2009; Zhang et al., 2015a; Pedro et al., 2006).

Habitat suitability index (HSI) model is suitable for describing and evaluating habitat qualities and species distributions (US Fish and Wildlife Service, 1981), including management of species, ecological environmental restoration (Duel et al., 1996; Gore and Hamilton, 1996; Maddock, 1999), and forecasting of central fishing grounds (Tian et al., 2009; Li et al., 2015). In addition, it has been applied to investigate relationships between fishing ground and relevant environmental factors effecting distribution of flying squid species in northwestern Indian Ocean (Yu et al., 2012), northwestern Pacific Ocean (Tian et al., 2009). Currently, large variation in spatial distribution of capture for purpleback flying squid fishery in the SCS existed, with overfishing in the coastal regions and underutilization in the open regions.

The HSI model for purpleback flying squid in the western Indian Ocean was applied to construct relationships between its catch and habitat factors (Chen et al., 2007), while, due to the dataset with short time series and mere catches of Chinese's vessels they used, their study is limited to illustrating general theoretical relationships to explore and protect this species. Thus, more accurate estimations are needed to identify the suitable habitat for purpleback flying squid. To aim this, using the HSI model, a full and specific fishery dataset (2010-2014) in the SCS were used to investigate optimal habitat with high production and corresponding habitat factors, including sea surface temperature (SST), sea surface height anomaly (SSHA), and net primary productivity (NPP). Additionally, the final goal is to effectively develop purpleback flying squid fishery by predicting locations of fishing grounds of purpleback flying squid in the open SCS.

Material and methods

Data

Commercial fishery data of purpleback flying squid were obtained from the SCS's dynamic fishing and monitoring network. This data recorded the date, location, catches and number of nets for each fishing activities from 2010 to 2014. In addition, the full ranges of these activities covered the area of 105°–120°E, 5–25°N (*Fig. 1*). Changes in monthly number of nets for purpleback flying squid over this time period were illustrated in *Figure 2*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

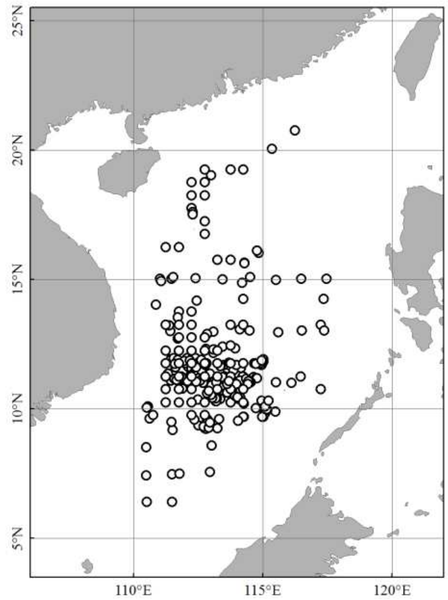

Figure 1. *The location of fishery data collected during 2010–2014 in the open South China Sea*

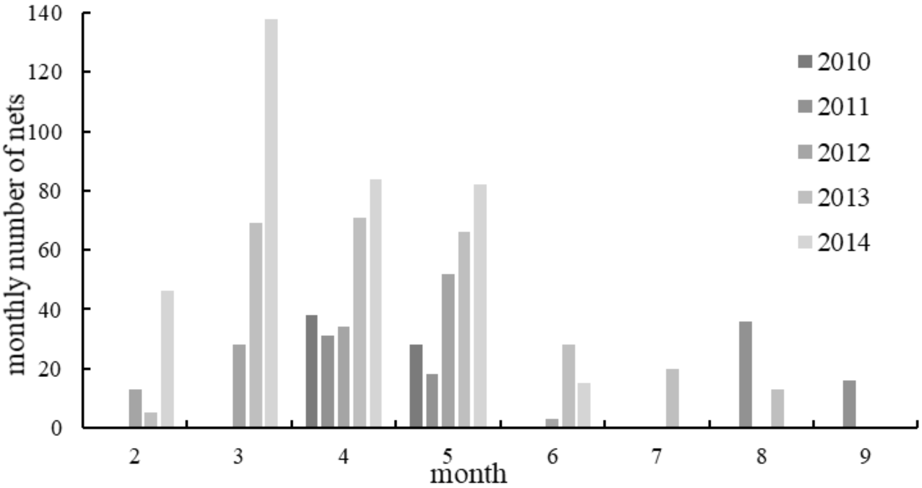

Figure 2. *Changes in monthly number of nets for purpleback flying squid during 2010–2014 in the open South China Sea*

Environmental data included SST, SSHA and NPP. The daily SST data were derived by the remote sensing system research company located in Northern California (http://www.remss.com/measurements/sea-surface-temperature). These data were the optimally interpolating SST products merging from microwave and infrared SST maps at an approximately 9 × 9 km resolution. The daily SSHA images were downloaded from Centre National d'Etudes Spatiales (CNES) data center (http://www.aviso.altimetry.fr/en/data/products/sea-surface-height-products.html), and merged based on altimeter data (Topex/Poseidon, JASON-1, Jason-2, Envisat, ERS-1, ERS-2, and Cryosat-2) at a 0.25 × 0.25 degree resolution. The 8 day average global ocean net primary production (NPP) data products were obtained from the Oregon State University College of Science

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DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

(http://orca.science.oregonstate.edu/1080.by.2160.monthly.hdf.vgpm.m.chl.m.sst.php), and were estimated based on MODIS at a 1/6 degree spatial resolution. All environmental data covered the full ranges of fishing grounds over the entire study period.

Methods

Data processing

All environmental data were processed at a 0.5 × 0.5 degree resolution and matched to the ranges of the fishing grounds using MATLAB 2012b (The MathWorks, Inc., Natick, Massachusetts, United States).

The nominal catch per unit effort (CPUE) for each cell was calculated in *Equation 1* as follows:

$$CPUE_{i,j} = \frac{\text{Catch}_{i,j}}{effort_{i,j}} \tag{Eq.1}$$

where $Catch_{i,j}$ and $effort_{i,j}$ is the total weight of catches, and numbers of nets for the ith and jth cell, respectively.

The nominal CPUE in *Equation 1* hardly indicates the true abundance of fishery over the entire fishing geographical locations and periods, because many factors can affect their accuracies (Zhang et al., 2015a; Maunder et al., 2006). Thus, to remover these effects, a standardized CPUE was computed using a support vector machine method following Yang et al. (2015). See Yang et al. (2015) for the detail processes for standardizing CPUE.

Building a suitability index (SI) model

A number of models and methods have been used to forecast the location of fishing grounds (e.g., Lee et al., 2005; Grant et al., 1988; Georgakarakos et al., 2006; Skov et al., 2008). HSI models appear to perform well for identifying the location of purpleback flying squid fishing grounds (Tian et al., 2009; Li et al., 2015). A number of methods have been used to calculate the Suitability index (SI) in HSI models, including the external envelope method, BP neural networks, and non-linear regression (Chen et al., 2009; Yu et al., 2012; Frenkel et al., 2017). Among these, the external envelope method appears more suited for cephalopoda (Fang et al., 2014; Gurkan, 2016).

Effort can be used as an index of fish presence or provide general information on the utilization of fishery resources (Andrade et al., 1999; Swamy et al., 2017). And we want to know whether swimming speed has an effect on catch, we adopted effort as a variable to build model. The correlation between effort and Standardized CPUE was analyzed, P value is 0.7724, which means there is no significant correlation between effort and standardized CPUE in other words.

Thus, we modeled the relationships between effort or standardized CPUE, and SST, SSHA, and NPP using the external envelope method during the years 2010–2013. When effort or standardized CPUE is highest, the SI value is 1, and when effort or standardized CPUE is 0, the SI value is 0 (Mohri et al., 1999). We calculated SI using *Equations 2* and *3:*

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$$SI_{effort} = \frac{effort_{i,j}}{\text{effort}_{\max}} \quad \text{(Eq.2)}$$

$$SI_{CPUE} = \frac{CPUE_{i,j}}{CPUE_{\max}} \quad \text{(Eq.3)}$$

where SI_{effort} (SI_{CPUE}) represents SI values computed based on effort (CPUE), $effort_{ij}$ ($CPUE_{ij}$) represents the effort (CPUE) at the location represented by longitude i and latitude j, and $effort_{\max}$ ($CPUE_{\max}$) represents the maximum of all effort (CPUE) data.

Building the HSI model

Prior research suggests that the arithmetic mean method (AM) is a better predictor of the fishing ground location than the geometric mean method (Li et al., 2015; Mejia-Sanchez et al., 2018). Thus, we used the AM method to calculate HSI values during HSI model establishment. The HSI was calculated using *Equations 4* and *5:*

$$HSI_{\text{effort}} = (SI_{SST\_effort} + SI_{SSHA\_effort} + SI_{NPP\_effort})/3 \quad \text{(Eq.4)}$$

$$HSI_{CPUE} = (SI_{SST\_CPUE} + SI_{SSHA\_CPUE} + SI_{NPP\_CPUE})/3 \quad \text{(Eq.5)}$$

where HSI_{effort}, and HSI_{CPUE} represent HSI values calculated based on effort and CPUE, respectively. $SI_{\text{SST\_effort}}$, $SI_{\text{SSHA\_effort}}$, and $SI_{\text{NPP\_effort}}$ represent SI values computed based on effort using SST, SSHA, or NPP as the variable, respectively. $SI_{\text{SST\_CPUE}}$, $SI_{\text{SSHA\_CPUE}}$, and $SI_{\text{NPP\_CPUE}}$ represent SI values computed based on CPUE using SST, SSHA, or NPP as the variable, respectively.

HSI model verification and comparison

The HSI values in 2014 were obtained using the models described above, and divided into five classes: 0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1 (Yu et al., 2012; Li et al., 2015). Then, the cumulative effort and catch was counted separately in each class (Tian et al., 2009). The habitats with HSI values >0.6 were considered to represent habitat near the center of the fishing grounds (Yu et al., 2012; Yuan et al., 2017). Percentages of effort with HSI values >0.6 computed by HSI models based on effort and CPUE, respectively were compared to get an optimal model for studying habitant of purpleback flying squid.

Results

Relationships between fishing effort, CPUE, and environmental factors

Figure 3 illustrated relationships between the SI based on fishing effort (*Fig. 3a-c*) and CPUE (*Fig. 3d-f*) and environmental factors. For the SI from the fishing effort, the optimal ranges for SST, SSHA, and NPP are 27.1–30.4 °C, 0–0.15 cm, and 236.2–492.8 mg C/m^2 d., respectively. For the SI from CPUE, the corresponding suitable ranges are 27.4–30.7 °C, 0.03–0.18 cm, and 238.4–475.5 mg C/m^2 d, respectively.

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DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

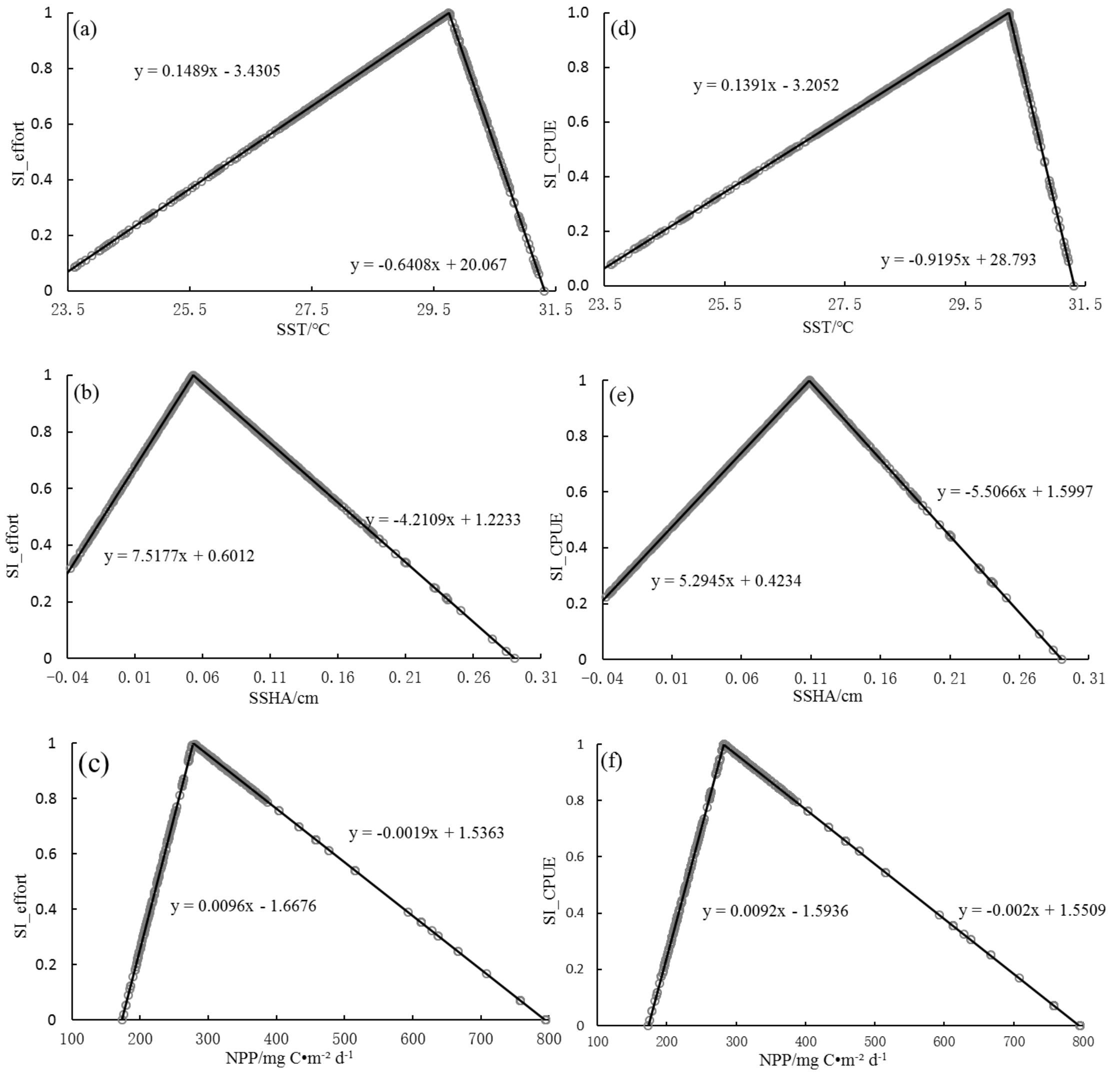

***Figure 3.** The suitability index curves based on fishing effort and catch per unit effort (CPUE) during 2010–2013 in the open South China Sea*

Models comparisons

Regions with a HSI value greater than 0.6, are considered as the preferred fishing grounds for purpleback flying squid in the open SCS. Thus, in this study, 72.45% of the catches and 71.88% of fishing effort in whole fishing grounds is with HSI over 0.6 based on HSI model from fishing effort during the period from 2010 to 2013 (*Table 1*), while, 68.86% of the catches and 70.24% of fishing effort in whole fishing grounds is with HSI over 0.6 based on HSI model from CPUE.

Verification and comparison of fishing ground distribution

When HSI is >0.6, the HSI model based on fishing effort verifies fishing effort in 2014 with 72.6% accuracy and the average CPUE is 4.29 t/d. Similarly, the model based on CPUE is 66.39% accurate and the average CPUE is 4.23 t/d (*Table 2*). The accuracy of the forecasting model based on fishing effort is higher than that based on CPUE. Thus, the HSI model based on fishing effort is the best available model for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

forecasting the distribution of habitats suitable for purpleback flying squid in the open SCS. The precision of this forecasting model is 72% when actual fishing effort can be used as an index to judge the accuracy of main fishing grounds. Catch and HSI values of SST, SSHA and NPP in 2014 were calculated using above HSI model based on fishing effort illustrated in *Figure 4*.

Table 1. *Percent of catch and fishing effort explained by the HSI models based on fishing effort or catch per unit effort (CPUE)*

HSI	HSI model based on fishing effort			HSI model based on CPUE		
	Percentage of catch /%	Percentage of fishing effort /%	Average CPUE /(t/d)	Percentage of catch /%	Percentage of fishing effort /%	Average CPUE /(t/d)
0–0.2	4.77	4.39	1.73	5.08	4.51	1.79
0.2–0.4	7.46	8.73	1.36	14.02	12.07	1.85
0.4–0.6	15.32	15.00	1.63	12.04	13.18	1.45
0.6–0.8	22.31	24.96	1.42	23.91	24.60	1.55
0.8–1	50.14	46.92	1.70	44.95	45.64	1.57

We used sea surface temperature (SST), sea surface height anomaly (SSHA), net primary productivity (NPP) as explanatory variables for the years 2010–2013 in the open South China Sea

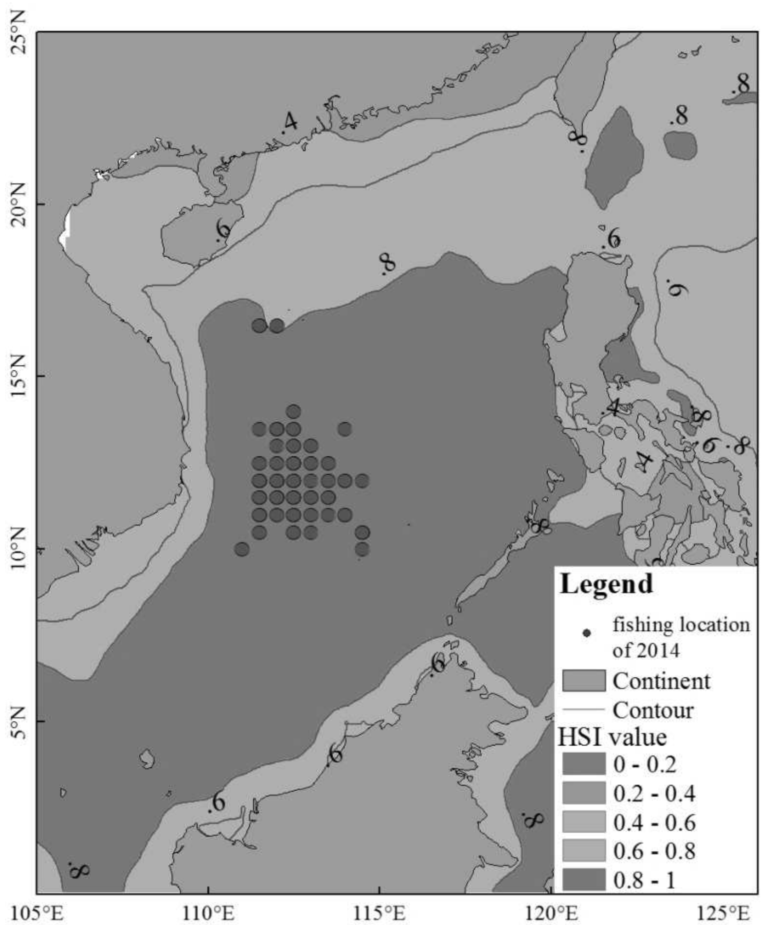

Figure 4. *Distribution of projected HSI values and fishing location of 2014 in the open South China Sea*

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DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

Table 2. *Percentage of catch and fishing effort in 2014 explained by the HSI model based on fishing effort and catch per unit effort (CPUE)*

HSI	HSI model based on fishing effort			HSI model based on CPUE		
	Percentage of catch /%	Percentage of fishing effort /%	Average CPUE /(t/d)	Percentage of catch /%	Percentage of fishing effort /%	Average CPUE /(t/d)
0–0.2	0.88	1.28	1.41	0.51	0.73	1.43
0.2–0.4	2.89	5.30	1.11	5.84	7.31	1.63
0.4–0.6	19.05	20.82	1.87	24.01	25.57	1.92
0.6–0.8	27.85	27.85	2.04	26.94	28.13	1.95
0.8–1	49.32	44.75	2.25	42.70	38.26	2.28

We used sea surface temperature (SST), sea surface height anomaly (SSHA), net primary productivity (NPP) as explanatory variables for the years 2014 in the open South China Sea

Discussion

Changes in the fishing ground were impacted by multi-factors, e.g. life history for each fish species, supply of food, and changes in environmental factors (Chen et al., 2007; Yan et al., 2012). Among these factors, the later one can directly affect fish's life cycle by, e.g. food supplication or distribution of prey resources (Yu et al., 2016). For detail, SST can determine the general distribution patterns by affecting the processes of birth, reproduction, and migration (Ji et al., 2015; Zhang et al., 2015a).

Sea surface height (SSH) data reflects the influence of the direction and velocity of water flow, the interaction of cold and warm water masses, and other dynamic environmental variables (Shao et al., 2008; Song, 2014), which, in turn influence the distribution of fauna and the formation of fishing grounds (Shao et al., 2008). SSHA reflects the difference between sea surface height and mean sea level. Thus, SSH or SSHA data are often used in assessments of the relationship between environmental factors and fisheries (Song, 2014). NPP data have been used to model tuna, mackerel, and squid resources in other regions (Zainuddin et al., 2006; Yu et al., 2016). To our knowledge, this is the first study to evaluate the utility of using NPP to predict purpleback flying squid distribution in the open SCS. Our analysis suggests that it is feasible to build an HSI model based on SST, SSHA, and NPP to describe the fishing distribution of purpleback flying squid in the open SCS. For the period 2010–2013, the suitable range of SST, SSHA, and NPP was 27.1–30.4 °C, 0–0.15 cm, and 236.2–492.8 mg C/m^2 d, respectively. This is consistent with prior studies that estimated the range of suitable SST for purpleback flying squid was 27–29°C in the northwestern waters of the Indian Ocean (Yu et al., 2012; Luo et al., 2015) and an optimal SSHA of 0.00 in the northwest Indian Ocean (Shao et al., 2008).

Conclusions

Analysis of the HSI models

Because of sovereignty disputes in the SCS, the spatial scale of fishing is limited. To ensure forecasting accuracy, we used daily records of environmental data rather than monthly. Additionally, because of the influence of atmospheric climate on oceanic variables, the environmental factors that exhibit high spatial and temporal resolution with respect to the distribution of purpleback flying squid should also reflect the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49034913

influence of season on distribution of purpleback flying squid. We compared two HSI models, one based on fishing effort and the other based on CPUE using the external envelope method. The precision of the HSI model based on fishing effort (72.6%) was higher than the HSI model based on CPUE (66.39%). Thus, the HSI model based on fishing effort appears to provide more realistic results when his values >0.6.

Improvement of the HSI model

Although the HSI model described in this paper has high forecast precision, it could be improved by increasing the amount of fishing data used to parameterize the model. Additionally, although we used three commonly referenced environmental variables, purpleback flying squid may be influenced by other environmental factors that were not considered (Jia et al., 2004; Yu et al., 2012). Thus, we recommend developing the current HSI model to incorporate more fishery data and test the influence of other environmental factors (e.g. currents, sea surface salinity) and interactions among variables.

Acknowledgements. This research was financially supported by Project 31602206 supported by National Natural Science Foundation of China, the Natural Science Foundation of Shanghai under contract NO. 16ZR1444700, the Key Technologies R&D Program of China under contract No. 2013BAD13B06 and Project NO.2016T05 Supported by Special Scientific Research Funds for Central Non-profit Institutes (East China Sea Fisheries Research Institute). And we also acknowledge China Scholarship Council (File No.201803260014).

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OVIPOSITION DETERRENCE AND ADULT EMERGENCE INHIBITION ACTIVITIES OF *CYMBOPOGON NARDUS* AGAINST *CULEX QUINQUEFASCIATUS* WITH STUDY ON NON-TARGET ORGANISMS

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(Received 5th Nov 2018; accepted 25th Jan 2019)

Abstract. The present study aimed to investigate the oviposition deterrent and adult emergence inhibition activities of non-polar solvent extract of *Cymbopogon nardus* whole plant against *Culex quinquefasciatus* mosquito with effect on non-target organisms. During the oviposition deterrent activity, the 1000 ppm concentration of extract showed maximum (71.9±3.3 %) effective repellence (ER). The lowest concentration (31.25 ppm) caused 13.4±1.3 % ER. The oviposition activity index (OAI) of the gravid female *Cx. quinquefasciatus* was estimated. For each concentration, negative OAI value was found. The negative OAIs values indicated that the n-hexane extracts of *C. nardus* whole- plant is oviposition repellent. Lowest OAI value was exhibited by 1000 ppm extract solution. During the adult emergence inhibition (EI) activity, the *C. nardus* n-hexane extract solutions restricted adult emergence. There occurred 31±17.9 % adult emergence from the containers containing 1000 ppm extract solution. From the container containing 125 ppm extract solution, 82.3 ± 5.6 % adult emerged. The 1000 ppm of extract solution caused 66.2 ± 19.5 % EI. At 125 ppm extract solution, 12.2 ± 6.5 % EI was observed. There was a positive correlation between extract concentration and EI % (R square =0.86). The EI_{50} value of *C. nardus* was 515.2 ppm. During the study of effect of *Cymbopogon nardus* whole-plant n-hexane extract on non-target aquatic insects i.e., damselfly and dragonfly nymphs, the extract caused no mortality of any nymph up to 500 ppm. However, at 1000 ppm concentration, the plant extract caused some mortality (5 %). During the study of effect of extract on grass carp fish (*Ctenopharingodon idella*), there occurred no mortality during 24-hour exposure. During the study of effect of extract on rabbits, there occurred no mortality and found no significant alterations in the normal biochemical and hematological parameters. Photomicrographs of microtome sections of liver and kidney of extract treated rabbit groups showed normal histoarchitecture. From the findings of the present research, it was concluded that the n-hexane extract of *C. nardus* whole-plant possesses oviposition deterrent and adult emergence inhibition activities against *Cx. quinquefasciatus*. It was also concluded that the n-hexane extract of *C. nardus* whole plant is least toxic to damselfly and dragonfly nymphs. The extract does not cause mortality or behavioural

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

abnormality in fish. The ingestion of *C. nardus* whole-plant n-hexane extract in rabbits do not cause mortality or alterations in the normal biochemical and haematological parameters of rabbits.
Keywords: *effective repellence, oviposition activity index, damselfly and dragonfly nymphs, grass carp, rabbit, biochemical and haematological parameters*

Introduction

Mosquitoes are constant threats to human by transmitting several diseases such as malaria, filariasis, West Nile Virus infection, dengue fever and yellow fever (Farajollahi et al., 2011). Three genera viz; *Anopheles*, *Aedes* and *Culex* are more important as many of their species are vectors of diseases in human and other animals. *Culex quinquefasciatus* is a well-known culicine mosquito that causes serious nuisance through its irritating biting and is the vector of *Wuchereria bancrofti,* that causes lymphatic filariasis in humans (Rajasekariah et al., 1991). This disease has affected more than 100 million people in Asia, Africa, Central- and South America and the Pacific (Simonsen, 2009). In Pakistan, Beg et al. (2001) reported confirmed cases of tropical pulmonary eosinophilia in indigenous patients however this disease is very rare in Pakistan.

Synthetic chemical insecticides such as organochlorine and organophosphate compounds are commonly applied for the control of mosquitoes (Ghosh et al., 2012). The frequent application of synthetic chemical insecticides has caused the development of insecticide resistance in insect pests, contamination of the environment, and adverse effects on non-target organisms (Lee et al., 2001). Alternative approaches which are environment friendly should be adopted for controlling mosquito population (Ghosh et al., 2012). Biological control is the environment friendly and effectual means of managing pest. The biological control agents include some larvivorous fish (Walton, 2007), predatory insects (Mandal et al., 2008), protozoans (Das et al., 2016), bacteria (Phillips, 2001) and plants (Ajaegbu et al., 2016).

Plant-based insecticides have got attraction (Sivagnaname and Kalyanasundaram, 2004; Singh et al., 2006) as they are biodegradable, effective and environment friendly (Wang et al., 2000; Nerioa et al., 2010). Extracts of some plant have been reported for their larvicidal (Al-Mehmadi et al., 2010; Rawani et al., 2014), pupicidal (Kovendan et al., 2012), adulticidal (Jayapriya and Gricilda-Shoba, 2015; Ajaegbu et al., 2016) and insect repellent (Bekele and Petros, 2017) activities against mosquitoes.

In addition to the use of plants as general toxicants against mosquito larvae or adults, plant-based insecticides also deter the female adult mosquitoes from egg laying (Prajapati et al., 2005). The oviposition deterrent effect of plant extract may be due to the changes induced in the physiology and behavior of the female adult mosquitoes reflected by their egg-laying capacity. Some phytochemicals act as growth regulators or chemosterilent while some produce olfactory stimuli acting as repellent or attractant (Prathibha et al., 2014). The plants that possess insect repellent property may also exhibit oviposition deterrent and adult emergence inhibition properties (Rajkumar and Jebasan, 2009). The oviposition deterrent and adult emergence inhibition activities of some insecticidal repellent plant have been reported (Prathibha et al., 2014; Elango et al., 2012). The plant *Cymbopogon nardus* (Linn.) belongs to the family Poaceae and locally called Sargarai in Dir Lower, Khyber Pakhtunkhwa, Pakistan. The essential oil of *C. nardus* has been reported for its insect repellent activity against mosquitoes (Silva et al., 2011). The present study aimed to determine the oviposition deterrent and adult emergence inhibition activities of *Cymbopogon nardus* against *Culex quinquefasciatus*.

Synthetic chemical insecticides also kill non-target organisms along with the target insect pests (Zacharia, 2011; Morrissey et al., 2015), but botanical pesticides are claimed to be safe or least toxic for non-target organisms (Rawani et al., 2014). Few studies have been conducted on the mosquitocidal activity of plant extracts in which the effect of plant extract on non-target aquatic insects (*Chironomus*) (Chowdhury et al., 2009), fish (*Gambusia affinis*) and tadpole (*Bufo*) (Adhikari et al., 2012) and even mammal (mice) (Carvalho et al., 2003) have been studied. According to the results of these studies, plant extracts appeared safe for non-target organism. The present research also aimed to study the effect of *Cymbopogon nardus* whole-plant n-hexane extract on non-target organisms i.e., damselfly and dragonfly nymphs and grass carp fish due to their habitat similarity with mosquito larvae. Synthetic chemical insecticides damage body organs in mammals if ingested (Tomlin, 2000; Soni et al., 2011). During the present research, the effects of oral administration of *Cymbopogon nardus* whole-plant n-hexane extract on some biochemical and haematological parameters of mammal i.e., rabbits were also studied.

Materials and Methods

Preparation of plant extract

Cymbopogon nardus (Linn.) whole-plant was collected from non-cultivated fields in Chakdara, Dir Lower Khyber Pakhtunkhwa, Pakistan and authenticated by an expert in plant taxonomy at the Department of Botany University of Malakand. The plants were shade dried and then ground into powder form by using electric grinder. During the present research, 1500 grams powder of *C. nardus* was soaked in 7500 ml non-polar solvent i.e., n-Hexane in 15-liter plastic bucket. After soaking for three days, the plant material was filtered through Whatman filter paper 42. The filtrate was evaporated through rotary vacuum evaporator and extract in dense solution form was then poured from the bulb of rotary evaporator into a clean dry glass beaker and placed under running fan for an hour. Finally, n-hexane extract of *C. nardus* whole-plant was obtained in oily paste form.

Laboratory rearing of Culex quinquefasciatus mosquito

Colony of *Cx. quinquefasciatus* was established in the laboratory (*Figure 1*) at the University of Malakand, Chakdara, Dir Lower, Khyber Pakhtunkhwa, Pakistan. For this purpose, larvae of *Cx. quinquefasciatus* were collected by using a rectangular plastic dipper (38 cm length, 28 cm width and 6.5 cm height) from a ditch containing stagnant water at the campus of university of Malakand. The larvae were brought in 700 ml plastic containers with water from the collection site to the laboratory and reared for establishing a colony. The larvae were provided with larval food comprising of dog biscuit and dry yeast powder in the ratio of 3:2. The pupae emerged were transferred to a 500 ml plastic jar containing 300 ml non-chlorinated tap water and placed in mosquito cage (45 cm × 45 cm × 45 cm). The adults emerged were fed with carbohydrate food by providing cotton pad soaked in 10 % sucrose solution. The female adult mosquitos laid eggs in the jar containing water inside the cage which then hatched into larvae. For confirmation of species proper literature was used for identification of both larvae and adults (Harbach, 1988). Powdered yeast and dog biscuit in 2:1 ratio was provided as food to the larvae. The adults obtained were initially provided with 10% sucrose and

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later blood fed periodically by allowing mice for eggs development. Larvae and adults of *Cx. quinquefasciatus* were available.

Figure 1. *Picture of laboratory reared colony of Cx. quinquefasciatus established during the present experiments*

Oviposition deterrent bioassay

During this bioassay, guidance was taken from the method of Xue et al. (2001). Three concentrations i.e., 1000, 250 and 31.25 ppm of *C. nardus* whole-plant n-hexane extract were selected during this study. The selection was based on the fact that these concentrations showed highest, moderate and lowest larval mortality during our study on larvicidal activity of *C. nardus* whole-plant n-hexane extract against *Cx. quinquefasciatus* (Ilahi and Yousafzai, 2017). 1000 ml stock solution of 2000 ppm extract was prepared in non-chlorinated tap water in glass flasks containing 1.6 % acetone and 0.05 % tween 80. From the stock solution, 200 ml solution of 1000 ppm, 250 ppm and 31.25 ppm were prepared in three 400 ml polyethylene containers by applying dilution equation, $C1V1=C2V2$. The 1000, 250 and 31.25 ppm extract solutions were placed inside the mosquito cages A, B and C, respectively. A control container containing 200 ml non-chlorinated tap water with 1.6 % acetone and 0.05 % tween 80 was also placed inside each cage. The experiment was run in triplicates. Into each mosquito cage, 100 blood fed and gravid female *Cx. quinquefasciatus* mosquitoes caught from the existing laboratory colonies were introduced. The mosquitoes laid eggs after 2 or 3 days of introduction. For each jar, total number of eggs and rafts were counted under dissecting microscope. The oviposition data was presented as percentage of effective repellence (ER %) and oviposition activity index (OAI). Positive OAIs are considered as attractants, while negative OAIs are considered as repellents (Govindarajan et al., 2011). The ER % was calculated by using the following method of Rajkumar and Jebasan (2009) (*Eq.1*):

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$$ER\% = \frac{NC - NT}{NC} \times 100 \qquad \text{(Eq.1)}$$

Where ER: Effective repellency; NC: Number of eggs in control; NT: Number of eggs in treatment. The oviposition activity index (OAI) was calculated by using the following formula of Kramer and Mulla (1979) (*Eq.2*):

$$OAI = \frac{NT - NC}{NT + NC} \qquad \text{(Eq. 2)}$$

Where Nt: total number of eggs in the treatment container, Nc: total number of eggs in the control container. The oviposition data for each extract was presented as mean ER % and mean OAI.

Egg rafts and eggs of *Cx. quinquefasciatus* are shown in *Figure 2* and *Figure 3*, respectively.

Figure 2. *Egg rafts deposited by Culex quinquefasciatus during the present experiments*

Figure 3. *Microscopic picture of Culex quinquefasciatus eggs in rafts taken during the present experiments*

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DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

Adult emergence inhibition bioassay

During the present study, the effect of n-hexane extracts of *C. nardus* whole- plant on the adult emergence of *Cx. quinquefasciatus* was studied in the laboratory at 1000, 500, 250 and 125 ppm. The selection of theses concentrations was based on the fact that these concentrations showed highest, moderate and lowest mortality during our study on larvicidal activity of *C. nardus* whole-plant n-hexane extract against *Cx. quinquefasciatus* (Ilahi and Yousafzai, 2017). Guidance was taken from the work of Elimam et al. (2009) who followed the procedure for testing insect growth regulators. 300 ml extract solutions of 1000, 500, 250 and 125 ppm were prepared in four 600 ml polyethylene containers from a 2000 ppm stock solution containing 1.5 % acetone and 0.05 % tween 80. A 600 ml control polyethylene container containing 300 ml non-chlorinated tap water (with 1.5 % acetone and 0.05 % tween 80) was also placed along each concentration. One hundred 3rd instar larvae of *Cx. quinquefasciatus* were transferred from the mosquito breeding containers to each treatment container. One hundred 3rd instar larvae of *Cx. quinquefasciatus* were also transferred to the control containers. This experiment was run in four replicates. The duration of the test was long therefore yeast was provided to each container as larval food at interval of two days. The jars were capped with gauze to prevent the escape of emerging adult mosquitoes. The jars were daily checked for the appearance of pupae and adults. The adults appeared were caught with the help of mouth aspirator and then put into a clean dry reagent bottle and anaesthetized by applying cotton swab soaked in diethyl ether. The experiment was conducted during 14-25 August 2016, and the maximum temperature inside the laboratory was 30°C to 34°C. The observations were continued till all the larvae or pupae in the control have died or emerged as adults. At the end of experiment the number of adults emerged was noted for each treatment and control container. The number of adults emerged in control and each of the treatment containers was noted. The effect was expressed as percentage of emergence inhibition (EI %). The EI % was calculated by using the following formula used by Elimam (2007) (*Eq.3*):

$$EI = 100 - \left[\frac{T \times 100}{C} \right] \quad \text{(Eq. 3)}$$

Where T represents percentage emergence in treatment container and C represents percentage emergence in the control container.

Adult emergence in control was more than 95 % therefore there was no need of correction of data by abbot formula. Adult emergence inhibition data was presented as mean EI %. The mean EI % data was subjected to log probit analysis (Finney, 1971) for calculating EI_{50} (a measure of the extract concentration that caused 50 % EI) value using SPSS 16 software.

Effect of plant extract on non-target organisms

During the present research, the effect of *C. nardus* whole- plant n-hexane extract on the following non-target organisms was also studied:

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Effect on damselfly and dragonfly nymphs

Damselfly (order Odonata, sub order Zygoptera) and dragonfly (order Odonata, sub order Anisoptera) nymphs of early instars (4 to 5 instar) were collected from the puddles on the bank of River Swat near the campus of University of Malakand. Collection of nymphs and experiments were conducted during August 2016 (maximum temperature 30-33^{0}C). Damselfly and dragonfly nymphs were collected from pond at the campus of University of Malakand by using a rectangular plastic dipper. The nymphs were transported in plastic jars containing water of the collection site to the laboratory at University of Malakand within 30 minutes of capture. In the laboratory, the nymphs along with water of collection site were transferred to a wide plastic tray (40 cm length, 30 cm width and 8 cm height). Before conducting experiments, the nymphs were fed with dried yeast powder and mosquito larvae. The specimens were identified to the species level with the help of literature (Gardner, 1960; Yousuf et al., 1996; Anjum, 1997; Mitra 2002; Din et al., 2013). Large number of nymphs were belonging to a damselfly species, *Ischnura elegans* and a dragonfly species, *Sympetrum decoloratum*.

During this study, 1000 ppm solution of *C. nardus* whole- plant n-hexane extract was prepared in tap water containing 0.8 % acetone and 0.02 % tween 80. This 1000 ppm extract solution was serially diluted by factor of two, thus six solutions of 1000, 500, 250, 125, 62.5 and 31.25 ppm were arranged in 400 ml polyethylene containers. Volume of testing solution in each polyethylene container was 250 ml. A 400 ml polyethylene control container containing 250 ml non-chlorinated tap water with 0.8 % acetone and 0.02 % tween 80 was also kept. For exposing the nymphs to the toxic effect of plant extract, the following method of Hardersen and Wratten (1996) was followed: seven intact 6th to 7th instar nymphs of each nymph species were placed separately in seven plastic containers (six extract concentrations and one control). In short, 14 containers were arranged for the nymphs of two odonate (damselfly and dragonfly) species, seven for each species. The experiment was run in triplicate and the period of exposure was 72 hours. Following standard toxicity protocols, the nymphs were not fed during the 72 hours exposure (ASTM standard E47, 2008). After 72 hours of exposure period, the numbers of dead and live nymphs were noted. The criterion for death was lack of response to prodding.

Effect of plant extract on freshwater fish

During this study, the toxic effect of *C. nordus* whole-plant n-hexane extract on fresh water fish was studied. Grass carp (*Ctenopharingodon idella*) was selected as test fish for this study because it is commercially important and is widely cultured in fish farms. Healthy grass carp fish of 11.6±1.3 cm length were brought from fish hatchery at Thana Malakand Agency to the laboratory in round plastic jar of 5.5 L volume. The jar was containing water of pond from which fish were collected. The time taken in bringing the fish to laboratory was less than 30 minutes. The fish were brought safe to the laboratory (no fish was died or sluggish). In the laboratory the fish were maintained in small fish aquaria (45cm length, 40 cm width and 40 cm height) containing non-chlorinated tap water. The aquaria were receiving solar illumination through windows and oxygenated by using air pumps. Fish were exposed to a single concentration of *C. nardus* whole-plant n-hexane extract that was ten times higher than its LC50 value for *Cx. quinquefasciatus* 4th instar larvae during our study on larvicidal activity of *C. nardus*

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DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

whole-plant n-hexane extract against *Cx. quinquefasciatus* (Ilahi and Yousafzai, 2017). The LC50 value of *C. nardus* whole- plant n-hexane extract against *Cx. quinquefasciatus* 4th instar larvae was 599.6 ppm. The ten times value of LC50 was 5996 ppm. 20 liters extract solution of 5996 ppm concentration (containing 1.5 % acetone and 0.05 % tween 80) was prepared in non-chlorinated tap water in a fish aquarium (45 cm × 40 cm × 40 cm). A control aquarium containing non-chlorinated tap water with 1.5 % acetone and 0.05 % tween 80 was also arranged. Six grass carp fish were placed in each aquarium (total 12 fish in 2 aquaria). The behavior and mortality in fish of each aquarium was checked for 24 hours. After 24 hours, the extract solution in the aquarium was replaced with clean non-chlorinated tap water and observed for mortality for a further period of 24 hours. Experiment was conducted during August 2016 (maximum temperature <33°C).

Effect of plant extract on rabbit (Oryctolagus cuniculus)

During the present research, the effect of oral administration of high dose of *C. nardus* whole-plant n-hexane extract on some biochemical and hematological parameters were studied in rabbits. For this purpose, male domestic rabbits (*Oryctolagus cuniculus*) weighing 700-900 grams and 5-6 months of age were purchased from local market. They were housed in wide and well-ventilated chambers in Animal House at the University of Malakand, Khyber Pakhtunkhwa, Pakistan. The rabbits were fed on green vegetables and chaw pellets and allowed tap water ad libitum. The animals were kept in such condition for five days for acclimation before start of the experiments. In total, eight rabbits were divided into two groups (A and B), four in each. Rabbits of group A were orally administered *C. nordus* n-hexane extract in 4 ml vegetable oil at a dose of 1000 mg per kg body weight per oral. Group B was control and the rabbits of this group were orally administered only 4 ml vegetable oil per Kg body weight per oral. The mortality and behavior of rabbits were monitored for 48 hours. This study was approved by University of Malakand Animal Ethics Committee. Experiment was conducted during September 2016 (maximum temperature <31°C).

After 48 hours, the animals were sequentially anesthetized with inhaled diethyl ether. Each rabbit restricted on the dissecting board was dissected and 3 mL blood was collected from the heart chambers by cardiac puncture with a 21 Gauge needle mounted on 5 mL syringe and then expelled gradually into Ethylene Diamine Tetra-acetic Acid (EDTA)-coated tubes for estimation of hematological parameters i.e., blood cells count (RBCs, WBCs and platelets count), and hemoglobin concentration. Another 3 ml blood was collected by the same method in sterile tubes with coagulant for estimation of biochemical parameters i.e., ALT, AST, ALP, albumen and globulin. The data was presented as mean with standard error. The data was analyzed by un-paired sample T-test for determining significant difference between the extract treated and control rabbit groups. For these analyses a computer software, SPSS 16 was used.

During this study the, liver and kidney tissues were processed for paraffin embedding and sections of 5-micron thickness were taken by a microtome. The sections were stained with hematoxylin and eosin, slides were prepared and then examined under microscope for histopathological changes and images were captured through attached CCTV camera. The data was presented as mean with standard error. The data was analyzed by un-paired sample T-test in One-Way ANOVA for determining significant difference between the extract treated and control rabbit groups. For these analyses a computer software, SPSS 16 was used.

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Results

Oviposition deterrence

Table 1 shows the range of number of eggs laid by the gravid female *Cx. quinquefasciatus* adults in control containers and in extract solution containers (1000 ppm, 250 ppm, 31.2 ppm). In control containers, the number of eggs was in the range 997-1937, in containers with lowest concentration (31.2 ppm), the number of eggs was in the range of 887-1614 but in containers with highest concentration (1000 ppm) of extract, the number of eggs was in the range of 287-707. *Figure 4* shows the percentage of effective repellency (ER %) of *C. nardus* whole-plant n-hexane extract against *Cx. quinquefasciatus*. The highest concentration (1000 ppm) of *C. nardus* n-hexane extract greatly affected the egg laying capacity of the gravid female *Cx. quinquefasciatus* mosquito. Maximum ER % was observed for the container containing 1000 ppm extract solution (ER %=71.9±3.3) followed by 250 ppm (ER %=44.3±7.4) and 31.25 ppm (ER %=13.4±1.3). *Table 1* also shows the oviposition activity indices (OAI) of each concentration of the *C. nardus* whole- plant n-hexane extract. The 1000 ppm concentration of the plant extract exhibited minimum OAI value (mean OAI= -0.5±0.1) followed by 250 ppm (mean OAI= -0.3±0.1) and 31.25 ppm (mean OAI= -0.1±0).

Table 1. *Effect of C. nordus whole-plant n-hexane extract on oviposition activity indices (OAI) of Cx. quinquefasciatus*

Concentration (ppm)	No. of eggs (range)	OAI	Statistics
1000	287-707	-0.5 ± 0.1^{a}	Sig= 0.005
250	409-803	-0.3 ± 0.1^{ab}	Df within
31.2	887-1614	-0.1 ± 0^{bc}	groups= 9
Control	997-1937	---------------	F= 9.4

Mean values with different letters represent significant difference at P<0.05 significance level.

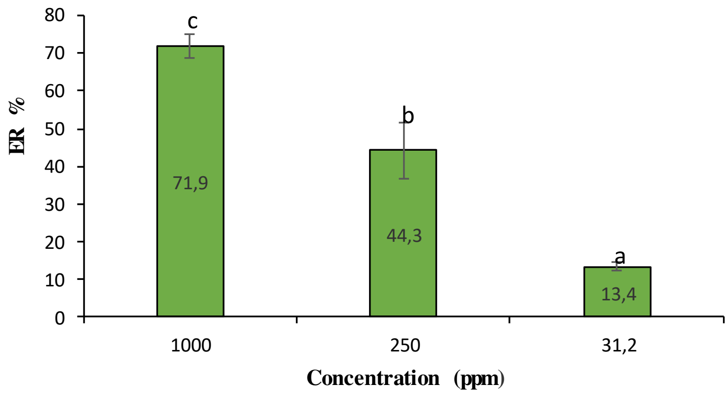

Figure 4. *Effective repellency (ER %) of C. nordus whole-plant n-hexane extract against Cx. quinquefasciatus. Tukey test in One Way Anova. Df within group= 9, F= 38.2, Sig= 0.000. Mean values with different letter represent significant difference at P<0.05 significance level*

Adult emergence inhibition

Table 2 shows the adult emergence inhibition activity of *C. nardus* n-hexane extract against *Cx. quinquefasciatus.* More than 90 % emergence of adults occurred from the control containers. The emergence of adults was restricted in those containers that were

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containing *C. nardus* extract solutions. There occurred 31±17.9 % adult emergence from the containers containing the highest extract concentration (1000 ppm). There occurred 82.3 ± 5.6 % adult emergence from the containers containing the lowest extract concentration (125 ppm). The highest extract concentration caused 66.2 ± 19.5 % emergence inhibition (EI), while the lowest extract concentration caused 12.2 ± 6.5 % emergence inhibition (EI). There was a positive correlation between extract concentration and EI % (R square =0.86). The EI_{50} value of *C. nardus* was 515.2 ppm.

Table 2. *Adult emergence inhibition activity of C. nordus against Cx. quinquefasciatus*

Concentration (ppm)	E % in treatment (Mean± SE %)	E % in control (Mean± SE)	EI % (Mean± SE)	R Square	EI_{50} (ppm)
1000	31 ± 17.9	94.3 ± 2.4	66.2 ± 19.5	0.86	515.2
500	43.8 ± 16.8	95.0 ± 1.8	54.0 ± 17.7		
250	66.5 ± 14.4	93.8 ± 3.3	29.3 ± 13.8		
125	82.3 ± 5.6	94.0 ± 1.4	12.2 ± 6.5		

Mean values with different letter represent significant difference at P<0.05 significance level.

Effect on damselfly and dragonfly nymphs

The effect of different concentrations of *C. nardus* whole- plant n-hexane extract on damselfly and dragonfly nymphs has been shown in *Table 3*. Up to 500 ppm concentration, the *C. nardus* whole- plant n-hexane extract caused no mortality of damselfly or dragonfly nymphs. Only at 1000 ppm concentration, the extract caused 3.3 ± 1.6 % mortality of nymphs of each species, therefore table was not arranged for showing the effect of plant extract on non-target organisms. There also occurred no mortality in nymphs of control group.

Table 3. *Effect of C. nardus whole plant n-hexane extract on non-target damselfly (Ischnura elegans) and dragonfly (S. decoloratum) nymphs*

Concentration (ppm)	*I. elegans*	*S. decoloratum*
1000	3.3±1.6	3.3±1.6
500	0	0
250	0	0
125	0	0
Control	0	0

Effect on freshwater fish

Grass carp fish, *Ctenopharingodon idella*, were exposed to 2040, 5007 and 5996 ppm concentration of *C. ambrosioides*, *C. botrys* and *C. nardus* whole- plant n-hexane extracts, respectively. The behavior and mortality of fish in each aquarium was checked for 24 hours. The extracts did not cause mortality in fish for 24 hours period of exposure.

Effect of plant extract on rabbit

During the present research, the effect of oral administration of high dose of *C. nardus* whole- plant n-hexane extract on the normal levels of some biochemical parameters i.e.,

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ALT, AST, ALP and creatinine and hematological parameters i.e., RBCs, WBCs and platelets count, and hemoglobin concentration, of male domestic rabbits (*Oryctolagus cuniculus*) were studied. Each extract was orally administered to rabbit group (four rabbits in a group) at a dose of 1000 mg per Kg body weight per oral. There occurred no significant change in the serum level of ALT, AST, ALP and creatinine of extract treated rabbit group when compared to control rabbit group (P>0.05) (*Table 4*). Similarly, there occurred no significant change in the RBCs, WBCs and platelets count and hemoglobin concentration of extract treated rabbit group when compared to control rabbit group (P>0.05) (*Table 5*). The photomicrographs of liver and kidney microsections of extract treated rabbit groups showed normal histoarchitectures (*Figure 5*).

Table 4. *Effect of larvicidal extracts (n-hexane extracts) of three whole- plant on some biochemical parameters of normal rabbits. N=4*

Plants	ALT (U/L)	AST (U/L)	ALP (U/L)	Creatinine mg/dl
C. nardus	47.3±4.4[a]	47.8±8.9[a]	116.6±24.3[a]	0.5±0.2[a]
Control	51.0±3.2[a]	52.3±6.1[a]	113.7±11.6[a]	0.4± 0.05[a]
t value	–0.961	–0.620	–0.092	0.570
DF	6	6	6	6
Significance (2-tailed)	0.374	0.558	0.930	0.589

Mean values with similar letter represent that there is no significant difference at P<0.05 significance level.

Table 5. *Effect of larvicidal extracts (n-hexane extracts) of three whole- plant on some haematological parameters of normal rabbits. N=4*

Plants	RBCs (X 10^6/µl)	WBCs (X 10^3/µl)	Platelets (X 10^3/µl)	Hb (g/dl)
C. nardus	6.0±0.1[a]	11.5±0.8[a]	263.7±3.2[a]	11.8±0.9a
Control	5.9±0.2[a]	11.9±0.7[a]	273.3±14.3[a]	11.6±0.5[a]
t value	0.548	–0.617	–0.940	0.291
DF within groups	6	6	6	6
Significance (2-tailed)	0.604	0.560	0.383	0.781

Mean values with similar letter represent that there is no significant difference at P<0.05 significance level.

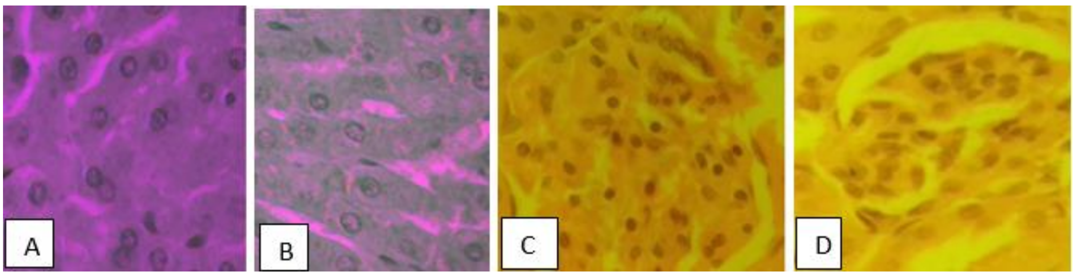

Figure 5. *Photomicrographs showing normal histoarchitecture of rabbit liver and kidney microtome sections. The alphabets A to B represent photomicrographs of liver microtome sections of control and extract treated groups, respectively. These photomicrographs show normal hepatic histoarchitecture. The alphabets C to D represent photomicrographs of kidney microtome sections of control and extract treated rabbit groups, respectively. These photomicrographs show normal renal histoarchitecture*

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DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

Discussion

During the present research, the n-hexane extract of *C. nardus* whole- plant showed oviposition deterrent activity against *Cx. quinquefasciatus* mosquito. During the oviposition deterrent activity, most of the gravid female *Cx. quinquefasciatus* mosquitoes preferred to lay eggs in the control container or in the container that contain extract solution of lower concentration (*Table 1*). The extract solution of highest concentration was least preferred by the gravid female mosquitoes for oviposition. The oviposition deterrent activity was expressed in percentage of effective repellency (ER %) and oviposition activity indices (OAI). Extract solution of highest concentration (1000 ppm) showed high ER % whereas the extract solution of lowest concentration (31.2 ppm) showed low ER % (*Figure 4*). The oviposition activity index (OAI) of the gravid female *Cx. quinquefasciatus* at each tested concentration of extract was estimated (*Table 1*). Negative OAI value was observed at each tested concentration of plant extract. The negative OAIs values indicated that the n-hexane extracts of *C. nardus* whole- plant is oviposition deterrent (Govindarajan et al., 2011). Lowest OAI value (-0.5 ± 0.1) was exhibited by highest concentration (1000 ppm) of extract solution. The oviposition deterrent activity of some plants against mosquitoes has been reported. For example, Reegan et al. (2015) reported that all the tested concentration of n-hexane extract of *Limonia acidissima* cause 100 % ER against *Cx. quinquefasciatus* and *Ae. aegypti*. Elimam et al. (2009) studied the oviposition deterrent activity of aqueous leaves extract of *Calotropis procera* against gravid female *Anopheles arabiensis* and *Cx. quinquefasciatus*. All the tested concentrations (1000, 500 and 200 ppm) caused more than 95 % ER against *An. arabiensis* and *Cx. quinquefasciatus*. In the reported study of Elango et al. (2009), the OAI values of acetone, ethyl acetate, and methanol extracts of *Aegle marmelos*, *Andrographis lineata* and *Cocculus hirsutus* against *Anopheles subpictus* at 500 ppm were -0.86, -0.87, -0.90 -0.78, -0.87, -0.86, -0.91, -0.94, and -0.86 respectively. Prathibha et al. (2014) studied the OAI value of *Eugenia jambolana*, *Solidago canadensis*, *Euodia ridleyi* and *Spilanthes mauritiana* against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus.* The OAI value of *E. jambolana*, *S. canadensis*, *E. ridleyi* and *S. mauritiana* at 100 ppm against *Cx. quinquefasciatus* were −0.81, −0.84, −1.0 and −1.0, respectively. The oviposition deterrent effect of the plant extract may be due to the changes induced in the physiology and behavior of the adult mosquito species reflected by their egg-laying capacity (Prathibha et al., 2014). Some phytochemicals act as growth regulators or chemosterilent while some produce olfactory stimuli acting as repellent or attractant (Prathibha et al., 2014). Rajkumar and Jebasan (2009) reported that the plant extarcts that exhibited appreciable insect repellency were oviposition deterrent. Similarly, Mehra and Hiradhar (2002) reported that those plant extracts showed appreciable oviposition deterrent activity which were insect repellent.

During the present research, the n-hexane extract of *C. nardus* whole- plant showed adult emergence inhibition activity against *Cx. quinquefasciatus* mosquito (*Table 2*). During this study, maximum emergence of adults occurred in the control containers (>90 %). Emergence of adults was restricted in those containers which were containing extract solutions. The percentage of adult emergence inhibition (EI %) increased with increasing the concentration of extract solution. The EI_{50} value of *C. nardus* whole-plant n-hexane extract was EI_{50}=515.2 ppm. The biological activity of the plant extract might be due to the presence of various bioactive phytocompounds, including phenolics, terpenoids, and alkaloids, existing in plants, may jointly or independently contribute to

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produce adult emergence inhibition activity (Arivoli and Tennyson, 2011). The plant extracts have the potential to inhibit the growth of various developmental stages during the life historey of mosquitoes (Arivoli and Tennyson, 2011). Plant extracts have the potential to delay larval development, extend pupal duration, inhibit moulting, cause morphological abnormalities and mortality during moulting and melanization processes in mosquitoes (Shaalan et al., 2005). The adult emergence inhibition activity of some plants such as *Abutilon indicum* (Arivoli and Tennyson, 2011), *Aegle marmelos*, *Andrographis lineata*, *Andrographis paniculata*, *Cocculus hirsutus*, *Eclipta prostrate* and *Tagetes erecta* (Elango et al., 2012), *Azadirachta indica* (Howard et al., 2009), *Eucalyptus citriodora* (Singh et al., 2007) and *Balanites aegyptiaca* (Wiesman and Chapagain, 2006) has been reported against mosquitoes.

During the present study, the effect of *C. nardus* whole-plant n-hexane extract on non-target insects i.e., damselfly and dragonfly nymphs, fish i.e., grass carp and mammal such as rabbits was also studied. During the study of effect of extract on non-target insects i.e., damsel and dragonfly nymphs, the extract caused no mortality of any nymphs up to 500 ppm. However, at 1000 ppm concentration, the plant extract caused some mortality (5 %) of the nymphs (*Table 3*). This concentration of *C. nardus* whole-plant n-hexane extract caused 71.9 % effective repellence during oviposition deterrent activity and 66.2 ± 19.5 % inhibition during adult emergence inhibition activity against *Cx. quinquefasciatus*. These findings showed that *C. nardus* whole plant n-hexane extract is efficient ovipositor deterrent and adult emergence inhibitor against mosquitoes but least toxic for non-target insects such as damselfly and dragonfly nymphs.

During the study of effect of extracts on non-target grass carp fish (*Ctenopharingodon idella*), the extracts did not cause mortality in fish for 24 hours exposure. Few such studies have been reported in which the effect of plant extracts has also been studied on non-target organisms. For example, Chowdhury et al. (2009) studied the larvicidal activity of *Solanum villosum* leaves against *Anopheles subpictus* with effect on non-target *Chironomus circumdatus* larvae. The extract was found safe for the non-target *C. circumdatus* larvae. Adhikari et al. (2012) studied the repellent and larvicidal activities of *Swietenia mahagoni* against the *Cx. quinquefasciatus* larvae. They also studied the effect of the same extract on some non-target aquatic organisms i.e., *Gambusia affinis*, tadpole of *Bufo* and *Chironomus* larvae. They observed no toxicity of plant extract in these non-target organisms.

During the study on non-target mammals, the effects of oral administration of high dose of extract on some biochemical parameters i.e., ALT, AST, ALP and creatinine (*Table 4*) and some hematological parameters i.e., RBCs, WBCs and platelets count, and hemoglobin concentration (*Table 5*) were studied in domestic rabbit. There occurred no mortality and found no significant change in the biochemical and hematological parameters of rabbits. Photomicrographs of microtome sections of liver and kidney of extract treated rabbit groups showed normal histoarchitecture (*Figure 5*). Carvalho et al. (2003) reported the larvicidal efficacy of the essential oil from *Lippia sidoides* against *Aedes aegypti*. In addition, they also studied the toxicity of the essential oil in mice. They injected intraperitonially the diluted and pure form of hydrolate in amount of 30 ml per kg body weight into the mice. The injection did not cause any adverse effects or mortality. The results of the present study revealed that *C. ambrosioides*, *C. botrys* and *C. nardus* whole- plant n-hexane extracts exhibit strong mosquitocidal activities but have no apparent deleterious effect on mammals (rabbit).

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DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

Conclusion and Recommendations

1.) From the findings of the present research, it was concluded that the n-hexane extract of *C. nardus* whole-plant possesses oviposition deterrent and adult emergence inhibition activities against *Cx. quinquefasciatus*. Further studies are recommended for elucidating the oviposition deterrent and adult emergence inhibition activities of *C. nardus* whole-plant n-hexane extract against a wide range of mosquito species and exploring active compound responsible for such activities.

2.) It was also concluded that the n-hexane extract of *C. nardus* whole plant is least toxic to non-target insects i.e., damselfly and dragonfly nymphs. There occurs no mortality or behavioural abnormality in fish i.e., grass carp when exposed to high concentration of *C. nardus* whole-plant n-hexane extract. The ingestion of *C. nardus* whole-plant n-hexane extract do not cause mortality or alteration in the biochemical and haematological parameters of rabbits. The findings of our research encourage the use of botanical insecticides for mosquito control because they are ecofriendly and safe for non-target organisms.

Acknowledgements. This article has been prepared from a part of my (Ikram Ilahi) PhD thesis entitled "Ecofriendly control of *Culex quinquefasciatus* (say, 1823) and susceptibility of its larvae and predators to different environmental pollutants" at the Department of Zoology, Islamia College, Peshawar, Khyber Pakhtunkhwa, Pakistan.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49154931

COMPARISON OF THE GENETIC VARIABILITY OF THREE REGIONS OF CHLOROPLAST DNA AND NUCLEAR DNA IN ISHPINGO (*OCOTEA QUIXOS*) COMING FROM FIVE PROVINCES OF THE ECUADORIAN AMAZON

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(Received 8th Nov 2018; accepted 5th Mar 2019)

Abstract. *Ocotea quixos* (Lam.) Kosterm. (Ishpingo) is a tree that grows in humid tropical forests in South America (310-1200 masl). It is used for food and medicine, since it possesses essential oils with antiplatelet and antithrombotic properties, among others. In Ecuador, the wild populations of Ishpingo have been reduced ecosystem transformation, deforestation and clonal cultivation. This study compared the genetic variability of three regions of chloroplast DNA (i.e., matK, rbcLa and psbA-trnH) and one nuclear region (i.e., ITS) in Ishpingo from 5 provinces of the Ecuadorian Amazon. Based on the obtained alignments, the phylogeny was performed by using the maximum likelihood criterion with a bootstrap of 1000 replicates and with the Jukes-Cantor correction. The genetic variability was determined using the DNA conservation coefficient and the Nei (π) nucleotide variability coefficient. The nucleotide variability of matK, rbcLa and psbA-trnH regions was low ($\pi \leq 0.005$) due to their high conservation degrees. The ITS region presented a superior variability ($\pi = 0.11$) by grouping the samples that were analyzed into two clades. The regions that were analyzed were not useful for the study of genetic variability in Ishpingo. Therefore, it is recommended that new chloroplast and nuclear DNA regions be analyzed to allow studies on the population genetics of this species in Ecuador to be carried out.
Keywords: *Ocotea, chloroplast, DNA, Amazonia, genetic variability*

Introduction

Ocotea quixos (Lam.) Kosterm. (Ishpingo, Ishpink or Canela Amazónica), is a perennial tree that reaches a height of 2 to 25 m (Cazorla, 2013; Palacios, 2016). It is distributed in the humid tropical forests of the Amazonian region of Colombia, Ecuador, Peru and Brazil (310-1200 masl) (Cárdenas et al., 2015).

In 2007, *Ocotea quixos* was banned from commercialization for six months due to the significant reduction from wild populations (Grijalva et al., 2012). In the Ministerial Agreement No. 167, published in the official Register No. 18 on February 8, 2007, it was reported that the main causes of the population reduction of *Ocotea quixos* are the

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DOI: http://dx.doi.org/10.15666/aeer/1702_49334948

expansion of the road network, the transformation of ecosystems, deforestation and the clonal cultivars of the species (Ministerio del Ambiente de Ecuador, 2010).

The lack of genetic studies on the *Ocotea quixos* populations of Ecuador suggests that the genetic variability of Ishpingo may be declining. The objective of this study is to compare the genetic variability of the three regions of chloroplast DNA (i.e., MatK, rbcLa, psbA-trnH)) and a region of nuclear DNA (i.e., ITS) in *Ocotea quixos* to establish possible regions for future population genetics studies on Ishpingo.

Materials and methods

Botanical samples and the 38 foliar tissue samples of Ishpingo (*Ocotea quixos*) species were collected. The samples were obtained from 11 locations in 5 provinces of the Ecuadorian Amazon, where Ishpingo is traditionally cultivated. Botanical specimens were morphologically identified with the help the herbarium of Pontificia Universidad Católica del Ecuador.

Prewashes were performed according to the division protocol of polyphenols and polysaccharides as proposed by Porebski et al. (1997). After washing, Doyle and Doyle's simple foliar DNA extraction protocol (1987) was performed. To determine the presence of the DNA, a horizontal electrophoresis was performed in agarose gel at 1%.

The chloroplast DNA regions (i.e., matK, rbcLa, psbA-trnH) and the nuclear ITS region, defined for the Barcode system, were amplified by specific primers (*Table 1*).

Table 1. *Primers for the amplification of DNA regions*

Primers	Sequence (5'->3')	Reference
matK-1RKIM (F)	ACCCAGTCCATCTGGAAATCTTGGTTC	Lahaye et al. (2008)
matK-3FKIM (R)	CGTACAGTACTTTTGTGTTTACGAG	Lahaye et al. (2008)
rbcLa (F)	ATGTCACCACAAACAGAGACTAAAGC	CBOL Plant Working Group et al. (2009)
rbcLa (R)	GTAAAATCAAGTCCACCRCG	Levin et al. (2003)
psbA (F)	GTTATGCATGAACGTAATGCTC	Kress and Erickson (2007)
trnH (R)	CGCGCATGGTGGATTCACAAATCC	Kress and Erickson (2007)
ITS leu1 (F)	GTCCACTGAACCTTATCATTTAG	Bolson et al. (2015)
ITS 4 (R)	TCCTCCGCTTATTGATATGC	White et al. (1990)

The sequencing and purification of the samples were carried out at the company Macrogen (South Korea) using Sanger's simple sequencing method.

The sequencing analysis was carried out using the MEGA7 program. The MUSCLE algorithm was used to align the sequences, maximum likelihood trees were used for the phylogeny, and the Bootstrap method was performed with 1000 replications. The substitutions that were assessed were nucleotides, and Jukes-Cantor's substitution model (Jukes and Cantor, 1969) was used. The trees were rooted following the criteria of external groups or "outgroups", using the species Ocotea veraguensis and Ocotea porosa. The genetic variability was analyzed with the conservation coefficient, and Nei's variability nucleotide coefficient was determined by the "estimation analysis of

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diversity with the specific data model" (Nei and Li, 1979) and was estimated with Tamura-Nei's model (Tamura et al., 2013).

Results and discussion

The DNA of 35 samples was obtained (*Fig. 1*) once the washings were done. The DNA obtained had no signs of degradation and was pollution-free; the band obtained corresponded to the expected size: > 2000 pb with a concentration of approximately 40-100 ng/µL.

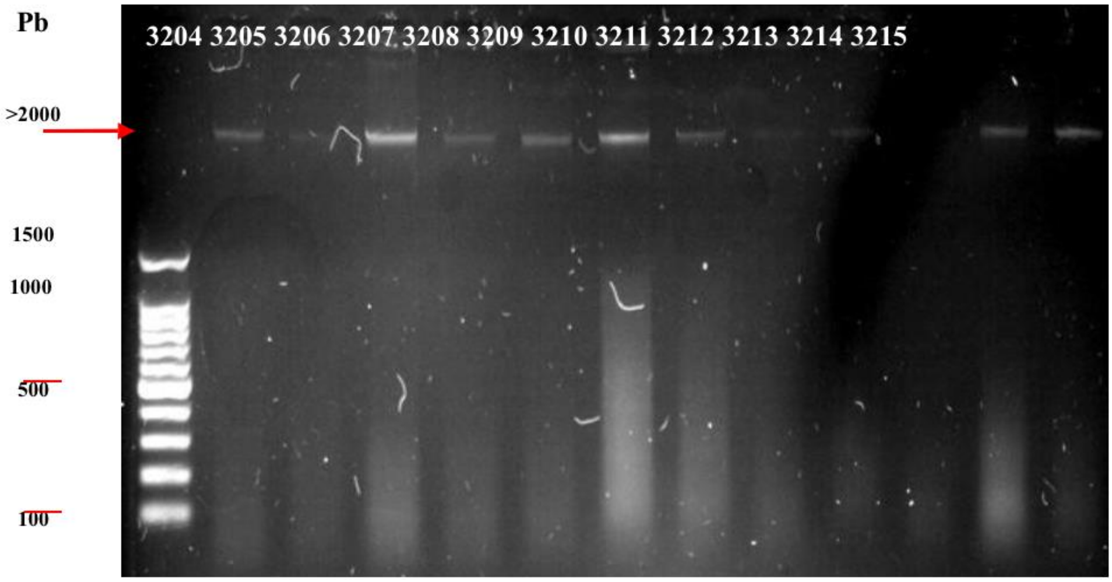

Figure 1. *DNA extraction with previous washings following the protocol of Porebski et al. (1997), and the simple extraction protocol of foliar DNA by Doyle and Doyle (1987), agarose gel at 1%. (Note: First molecular marker, samples 3204-3215 (+), sample 3213 (-))*

Out of the 35 sequences of the ITS region obtained, 28 consensus sequences were achieved. The average length of the sequence was 777 pb.

The region presented 428 polymorphic areas, a conservation region coefficient of 0.298 and a nucleotide variability coefficient of 0.1137. ITS presents a low conservation rate due to the great length variability of the sequence, and its incomplete concerted evolution led to the presence of divergent homologous copies within the samples (China Plant BOL Group et al., 2011).

The phylogenetic tree of the ITS region presented two clades; the samples grouped in the first clade were associated with the *Ocotea quixos* control sequence, and in the second clade, a polyphyletic grouping was observed with a branch support of 100% (*Fig. 2*). The presence of two clades may be because ITS has higher discrimination levels and interspecific variability followed by Gao et al. (2010); however, new studies have proven that there are several limitations associated with the ITS region, especially in phylogenetic inference (Gardes and Bruns, 1993), such as in the existence of extensive variations in genome sequences, which are determined by the formation events of duplications, the genomic accommodation of pseudogenes and an incomplete homogenization arrangement). These phenomena create relationships of paralog

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sequences that potentially confuse the accuracy of phylogenetic reconstruction. Homoplasy is higher in ITS than in other DNA regions due to the orthologous/paralogs confusion, compensatory change in bases, problems in alignment due to the accumulation of deletions, and errors in the sequence (Grudinski et al., 2014). Despite being a quasi-universal sequence used in plants for phylogenetic studies, its complex and unpredictable evolutionary behaviour reduces its usefulness in phylogenetic analyses. The use of single-copy nuclear genes is suggested by Alvarez and Wendel (2003).

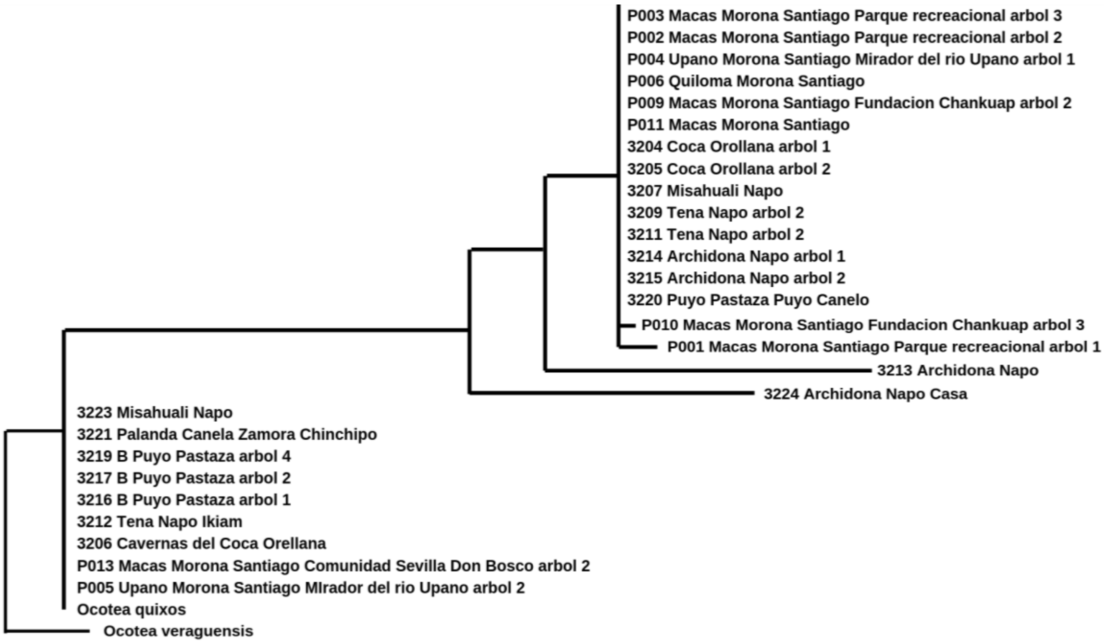

Figure 2. *Maximum likelihood tree ITS region. (Note: A defined clade and a grouped clade can be observed with the O. quixos sequence obtained from GenBank using the MEGA 7 program)*

Comparison of nucleotide variability among the regions matK, rbcLa, psbA-trnH and ITS: The psbA-trnH region has a variability coefficient of $\pi = 0.0051$ higher than those of the matK and rbcLa regions. This region has mononucleotide repetitions that increase its variability (Kress and Erickson, 2007). Despite this increase, the nucleotide variability coefficient is low <0.1, as mentioned by Jakobsson et al. (2013), and does not provide high nucleotide substitution rates due to its short length in the *Ocotea genus*, so its nucleotide variability is low (Liu et al., 2012).

The ITS region presented the highest variability coefficient ($\pi = 0.1137$) among the four regions. One of the main reasons for this increase in the genetic variability is due to the homology problem of the sequences that this region has; i.e., the appearance of divergent copies in the samples, which can lead to an increase in the genetic variability, stated by Hollingsworth (2011) and Hollingsworth et al. (2011).

Conclusions

DNA regions matK, rbcLa and psbA-trnH in *Ocotea quixos* are preserved (nucleotide variation coefficient $\pi \leq 0.005$) for the study of genetic variability in this

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DOI: http://dx.doi.org/10.15666/aeer/1702_49334948

population. The ITS region had highest substitution rates in nucleotides ($\pi = 0.1137$) in the sequences of the *Ocotea quixos*. This variability is associated with the amount of inscriptions in the DNA sequence that increase the nucleotide variability. The genetic variability coefficient obtained in the DNA regions of *Ocotea quixos* was low ($\pi = 0$-0.1137), which suggests that the genetic variability of the species is threatened by deforestation and the cultivation of clones.

Considering our study findings, we highly recommend to continue our research in order to establish possible regions for future population genetics studies on Ishpingo.

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APPENDIX

Primers for the amplification of DNA regions

#	Query length	Description	Query cover	Identity	Reference
1D *matK*	895	*Nectandra sp*. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	94%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
2D *matK*	902	*Nectandra sp*. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	94%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3D *matK*	856	*Nectandra sp*. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
4D *matK*	859	*Nectandra sp*. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
5D *matK*	859	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
6D *matK*	845	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
7D *matK*	851	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
8D *matK*	845	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
9D *matK*	855	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
11D *matK*	857	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
12D *matK*	860	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
13D *matK*	847	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	96%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
14D *matK*	812	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
17A *matK*	813	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
18A *matK*	800	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
19A *matK*	727	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
20A *matK*	850	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
21A *matK*	849	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
22A *matK*	782	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
23A *matK*	811	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
24A *matK*	821	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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25A *matK*	762	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
26A *matK*	820	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
27A *matK*	778	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
28A *matK*	807	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
29A *matK*	778	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
30A *matK*	852	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
31A *matK*	797	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
32A *matK*	793	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
33A *matK*	858	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
34A *matK*	809	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
35A *matK*	826	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_9931066
36A *matK*	784	*Nectandra* sp. 1 AN410 maturase-like (*matK*) gene, partial sequence; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
1A *rbcLa*	564	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
2A *rbcLa*	572	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3A *rbcLa*	565	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
4A *rbcLa*	565	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
5A *rbcLa*	524	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
6A *rbcLa*	565	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
7A *rbcLa*	565	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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8A *rbcLa*	540	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	540	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
9A *rbcLa*	565	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	565	*Ocotea quixos* voucher COAH:81063 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
10A *rbcLa*	563	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	563	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
11A *rbcLa*	521	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	521	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
13A *rbcLa*	566	*Ocotea quixos* voucher COAH:81063 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
14A *rbcLa*	567	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
16A *rbcLa*	389	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	389	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
17A *rbcLa*	563	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	563	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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18A *rbcLa*	504	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	504	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
19A *rbcLa*	564	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	564	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
20A *rbcLa*	519	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	519	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
21A *rbcLa*	510	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	510	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
22A *rbcLa*	556	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	556	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
23A *rbcLa*	564	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	564	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
24A *rbcLa*	507	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	507	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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25A *rbcLa*	559	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	559	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
26A *rbcLa*	562	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	562	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
27A *rbcLa*	564	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	564	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
28A *rbcLa*	507	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	98%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	507	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	98%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
30A *rbcLa*	567	*Ocotea quixos* voucher COAH:81083 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
31A *rbcLa*	559	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	559	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
33A *rbcLa*	560	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	560	*Ocotea quixos* voucher COAH:81062 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
34A *rbcLa*	565	*Ocotea cymbarum* voucher COAH:66878 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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	565	*Ocotea quixos* voucher COAH:81063 ribulose-1,5-bisphospahte carboxylase/oxygenase large subunit (rbcL) gene, partial cds; choloplast	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
1B *psbA-trnH*	488	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	99%	97%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
2B *psbA-trnH*	494	*Ocotea quixos* choloplast *psbA-trnH* intergenic spacer region	96%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3B *psbA-trnH*	433	*Ocotea moschata* photosystem II protein D1 (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
5B *psbA-trnH*	499	*Ocotea botrantha* vouvher s.n. PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	97%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
6B *psbA-trnH*	498	*Ocotea botrantha* voucher Wernisch s.n. PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	97%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
7B *psbA-trnH*	498	*Ocotea botrantha* voucher Wernisch s.n. PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	97%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
8B *psbA-trnH*	441	*Ocotea cuneata* voucher 1079133346 PsbA 9psbA) gene, partial cds; and *psbA-trnH* intergenic spacer, complete sequence; chloroplast	97%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
9B *psbA-trnH*	489	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	98%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
10B *psbA-trnH*	440	*Ocotea cuneata* voucher 1079133346 PsbA 9psbA) gene, partial cds; and *psbA-trnH* intergenic spacer, complete sequence; chloroplast	97%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
11B *psbA-trnH*	1078	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	44%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
12B *psbA-trnH*	573	*Ocotea* quixos	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
13B *psbA-trnH*	496	*Ocotea botrantha* voucher Wernisch s.n. PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	98%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
14B *psbA-trnH*	495	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	97%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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16A *psbA-trnH*	506	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
17A *psbA-trnH*	500	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	97%	97%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
18A *psbA-trnH*	504	*Ocotea botrantha* voucher Wernisch s.n. PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	96%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
19A *psbA-trnH*	497	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
20A *psbA-trnH*	494	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	98%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
21A *psbA-trnH*	492	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	98%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
22A *psbA-trnH*	497	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
23A *psbA-trnH*	505	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
24A *psbA-trnH*	501	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
25A *psbA-trnH*	467	*Lindera benzoin* voucher SERC-1076419278 trnH-psbA intergenic spacer, partial sequence; choroplast	96%	95%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
26A *psbA-trnH*	505	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
27A *psbA-trnH*	504	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
28A *psbA-trnH*	488	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	97%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
29A *psbA-trnH*	496	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
30A *psbA-trnH*	505	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
31A *psbA-trnH*	422	*Ocotea quixos* chloroplast *psbA-trnH* intergenic spacer region	98%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
32A *psbA-trnH*	401	*Ocotea cuneata* voucher 1079133346 PsbA (psbA) gene, partial cds; and *psbA-trnH* intergenic spacer, complete sequence; chloroplast	98%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
33A *psbA-trnH*	474	*Ocotea purpurea* voucher Lundell 21170 PsbA (psbA) gene, partial cds; *psbA-trnH* intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial sequence; chloroplast	100%	97%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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34A *psbA-trnH*	402	Lauraceae sp. MAG2009 voucher NL 110192 PsbA (psbA) gene, partial cds; and *psbA-trnH*intergenic spacer, partial sequence choroplast	99%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
35A *psbA-trnH*	330	*Ocotea cuneata* voucher 1079133346 PsbA (psbA) gene, partial cds; and *psbA-trnH* intergenic spacer, complete sequence; chloroplast	97%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
36A *psbA-trnH*	445	*Lindera benzoin* voucher SERC-1076419278 trnH-psbA intergenic spacer, partial sequence; choroplast	99%	95%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
1C *ITS*	405	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	71%	96%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
2C *ITS*	734	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3C *ITS*	495	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
4C *ITS*	671	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	98%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
5C *ITS*	683	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	90%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
6C *ITS*	460	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cg
9C *ITS*	507	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
10C *ITS*	611	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	61%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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11C *ITS*	627	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
13C *ITS*	694	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	89%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3204 *ITS*	593	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3205 *ITS*	712	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3206 *ITS*	647	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	94%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3207 *ITS*	559	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3209 *ITS*	709	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3211 *ITS*	620	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3212 *ITS*	513	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	86%	94%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3213 *ITS*	719	Uncultured eukaryote clone CMH360 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 28S ribosomal RNA gene, and 45S rDNA intergenic spacer, partial sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

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3214 *ITS*	709	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3215 *ITS*	701	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	100%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3216 *ITS*	591	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	100%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3217 *ITS*	678	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	89%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3219 *ITS*	512	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3220 *ITS*	359	*Brassica oleraceae* var. capitata 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 45S rDNA intergenic spacer, complete sequence	99%	100%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3221 *ITS*	668	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	91%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3223 *ITS*	641	*Ocotea quixos* internal trnascribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	95%	99%	https://blast.ncbi.nlm.nih.gov/Blast.cgi
3224 *ITS*	542	*Tripodanthus belmirensis* voucher Nickrent 5050 internal transcribed spacer 1, partial sequence; 5.8 ribosomal RNA gene, internal transcribed spacer 2, 26S ribosomal RNA gene, and 26S rDNA intergenic spacer, partial sequence	99%	92%	https://blast.ncbi.nlm.nih.gov/Blast.cgi

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49334948

A STUDY OF BIODIVERSITY AND WATER QUALITY BY ANALYSING AQUATIC MACROINVERTEBRATES IN THE PASOCHOA WILDLIFE REFUGE, ECUADOR

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(Received 8th Nov 2018; accepted 5th Mar 2019)

Abstract. The Province of Pichincha, Ecuador is home to the calderas and flanks of the Pasochoa volcano, where the Pasochoa Wildlife Refuge (RVSP for its Spanish acronym) is located. This area is one of the few places that conserves some of the most important remnants of the Andean native forest. This study seeks to determine the biodiversity and water quality of the Pasochoa Wildlife Refuge by studying its aquatic macroinvertebrates. The data for this work were obtained from five sampling points located between the caldera and outside the RVSP. This effort was carried out along the stream that descends from the Pasochoa volcano at an altitude of 3014 meters above sea level (masl) to 2667 masl. Water samples were also obtained for a physical-chemical analysis. Preliminarily, a total of 6149 invertebrates, corresponding to 3 phyla, 10 orders and 36 families, were captured as indicators of water quality. The most abundant phylum was Arthropoda, with 6130 individuals. Additionally, specimens of annelids and mollusks were recorded. The greatest abundance was recorded for the families of Elmidae (Coleoptera) and Leptoceridae (Trichoptera), with 2059 and 1071 individuals, respectively. According to the families that indicate water quality, it fluctuates between very good to regular in PMM-01, which is an area with anthropic influence.
Keywords: *environmental indicators, water quality, species richness, abundance, environmental quality*

Introduction

Modelling the environment and water resources are essential to improve and protect water quality (Eurie et al., 2015; Forio et al., 2016). Today, water monitoring has become particularly important due to the increasing human activity that often leads to water quality deterioration (Yillia et al., 2008; Holguin-Gonzalez et al., 2013; Kibena et al., 2014). Selecting the right indicators for monitoring has become essential and often the most challenging task of environmental studies and applied conservation science (Game et al., 2016). We have chosen macroinvertebrates for determining water quality considering that they are well-known indicators in environmental monitoring and assessment worldwide (Forio et al., 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49494956

The Pasochoa Wildlife Refuge (RVSP) is located in the caldera and in the flanks of the Pasochoa volcano, in the province of Pichincha, Ecuador. This area is one of the few places that conserves some of the most important remnants of the Andean native forest.

According to our bibliographic review, quantitative-type floristic investigations have been carried out within the protected area, such as those by Valencia and Jorgensen (1992), while other studies have consisted of rapid sampling where physical backups are not available. In terms of fauna, bird lists have been compiled by Sierra (1996), and the baseline list was recorded by Fundación Natura (1990). Studies referring to both terrestrial and aquatic insects are practically nonexistent for the sector. According to the map of vegetal coverage by the MAE (2013), the vegetal formation is a high-altitude evergreen mountain forest in the northern part of the Eastern Cordillera of the Andes. Its altitude range is from 3000 to 3700 masl, and the grassland of the moors ranges from 3400 to 4300 masl. The area has an average annual rainfall of 1200 mm, an average annual temperature of 10°C and a relative humidity that is almost higher than 80% (Valencia and Jørgensen, 1992).

In this study, aquatic macroinvertebrates were used as indicators of environmental quality. Aquatic macroinvertebrates have received much attention in studies regarding the ecosystems of running waters because they are transformers and integrators of allochthonous organic matter (leaves, seeds, branches, fallen trunks, etc.), the main inflows of energy to the fluvial systems and useful biological indicators.

Materials and methods

The Pasochoa Wildlife Refuge is located in the Province of Pichincha, Cantón Mejía, in the parish of Uyumbicho, Ecuador. It contains 520 hectares, which are covered mostly by Andean forest and, to a lesser extent, by herbaceous moorland. For the sampling points, the area of the caldera was prioritized because it is a pristine area, has been conserved and contains remnants of a mature forest. The second sampling point corresponded to the area outside the wildlife refuge where anthropic influence exists due to the presence of livestock and pastures. Then, the two areas were compared and evaluated. The data for this work were obtained from five sampling points along the stream that descends from the Pasochoa volcano from an altitude of 3014 to 2667 masl. The sampling points can be seen in *Figure 1* (López-López and Sedeño-Díaz, 2014).

The macroinvertebrates were collected through a “Surber” network in five sampling points (*Fig. 1*) along a 100 m-long transect according to the proposal of Roldán (2003) (Brito and Pinto, 2014). At each sampling point, we examined the riverbed of each body of water for a lapse of one minute to capture the existing macroinvertebrates, covering all possible microhabitats (riparian vegetation, sandy areas, rocky areas and algae). In addition, the ecological quality protocols for Andean rivers that were proposed by Encalada et al. (2011) were applied (Damanik-Ambarita, 2016; Carter, 2017; Mariadoss and Abril, 2015).

The samples were cleaned to later extract the macroinvertebrates and deposit them in correctly labeled plastic containers with 70% alcohol. Field work was executed following the rules and regulatory guidelines outlined by the Environment Ministry’s research permit.

To determine aquatic invertebrates, which were deposited in the aforementioned containers, the specimens were separated and identified at the family taxonomic level according to the protocols proposed by Encalada et al. (2011). To identify the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49494956

invertebrates, we used the taxonomic keys of Fernández and Dominguez (2009). The invertebrate collections will be displayed in the Gustavo Orcés Natural History Museum at the Escuela Politécnica Nacional, under patent No. 04-2014-FAU- DPAP-MA (Knowl, 2016; Stancheva and Seath, 2016).

The analyses related to the abundance, diversity, and species accumulation curves were performed according to Colwell (2006) and Oksanen, 2015 using the EstimateS 8.2.0 programs and PAST (Paleontological Statistics version 1.12) according to Hammer et al. (2003).

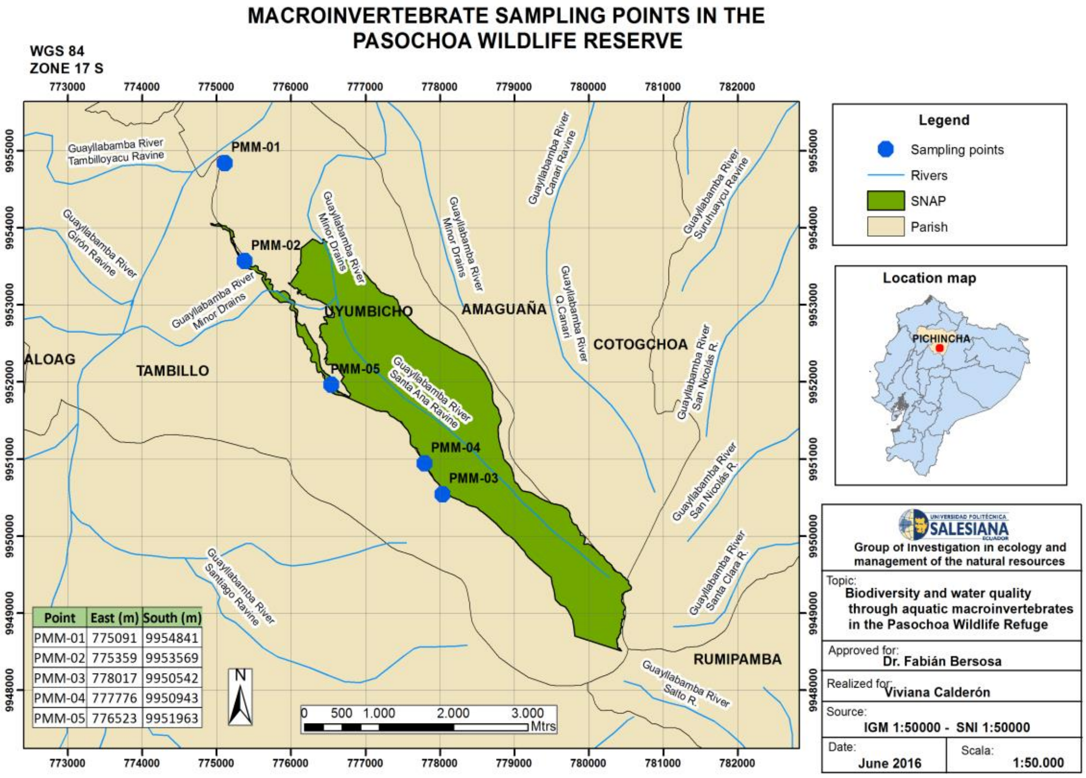

Figure 1. *Sampling points for macroinvertebrates in the Pasochoa Wildlife Reserve. "PPM-01" = Point 1; "PPM-02" = Point 2; "PPM-03" = Point 3; "PPM-04" = Point 4; "PPM-05" = Point 5; "Este" = East; "Sur" = South. Legends: "Puntos de muestro" = Sampling point, marked with blue dots; "Ríos" = Rivers, marked with green line; "Parroquias" = Parishes*

The analyzed parameters were divided into two groups. The first group is related to finding out the level of intromission of human effluents, such as gray and black waters, discharges from tourism or agriculture, and so on. Here, we measured the 5-day dissolved oxygen, Biochemical Oxygen Demand (BOD5), phosphorus, ammonia, nitrate and nitrite, the last three of which are indicators of pollution temporality (Cheeme, 2018). The second group of indicators focused on change in water quality due to human or natural causes. The indicators used were pH, calcium hardness, turbidity and total solids. The presence of calcium hardness, increased BOD5 and decreased dissolved oxygen could be the result of diffuse soil contamination from fertilizers (Schahrakane, 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49494956

Results

A total of 6149 invertebrates were captured, corresponding to 3 phyla, 10 orders and 36 families, that indicate the water quality. The most abundant phylum was Arthropoda, with 6130 specimens. Additionally, specimens of annelids and mollusks were recorded (*Table 1*).

The greatest abundance was recorded in the families of Elmidae (Coleoptera) and Leptoceridae (Trichoptera), with 2059 and 1071 individuals, respectively. The greatest abundance was recorded in Point 5.

Table 1. *Diversity and abundance*

Parameter	Point 1	Point 2	Point 3	Point 4	Point 5
Taxa_S	11	20	20	27	29
Individuals	197	253	465	2468	2766
Dominance_D	0.3141	0.1245	0.1764	0.2585	0.2303
Simpson_1-D	0.6859	0.8755	0.8236	0.7415	0.7697
Shannon_H	1383	2.35	1999	1792	1888

The determination of the quality of water in the sector by means of physical chemical parameters has not been done previously, but as regards the diversity in the present study, 3 phyla, 10 orders and 36 families were found in front of the study by Gallegos, 2013, which mentions 4, 13 and 34 respectively

Low values of dissolved oxygen were observed for all points, which are not due to temperature but to the existence of an organic load from three thousand meters high. This is corroborated by the BOD5 measurements that are accentuated in Point 4, where it reached 36 mg/l. With these levels of dissolved oxygen, fish cannot exist. From Point 2 onwards, there is a continuous presence of ammonia, which causes the depletion of dissolved oxygen and an increase in the nitrogen-bearing BOD5, which is oxidized slowly into nitrate and quickly into nitrite.

The increase in BOD5 and calcium hardness and the decrease in dissolved oxygen demonstrate the diffused intrusion of runoff water from crops with agrofertilizers.

The pH is basic, probably due to the type of riverbed in the upper part of the volcano, which decreases downstream. Turbidity increases as the water descends; the same is true for solids in the water, probably because of erosion and an increase in the organic load.

Current water quality does not present levels of eutrophication, but its decontamination is required for the water habitable by fish, and safe for human use. The results of the analyses, location and distance between the points are presented in *Table 2*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 2. *Results of the water quality analysis*

Parameter	Point 1	Point 2	Point 3	Point 4	Point 5
Altitude (masl)	3,014	2,995	2,864	2,718	2,667
Length from the previous point (m)	0	464	1,618	1,983	1,339
Dissolved oxygen (mg/l)	2.3	2.3	3	1	0.9
BOD5 (mg/l)	1.9	0.9	2.16	36	7.2
Total phosphorus (mg/l)	0.4	0.4	0.4	0.5	0.4
Ammonia (mg/l)	0	0.01	0.09	0.07	0.57
Nitrate (mg/l)	0	0	0	0	0
Nitrite (mg/l)	14	10	13	9	19
pH	8.35	8.82	8.5	8.1	7.41
Calcic hardness (mg/l)	0.38	0.63	1.09	0.79	1.3
Turbidity (NTU)	1.5	2.29	0.93	3	4.28
Total solids (mg/l)	0.06	0.06	0.07	0.08	0.1

Conclusions and recommendations

Values of BOD5 vary between 0.9-36 mgL^{-1} in our study that are comparable to other study (Naciph, 2016) findings (BOD5 vary between 5-40 mgL^{-1}) where San Pedro River was analysed. It is reassuring that the diversity and abundance values in our study are significantly higher than the values in Naciph's study where the same method (Encalada et al., 2011) was applied for specimens separation and for family taxonomic level identification. In our study 36 families were detected, while in Naciph's study only 12. This is due to the sad fact that San Pedro River is highly contaminated (FONAG, 2011) and can not be used for irrigation without pre-treatment (Naciph, 2016).

To aid reader understanding in the topic our preliminary study should be extended to other study areas, such as the lower part of the volcano, where areas of influence, such as cattle, urbanisation and plantations, exist.

According to the families that indicate the quality of water, the water quality fluctuates between very good to regular in Point 2, which is an area with anthropic influence. The current water quality does not present levels of eutrophication, but decontamination is required for the water to be safely used by humans.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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APPENDIX

Abundance of the different families within the sampling points

Order	Family	Point 1	Point 2	Point 3	Point 4	Point 5	Number of individuals
Oligochaeta	N.d	2	16	1			19
Amphipoda	Hyalellidae	3	50		30	31	114
Coleoptera	Dytiscidae		1		3	1	5
	Elmidae	1	6	80	1006	966	2059
	Ptilodactylidae		3	2	1	3	9
	Scirtidae	1	26	3	29	11	70
	Staphylinidae				6	3	9
	Blepharoceridae				2	4	6
	Ceratopogonidae	4	51	22	50	50	177
	Chironomidae	36	25	124	117	163	465
Diptera	Culicidae			1			1
	Dixidae					1	1
	Empididae		1				1
	Limoniidae		4		4	8	16
	Muscidae				1	5	6
	Psychodidae		1		4	6	11
	Simuliidae	83	29	76	30	16	234
	Tabanidae			5	41	35	81
	Tipulidae	2	14	25	678	368	1087

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49494956

Ephermeroptera	Baetidae	63	13	96	121	122	415
	Leptohyphidae	1		1	35	69	106
	Leptophlebiidae	1			1	1	3
Heteroptera	Veliidae		1				1
Hydracarina	N.d		2				2
Plecoptera	Perlidae		1	1	12	26	40
Trichoptera	Anomalopsychidae		5	2	19	28	54
	Glossosomatidae				1	3	4
	Helicopsychidae			2	9	7	18
	Hidropsychidae			5			5
	Hydrobiosidae			1	1		2
	Hydropsychidae				6	9	15
	Hydroptilidae			4		14	18
	Leptoceridae		2	13	256	800	1071
	Limnephilidae				1	11	12
	Odontoceridae			1			1
	Philopotamidae				4	4	8
Veneroidea	N.d		2				2
Basommatophora	Limnaeidae					1	1
Total:		197	253	465	2468	2766	6149

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49494956

THE EFFECTS OF CLIMATE CHANGE ON DECOMPOSITION PROCESSES IN ANDEAN PARAMO ECOSYSTEM–SYNTHESIS, A SYSTEMATIC REVIEW

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(Received 8th Nov 2018; accepted 5th Mar 2019)

Abstract. The paramo is a high mountain ecosystem with cold and humid climate, it has high amount of sunlight and cloudiness, furthermore, organic matter has low decomposition rates, hence, leaf litter degradation gets 40% per year as maximum. In the last 70 years, the air temperature near the surface of the Tropical Andes has increased significantly up to 0.34 °C/decade. In the same region, it is estimated that in this century the average temperature will vary from <+ 1.60 °C to >+ 2.61 °C, which exceeds the threshold of natural climatic variability. On the other hand, although precipitation along the Andes has not shown an increase or diminish pattern between 1955 and 1994, it is projected that by the year 2100, it will decrease in the outer tropics and will increase in the interior tropics. In this paper, we discuss how the decomposition of organic matter in paramo areas is influenced by climate change and analyze how current trends of variation in temperature are projected to affect ecosystem processes. Previous studies have determined that the increase of the atmospheric temperature (as long as the humidity remains stable or increases), generates higher decomposition rates, so that an increase of 1 °C in the atmospheric temperature would also mean an increase in the rate of decomposition of up to 10%. These changes would cause the release of the carbon accumulated in the soil to the atmosphere as CO_2, which would be a determining factor for climate change.
Keywords: *CO_2, global warming, greenhouse gases, organic matter, temperature*

Introduction

The paramo is a high mountain ecosystem in the humid tropic, it is located in the Andes, the Afroalpine zone, Indonesia and Papua New Guinea (Buytaert et al., 2011). This ecosystem has a cold and humid climate, with high amount of sunlight and cloudiness, its temperature gets an anual average of 10 °C at 3000 m a.s.l. and reaches 0 °C at 4600 m a.s.l. (Llambí et al., 2013). Climate conditions in this region are variable, so that, the daily thermal amplitude ranges between 10 and 15 °C in the air and in clear days it reaches 50 °C in the soil surface (Llambí et al., 2013), while precipitation ranges between 700 mm to 3000 mm/year (Llambí et al., 2013). Despite climate conditions, there is a high biological diversity and endemism, approximately 3400 species of vascular plants inhabit the paramo of South America and 60% are endemic (Luteyn, 1999; Rangel Churio, 2000).

Paramo is considered as a carbon sink, it can store a higher quantity of carbon per hectare than tropical forests (Hofstede et al., 2014). It accumulates between 119 and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

397 tons per hectare (t/ha) in 0 to 40 cm depth, (Castañeda–Martín and Montes–Pulido, 2017) meanwhile bofedales show an approximate accumulation of carbon of 30 kg m² (Segnini et al., 2010).

In addition to carbon storage in mineral soils, there is also accumulation of organic matter in peat bogs (Hofstede et al., 2014). In the north of Ecuador, it was estimated the average organic matter quantity in two peat bogs is 1282 t/ha (Hribljan et al., 2016), with an accumulation of 4.6 t/ha/year (Chimner and Karberg, 2008). Moreover, paramo vegetation contributes with fresh organic matter to soil, which means about 6 to 13 t/ha/year (Escobedo–Urquizo, 1980).

On the other hand, despite the importance of paramo through supplying environmental services such as water provision, climate regulation and carbon accumulation in soil), it is one of the more vulnerable terrestrial ecosystems to global climate change (Buytaert et al., 2011; Ruiz et al., 2008). It is found within alpine life zones that are highly sensitive to climate change because its distribution has been closely related to temperature and precipitation patterns (Cuesta et al., 2017). Likewise, if high mountain ecosystems lose humidity and the annual average temperature gets higher, it would cause an increasing conversion of organic matter into atmospheric carbon, which would favor climate change conditions (Buytaert et al., 2011; Jones et al., 2005).

In the same way, an increasing environmental temperature would augment the evaporation and evapotranspiration, altering the precipitation patterns (Arnell, 1999), which could cause a deficit in water supply to populations that depend directly on water from the paramo, like Quito and Bogotá (Buytaert et al., 2011).

In the Tropical Andes region, environmental temperature has shown an increasing trend of 0.11 °C/decade from 1939 to 1998 and 0.34 °C/decade from 1974 to 1998 (Buytaert et al., 2014). Regarding precipitation, changes have not been significant in all the region (Vuille et al., 2003). Nevertheless, records from weather stations indicate higher precipitation levels for Ecuador, but lower levels Peru and Bolivia (Buytaert et al., 2014).

The aim of this paper is to comprehensively review the state of knowledge of decomposition and climate change in paramo ecosystems to understand the consequences on ecological processes, and to obtain ideas to develop strategies to mitigate the effects of climate change.

Decomposition and climate projections

Climate projections for the Andean Region show an increase in the average temperature up to 5 °C on the eastern flank of Ecuador and Peru (Herzog et al., 2012). Precipitation has a mixed pattern: increasing in the eastern and western flanks and decreasing in the inter Andean region up to 15% (Vuille et al., 2008; Urrutia, 2008; IPCC, 2013; Kirtman et al., 2013)

Predictions of global climate models and regional models for the Andean region have a high range of uncertainty due to lack of meteorological stations at altitudes above 3000 m a.s.l. (Buytaert et al., 2014). However, these projections are important to analyze the effects that climate change could have on the stability of ecosystems, environmental services and ecological processes (IPCC, 2007), especially because climate change has the capacity to alter physical and biological processes, threatening survival of endangered species (Carroll et al., 2015). For example, many studies have

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

focused on assessing the influence of climate change on certain species, and some results suggest that climate change conditions could lead to changes in communities composition, populations declines, range shifts, changes in relative abundance and high extinction risks (Gillings et al., 2015; Bush et al., 2016; Wang et al., 2016; Dalamsuren et al., 2017; Pecl et al., 2017; Urban, 2015).

Decomposition comprises physical and chemical processes that allow the reduction of macromolecules into their essential components (Aerts, 1997) generating CO_2 and soluble forms of nutrients that can be recycled in plants and other organisms (Cronan, 2018). Most of the organic matter that is involved in this process in terrestrial ecosystems are derived from plants (leaf litter, stems, root exudates) (Carter et al., 2007), while influential factors are: weather, chemical composition of leaf litter and soil organisms (Coûteaux et al., 1995; Aerts, 1997, 2006).

Concerning climate, temperature and soil moisture (commonly referred to as climate decomposition index) have shown to be the best predictors of decomposition rates (Parton et al., 2007)), they can slow or accelerate organic matter decomposition (Davidson and Janssens, 2006; Coûteaux et al., 2002).

As shown, it is evident that decomposition is different in every ecosystem (Zhang et al., 2008; Djukic et al., 2018). This was verified by assessing decomposition rates in three regions of the planet: Tropical, Temperate and Mediterranean, the average values obtained per year were 2.33, 0.36, 0.35 respectively, showing that in humid tropics the decomposition rate is higher than in the temperate and Mediterranean zones (Aerts, 1997).

Regarding chemical composition of organic matter, leaf litter quality has a significant and predictable influence on the temperature sensitivity of organic matter decomposition (Fierer et al., 2005), besides, it is also important during decomposition phases due to its effects on humus formation (Coûteaux et al., 1995). Likewise, certain plant species have low decomposition rates compared to their high lignin content (Zhang et al., 2008) or presence of polyphenols that inhibit degradation (Hättenschwiler et al., 2005).

Although bacteria and fungi are the main decomposer organisms, macroinvertebrates also play a fundamental role, starting the process of litter crushing (Ulyshen, 2016). While more organisms participate in degrading organic matter, an increase in the rate of decomposition is generated (Smith and Bradford, 2003; Wall et al., 2008).

Given the sensitivity of decomposition to climatic conditions, it is understood that global warming will increase litter decomposition rates, through direct effects and through indirect effects on leaf litter quality and soil organisms (Aerts, 2006).

Organic matter decomposition in paramo ecosystems

Decomposition process in paramo ecosystems is mainly limited by atmospheric temperature that shows a high daily variation (temperature differences higher than 20 °C between night and day are common) (Hofstede et al., 2014). On the other hand, in paramo ecosystems with volcanic origins, an important factor is the presence of vesicles that organic matter generates with aluminum from volcanic ashes, turning into chemical complexes that are resistant to organisms degradation (Hofstede et al., 2003, 2014; Bottner et al., 2006).

As mentioned before, although bacteria communities are important for decomposition (Bottner et al., 2006; Pansu et al., 2004), invertebrates also play an

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

important role in the disintegration of organic matter (Ulyshen, 2016), its diversity is associated with the presence of vegetation (Ewers et al., 2015) and, richness of soil fauna together with climate are associated with decomposition rates globally (Wall et al., 2008).

Concerning leaf litter quality, decomposition process in paramo do not depend on lignin concentration. As paramo vegetation is mainly composed by rosettes, cushions, shrubs and herbs (Ramsay and Oxley, 1997) it is not woody, then its lignin content would be low (Coûteaux et al., 1995). Nevertheless, some species contain aromatic resins that decrease the rate of decomposition (Smith, 1981).

Several studies have analyzed decomposition processes in High Andean ecosystems focusing research in: 1) the effect of human activities on decomposition (Jiang et al., 2015; Ossola et al., 2016; Urbina and Benavides, 2015), 2) influence of altitude in organic matter decomposition (Coûteaux et al., 2002; Röderstein et al., 2005) and 3) factors that influence on organic matter decomposition (Bottner et al., 2006; Pansu et al., 2004, 2007; Ibrahim et al., 2015; Martinez et al., 2007; Pinos et al., 2017).

Climate change and organic matter decomposition

It is expected that in this century, the increasing CO_2 concentrations and other greenhouse gases in Earths atmosphere cause warmer surface temperatures and changes in precipitation patterns (IPCC, 2013). These environmental changes could affect the carbon cycle, modifying the function and ecosystem services (Dukes et al., 2005).

In order to determine the influence of climate change on ecosystems, models have been generated to simulate the response of the decomposition process to climate change, which have made it possible to determine that an increasing atmospheric temperature will produce a decrease of 54 gigatons (Gt) in the global carbon stock of the soil for 2100 (Jones et al., 2005).

In addition, some studies have shown the influence of climate change on the decomposition process, so that, it was determined that the increase in soil temperature accelerates the decomposition process in Arctic soils (Robinson et al., 1995), while in other study, researchers determined that the decomposition process is limited by moisture in the xeric zones and in the mesic zone it is determined by temperature (Shaw and Harte, 2001). On the other hand, a research using experimental microcosms, determined that the change of climatic conditions in a short term (from days to decades) strongly influences the decomposition process of litter (Strickland et al., 2015).

Climate change and organic matter decomposition in Andean paramos

In the Tropical Andes, atmospheric temperature near surface has increased significantly in the last 70 years (Vuille et al., 2008). A regression analysis with ordinary least squares indicates a warming of 0.10 °C/decade and a general temperature increase of 0.68 °C since 1939 (Vuille et al., 2008). Furthermore, it is suggested that in this century, in a scenario with a balanced use of fossil fuels and energies of non–fossil origin (scenario of A1B emissions), the temperature for Tropical Andes region will probably vary from <+ 1.60 °C to >+ 2.61 °C, exceeding the threshold of natural climatic variability (+ 1.78 °C) (Ruiz–Carrascal et al., 2017).

In the case of precipitation, there are no reports of increasing or decreasing patterns at a regional levels between 1955 and 1994 (Vuille et al., 2003). For the period 2071–2100 in a scenario of A1 emissions (rapid economic growth at global level, a maximum

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

of the worlds population by the middle of the century and a rapid introduction of new and more efficient technologies) precipitation along the Andes would present a mixed pattern of increase and decrease that goes from –50 to 250% (Urrutia, 2008). In general terms, it is projected that for the second half of the 20th century, rainfall will decrease in the outer tropics and increase in the interior tropics (Urrutia, 2008; Vuille et al., 2008).

According climatic projections for the Andean Region (10°–20°S) (Herzog et al., 2012), with a high population growth (scenario of A2 emissions), an increase in average temperature and precipitation would occur by the end of the 21st century, for example, on the western flank of the northern Peru, the increase in rainfall would be up to 70%, while in the Inter Andean region of Colombia a decrease of up to 15% will occur; and an increase of 5 °C of temperature is projected in the eastern flank of Ecuador and Peru (Herzog et al., 2012).

In the Fifth Report of the Intergovernmental Panel on Climate Change (IPCC) results of projections for an immediate climate change (Period 2016–2035 regarding 1986–2005 in RCP4.5 scenario comparable with B1) in the Andean Region suggest an increase in temperature of up to 1.5 and an increase in precipitation of up to 10% (IPCC, 2013; Kirtman et al., 2013).

An eventual change in the temperature and humidity of the paramo soil would lead to a modification in decomposition processes. It is necessary to consider that high Andean ecosystems would be more sensitive than the humid tropical forest in conditions of increasing temperature, with a trend to increase the rate of decomposition, therefore carbon sink ecosystemic services would be affected. In addition, it is important to mention that it is unknown how microbial communities would participate in different climate change scenarios (Hofstede et al., 2014).

While preparing this document, a considerable amount of information about the impact of climate change on soil and biodiversity was found. However, in the few investigations found about the effect of climate change on the decomposition process in the paramo (*Appendix 2*), it was determined that the increase in atmospheric temperature accelerates decomposition rates.

Climate change in the Andean Region will increase decomposition and mineralization rates up to 10% (Salinas et al., 2011). In situ studies through an altitudinal gradient, have allowed to project the changes of climatic conditions, being able to determine that the decomposition rate in the Andean paramo will be influenced by an increase in the air temperature (Espín Meneses, 2012). Furthermore, even if atmospheric temperature increases, when humidity decreases, decomposition rate decreases too, it happens not only due to its direct incidence on degradation process but also because it alters abundance and richness of decomposing organisms (Looby and Treseder, 2018).

Conclusions

Despite the importance of decomposition process in the functioning of ecosystems and although since 1939 there have been alarming reports of a general increase in temperature of 0.68 °C for Tropical Andes region, there are few studies about the influence of climate change on decomposition in paramo areas, in other words, knowledge about this issue is currently scarce.

Decomposition process at biomes scale is controlled by climate, then climate change can directly affect the functioning of ecosystems. Studies about this issue mention that

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

the rate of decomposition increases with higher temperatures (only when humidity is maintained or increased), then an increase of 1 °C in atmospheric temperature could cause the decomposition rate to increase by up to 10%.

Projections suggest that in this century, paramo areas will have constant humidity and increasing temperatures exceeding 2 °C. According to this climate scenario, the rate of decomposition would increase, causing the carbon accumulated in the organic matter of the soil to be emitted back into the atmosphere

Exploring studies about the effect of climate change in decomposition rates in paramo ecosystems is still a challenge, we need to conduct experimental research in order to understand how ecological processes work in this ecosystem. Generating knowledge will provide people new ideas to mitigate effects of climate change for present and future scenarios.

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APPENDIX

Appendix 1. *Studies about decomposition process in paramo ecosystems*

Authors	Year	Title	Conclusions	Decomposition %
Hofstede	1995	The effects of grazing and burning on soil and plant nutrient concentrations in Colombian paramo grasslands.	There is greater decomposition in sites with high affectation by grazing and burning than in sites without disturbances.	In areas without disturbance, litter loss is up to 11.3 ± 4.64% per year. While in areas exposed to livestock and fire, the decomposition rate is 34.3 ± 8.03% per year.
Chapela et al.	2001	Ectomycorrhizal fungi introduced with exotic pine plantations induce soil carbon depletion.	Paramo soil with Pinus radiata plantations contain less carbon than paramo grassland. It is caused by the introduction of ectomycorrhizas in the roots of the pines.	The soil of the pine plantations of 10 to 20 years, show 30% less C than soils of pasture paramos.
Coûteaux et al.	2002	Decomposition of standard plant material along an altitudinal transect (65–3968 m) in the tropical Andes.	Decomposition rates decrease along with decreasing temperatures (while altitude is higher).	Five altitudes were assessed: 95, 165, 780, 1800, 3400 y 3968 m a.s.l., carbon loss percentage in the first year was 78.7, 76.6, 67.5, 62.9, 40.4 and 42.8 respectively.
Sarmiento and Bottner	2002	Carbon and nitrogen dynamics in two soils with different fallow times in the high tropical Andes: indications for fertility restoration.	In an agricultural area of the Andean region above 3000 m a.s.l., decomposition of straw in a restored soil is significantly faster than in a degraded soil.	Soil carbon loss was expressed as the initial percentage of 14C added to the soil (mineralized). The percentage of mineralization of 14C after 12 weeks of incubation was 43.9% in the restored soil and 42.9% in the degraded soil.
Pansu et al.	2004	Comparison of five soil organic matter decomposition models using data from a 14C and 15N labeling field experiment.	The best model of organic matter decomposition of soil (MOMOS) uses kinetic constants: three entries of microbial biomass MB and two MB outputs mortality and respiration constants. This model significantly improved the predictive quality and robustness of MB-14C and -15N predictions.	In 720 days, 80% of 14C was lost in the analyzed samples. This decomposition percentage was obtained in the five models of soil organic matter decomposition.

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Röderstein et al.	2005	Above-and below-ground litter production in three tropical montane forests in southern Ecuador.	Decrease in temperature while altitude increases, influence the carbon cycle of montane forests. The decrease in temperature along the slope affects decomposition.	The litter mass decreased to less than one third (862 to 263 g m^{-2} y^{-1}) with increasing altitudes (1890 m to 3060 m). While litter production of fine roots increased in an approximated factor of four (506 to 2084 g m^{-2} y^{-1}).
Bottner et al.	2006	Factors controlling decomposition of soil organic matter in fallow systems of the high tropical Andes: a field simulation approach using 14 C- and 15 N-labelled plant material.	The relation between C and N is determinant in decomposition. If the C:N relation in the plant material increases, the decomposition rate decreases and the mortality of the microbial biomass increases.	For N-rich plant material (N + treatment), the total of 14 C remaining at the end of the experiment was similar in the Gavidia paramo and in Puna de Patacamaya: 25% of the initial 14 C.
Pansu et al.	2007	Modelling the transformations and sequestration of soil organic matter in two contrasting ecosystems of the Andes.	The dynamics of 14C and 15N were very different in the two systems. In the puna, the transformation processes stop during the long dry periods, although the annual total mineralization is greater than in paramo.	In dry puna, the 66% of the 14C initially added was mineralized during the first wet season (first 120 days). Subsequently, the mineralization was almost paralyzed until the end of the dry season (on day 400), then 73% of the aggregate 14C was mineralized after the second wet season (on day 500) and 75% on day 690 after the first rain that followed the last long dry period. In humid paramo, only 42% of the 14C added was mineralized during the initial phase of the process, then mineralization progressed slowly, until approximately 65% of the 14C was mineralized after 1 year, and the 75% after 2 years.
Martinez et al.	2007	Succession pattern of carrion-feeding insects in Paramo, Colombia.	The succession of scavenger insects can be an indicator of the state of decomposition and the time elapsed after an organism death.	After 83 days, 83% of the initial weight of Sus scrofa corpse had been lost. Five stages of decomposition were identified with indicator species: Calliphora nigribasis in the fresh

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

				stage; Compsomyiops verena in the swollen stage; Compsomyiops Bolivian during active decomposition; Stearibia nigriceps and Hydrotaea sp. during the advanced decay and Leptocera sp. for dry remains.
Garrido	2011	Study on radial growth, exchange and leaf decomposition of three Polylepis species (Rosaceae) in two locations of the Ecuadorian Andes.	There is a highly significant difference among species. Polylepis reticulata had the least decomposition, attributed to climatic conditions as soil moisture and temperature, since they are better predictors of decomposition than quality of its organic matter.	The global average for the remaining biomass percentage of leaf decomposition is 75.44%. P. reticulata shows a lower decomposition with a mean of 86.67 + 7.015% Standard deviation (D.E.), P. pattern shows an average of 75.809 + 12.183% D.E. and P. incana had a greater decomposition with an average of 63.83 + 16.467% D.E.
Ibrahim et al.	2015	Modelling carbon turnover through the microbial biomass in soil.	Comparison of predictions with collected data in the field showed that the model is capable of simulating transformations and movement (plant-soil-atmosphere) of the carbon depending on the parameters previously defined	The predictions of the model showed a higher microbial content of C and a higher microbial respiration activity in the wheat plots than in vean plots. The metabolic coefficient (mg CO^2/g MB C) after 9 months was 0.04 in wheat and 0.025 in bean.
Urbina and Benavides	2015	Simulated small scale disturbances increase decomposition rates and facilitates invasive species encroachment in a high elevation tropical Andean peatland.	Decomposition rate in soil surface (0-10 cm) triples in fertilized soils with physical disturbances in comparison with the control (soil without additions of foreign materials or physical disturbances).	Decomposition rate in the control was 0.09 ± 0.001 and in the plots with fertilized soils with physical disturbances was 0.25 ± 0.005.
Pinos et al.	2017	Leaf litterfall and decomposition of polylepis reticulata in the treeline of the Ecuadorian Andes.	Temperature was one of the main regulators for decomposition rates of litter. Humidity was not controlled, then it is not a limiting factor in decomposition.	Decomposition rate of litter of Polylepis reticulata was 0.38 ± 0.02 per year. The study was conducted in an altitudinal range of 3700 to 3900 m a.s.l.

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Appendix 2. *Studies about the effect of climate change in decomposition process in paramo ecosystem*

Authors	Year	Title	Conclusions	Decomposition %
Salinas et al.	2011	The sensitivity of tropical leaf litter decomposition to temperature: results from a large-scale leaf translocation experiment along an elevation gradient in Peruvian forests.	When samples were grouped between species and elevations, soil temperature explained 95% of the variation in the rate of decomposition, but no direct relation with soil humidity or precipitation was observed.	Heating of 0.9 °C in the last decades could have increased decomposition and mineralization rates of nutrients by 10%.
Espín	2012	Altitudinal patterns of litter decomposition in two paramo localities with different climatic regimes.	Using an altitudinal gradient to outline possible in situ scenarios on climate change is valid. It is useful to determine that there is a strong relation between temperature and decomposition rate in the Andean paramo.	Decomposition rates of litter could increase up to 5% per year with every 1 °C additional average diurnal temperature.
Salcedo	2014	Effects of the increasing temperatures and fires on Mesofauna (Arthropods) of the Soil and the Decomposition of Litter in a paramo Ecosystem in the North of Ecuador.	In a cold ecosystem such as paramo, an increase in temperature could be expected to determine greater decomposition of litter; however, the results of the study showed an opposite pattern, perhaps as a result of a decrease, at least temporarily, in the moisture content of the ground.	Decomposition rate in the treatments that were heated with cameras (which increased the temperature between 0.89 °C and 2.06 °C in the day and between 0.66 °C and 0.86 °C at night) They were between 3 and 4% slower than treatments that were not heated with cameras. The treatment with the slowest decomposition was camera/burning (54.5% ± 1.38), while the highest decomposition was recorded in the plots with the control treatment (49.7% ± 1.2).
Looby and Treseder	2018	Shifts in soil fungi and extracellular enzyme activity with simulated climate change in a tropical montane cloud forest.	Fungi abundance and alpha diversity increased significantly with warmer temperatures and drier conditions. Concerning composition of fungal community, it showed a change with increasing temperatures	With a 4 °C increase and 20% of humidity decrease, fungal abundance and richness grew in 50%.

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

			($F = 4.31$, $R2 = 0.170$, $P < 0.001$) and decreasing humidity ($F = 4.30$, $R2 = 0.170$, $P < 0.001$). Alterations in the composition of the community was associated with changes in the richness of certain fungal phylum. There was a general increase in Ascomycota richness with warmer temperatures and drier conditions. While, the richness of Cryptomycota decreased in translocated soils to warmer and drier conditions.	

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DOI: http://dx.doi.org/10.15666/aeer/1702_49574970

COMPARISON OF LEARNING AND MEMORY OF EASTERN (*APIS CERANA CERANA*) AND WESTERN HONEY BEES (*APIS MELLIFERA* L.)

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(Received 20th Nov 2018; accepted 14th Feb 2019)

Abstract. Honeybees are valuable pollinators strongly influencing ecosystem conservation, stability, genetic variation and ecological relationships in the floral diversity, plant community, evolution, and specialization. Honeybee is an excellent model organism for research on learning and memory among invertebrates. In their behavior, the subjective evaluation of a sucrose stimulus influences the behavioral performance. Here we report comparative behavioral data on the sensitization, habituation, short-term and long-term memory of *Apis cerana* and *A. mellifera* in China, using different sucrose concentrations. *A. mellifera* foragers have higher sucrose responsiveness than *A. cerana* foragers when tested using a proboscis extension response (PER) assay. Sensitization and habituation are well-known forms of non-associative learning. *A. cerana* took less number of habituation trials as compared to *A. mellifera*. Thus, these results significantly showed that *A. cerana* took less time to dishabituate. The sensitive stimuli of *A. cerana* against lemon extract were more than *A. mellifera*. In addition, although *A. mellifera* showed more sensitive stimuli against apple extract than *A. cerana cerana*, *A. mellifera* displayed significantly more learning and memory behavior than *A. cerana* after 2 and 24 hours.
Keywords: *olfactory learning, honeybee, proboscis extension response, odors, sensitization*

Introduction

In ecosystem functioning, diversity is a major and important component (Tilman et al., 2001; Duffy et al., 2007). Honey bees are key contributors and the best pollinator species in the world in natural ecosystems and functions (Ebeling et al., 2011). Species richness can stabilize and play an important role in ecosystem services, including pollination to environmental disturbance and variability pollination. Honeybees are crucial to ecosystems and environment, wild plant growth, wildlife habitats, and biodiversity as a pollinator and food source. The eastern honeybee (*Apis cerana*) is a species of honeybee found in southeastern and southern Asia, including China, Pakistan, Korea, Malaysia, Japan, Bangladesh, Nepal, Papua New Guinea, Australia Solomon Islands and India (Engel, 1999; Oldroyd and Wongsiri, 2009). The western honeybee or European honeybee (*Apis mellifera*) is the most common of the 7–12 species of honeybee worldwide. The genus name *Apis* is Latin for "bee", and *mellifera* is the Latin for "honey-bearing", referring to the species' production of honey. As the wasps come close to the honeybee nest (*Apis cerana cerana*), extra guard bees are alerted, which in turn increases their chance of being killed by heat-balling bees. Heat balling is a distinctive defense system in which several hundred bees surround the wasp in a tight ball and vibrate their muscles in an effort to produce heat and effectively kill the wasp inside. *Apis mellifera* colonies can reach sizes of up to 50,000 or more individuals, *Apis cerana* colonies are relatively small, with only around

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DOI: http://dx.doi.org/10.15666/aeer/1702_49714984

6,000 to 7,000 workers. *Apis cerana* is found predominantly in the Eastern Asian region of the world, while *Apis mellifera* is found predominantly in the Western European and African region of the world (Winston, 1991). *A. cerana* recruits threefold more guard bees to stave off predation than *A. mellifera*. Honeybees avoiding wasps' predation behavior, *A. cerana* can efficaciously escape from wasp's predation through changing flying behavior, but *Apis mellifera* cannot. *Apis cerana* has been recognized as a lower pathogen occurrence species versus *Apis mellifera*. Hygienic response (removing freeze killed brood and opening cell caps) of *Apis cerana* is faster and better than *Apis mellifera* (Lin et al., 2016). *A. cerana* visited more flowers and took greater time for completing a single foraging trip on apple bloom than *A. mellifera* (Ahmad et al., 2017). The honeybees must have to learn and memorize the color of flower, how to navigate, and what plants are rich food source. Learning and memory are basic and essential capabilities for dance communication and foraging behavior. Flowers shape, odor, and color are learned during foraging (Sandoz et al., 2002; Hooper et al., 2005; Haddad et al., 2011).

Most animal species are capable of discriminating between varieties of odors. This ability is usually crucial for the organization such as feeding, mating, and social communication, as well as for the processes of learning and memory that are associated with these behaviors (Leonard et al., 2011). Insects constitute successful models for the study of learning and memory due to their remarkable learning abilities (Menzel, 1999; Mizunami et al., 2004; Davis, 2005; Giurfa, 2007). Among insects, honey bees (*Apis mellifera*) are reported to have the highest and broadest range of learning abilities (Menzel, 1999; Giurfa et al., 2001; Giurfa, 2007; Sandoz, 2011; Giurfa and Sandoz, 2012). Honey bees are able to associate a food reward with different sensory stimuli such as odors, colors and visual patterns, tactile or thermal stimuli (Menzel, 1999; Giurfa, 2007). Both *A. mellifera* and *A. cerana* are kept in China, with about four million colonies and three million colonies, respectively (Hongliang, 2007). *A. cerana* colonies are mainly kept in mountainous locations, while *A. mellifera* colonies are usually transported across the country to follow the blooming of flowers (WANG et al., 2007). Honey bees (*A. mellifera*) are well known for their communication and orientation skills and for their impressive learning capability (Menzel and Giurfa, 2001; Menzel et al., 2006). Because the survival of a honeybee colony depends on the exploitation of food sources, forager bees learn and memorize variable flower sites as well as their profitability. Forager bees can be easily trained in natural settings where they forage at a feeding site and learn the related signals such as odor or color. Appetitive associative learning can also be studied under controlled conditions in the laboratory by conditioning the proboscis extension response (PER) of individually harnessed honey bees (Bitterman et al., 1983; Kesner and Olton, 2014).

The proboscis extension reflex is a classical behavioral trait used to determine the learning behavior of honeybees' response to odors. The PER is the behavior of a honey bee that extends her proboscis when a drop of sucrose solution at sufficient concentration is applied to the antennae (Pankiw and Page, 2003). However, studies on learning and memory of honeybees have mostly used harnessed individuals and olfactory learning protocols when the goal was to achieve full control of behavior by the experimenter. The most popular protocol used to this end is the olfactory conditioning of the PER, which is a case of classical conditioning (Takeda, 1961; Vareschi, 1971; Bitterman et al., 1983; Giurfa and Sandoz, 2012). The PER is a reflexive response of hungry bees which is part of their feeding behavior while foraging or within the hive (Frings and Frings, 1949). It occurs when the antennae, tarsi or mouthparts come in contact with sucrose solution; the bee then reflexively extends its proboscis (PER) to

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reach the sucrose solution and drink it. Odors generally do not evoke the PER in bees naïve to the experimental conditions. During conditioning, an odor (CS) is presented in close temporal association with sucrose solution (US). At the end of training, the odor alone elicits the PER, indicating that the bee has learned the odor-sucrose association (Takeda, 1961; Bitterman et al., 1983). PER is usually recorded as a dichotomous response (1 or 0), which can thus be used as an index for learning and memory performances. The protocol of olfactory PER conditioning, first established by (Takeda, 1961) and was later standardized by (Bitterman et al., 1983).

Habituation and sensitization are two well-known forms of non-associative learning, which change the response probability of an animal towards a stimulus following repeated exposure to the same stimulus (habituation) or exposure to a strong yet different stimulus (sensitization). Habituation is a response decrement to a monotonously repeated stimulus (Braun and Bicker, 1992). While sensitization implies that a strong or particularly salient stimulus enhances the response of the bee to a test stimulus (Menzel, 1999). A honeybee extends its proboscis when its antennae are stimulated with a sucrose solution whose concentration exceeds its sucrose response threshold. When low-concentrated sucrose solutions are repeatedly applied to the antennae of a bee with a short interval between two stimulations ("inter-trial interval"), the proboscis extension response habituates (Braun and Bicker, 1992; Menzel., 1993; Scheiner et al., 2004). The bee no longer shows proboscis extension when its antennae are stimulated with this sucrose stimulus. Similar to habituation, the degree of sensitization depends on the gustatory responsiveness of the bee and the strength (i.e. the sucrose concentration) of the sensitizing stimulus (Menzel el al., 1993; Scheiner el al., 2004).

Sensitization can be easily tested in honeybees using an odorant and a sucrose stimulus. Most bees do not show spontaneous proboscis extension when their antennae are stimulated with an odorant such as carnation, citral or geraniol. When their antennae are briefly stimulated with a high-concentrated sucrose solution (30% or 50% for example) and with an odorant immediately afterward, the bees become sensitized to the odorant (Menzel et al., 1993; Hammer et al., 1994). Honey bees display proboscis extension in response to antennal stimulation with the odorant, which previously did not evoke a visible response. Note that sensitization to an odor is different from classical olfactory conditioning because: (1) it does not require repeated pairings of odor and sucrose solution, as well as (2) it only occurs up to two minutes after olfactory stimulation. For a century now, honeybee (*A. mellifera*) has been a key insect model in which behavioral, neuroanatomical, and neurophysiological approaches have been performed to unravel the basis of olfaction and olfactory learning. Honey bees are social insects which present a wide range of behaviors relying on olfaction both within and outside of the colony (Seeley, 1995).

The honey bee is well-known for its excellent associative learning capacities in a wide range of assays (Menzel and Müller, 1996; Menzel and Giurfa, 2001; Giurfa, 2007; Matsumoto et al., 2012). Both in the field and under the controlled conditions of a laboratory, bees reliably learn and remember odors, shapes and surface structures. Learning of colors is much more complicated under laboratory conditions (Gerber and Ullrich, 1999; Mota et al., 2011). Bees can be trained in classical and operant conditioning paradigms. In classical conditioning, honey bee learns to associate an originally neutral stimulus (conditioned stimulus, CS) with a biologically relevant stimulus (unconditioned stimulus, US), while in operant conditioning, a bee evaluates its own behavior and its consequences (Menzel, 2012).

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Any study of memory must carefully separate the effect of storage and recall mechanisms (Spear et al., 1990). The results reported here indicate a complex relationship between olfactory storage and recall mechanisms in the honeybee. That is, what is recalled after memory consolidation (Erber et al., 1980) can be slightly different from the conditioning event, and a lack of a response does not necessarily imply forgetting (Spear et al., 1990). Associative learning is an essential component of the bee's central place foraging behavior and dance communication.

Materials and Methods

Insect

Experiments and observations were conducted with colonies of *Apis cerana* and *A. mellifera* from March to August 2017. Three colonies of each species (*A. cerana* and *A. mellifera*) were set up at an apiary of the College of bee science, Fujian Agriculture and Forestry University, Fuzhou, China. All colonies were queen right and housed in standard Lang troth hives.

Catching bees

A pyramid (height 24.5 cm, apex 3.5 cm × 3.5 cm, base 18 cm × 18 cm) made of UV-translucent Plexiglas was used to catch bees when they departed from the hive and flied toward the sky (*Fig. 1A*). The Plexiglas was UV-transparent; in order to offer a complete view of celestial cues and thus lure the departing bees into the pyramid, the pyramid was closable at the apex and at the base. For catching bees, the pyramid was held at a frontal distance of about 10–20 cm from the hive entrance, with the base open and the apex closed. Unexpected movements and standing directly in front of the hive entrance (instead of laterally) were avoided to prevent arousing guard bees. When enough bees were caught, the base was closed and the pyramid was taken to the laboratory. In the laboratory, the pyramid was darkened (except for its apex), for instance by placing a thick cloth over it. Because of their positive photo taxis behavior, bees then tended to leave the pyramid one by one through the apex. Honey bees were individually captured into glass vials. Vials were then placed in crushed ice as long as it is necessary to render the bees motionless (usually between 3 and 5 min) so that they were harnessed individually. Cooling time was kept to a minimum as extended cooling impair learning performances (Frost et al., 2011) and survival in the harness.

Harnessing bees

As soon as the bees ceased their movements, they were being placed in the harnessing tubes. Each bee was fixed within a tube using a piece of adhesive tape placed at the level of the neck, the rest of the body being concealed within the tube. Once fixed in the tube, the bee should only be able to freely move its mouthparts and antennae, thus hiding other body parts from possible contacts with sucrose stimulation. The forelegs of the bees, for instance, should not be able to move freely but should remain enclosed within the tube to avoid interference with olfactory and sucrose stimulation. Each harness tube should be numbered to allow individual identification of the bees throughout the experiment. A rack with numbered boreholes was useful for handling and identifying harnessed bees.

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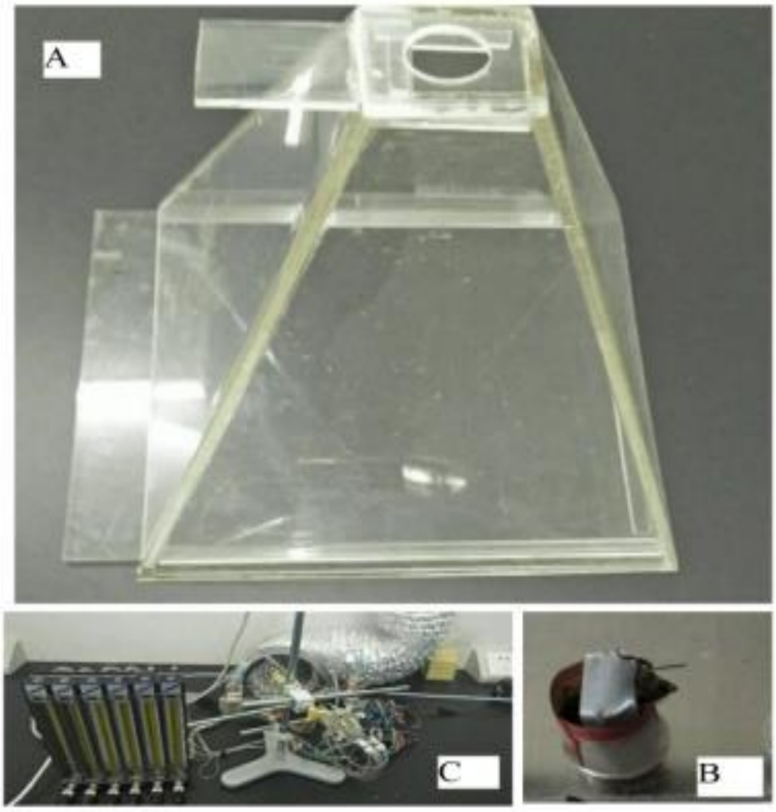

Figure 1. ***(A)*** *pyramid made of UV-translucent Plexiglas was used to catch bees when they departed from the hive and fly towards the sky,* ***(B)*** *each bee was fixed within a tube using a piece of adhesive tape placed at the level of the neck, the rest of the body being concealed within the tube. Once fixed in the tube, the bee should only be able to freely move its mouthparts and antennae,* ***(C)*** *the bee is set in front of an odorant Proboscis extension response setup which is controlled by a computer and which sends a constant flow of clean air to the bee*

Sucrose concentration

Explicit control of the bee caste used for conditioning is recommended because foragers are the individuals that exhibit the highest appetitive motivation for sucrose within the hive (Scheiner et al., 2001) and they are, therefore, more appropriate for appetitive olfactory conditioning. Catching and harnessing the bees were conducted as described in section 2.2 and 2.3. Capture of bees departing from the hive in the morning or late afternoon (avoiding mid-day times when young bees performed their first orientation flights) enhanced the probability of obtaining empty foragers for experiments. Empty foragers (i.e. with empty crop) were necessary to ensure highest appetitive motivation for the experiments. After immobilization, the foragers were mounted in the holding tubes like other bees. One hour after mounting, responsiveness to sucrose was tested using the PER. Water and the following six sucrose concentrations were applied to the antennae of each bee: 0.1%, 0.3%, 1%, 3%, 10% and 30% (w/v). With each sucrose concentration, it was recorded whether proboscis extension occurred. The inter-trial interval was 2 min. The sum of the responses to water and the six sucrose concentrations represents the gustatory response score (GRS) of an individual and is a measure of its responsiveness to sucrose (Scheiner et al., 2004). Forager's bees were tested sequentially with the series of sugar solution, starting with the lowest concentration. Water was used between each sugar solution to reduce possible sensory

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sensitization to antennal touch (Pankiw et al., 2001). The PER was recorded as '1' if a bee extended her proboscis after both of her antennae were touched by a toothpick pre-soaked with a sucrose solution, and '0' if she did not respond. The responsiveness of 30 bees to each sucrose concentration was expressed as "percent of bees showing PER (%PER)" by dividing the number of bees that responded to sugar by the total number of tested bees. For example, if 5 bees out of 15 responded to 10% sugar solution, then the %PER for this concentration was 5/15= 33%. The %PER is a more refined response as compared to PER score because the PER score is a summed response across all the sugar concentrations. Three colonies of each species were measured for this experiment (with N = 3 × 2 × 30 =180 bees tested for both species) (Friedrich, 2004; Yang et al., 2013).

Habituation

Catching and harnessing the bees were conducted as described in section 2.2 and 2.3. All the bees were fed three drops of 30% sucrose solution and put in an incubator for one hour. Bees were stimulated five times with 30% sucrose to induce dishabituation. Habituation of PER was tested by repeated stimulation of an antenna (30% sucrose Solution) at an inter-stimulus interval of 1 s. The number of PER occurring before five consecutive response failures defines the habituation criterion (Braun and Bicker, 1992; Müller and Hildebrandt, 2002; Scheiner et al., 2004; Pirk et al., 2013).

Sensitization

Catching and harnessing the bees were conducted as described in section 2.2 and 2.3.use olfactory learning machine for lemon extract for 4 seconds. Two minutes after testing the initial responsiveness to a lemon odor stimulus, the honeybee was sensitized by antennal stimulation with sucrose (30% sucrose solution). After 20 s, the second odor apple stimulus was presented to test for sensitization (Iqbal and Mueller, 2007).

Learning and memory

Catching and harnessing the bees were conducted as described in section 2.2 and 2.3.A conditioning trial contained pairing an odor stimulus (apple extract) (conditioned stimulus, CS) with a sucrose reward (30% sucrose solution) (unconditioned stimulus, US). After the animals received three successive conditioning trials at an inter-trial interval of 2 min, memory tests were performed 2 h and 24 h after training.

Statistical analysis

The significance of the finding for the sucrose responsiveness experiment was tested by two-way ANOVA, two-way ANOVA comparison shows response of *Apis mellifera* and *Apis cerana* of different sucrose concentrations (Pérez Claudio et al., 2018; Afik et al., 2006). All observed scores of both groups were compared using a *t*-test or nonparametric equivalents. More than two groups were compared using analysis of variance (ANOVA) or nonparametric equivalents with respective post-hoc tests. Response frequencies were compared using Chi-square tests or multiple-measurement ANOVA (for further details see the BEEBOOK paper on statistical methods (Pirk et al., 2013). The entire data of learning and memory were analyzed through One-Way Analysis of Variance (ANOVA) and the means were compared by Tukey's test ($P \leq 0.05$) with the help of KyPlot™ v5.0 program (KyensLab Inc., 2017).

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Results

Sucrose responsiveness in honey bee foragers

Apis mellifera showed significantly more proboscis extension response than *A. cerana* foragers (F=2.879, df=5 and P<=0.05). The mean proboscis extension response of *Apis mellifera* was significantly higher at 30% and 10% of sucrose respectively (*Fig. 1*). The lowest concentration causing proboscis extension was observed on *A. cerana* at 0.10% of sucrose (*Fig. 2*).

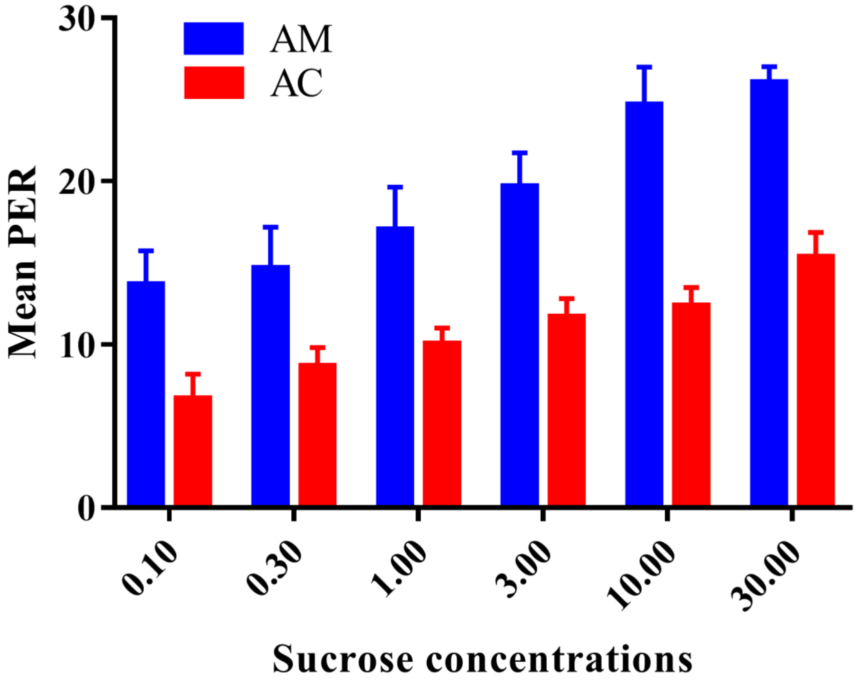

Figure 2. *Percentage of bees showing proboscis extension response (mean ± SE) of foragers of Apis mellifera and A. cerana across different sucrose concentrations. The following data based on three colonies of each species of honeybee. (N = 3 × 2 × 20 = 120 bees).N (total number of species), 3 (three colonies of each species), 2 (two species Apis cerana cerana, Apis mellifera L. were studied) and 20 (number of bees from each colony of both species)*

Habituation

Habituation represents the gradual decrease in responsiveness during a continuous series of repeated stimulations. When the bees were habituated, sucrose stimuli would no longer elicit PER. The habituation rate was assessed by counting the number of trials until no visible movement of the proboscis occurred in five subsequent trials (Braun and Bicker, 1992). *A. cerana* took less number of habituation trials as compared to *A. mellifera*. Thus, our results indicated that *A. cerana* took less time to dishabituate (*Fig. 3*). After A (1-20) and B (21-40), habituation trials 77.78% and 15.56% bees of *A. cerana* showed proboscis extension response respectively (*Fig. 3*).

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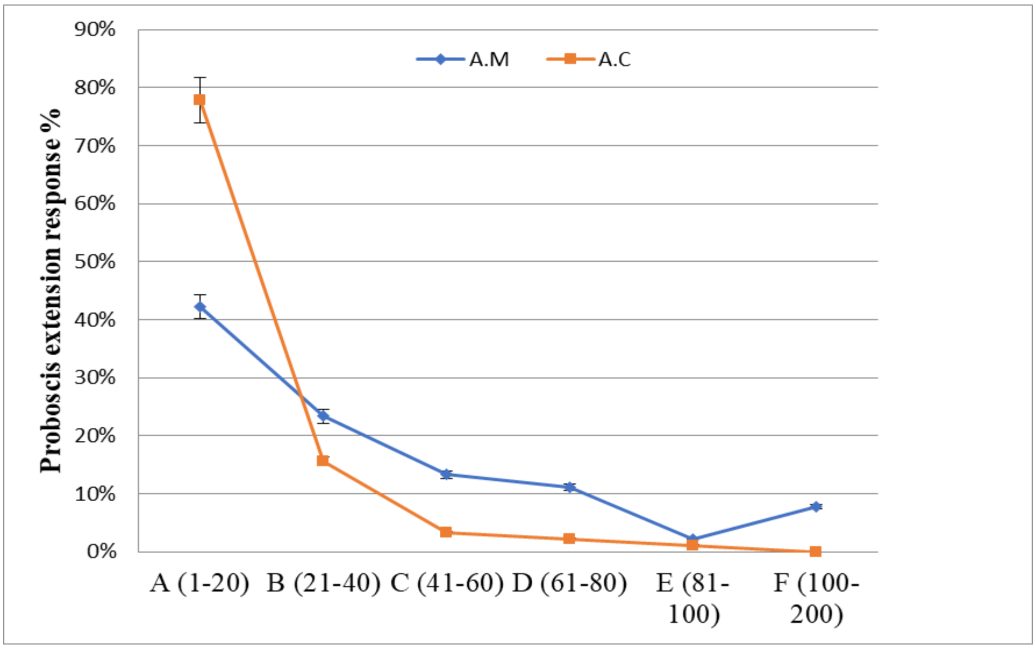

Figure 3. *Percentage of Apis cerana and A. mellifera responding to the appetitive stimulation during trials; (A) Habituation of PER (N=16); (B) Dishabituation of PER (N=10)*

Learning and memory

Apis mellifera showed significantly stronger learning and memory behavior than *A. cerana* after 2 h and 24 h (df=3, F=18.512821 and P<=0.05). The learning and memory behavior of *Apis mellifera* and *Apis cerana* after 2 h and 24 h was 62.22%, 27.28%, 51.11%, and 28%, respectively (*Fig. 4*).

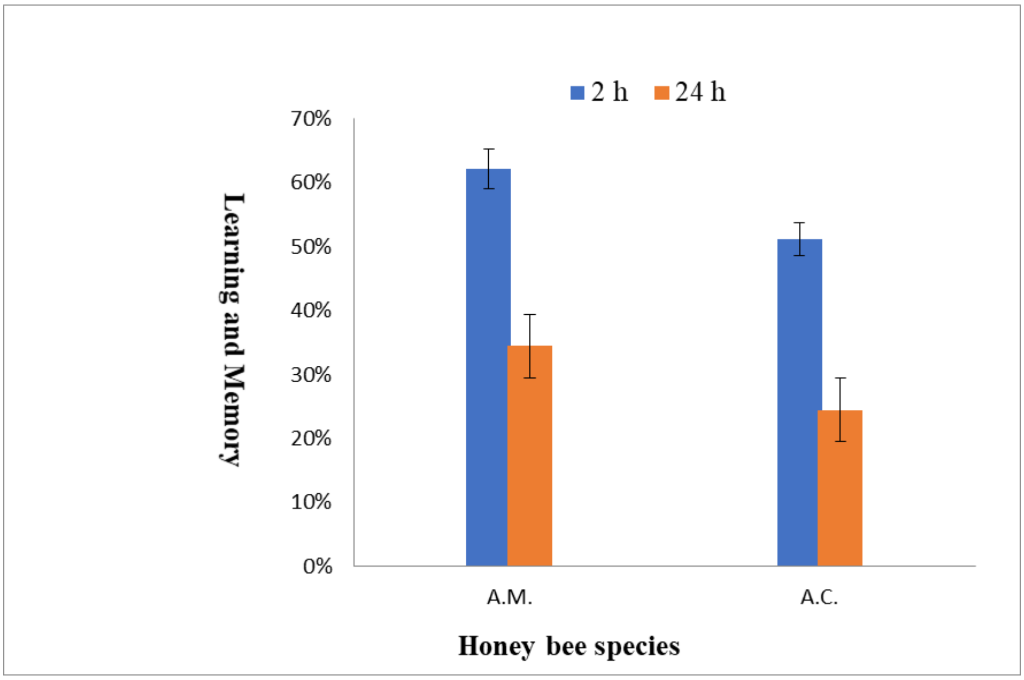

Figure 4. *Percentage of learning and memory (mean ± SE) of Apis mellifera and A. cerana after 2 h and 24 h treatment of 30% sucrose solution. The following data based on three colonies of each species of honeybee. (N = 3 × 2 × 2 0 = 120 bees)*

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Sensitization

The sensitive stimuli of *A. cerana* against lemon extract were more than *A. mellifera* (F=18.512821, df= 2 and P>0.05 NS). The percentage of the sensitivity of *A. cerana* and *A. mellifera* was 29.31% and 4.44% against lemon extract, but *A. mellifera* were more sensitive against apple extract than *A. cerana*. For apple extract, the sensitive responses of *A. mellifera* and *A. cerana* were 11.63% and 2.13% (*Fig. 5*).

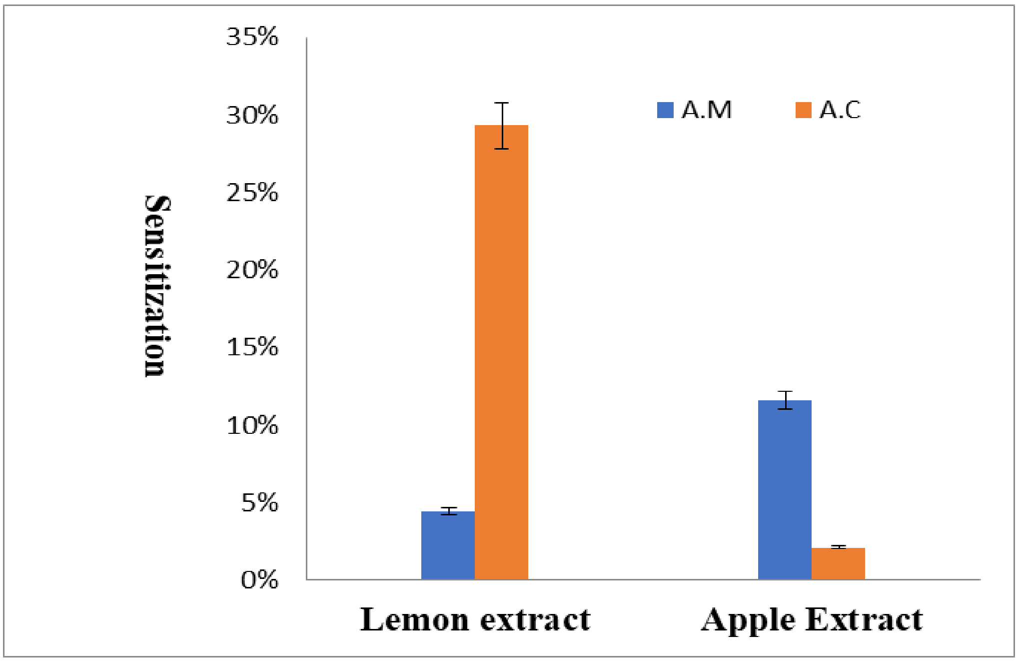

Figure 5. *Percentage of sensitization (mean ± SE) of Apis mellifera and A. cerana for lemon extract and apple extract*

Discussions

Highly responsive bees respond to sucrose solution at concentrations as low as 0.1% or even to water, whereas unresponsive bees only respond to 30% of sucrose or higher sucrose concentrations (Scheiner et al., 2004). This study reported that sucrose responsiveness between two species *A. mellifera* and *A. cerana*. We demonstrated proboscis extension response (PER) percentage of foragers. These two honeybee species showed different response patterns at different sucrose concentrations when sucrose concentration was low then its proboscis extension response is also low. However, the results in this study indicated that *A. mellifera* foragers had significant higher proboscis extension percentage than *A. cerana* foragers at 30% sucrose. Our finding fits well and strongly agreed with previous studies (Scheiner et al., 2004; Yang et al., 2013). *A. mellifera* was more responsive than *A. cerana*. According to previous research work, *A. mellifera* foragers were more responsive to sucrose. Our conclusion showed that *Apis mellifera* has higher sucrose responsiveness than *Apis cerana*. The results of Yang et al. (2013) helped to confirm our conclusion.

Non-associative forms of learning change the behavior of an animal because of the exposure to a stimulus. If the response decreases as a result of repeated stimulation, the animal may have habituated rather than undergone motor fatigue or sensory adaptation (Carew, 1987; Menzel, 1999; Kandel et al., 2000). Habituation signifies the gradually diminish in responsiveness during a continuous series of frequent stimulations. In our

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DOI: http://dx.doi.org/10.15666/aeer/1702_49714984

finding *Apis cerana* took less time as compared to *Apis mellifera*. The proboscis extension reflex is an appetitive component of the bee's feeding behavior that is elicited by touching one antenna with a droplet of sugar water. Repetitive stimulation leads to a decrement and finally to the disappearance of the response. This experiment was designed to analyze the role of individual sucrose responsiveness in non-associative habituation and to study the effect of sucrose concentration used as habituating stimuli on non-associative habituation. A wide range of habituation patterns in Drosophila have been investigated, as well as the proboscis extension response, visual escape jump landing response (Braun and Bicker, 1992). Honeybee foragers must fly many kilometers - even more than 6-10 kilometers away - try to collect nectar and pollen. Therefore, they must learn and remember not only the shape and color of the flowers, as well as how to reach them. This experiment was designed to analyze the role of individual sucrose responsiveness in non-associative habituation and to study the effect of sucrose concentration used as habituating stimuli on non-associative habituation (Abramson, 1997;) Giurfa, 2007; Giurfa and Sandoz, 2012; Matsumoto et al., 2012). Memory was developed in a trial and time dependent way that indicates processes of memory formation that can be transmitted to particular areas of the honeybee brain and cellular and molecular processes. *Apis mellifera* showed significantly stronger learning and memory behavior than *A. cerena* after 2 h and 24 h. The finding of this study demonstrated that the percentage of learning and memory behavior of *Apis melliferra* was significantly higher than *Apis cerana* after 2h and 24h as a resulted by Chen (2001), Qin et al. (2012). This could be due to *Apis mellifera* strain introduced in China has been subjected to intensive artificial selection to be better manageable, while *Apis cerana* bees were managed well in a half wild condition. These two species of honeybees may have distinct responses to harnessing.

The findings of sensitization study clearly determine that the sensitive stimuli of *A. cerena* against lemon extract were more than *A. mellifera*, but *A. mellifera* were more sensitive against apple extract than *A. cerena*. In addition, the results of this study showed significant differences between *Apis Cerana* and *Apis mellifera* in response to lemon and apple extract as revealed by Chen (2001), *Apis cerana* has an excellent sense of smell than *Apis mellifera*. Introduced over 100 years ago, in majority regions of China, *Apis mellifera* is completely developed and adapted to nectar sources and climate. Despite that, to be sure those *Apis mellifera* bees are not adapted pretty well such as *Apis cerana*, whose homeland and region is here Qin et al. (2012).Individual sucrose responsiveness and the sucrose concentration used as reward strongly affect associative PER learning. In previous studies comparative behavioral data of color and grating learning and memory for *Apis cerana* and *Apis melliferra* in China. This study provides the first evidence of the learning and memory difference between *Apis cerana* and *Apis melliferra* under controlled conditions, and it is important for the further study of the mechanisms of learning and memory in honeybees (Giurfa et al., 2001). Yang (2009) described that the niche between *A.cerana* and *A.mellifera* is overlap among *Apis cerana* and *Apis mellifera*; both of these species have their own characteristic to diminish competition in food. *Apis cerana* is able to collect sporadic nectar flowers, while *Apis mellifera* takes advantage of large flower fields. *Apis cerana* meets the significantly lower selectivity pressure to learn how odors and rewards are associated. This may be possible reasons why *Apis cerana* did poorer performance than *Apis mellifera*. After the achievement, there was no distinction in the memory retaining of different odors between these both sibling species. Both species distinguished between experienced scent and un-experienced scent. These findings are consistent with earlier odor learning and odor discrimination studies in bees (Bitterman et al., 1983).We still

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49714984

cannot exclude the possibility that rather unspecific parameters could have also influenced our experiments. For example, the two species may have had distinct abilities to adapt to the confined test conditions or may have required different amounts of food in order to achieve satiation meaning that the reward value of the sucrose solution could be different for each species. *Apis cerana* had lower PER scores than *A. mellifera* in the first three trials of the acquisition phase, suggesting that it was a slow learner. Which may attribute to the reasons that the strain of *A. mellifera* introduced in China had been subjected to intense artificial selection to be more docile. While the *Apis cerana* we utilized were managed in a half-wild situation. Thus, these two bee species could have different reactions to harnessing.

We suggested that *Apis Cerana* could be investigated by applying classic olfactory PER protocol. Moreover, further work is necessary to reveal the physiology of the olfactory learning method based on this cognitive research.

Conclusion and future

The most significant and our overall finding show that *Apis mellifera* foragers are able to store reward information and have better learning and memory versus *Apis cerana cerana*. But these experiments were first and basic step to understanding the learning, memory and behavior of both species. Various experiment and studies need to explore on different aspects of learning and memory for example different age bees, use of different odor, different kind of behavior, level of brain biogenic amines, and protein synthesis and gene expression in different age bees.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49714984

ASSESSING GROUND AND SURFACE WATER SCARCITY INDICES USING GROUND AND SURFACE WATER FOOTPRINTS IN THE TEHRAN PROVINCE OF IRAN

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(Received 1st Dec 2018; accepted 12th Feb 2019)

Abstract. Water consumption is increasing at an alarming rate all over the world. Iran is currently facing high water stress as a consequence of mismanagement. The aim of this study is to evaluate the agricultural ground and surface water footprints in the Tehran province of Iran in order to establish agricultural ground and surface water scarcity indices. Blue and green water footprints were evaluated based on the water footprint method. The volume at which ground and surface water is generated was obtained via fieldwork and GIS (Geographic Information System) data for the first time. In addition, an indicator for evaluating the agricultural ground and surface water scarcity index was established. The total groundwater footprint for the production of crops in Tehran was half of the total water footprint in 2014-2015. The results showed very high agricultural groundwater stress and moderate surface water stress. Groundwater abstraction for the agricultural sector was more unsustainable than surface water. Besides, agricultural ground and surface water scarcity indices are more suitable than existing indicators because they disclose environmental impact of crops production and help stakeholders to assess groundwater and surface water management policies. The agricultural water footprint should be reduced to sustain agricultural water consumption in Tehran.
Keywords: *agricultural water footprint, water management, CROPWAT model, GIS data, water scarcity*

Introduction

Freshwater as an essential resource for humans and the ecosystem should be considered a mandatory requirement for sustainable development (Chenoweth, 2008). Agriculture is the sector with the highest water consumption in the world (Rijsberman, 2006). The use of freshwater resources for agricultural activity contributes to the reduction in water availability for other users (Rijsberman, 2004). Iran is facing severe water scarcity, lack of precipitation, population growth and mismanagement, as well as inefficient water use in the agricultural sector (Madani, 2014). Moreover, per capita water consumption was 7 m^3 in 1956 but later decreased to 2.160 m^3 in 1996, which further dropped to 1.900 m^3 in 2006 (Zehtabian et al., 2010). Agricultural activity is responsible for more than 92% of freshwater consumption (Zehtabian et al., 2010). Moreover, the number of drying lakes and rivers is increasing in Iran. For instance, the Zaiyanderoad and Oromeyeh lakes have dried in recent years (Madani, 2005; Fathian et al., 2014). Besides that, the groundwater level in Iran is dramatically declining as a consequence of decreasing rainfall and water mismanagement (Khaki et al., 2018). The rate of reduction in groundwater level in Iran from 2002 to 2012 was -0.89 cm/year (Khaki et al., 2018). Groundwater in arid climates is mostly used for the irrigation of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

crops (Zektser and Everett, 2004). It is documented that groundwater is identified as a crucial natural resource in most countries due to low probability of pollution, uniform distribution, good quality, and not being affected by evaporation (Zektser and Everett, 2004). Besides that, increasing groundwater demand and inappropriate groundwater management create concerns for groundwater shortages and over-exploitation of this limited water resource (Gleeson et al., 2012). Most water management methods are interlinked with surface water and there is a lack of attention to groundwater management, resulting in an increase in water depletion (Gleeson et al., 2012). Unsustainable groundwater abstraction is evident in many regions (Umar, 2004). The agricultural sector is responsible for more than 70% of freshwater abstraction and 90% of consumptive water use in the world (Fiedler and Zhang, 2009). Thus, it is essential to know the type of water source for irrigation in order to know the anthropogenic impact on water resources (Burke, 2002). Recently, due to the rapid reduction in groundwater level, identifying the type of blue water (the volume of irrigation (Hoekstra et al., 2011)) resource is regarded as an important topic in terms of interest (Siebert et al., 2010). It is claimed that groundwater for irrigation accounts for 43% of total water consumption on the global scale (Siebert et al., 2010).

In the past decades, many studies have investigated water scarcity indicators to evaluate water scarcity and contribute water management methods. The Falkenmark indicator is based on water resources available per person at a national scale. It is described as a ratio of the total water availability for human needs. A water scarcity index (WSI) lower than 500 shows that the area is a water scarcity area (Falkenmark and Lundqvist, 1998). In the Smakhtin water scarcity method (2004), water scarcity is modified via considering environmental water requirement in the water stress formula. WSI equals to the amount of water extraction divided by available water quantity after taking away the environmental water requirement on an annual scale (Smakhtin et al., 2004). Water scarcity is defined as a function of water availability to the population (Rijsberman, 2004). Moreover, physical water stress areas are arid areas with dense populations such as Central Asia, West Asia, and North America, with accessibility to less than 1000 m^3 per capita per year (Rijsberman, 2004). Ma et al. (2015) investigated water stress as a fraction of total water footprint to water availability (Ma et al., 2015). Zeng provided a simple method to evaluate water stresses in different regions. This index is a component of quantity and quality of blue and green water (the volume of rainwater (Hoekstra et al., 2011; Zeng et al., 2013). Raskin index is based on water use per resource (WUPR), which is a suitable index for evaluating environmental impact on aquatic ecosystems (Raskin et al., 1996). Hoekstra evaluated monthly water stress at a global scale. Blue water stress is described as a fraction of blue water footprint to blue water availability after subtracting the environmental flow requirement (Hoekstra et al., 2012). Available water quantity is the amount of water in internal resources plus the amount of entering from outside resources in river basin (Liu et al., 2017). Moreover, Variability of monthly blue water scarcity evaluated at river basin scale by Zhuo et al. (2016, 2019). Green water stress equals to the amount of green water footprint divided by available green water quantity (Veettil and Mishra, 2016). Besides, in the Pfister method, a monthly water stress index is introduced, which is the ratio of water consumption to the modified water availability. This is the square root of the variation factor (Pfister and Bayer, 2014). Xinchun evaluated water stress in the agricultural sector using total water footprint. Water stress in the agricultural sector is found using

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DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

green, blue, and grey water footprints. Water stress is defined as the ratio of total water footprint in the agricultural sector to total water availability (Xinchun et al., 2017).

On top of those methods, different dimensions of water stress indexes should be considered to provide more information about water stress. There is a lack of attention on separately evaluating blue water stress as groundwater and surface water scarcity. In this study, ground and surface water footprints are assessed separately to evaluate surface and groundwater stresses in the agricultural sector. Besides, Groundwater and surface water footprint were evaluated in the water footprint method in one category as blue water footprint, while in this study, these kinds of waters were evaluated separately as groundwater footprint and surface water footprint.

The objective of this study is to evaluate the agricultural ground and surface water footprints in the Tehran province in order to establish agricultural ground and surface water scarcity indices.

This study yields useful information for managing and achieving sustainable ground and surface water consumption. Moreover, When WF was accounted with the water scarcity index, it could produce an effective investigation result for water management and decision makers by providing them with useful knowledge (White, 2015).

Materials and methods

Study area

The Tehran province is situated in the north of the central plateau of Iran with a population of more than 12 million people (approximately 18% of the Iranian population) (Statistical Center of Iran) and situated in 35.724842° N, 51.381653° E. The southern part of the province has a semiarid climate but the northern part experiences a mountain climate. The province covers an area of 18,909 km^2. *Figure 1* shows the average monthly precipitation and average monthly temperature during 2007–2015 obtained from the Mehrabad weather station (IMO, Iran Meteorological Organization). The Jajrud and Karaj rivers are the largest rivers in the Tehran province. Around 60% of water withdrawal in Tehran is abstracted from groundwater including wells, springs and qanats (Ministry of Energy in Tehran).

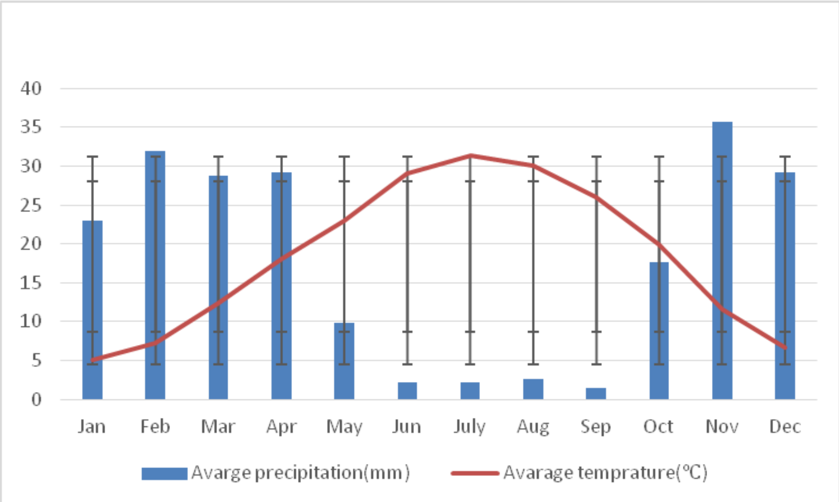

Figure 1. *The plot of average temperature and average precipitation with related standard deviation (2007-2015)*

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Data

Climate data were obtained from the Mehrabad Weather Station (Iran Meteorological Organization, 2018). The data for crop yield were obtained from the Ministry of Agriculture, Jahad, in Tehran (IMAJ, Iran's Ministry of Agriculture Jihad, 2018). Crop parameter data (KC [crop coefficient], day of crop growth, depth of root, critical depletion, yield response, and crop height) were obtained from OPTIWAT (Optimization Water use in agriculture) software (Alizadeh and Kamali, 2009), which is local databases for Iran. The volume of production by ground and surface water were obtained using field work and GIS data (*Fig. 2*) (obtained from the Ministry of Energy in Tehran). The ground and surface water use and availability were obtained from the Ministry of Energy in Tehran (*Fig. 3*).

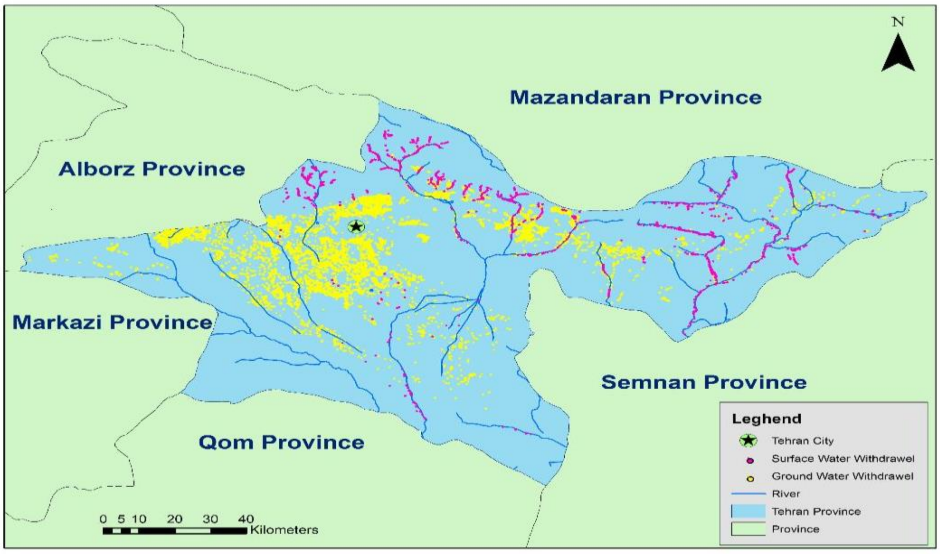

***Figure 2.** Surface and groundwater withdrawal for agricultural sector in Tehran province*

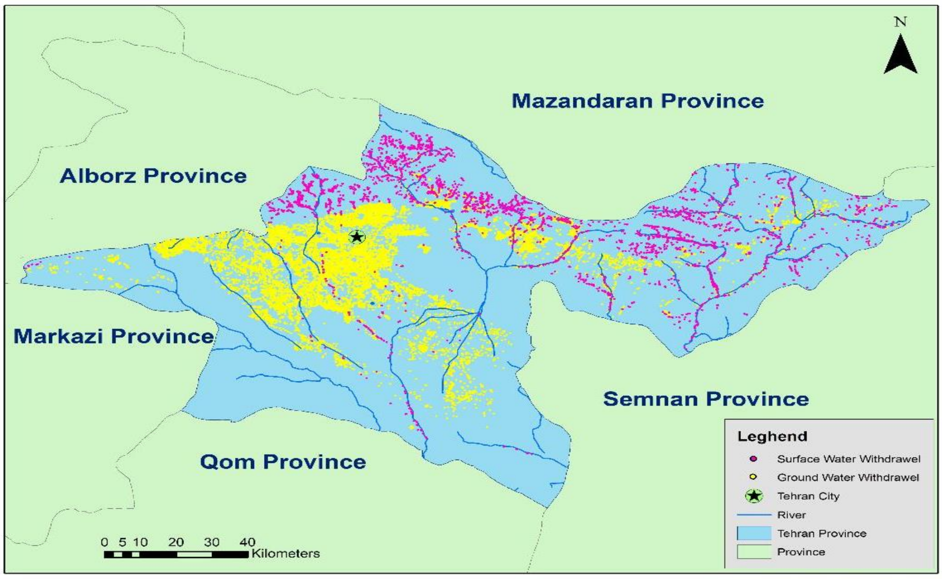

***Figure 3.** Total surface and groundwater withdrawal of Tehran province*

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DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

The primary crops cultivated in Tehran are apple, apricot, cherry, sour cherry, pear, plum, almond, walnut, pistachio, pomegranate, peach, wheat, alfalfa, barley, bean, cotton, maize, tomato, potato, grapes, melon, watermelon, and onion. In this study, the water footprint of these crops was evaluated from 2007 to 2015.

Evaluation of agricultural ground and surface water stress indices

The agricultural ground and surface water stress indices (AWSI ground and AWSI surface) were evaluated separately in this study (*Fig. 4*). The agricultural water stress index is the fraction of water used (WW) to water availability (WA), as per *Equation 1* (Xinchun et al., 2017).

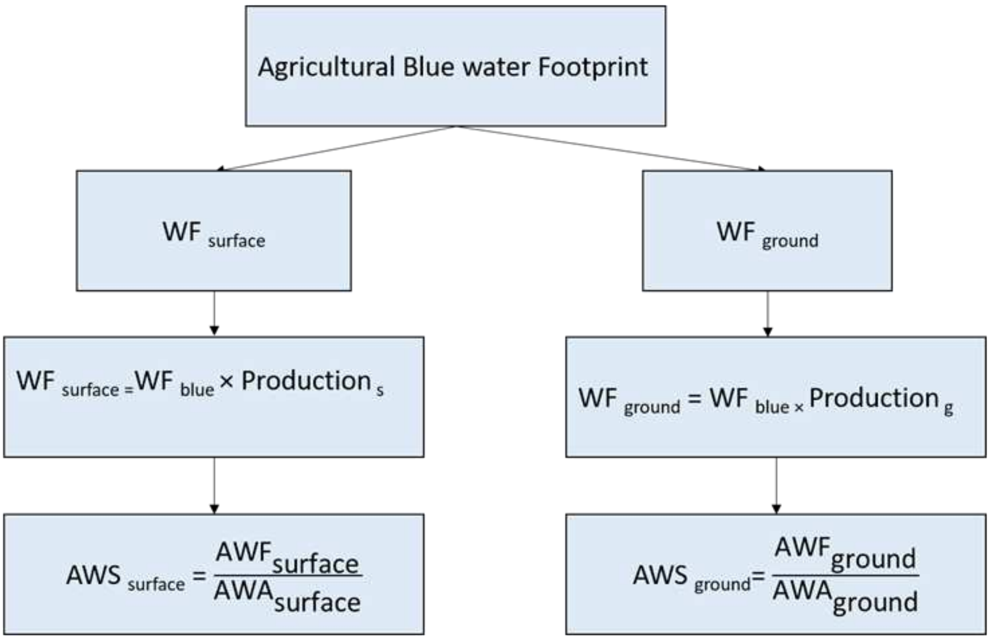

Figure 4. *The accounting framework for computing the ground and surface water stress in agricultural sector. $WF_{surface}$ = surface water footprint, WF_{ground} = groundwater footprint, Production $_g$ = the volume of production by groundwater, Production $_s$ = the volume of production by surface water, $AWS_{surface}$ = Agricultural Surface Water stress, AWS_{ground} = Agricultural Groundwater stress, $AWA_{surface}$ = Agricultural Surface Water Availability, AWA_{ground} = Agricultural Groundwater Availability*

$$WSI = \frac{WW}{WA} \quad \text{(Eq.1)}$$

In this study, AWSI was evaluated as the agricultural groundwater stress index (AWS ground) and agricultural surface water stress index (AWS surface). AWS surface is the fraction of surface water footprint (m^3/year) to the agricultural surface water availability (m^3/year), as per *Equation 2*. Similar consideration can be done for the evaluation of agricultural groundwater stress AWS ground.

$$AWS_{surface} = \frac{AWF_{surface}}{AWA_{surface}} \quad \text{(Eq.2)}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

$$AWS_{ground} = \frac{AWF_{ground}}{AWA_{ground}} \quad \text{(Eq.3)}$$

Water scarcity is ranked as presented in *Table 1.*

Table 1. *Baseline score for assessment of water stress index*

AWSI < 0.1	Low water stress
0.1 < AWSI < 0.5	Moderate
0.5 < AWSI < 0.9	Severe water stress
AWSI > 0.9	Extreme water stress

Evaluation of agricultural blue and green water footprints

The methodology used for the evaluation of water footprint was based on a method by Hoekstra The blue and green water footprints of crop production in the Tehran province was assessed for years 2014 to 2015. The CROPWAT software was used to evaluate crop water requirement and irrigation based on soil, climate and crop data (Allen et al., 1998).

The blue water and green water footprints of different crops were evaluated using *Equations 4* and *5* (Hoekstra et al., 2011).

$$WF_{blue} = \frac{CWU_{blue}}{Yeild} \quad \text{(Eq.4)}$$

$$WF_{green} = \frac{CWU_{green}}{Yeild} \quad \text{(Eq.5)}$$

where, WF_{blue} and CWU_{blue} are the blue water footprint and crop water use (m^3/ha), respectively, yield (ton/ha) (Hoekstra et al., no date). The reference crop evapotranspiration (ET_0) was evaluated based on the Penman-Monteith equation (Allen et al., 1998)

Crop water use was evaluated by multiplying 10 with ET (evapotranspiration) (Hoekstra et al., 2011; *Eqs. 6* and *7*).

$$CWU_{green} = 10\sum_{d}^{lgp} ET_{green} \quad \text{(Eq.6)}$$

$$CWU_{blue} = 10\sum_{d}^{lgp} ET_{blue} \quad \text{(Eq.7)}$$

where, ET_{blue} and ET_{green} were the evapotranspiration of blue water and green water, respectively. The assessment was based on growing time from planting to the harvesting period as a whole. To convert water depth in mm into water per surface area (m^3/ha) a factor 10 was applied (Hoekstra et al., 2011). The calculation of Evapotranspiration was started from the time of planing (d = 1) and continued until the harvesting time (lgp is the days of growing crops) (Hoekstra et al., 2011).

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ET_{blue} and ET_{green} were accounted for using the CROPWAT software (Allen et al., 1998). An assumption was taken with climate data and the crop parameter. Climate data included maximum temperature, sum of precipitation, wind speed, humidity, sunshine, and crop parameter. ETblue was accounted for by subtracting effective rainfall (eff_{rain}) from evapotranspiration (ETc). However, ETgreen was considered as the minimum effective rainfall and evapotranspiration, given by *Equations 8* and *9,* respectively (Hoekstra et al., 2011).

$$ET_{blue} = Eff_{rain} - ETc \qquad \text{(Eq.8)}$$

$$ET_{green} = Minimum(Eff_{rain} \& ETc) \qquad \text{(Eq.9)}$$

Evaluation of groundwater and surface water footprint

Groundwater footprint was calculated by multiplying the blue water footprint (m^3/ton) and the volume of production by groundwater P_{ground} (ton/year), as per *Equation 10*. The same method was used to evaluate the surface water footprint, as per *Equation 11*.

$$WF_{ground} = WF_{blue} \times P_{ground} \qquad \text{(Eq.10)}$$

$$WF_{surface} = WF_{blue} \times P_{surface} \qquad \text{(Eq.11)}$$

Evaluation of production by ground and surface water

The volume of production by ground and surface water was obtained via fieldwork and GIS data (obtained from the Ministry of Energy in Tehran) (*Fig. 2*). First, the volume of crop production in each region was accounted for. Then, GIS data were contributed to know the type of water used (groundwater and surface water) for the agricultural sector and volume of water used. Fieldwork contributed to discovering the volume of production by groundwater and surface water exactly.

Results

Table 2 shows the yield, the volume of production by ground and surface water, ground and surface water footprint, and cultivated area in the Tehran province. The production of wheat consumed was 158, 973,775.35 m^3/year of groundwater and 58,626,083.65 m^3 of surface water. Alfalfa consumed 92,250,581.5 m^3 groundwater and 75,477,748.5 m^3 of surface water. The result illustrates that wheat has the largest groundwater footprint. Alfalfa has the second largest groundwater footprint, followed by barley. Besides that, the groundwater footprint for the production of all crops was bigger than surface water footprint unlike pistachio, which had a somewhat larger surface water footprint (*Table 2*). Besides that, the largest groundwater was related to cereal (wheat, barley, and maize) production because cereal is produced in a large scale. After cereal production, apple consumed the largest groundwater footprint because it is produced on a large scale. Pistachio and cotton with large virtual water (m^3/year) have low groundwater and surface water footprint because the production is at a low scale.

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Table 2. *Crop production (ton), yield (kg/ha), groundwater footprint, surface water footprints, and cultivated area (ha) for all the cultivated crops in Tehran (2014-2015)*

Name of crops	Production by groundwater (ton)	Production by surface water (ton)	Yield (kg/ha)	WF_{ground} (m^3/year)	$WF_{surface}$ (m^3/year)	Cultivated area (ha)
Wheat	128,101	68,977	4,836	15897375.3	58626083.6	40750
Alfalfa	119,805	89,023	16,528	92250581.5	75477748.5	13179
Barley	98,583.5	50,583.46	4,137	66839640	34432541	36104
Bean	457	236	2,492	216855	291885	9
Cotton	2,704	2,814	1,810	53466	104842	11
Maize	916,495	515,529	50,177	56822690	31962798	28539
Tomato	74,880	40,320	47,242	8835840	4757760	2439
Potato	33,085	19,431	28,213	4830410	2836926	1861
Grapes	51,323	24,152	12,500	42341475	19925400	6188
Melon	10,291	11,149	47,120	679206	735834	455
Water melon	14,874	7,663	52,048	892440	459720	433
Onion	23,167	10,108	50,374	857179	385096	667
Apple	271,893	90,631	33,500	55466172	18488520	11101
Apricot	29,632	13,313	15,000	15764224	7082516	3329
Cherry	62,737	20,912	13,400	29047231	9682256	6385.5
Sour cherry	5,580	2,170	10,000	3465180	1347570	805
Pear	15,375	6,590	17,000	9209625	3946811	1395
Plum	11,816	5,064	15,630	5340832	2288928	1099
Almond	266	98	2,326	933660	343980	156.5
Walnut	2,226	954	2,100	10767162	4614498	1641
Pistachio	1,649	1,463	1,200	10397625	13057275	4350
Pomegranate	8,883	3,454	12,499	5827248	2265824	1070
Peach	17,280	6,720	24,000	5028480	1966520	1100

Figure 5 shows that green water footprint contributes the least to the total water footprint in the Tehran province. The production of grapes contributed to the largest green water footprint in comparison with other crops. Besides that, the production of all crops consumed larger groundwater. The production of cotton consumed the lowest groundwater and surface water because it was produced in a low volume.

Groundwater footprint makes up 52% of the total water footprint. This means that the production of crops in Tehran has a groundwater footprint that is one half of the total water footprint. Meanwhile, surface and green water footprints account for 28% and 20% of total water footprint, respectively (*Fig. 6*). The ground and surface water footprints for the production of crops were 585 and 322 million m^3/year, respectively, accounting for 59% and 28%, respectively, of total water consumption in the Tehran province (2014-2015). However, the green water footprint is the lowest contributor to the total water footprint in the Tehran province, accounting for 20% of total water footprint.

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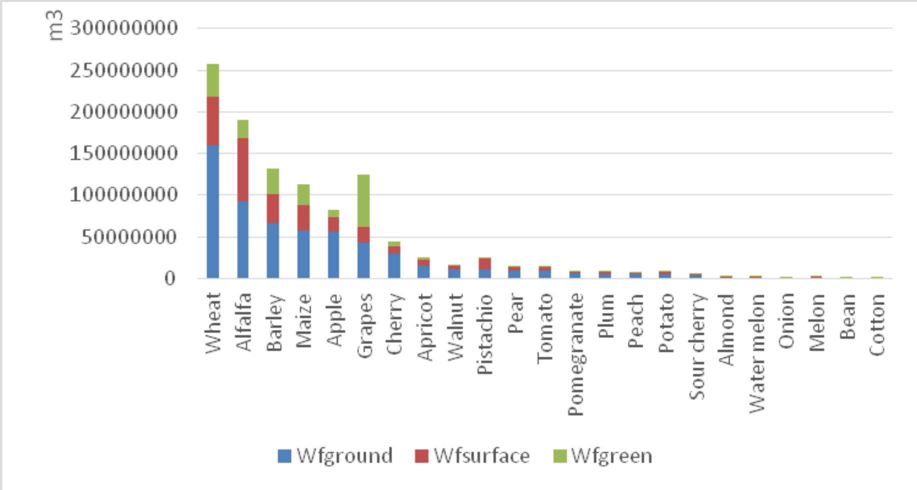

Figure 5. *Ground, surface and green water footprints (cube meter/year) in Tehran province (2014-2015)*

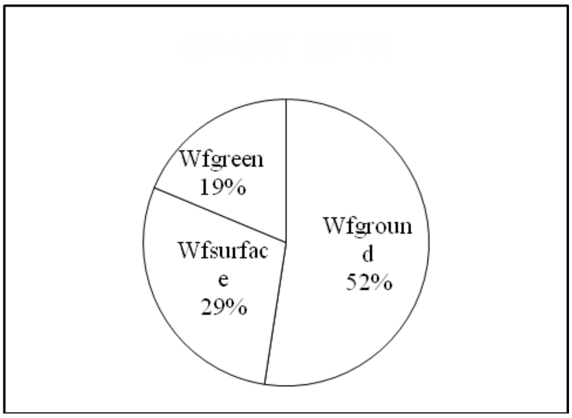

Figure 6. *The proportion of surface, ground, and green water footprints in the total water footprint (2014-2015)*

Ground and surface water scarcity

Ground and surface water scarcity is evaluated based on ground and surface water footprints. The result illustrates that the agricultural sector in the Tehran province is under high water stress. The production of agricultural crops relies on groundwater resources. The agricultural groundwater and surface water stresses accounted for 0.95 and 0.4, respectively. It can be observed, therefore, that groundwater stress is very high. The Tehran province is under moderate surface water stress for the production of crops. Additionally, agricultural water consumption is unsustainable since the production of crops contributes to high groundwater stress and moderate surface water stress.

Discussion

In this study, the agricultural ground and surface water stress indices were established and determined. Besides that, this indictor divided blue water stress into groundwater and surface water stresses. These indices are different from the previous

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water stress indicator, which was based on green and blue water footprints and water availability (Xinchun et al., 2017). Besides, the Falkenmark and Raskin indexes are based on water resources available per person and water use per resource (WUPR), respectively (Falkenmark and Lundqvist, 1998; Raskin et al., 1996). Currently, most water scarcity assessments focus on blue water and total water scarcity indexes (Pfister and Bayer, 2014; Hoekstra et al., 2012; Liu et al., 2017; Zhuo et al., 2019). Other evaluations are based on total water stress (Smakhtin et al., 2004; Ma et al., 2015; Zeng et al., 2013). However, in most methods, there has been a lack of attention on the evaluation of ground and surface water scarcity. In this study, ground and surface water footprints are assessed separately to separately evaluate surface and ground water stresses in the agricultural sector.

The investigations of groundwater and surface water footprints conducted separately as a component of blue water was a difficult process since it was time consuming and laboring. This study used GIS data which contains information about the sources of water consumption (groundwater and surface) and the volume of production in each region to evaluate the surface WF and ground WF. However, in some places, there were both groundwater and surface water, and evaluation was done according to field work and using GIS data.

The green water stress was not evaluated since it did not impact blue water availability (Pfister and Bayer, 2014). However, Savenije believed that it should be accounted for because it can reduce blue water consumption (Savenije, 2000). Evaluation of water footprint at the local scale can be produced more accurately than in the country or global scale (Ababaei and Etedali, 2016).

Having knowledge of groundwater will prevent over-exploitation of limited water resources. Additionally, it also contributes to optimal priority being given to ground and surface water consumption in the agricultural sector (Tillotson et al., 2014). Determining the kind of water resource can determine illegal groundwater withdrawal.

According to this research, the agricultural groundwater consumption in Tehran province is more unsustainable than its surface water's. Besides, Tehran province is under sever groundwater stress, similar to what found by (Karandish et al., 2018). Moreover, some other studies at country scale observed that groundwater consumption in Iran is unsustainable (Gleeson et al., 2012). Besides that, groundwater depletion occurs in Iran (Mekonnen and Hoekstra, 2016). Moreover, Iran as a semiarid region is under high water stress (Wada et al., 2011)

Besides, the result indicates that crop production in Tehran has the largest ground and surface WF compared to other countries, similar to Hoekstra's result who claims that water footprint of Iran is high due to low productivity and high evapotranspiration (Hoekstra and Chapagain, 2007; Fader et al., 2011). Moreover, wheat is produced on a large scale. In other words, the production of wheat should be reduced on a large scale since the production of wheat has consumed a large ground and surface water footprint. Furthermore, arid climates have larger water usage than humid regions (Ibidhi and Ben, 2018). Besides that, since the production of all the crops is highly reliant on ground and surface water, it will be beneficial to reduce the production of these crops inside Tehran.

Decision makers should increase water saving by importing water intensive crops such as wheat since Tehran province is under severe agricultural groundwater scarcity. Virtual water trade plays an important role in mitigating water shortages in water-stressed areas (Qian et al., 2018).

However, the production of grapes is consumed more in green water. It is recommended that the production of grapes be increased, which has lower ground and surface water footprint. Besides that, the volume of cereal production (wheat, barely, and maize) should be reduced since the production of these three crops consumed 282,636,105.5 and 125021423.53 groundwater and surface water respectively.

Conclusion

Tehran's total water footprint (excluding grey water footprint) in the agricultural sector was 1,115,962,823.8 m^3/year (2014-2015). Groundwater footprint was 585,041,121.97 m^3/year, accounting for 52% of total water footprint. Surface water footprint was 321,943,927.03 m^3/year, accounting for 29% of total water footprint. Green water footprint was 208,977,774.8 m^3/year, accounting for 19% of total water footprint. Groundwater consumption contributed the largest share of blue water footprint in the Tehran province. Additionally, more than half of the total water consumption in Tehran was due to the groundwater footprint. Groundwater resources include wells, qanat and springs. Groundwater consumption has led to water scarcity in many parts of the province. Green water footprint contributes to the lowest share of total water footprint. The production of crops in Tehran is relatively reliant on limited groundwater resources. Therefore, the environmental impacts of blue water footprint cannot be overlooked. Thus, it is essential to establish an indicator for blue water scarcity based on ground and surface water stresses at the regional scale. This indicator reveals information about the status of ground and surface water resources at the provincial scale. Moreover, the result of this research provides beneficial information for decision makers in the water sector to help alleviate pressure on ground and surface water consumption. For instance, policy makers can prioritize water consumption to reduce water scarcity.

The productions of wheat, alfalfa, and barley have the largest groundwater footprint because these crops are produced at a large scale. Moreover, agricultural groundwater stress in the Tehran province is very high, which is 0.95. Agricultural surface water stress was only at a moderate level, which was 0.4. Besides that, the production of agricultural crops in the Tehran province is unsustainable. Agricultural water footprint should be reduced in order to achieve sustainable agricultural water consumption in the Tehran province. It is suggested that future water stress assessments evaluate the environmental impact of ground and surface water consumption separately using the life cycle impact assessment method. Despite Tehran being under severe groundwater stress, some agricultural crops produced extra and are imported to other regions (apple and barley) (excluded from this paper) that are produced in a large scale. The decision maker should reduce the production of cereal, which consumes a large volume of groundwater and surface water footprint. Besides that, the production of apple and barley for exporting purposes should be reduced. Water management should relocate crop production in Tehran. The production of grapes can be increased while the production of cereal should be reduced. Besides that, agricultural ground and surface water scarcity indices are more suitable than existing indicators because they disclose environmental impact of crops production and help stakeholders to assess groundwater and surface water management policies.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

Acknowledgements. The authors would like to gratefully acknowledge Universiti Putra Malaysia (GP/2018/9592300) for supporting this research project.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49854997

FLORISTIC COMPOSITION OF BLACK PINE FORESTS ON SERPENTINITE IN THE TERRITORY OF SERBIA AND BOSNIA AND HERZEGOVINA (B&H)

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(Received 10th Dec 2018; accepted 27th Feb 2019)

Abstract. Black pine forests of the Western Balkans constitute a significant complex of azonally and orographically-edaphically conditioned forests, on the ophiolite massifs of central and eastern Bosnia, as well as western and, to a certain extent, central Serbia. CA and Cluster analysis showed that there was no overlap in the floristic composition, which means that there are significant differences between the studied stands. Stands in Bosnia and Herzegovina (B&H) are floristically richer with 152 taxa recorded, while stands in Serbia had 82 taxa recorded. On the territory of B&H, communities grow under the conditions of maritime and humid climate; while in Serbia they grow in continental climate with less rainfall and significant exposure to sub-Mediterranean weather. In black pine forests in B&H two types of soil were described: eutric humus-siliceous, and brown soil on serpentinite; while in Serbia, there was only one type - eutric humus-siliceous. No significant differences were found in the spectrum of life forms. In the spectrum of floral elements, mesophilic floral elements (Central and Sub-Atlantic) were dominant on the territory of B&H; while stands in Serbia, were richer in xerophilous floral elements (Mediterranean, Balkan, Balkan-Apennine). Shannon-Wiener index and evenness index showed higher values in B&H.
Keywords: *Balkan, Pinus nigra, forest plant communities, floristic similarity, phytosociology*

Introduction

Area of Southern Europe, Iberian, Apennine and the Balkan Peninsula, along with the Crimea, Anatolia and the Caucasus mountains, is generally accepted as a refuge area, within which many tree species managed to survive during the glacial and post-glacial periods (Hewitt, 1999; Jasińska et al., 2014). Balkan Peninsula was one of the most significant refuge areas in Europe, because it had a warmer climate due to its geographical position, thus the glaciation was not fully expressed. This especially pertains to the western parts of the peninsula, where temperature changes during the glaciation were relatively small (Bennett et al., 1991). The plant cover of serpentinite is

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49995010

a segment of flora and vegetation of the Balkan Peninsula extremely rich in species, many of which are endemic. Serpentinite has a high percentage of Mg (18-24%), Fe (6-9%) and heavy metals (Ni, Co, Cr, Mn), but a low content of Ca (1-4%), and Al (1-2%) (Alexander, 2004). Soils formed on ultramafic rock, which includes serpentinite, can contain several hundred times more nickel (Ni), an element toxic to plants (Altinözlü et al., 2012), than other soils. Soils on serpentinites have unfavorable chemical composition, are shallow, rocky, often formed on steep slopes, so they are unsuitable for most plants and represent a stressful environment for their growth (Kazakou et al., 2008).

On the Balkan Peninsula, on serpentine bedrock, population of black pine occupies a significant area. Black pine (*Pinus nigra* Arn.) belongs to the group of Mediterranean pines, has a disjunct range and is present in North Africa, western Europe (Spain), running across the northern Mediterranean and to the Black Sea on the east (Afzal-Rafii and Dodd, 2007; Rubio-Moraga et al., 2012). Black pine is a typical South European forest tree species that is ecologically and economically important in a very wide distribution area (Gülsoy and Çinaŗ, 2019). Black pine forests in the area of the Balkan Peninsula occupy different geological substrates. They represent a significant complex of azonallly and orographically-edaphically conditioned forests on the ophiolite massifs of central and eastern Bosnia and western and central Serbia. Many authors consider this forest a relic due to a dominant role of black pine (*Pinus nigra* subsp. *gocensis* Vid.), which is, along with disjunctive areas of some subspecies, a typical tertiary relic (Tatić and Tomić, 2006). Black pine forests on the serpentines are highly dynamical systems, and their development goes in two directions: towards sessile forests at low altitudes, and towards the beech-fir forests at higher altitudes (Jović and Tomić, 1985). The aim of the paper was to broaden the knowledge on the black pine forests on serpentine bedrock and to compare the floristic data in order to determine the differences between the studied forests in the territory of Serbia and B&H.

Material and methods

Study area

Research included natural stands of black pine on serpentine bedrock in Serbia and B&H (*Fig. 1*). *Figure 2* illustrate the habitat of studied stands. Black pine stands in the area of the B&H are described in three locations (*Table 1*): Dobojsko-Derventski (DD) and Usorsko-Ukrinski (UU), which belong to the Central Bosnian ophiolite range, as well as in the area of Višegrad (V), which is located in eastern B&H. The average value of precipitation in Višegrad, at 416 m a.s.l. is 738 mm, and in Doboj, at 147 m a.s.l. it is 972 mm.

Black pine stands in Serbia are described in Pešter (P), in southwestern Serbia, as well as on Kopaonik (K), which is located in central Serbia. On Pešter, at 1038 m a.s.l, the average value of precipitation is 712 mm, and on Kopaonik, at 1097 m a.s.l, it is 885 mm.

Field work and data analysis

The study of floristic composition of the investigated stands was based on a total of 28 phytocoenological relevés. They were made by the classical method of Zurich-Montpellier school of phytosociology (Braun-Blanquet, 1964) and they are taken from

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literature. 15 relevés (Novaković-Vuković, 2015) were sampled in the black pine forest on serpentinites of Pešter (P) and Kopaonik (K) - Serbia, and 13 relevés, also in the black pine forest, were recorded on serpentines of Višegrad (V), Doboj-Derventa (DD) and Usorsko-Ukrinska (UU) area - B&H (Blagojević, 2016). Field research was carried out in the period from 2009 to 2011 in Serbia and in the period from 2013 to 2015 in B&H. Syntaxonomic names were given according to Tomić and Rakonjac (2013). Spectra of floral elements were performed based on the systematization of phyto-geographical elements (Gajić, 1980); floral elements that were not found in this source were supplemented according to Stevanović (1992) and Oberdorfer (2001). The spectrum of life forms, which is based on the division of Raunkiaer (1934), was determined according to Kojić et al. (1997), and partially according to Mueller-Dombois and Ellenberg (1974).

Figure 1. Location of studied stands in Serbia and B&H. (Abbreviations for locations: DD - Dobojsko-Derventski, UU - Usorsko-Ukrinski, V - Višegrad - B&H; P - Pešter, K - Kopaonik – Serbia)

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a b

***Figure 2.** The habitat of Black pine: a) Serbia, Kopaonik (K) and b) B&H, Dobojsko-Derventsko (DD)*

***Table 1.** GPS coordinates of phytocoenological relevés*

Relevé number	Gauss-Kruger X	Gauss-Kruger Y
K_1	7490309	4781855
K_2	7490091	4782456
K_3	7490069	4782599
K_4	7489989	4782850
K_5	7489926	4782856
P_1	7408023	4783187
P_2	7407951	4783197
P_3	7407965	4783214
P_4	7407934	4783296
P_5	7407902	4783284
P_6	7407909	4783305
P_7	7407930	4783331
P_8	7407947	4783259
P_9	7407963	4783249
P_10	7408040	4783140
V_1	6599825	4857465
V_2	6604813	4856663
V_3	6600031	4858201
DD_1	6519554	4945626
DD_2	6519753	4945105
DD_3	6518250	4946100
UU_1	6477630	4935730
UU_2	6477770	4935723
DD_4	6517036	4945859
DD_5	6519300	4945550
UU_3	6477203	4936263
V_4	6599073	4857122

Abbreviations for locations: DD - Dobojsko-Derventski, UU - Usorsko-Ukrinski, V - Višegrad - B&H; P - Pešter, K - Kopaonik – Serbia

In order to determine the similarity in the floristic composition between the studied stands, Bray-Curtis cluster analysis (measuring similarity) with the option "Group Average" in BIODIVERSITY PRO (McAleece et al., 1997) was performed. DCA vegetation data analysis was performed using the statistical software CANOCO 4.5 (Lepš and Šmilauer, 1999). The cover-abundance score obtained for each species within a relevé was transformed using the method of Van Der Maarel (Van Der Maarel, 1979). Indices of diversity and evenness (Shannon-Wiener diversity index and Evenness) were calculated using the program JUICE 7.0.166 (Tichý, 2002).

Shannon-Wiener diversity index is a mathematical measure of species diversity in a community. It is derived from a formula:

$$H' = -\sum pi \ln pi \quad pi = ni / N,$$

where "N" represents total species count, and "*ni*" ground coverage of *i*-species.

Species evenness shows how close in numerical values each species in an environment is. Species evenness index is calculated using the formula (Pielou, 1975):

$$EH = H' / H'max = H'/\ln N,$$

where "N" is number of species (taxa) in a phitocenological sample.

E_H ranges between 0 and 1. Lower evenness between species in a community (and presence of dominant species) will result in lower E_H letter.

Results

The black pine stands in the Višegrad, Dobojsko-Derventska and Usorsko-Ukrinska area (B&H) were recorded at the altitudes ranging between 293 and 590 m and at all aspects, but preferably at warmer ones (S, SW). The community grows on slopes of varying steepness; it has been recorded on flat ground or on a very mild slope (2°), but the majority of stands have been recorded on very steep slopes (25-33°). At all three sites, stands of black pine were situated on two types of soil: eutric humus siliceous and brown soil on serpentinite. There were 152 taxa recorded in 13 phytocoenological relevés. For a complete inventory see *Appendix.*

Stands on Pešter and Kopaonik (SRB) were located at the altitude of between 900 and 1219 m, where stands on the Kopaoniku are located at altitudes between 900 and 963 m, while stands on Pešter occupy narrow range of altitudes between 1180 and 1219 m. Stands of black pine on Kopaonik occupy mainly western aspects (NW, SW), while stands on Pešter occupy various aspects, but more often cooler ones (N, E, NE). Common to both sites is that slopes are steep to very steep (20-35°). There were 82 taxa recorded in 15 phytocoenological relevés. Black pine stands in Serbia were recorded on one type of soil, eutric humus siliceous, provided that on Kopaonik it is medium skeletal eutric humus siliceous on serpentinite, while on Pešter it is colluvial eutric humus siliceous soil.

High floristic similarity of studied stands is visible on the graph (*Fig. 3*), which was expected because in both cases the pine forests grow on serpentine bedrock. However, it is notable that studied stands make two separate groups: relevés from Serbia are concentrated along the y-axis, relevés from B&H show greater dispersion along the x-axis. The graph shows following mutual species as isolated: *Pinus nigra,* cinquefoil

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(*Potentilla heptaphylla*), deutscher backenklee (*Dorycnium germanicum*), wood false brome (*Brachypodium sylvaticum*). Within relevés from Serbia, xerophilous species of the *Erico-Pinetalia* order: burnet rose (*Rosa spinosissima*), ironwort (*Stachys scardica*), Balkan daphne (*Daphne blagayana*), rabbit tail (*Sesleria serbica*), *Pinus nigra,* owl-head clover (*Trifolium alpestre*), etc. appear as isolated. Within relevés from B&H, species of wide ecological amplitude: common bracken (*Pteridium aquilinum*), *Festuca rupicola,* bird's-foot trefoil (*Lotus corniculatus*), wild strawberry (*Fragaria vesca*), mountain melick (*Melica nutans*) etc. appear as isolated, as well as some mesophilous species: tuberous comfrey (*Symphytum tuberosum*), sessile oak (*Quercus petraea*), blackberry (*Rubus hirtus*). The presence of mesophilous species and species of wider ecological amplitude in the black pine forests in the area of B&H indicates more mesophilous conditions within these forests in the Illyrian province. It should be noted that beech in Serbia is described as *Fagus moesiaca*, whereas in B&H it is described as *Fagus sylvatica.* Sessile oak is also treated dually, in Serbia as Balkan oak (*Quercus dalechampii*), in B&H as *Quercus petraea*, which slightly affected the arrangement of phytocoenological relevés in the coordinate system.

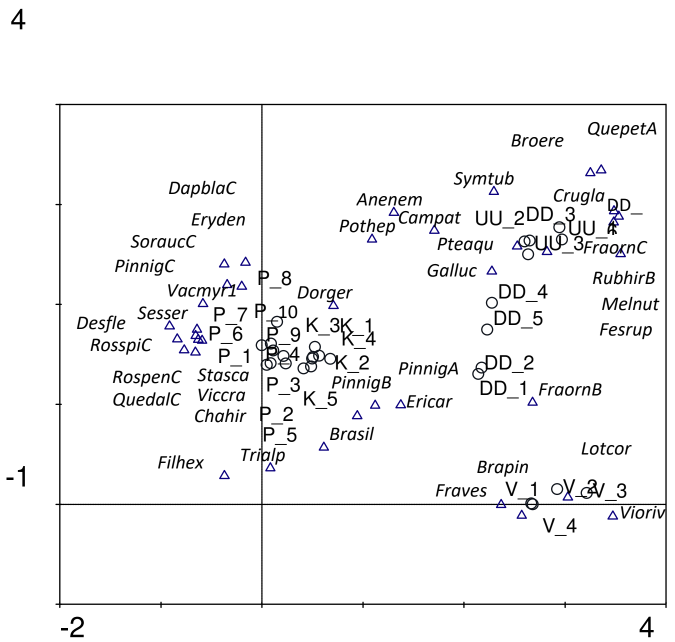

Figure 3. *DCA ordination bi-plot, fit range for species 30-100%, 38 species (○ - relevé representation, Δ - species representation). (Abbreviations for locations: DD - Dobojsko-Derventski, UU - Usorsko-Ukrinski, V - Višegrad - B&H; P - Pešter, K - Kopaonik – Serbia. Abbreviations for species: Quepet-Quercus petraea; Broere-Bromus erectus; Symtub-Symphytum tuberosum; Dapbla-Daphne blagayana; Anenem-Anemone nemorosa; Crugla-Cruciata glabra; Campat-Campanula patula; Eryden-Erythronium dens canis; Pothep-Potentilla heptaphylla; Sorauc-Sorbus aucuparia; Pteaqu-Pteridium aquilinum; Fraorn-Fraxinus ornus; Pinnig-Pinus nigra; Vacmyr-Vaccinium myrtillus; Dorger-Dorycnium germanicum; Rubhir-Rubus hirtus; Sesser-Sesleria serbica; Melnut-Melica nutans; Fesrup-Festuca rupicola; Desfle-Deschampsia flexuosa; Rosspi-Rosa spinosissima; Rospen-Rosa pendulina; Stasca-Stachys scardica; Quedal-Quercus dalechampii; Viccra-Vicia cracca; Chahir-Chamaecytisus hirsutus; Filhex-Filipendula hexapetalla; Triapl-Trifolium alpeste; Ericar-Erica carnea; Brasil-Brachypodium silvaticum; Brapin-Brachypodium pinnatum; Lotcor-Lotus corniculatus; Fraves-Fragaria vesca; Vioriv-Viola riviniana. The abbreviation following the species denotes A - tree layer, B - shrub layer, C - ground flora layer)*

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Cluster analysis (*Fig. 4*) also showed that studied stands compose two separate groups: one group includes phytocoenological relevés from Serbia and the other group includes phytocoenological relevés from B&H. There is no overlapping between the studied stands of two countries, which suggests that there is clear floristic differentiation between them.

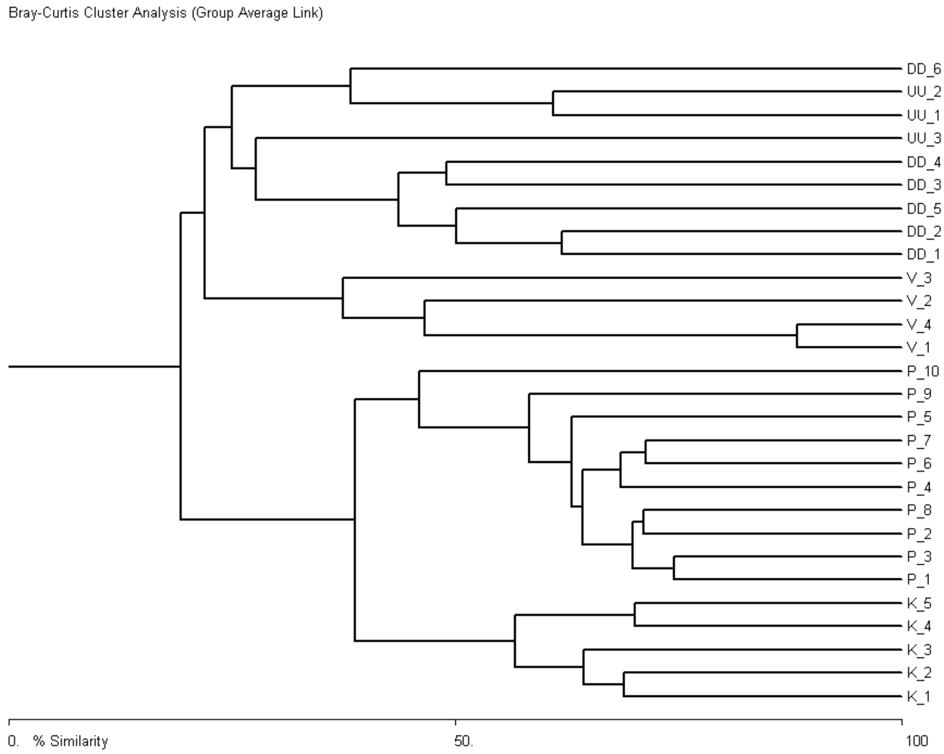

Figure 4. *Cluster analysis of studied stands of black pine in Serbia and B&H. (Abbreviations for locations: DD - Dobojsko-Derventski, UU - Usorsko-Ukrinski, V - Višegrad - B&H; P - Pešter, K - Kopaonik – Serbia)*

In the spectrum of life forms no significant differences between two countries (*Table 2*) were observed. Hemicryptophytes are a dominant life form in both Serbia and B&H, with nearly equal representation. Phanerophytes are slightly more numerous in Serbia than in B&H (22% to 15.8%), while chamaephytes are more common in B&H than in Serbia (15.7% to 12.2%). Other life forms are almost evenly represented.

Table 2. *Spectrum of life forms in the black pine forest in Serbia and B&H*

Life form	Serbia	B&H
Phanerophytes	22%	15.80%
Chamaephytes	12.20%	15.70%
Hemicryptophytes	50%	53.30%
Geophytes	12.20%	11.20%
Therophytes	1.20%	0.70%
Lianas	0.00%	0.70%
Therophytes/chamaephytes	2%	2.60%

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In the spectrum of floral elements (*Fig. 5*) more significant differences were observed, in comparison to the spectrum of life forms. In the spectrum of floral elements, the collective group of Central-European floral elements is dominant in both countires, with larger abundance in B&H than in Serbia (27%: 22%). A high presence of this group of floral elements indicates syndinamic connection between xerophilous black pine forest with more mesophilous sessile oak and beech forest. It is followed by a group of species of broad ecological amplitude of the Eurasian distribution type, with equal presence in both countries. If more xerophilous floral elements are observed separately (Sub-Mediterranean, Balkan, Balkan-Apeninian), it is noted that their presence is significantly higher in Serbia than on the territory of B&H (24.5% to 19%). The largest presence of sub-Mediterranean floral element is on Kopaonik (Serbia), as a result of exposure of this mountain massif to sub-Mediterranean. On the other hand, the mesophilous floral elements (Central-European and Subatlantic) are more numerous in B&H than in Serbia (31.6% to 24.4%). Greater presence of mesophilous floral elements in B&H and xerophilous in Serbia is a consequence of, first of all, different climatic influences - higher humidity in Illyrian province, to which study stands in the B&H belong.

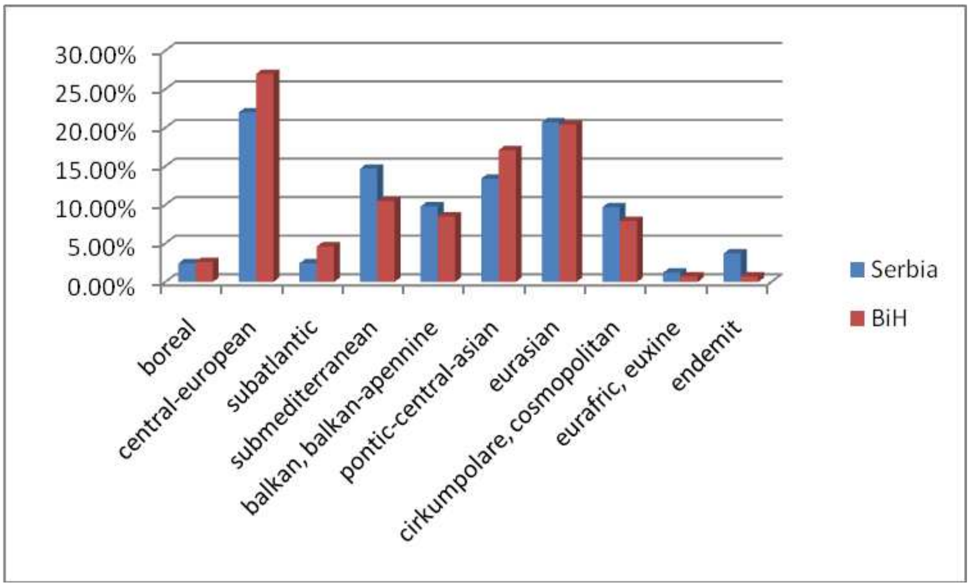

Figure 5. *The spectrum of floral elements in the black pine forest in Serbia and B&H*

For diversity analysis (*Table 3*) Shannon-Wiener diversity index of ground flora was used, which is sensitive to rare species (Heuserr, 1998). Diversity index (Shannon-Wiener index) shows higher value in B&H than in Serbia, which was expected, because the black pine stands in B&H have more species per phytocoenological relevé. Evenness index also shows higher value in B&H, where the spatial distribution of species is more regular than in Serbia, given that the index value approaches the number 1. Species winter heath (*Erica carnea*), *Brachypodium silvaticum,* bilberry (*Vaccinium myrtillus*)*, Sesleria serbica*, may be denoted as species that reduce the Evenness index value in Serbia, considering their number and coverage; while in B&H these are *Erica carnea,* heath false brome (*Brachypodium pinnatum*)*, Pteridium aquilinum,* erect brome (*Bromus erectus*)*,* etc.

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***Table 3.** Indices of diversity and evenness in researched stands*

Locality	Average Shannon-Wiener index	Average Evenness index
Serbia	2.35	0.64
B&H	2.57	0.74

Discussion

Black pine is one of the main important tree species in the Mediterranean region, but, this species is also tolerant to cold temperatures and can occur at higher elevations of up to 1800 m a.s.l. (Kara and Topacoglu, 2018). Although the observed stands in Serbia and B&H are located at different elevations, distribution of pine forest types is not primarily determined by elevation, but rather by relief, exposure, slope, character of the soil, as well as exposure to the winds (Pavlović, 1951). Since black pine stands on the territory of B&H belong to the Illyrian province, and stands in Serbia to Moesian and transitional Illyrian-Moesian province, significant differences in floristic composition are observed between them. Illyrian province is characterized by significantly higher amount of precipitation, compared to Moesian province, so ti is considered more humid. On the other hand, Moesian province climate is more continental: average temperature differences between summer and winter are more extreme, yearly amounts of precipitate are lower, and summer heats are followed by the lowest amounts of water residues. Generally speaking, Moesian vegetation, compared to Illyric, is much more xero-termophillic, and notably more resistant to high summer temperatures and drought (Jankovic, 1984). Of course, besides the macroclimate, microclimate also exhibits impact on vegetation. The elements of microclimate are interdependent, so that the changes in one of the elements affect other elements. The intensity of the trend of these changes depend on the type of forest, its structure, geographical location, canopy closure and other features (Babić et al., 2015).

The main characteristic of black pine forests on serpentine is presence of winter heath (*Erica carnea*), which the whole class of pine forests is named after (*Erico-Pinetea* Ht. 1959). *Erica carnea* has Illyrian geographical distribution, so the climate of Kopaonik (Serbia) does not suit it, hence this species was recorded in low numbers and coverage in this locality. In the area of B&H in 4 phytocoenological relevés typical Illyrian species alpine barrenwort (*Epimedium alpinum*) was recorded, with its presence decreasing going from the west towards the east, so it was not recorded in relevés in Serbia. Regular satelite of pine forests of Kopaonik (Serbia) is prickly juniper (*Juniperus oxycedrus*), representative of sub-Mediterranean flora, which was not recorded in stands of B&H, as another evidence of xerophillity of studied stands. Although these pine forests are on ultrabasic bedrock, in the floristic composition, two noticably acidophilous species stand out with their abundance and coverage: *Vaccinium myrtillus* in Serbia and common heather (*Calluna vulgaris*) in B&H. This phenomenon is a result of bases from the soil having been washed out due to the high amount of precipitation that led to acidification. This applies in particular to the species *Calluna vulgaris*, which belongs to the Atlantic areal type and its presence is, as expected, greatest in western and northern Europe, and decreases going towards the east. In the area of ex Yugoslavia, this species has the largest presence in Slovenia and Croatia, somewhat less in the B&H, and it is recorded at only four locations in Serbia (Cvjetićanin et al., 2014). Serpentine bedrock has low number of species compared to

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limestone (Ritter-Studnička, 1963; Brković et al., 2015), but is rich in endemic and relic species. Following species were recorded in studied stands as relic: *Pinus nigra,* European hop-hornbeam (*Ostrya carpinifolia*), mahaleb cherry (*Cerasus mahaleb*), *Cardamine glauca* and spurge (*Euphorbia glabriflora*). According to Stevanović et al. (2003), following endemics within studied forests of black pine in Serbia were recorded: *Allysum markgrafii, Sesleria serbica, Crocus veluchensis, Stachys scardica, Euphorbia glabriflora* and *Melampyrum hoermannianum*; while on the territory of B&H *Thymus jankae* is present as an endemic species, and *Scabiosa leucophylla* as a sub-endemic species. Black pine stands in Serbia are poorer in floristic composition in comparison to stands in B&H. The explanation might be that in terms of the continental climate on Pešter (Serbia), where winters are long and cold and vegetation period is short, only those species within the black pine forest that had adapted to extreme temperature conditions have survived. On Kopaonik (Serbia) there is a strong influence of the Mediterranean, so stands of black pine contain largest number of sub-Mediterranean species and chamaephytes, because these species are able to withstand the harsh life conditions. Clyster analysis of beech forests of SE Europe also showed that there was a significant increase in the proportion of Stenomediterranean, Eurymediterranean, Mediterranean-Montane, and Eurasian species, while Boreal species, decreased toward the southeast (Marinšek et al., 2013). Ritter-Studnička (1963) came to conclusions about the presence of individual life forms studying the serpentine complex in Bosnia, where decreasing presence of chamaephytes with increasing mesophilous conditions is observed, while geophytes are equally present on both bare substrate and in mesophilic conditions. Slopes are generally steeper than at other sites, soils are more skeletal and shallower, exposures are warmer, mainly west and southwest, all of which have an effect on poorer floristic composition. On the other hand, stands of black pine in B&H grow in more humid conditions, have developed soils, mild slopes, lower altitudes, and therefore living conditions are more favorable. The study of transition from warm to mesic forest vegetation showed that the model based on all variables has the best explanatory power and it is followed by models based on groups of variables, as: ecology, chorotypes, geomorphology, structure and function and soil properties (Čarni et al., 2016).

Conclusion

Comparison of floristic characteristics of Black pine forests on the territories of Serbia and B&H was conducted. Stands in B&H are floristically richer with 152 taxa recorded, while stands in Serbia had 82 taxa recorded. In the spectrum of floral elements, mesophilic floral elements (Central and Sub-Atlantic) were dominant on the territory of B&H, while stands in Serbia were richer in xerophilous floral elements (Mediterranean, Balkan, Balkan-Apennine). Results indicate the need for further research of flora and vegetation of the aforementioned localities. Multidisciplinary research, especially monitoring microclimate characteristics of these territories, could provide explanation for the distribution of certain plant species, as well as forest phytocenoses of researched territories.

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APPENDIX

Electronic Appendix 1. Phytocoenological table of the community of Black pine

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_49995010

A FUZZY LOGIC MODEL FOR ZONE DELINEATION IN A PRESERVATION AREA IN BRAZIL

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(Received 14th Dec 2018; accepted 27th Feb 2019)

Abstract. Territorial planning plays a fundamental role in preservation of areas for ecological conservation and environmental management as well as in providing adequate spaces for visitation and research and prioritizing sustainable relationships between man and nature. The present work exhibits a methodological proposal that allows the standardization of territorial classifications in preservation areas. Based on fuzzy logic, the proposed model observes the particularities of a preservation area; hence, the decision generated in the classification of zones represents the work that is currently being developed in the areas of biology, geography, environmental engineering, among other research groups. The modeling combines the evaluation of the data collected in the field (variables: quantitative and qualitative) with vegetation indices obtained from satellite imagery. Combining this information enables each sampling point to be categorized, thereby generating preservation area zones. The results showed that the methodology can distinguish border areas as transition zones, allowing appropriate management to offer detailed information of the study area. The case study, in which the methodology was applied in a preservation area in southern Brazil, showed that the methodology assists in territorial planning, with a clear indication of what actions have been performed and simple data collection.
Keywords: *environmental preservation, southern Brazil, standardized classification, territorial planning, zone of transition*

Introduction

The recent decades have seen a growing discussion by political authorities and scholars regarding the need to delimit natural areas for conservation and increased coverage and effectiveness of protected areas (Watson et al., 2014). The meaning of sustainability is often debated, which can be conceptualized as the management of resources such that their contribution to human welfare is conserved or improved for the next generations (Kennedy, 2007). Brazil, with its vast territory of more than 8.5 million km² (IBGE, 2019), is responsible for protecting its natural environment, which is a difficult task when facing pressures for the expansion of land use for economic purposes, such as agriculture, pasture, and mining (IBGE, 2015). From 2003 to 2009, Brazil stood out in relation to the expansion of the network of preservation areas (PA). Brazil accounts for 73% of world expansion out of the entire legally protected area of the planet that was established in 2003 (Jenkins and Joppa, 2009).

In Brazil, PAs are called conservation units; hence, the designation to the National System of Nature Conservation Units (SNUC in Portuguese) established in 2000 by federal law aims to ensure that all conservation units represented significant ecological samples from different populations, safeguarding the existing biological patrimony

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

(Ministério do Meio Ambiente, 2011; Bernard et al., 2014). In 2002, the Brazilian government established several procedures and criteria for the creation of each PA, establishing its management plan, protection, and supervision actions that must be formalized and implemented. The management plan is a document that works as the identification of the area of preservation, having the zoning definition of a PA observing the particularity of each zone as a fundamental part with adequate management to the territorial ordering, such that the administration of the entire of the area reaches objectives with consistency and effectiveness. Although the legislation deals with zoning, the distinction between legislation and scientific knowledge is still quite high (Azevedo-Santos et al., 2017). The process of how to do this territorial division is guided by documents with a methodological superficial purpose; however, when we looked for this information in the management plans, we observed that zoning occurs in different ways because no methodology standardizes this classification.

In terms of the effectiveness of classification, many questions can still be asked regarding zoning in preservation areas. Liu and Li (2008) highlighted several known problems with the implementation of zoning schemes in China and elsewhere, including the lack of clear regulations on how to structure the spatial arrangement of zones and the lack of guidelines that determine the factors that should be considered since the creation of the PAs (Fendrich et al., 2019).

In study by Lima and Ranieri (2018), zoning can be approached in many ways. Land use planning is done with respect to only one type of zone. The study was done through a documentary analysis using the management plans. Zhang et al. (2013) used the methodology of combining interviews with geographic information systems (GIS) using a multi-criteria decision analysis based on the GIS method to formulate the zoning areas in the national park, with a wise process used to identify the priorities and prior knowledge of the area. Interviews were then conducted for the case study. The study of Vardarman et al. (2018) observed that the invasion of exotic species generated a zoning according to the degree of invasion by making field observations and performing statistical analyses. The main source of data for spatial delimitation of maps was provided by a nature conservation agency in the Czech Republic.

A natural phenomenon related to sustainability can be described in many ways, including visualization, physical models based on the creation or recreation of ecosystems, and conceptual and quantitative models. The last method, where the fuzzy logic theory can be applied, is the most informative for decision makers (Todorov and Marinova, 2011). An eminent factor of the fuzzy logic theory is the ability to capture intuitive concepts in addition to considering the psychological aspects used by humans in their usual reasoning; this has not been represented in traditional models (Zadeh, 1965; Oliveira, 1999; Zimmermann, 2001).

Multi-criteria analyses are examples of techniques that consider several perspectives and can integrate several actions in the decision process in a certain complex situation. From the operational point of view, multi-criteria methods have the ability to adapt to problems on several fronts, which can be described as conflicting interests. Although the multi-criteria evaluation technique does not solve the problem, it can help detail the problem, making the complex situation more transparent for decision makers (Munda et al., 1994; Hsueh and Cheng, 2017).

The objective of this study was to develop a quantitative model based on fuzzy logic to delineate the management zones in PA in Brazil. For this purpose, the possible variables (i.e., quantitative and qualitative) must be combined such that the map of the

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DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

area emphasizes its relationship with the surrounding areas. A case study was performed in a PA in southern Brazil to test the efficiency of the model.

Materials and Methods

Preservation area establishment

The establishment of all possible zones for a Brazilian PA covers the following: zone intangible, natural, extensive use, intensive use, historical-cultural, recovery, special use, conflicting use, temporary occupation, indigenous superposition, experimental interference, buffer, and presented in the Methodological Guideline according to the national legislation (IBAMA, 2002; Mazza et al., 2016; Simardi and Souza, 2018).

A PA can be classified in two ways. Some PAs need only a specific element for their classification (e.g., in the case where buildings are identified, the PA is automatically classified as a "zone of special use"). These zones in PAs include zones of special use, zones of conflicting use, zones of temporary occupation, indigenous superposition, and zones where historical/cultural heritage is found (*Fig. 1*).

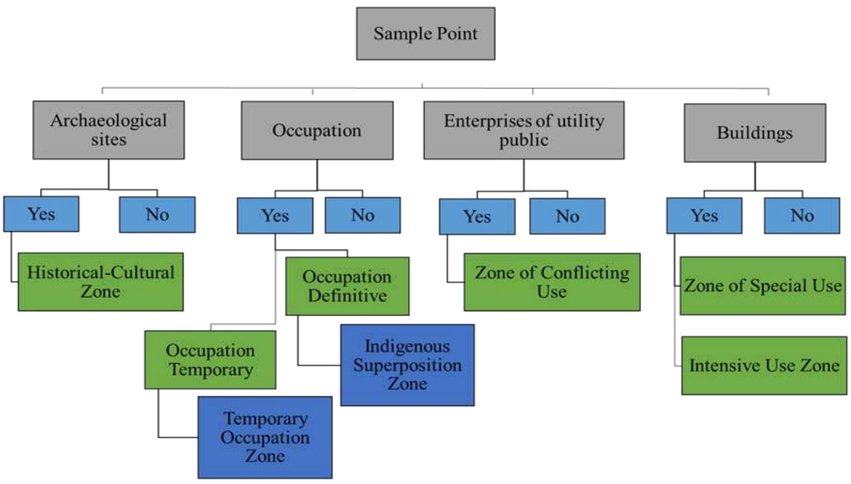

***Figure 1.** Scheme for the classification of areas with specific characteristics*

However, such specific elements will not always appear to generate the classification, which makes it necessary to seek the criteria for classifying intangible zone (IZ), natural zone (NZ), extensive use zone (EUZ), and recovery zone (RZ). The classification of this second group of PAs is much more complex and relies on the criteria not always clear for a decision maker.

Study area and data

The study area is the Santa Helena Relevant Ecological Interest Area (ARIE-SH), a 1.515 ha preservation area in the phytogeographic domain of the Atlantic Forest, which, in ecology, is considered a hotspot (Myers et al., 2000). According to the Brazilian legislation, this PA is classified as a Relevant Ecological Interest Area, a permanent category to sustainable use, with characteristics of possessing small areas with little or no human occupation and a singular natural feature with the purpose of protecting local ecosystems.

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ARIE-SH is located in the southern region of Brazil in the municipality of Santa Helena, state of Paraná (*Fig. 2*). It was officially established in 1984 on the banks of the Paraná River at the border between Brazil and Paraguay after the construction of the hydroelectric power plant of Itaipu (Management Plan, 2010).

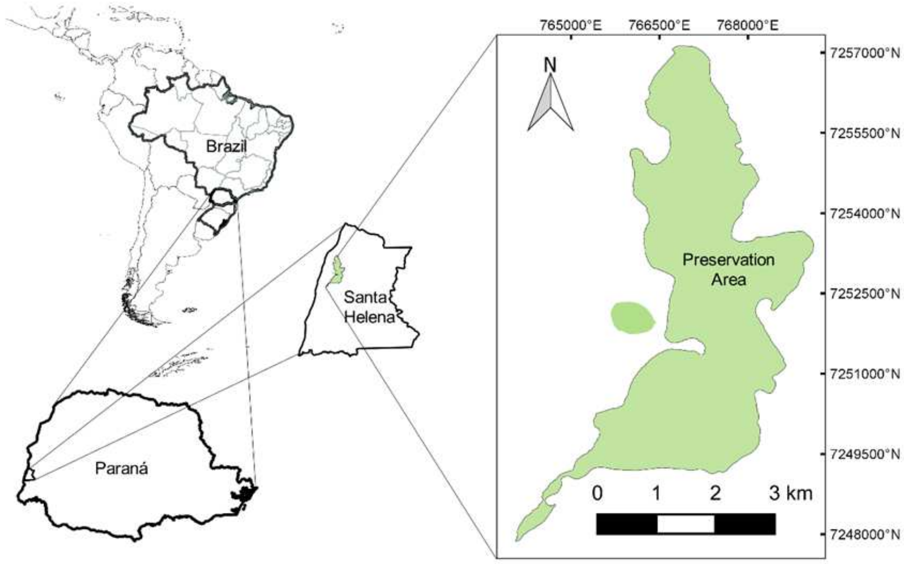

Figure 2. *Location of the Santa Helena Relevant Ecological Interest Area (images projected in the Universal Transverse Mercator (UTM) projection zone 21S and WGS 84 (World Geodetic System 84) ellipsoid)*

At this time, many areas along the river were expropriated, and reforestation with exotic and native trees species took place. Nowadays, the ARIE-SH is covered by a semi-deciduous seasonal forest according to the Brazilian classification of vegetation provided by IBGE (2012) in different stages of ecological succession. The main exotic species present include mango (*Mangifera indica* L.), jambolão or Java plum (*Syzygium cumini* (L.) Skeels), and Japanese raisin tree (*Hovenia dulcis* Thunb.). Meanwhile, the areas, where native species were planted, show a high diversity with several native herbaceous and shrub species, including ferns, growing in the shade sub-forest. The areas, where native species were planted, such as pitanga or Surinam cherry (*Eugenia uniflora* L.) and gabiroba (*Campomanesia xanthocarpa* Mart. ex O. Berg), are the richest in the number of trees species and present an intermediate successional stage.

Fieldwork was performed between March 2018 and June 2018. A total of 87 plots measuring 5 × 10 m² were sampled using the method proposed by Muller–Dombois and Ellenberg (1974). The location of the plots was selected with a distance of approximately 400 m. During the survey, the circumference at breast height (CBH) parameter was used in reference to the circumference of the trunk at 1.30 m height. Each plot was georeferenced at its center point using a global navigation satellite system (GNSS) (Etrex 30, Garmin, Garmin International, Inc., Kansas City, USA) receiver with a positional accuracy of approximately 5 m. The vegetation data collected in each plot consisted of (a) the number of trees with a CBH greater than or equal to 0.30 m, (b) the diversity of species, that is, how many species have a CBH greater than or equal to 0.30 m, and (c) the regeneration level registered by photography.

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DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

Model structure

The interpretability of the classification systems refers to their ability to express their behavior in a manner that is easily understood by a user (Jim and Mart, 2019). Based on this indication and to find means of facilitating the systematization of the process, a model was developed to propose to classify the sampled areas. The chosen variables were based on vegetation because "the lower degree of degradation of vegetation generally leads to the lower degree of degradation of fauna and soils" (IBAMA, 2002).

The input variables for the study are as follows: vegetation density index, diversity of species, and regeneration index. The expected output variable refers to "zoning," where each investigated point will be associated with a zone. The general approach is to first quantify the linguistic statement then retransmit the quantized logical input and output ratios using mathematical operators (Chen and Pham, 2000).

The fuzzy sets for the input and output variables considering the working hypothesis can be represented in several ways by pertinence functions, the most common being the triangular, trapezoidal, Gaussian, bell-shaped, Z-shaped, and S-shaped functions (Ibrahim, 2004). The pertinence functions chosen to describe the variables in this work exclude the triangular and trapezoidal functions considering that in nature, the behavior changes are not abrupt.

The membership function in the "Z" form is presented as follows:

$$f(x,a,b)=\begin{cases}1, & x<a\\ 1-2\left(\dfrac{x-a}{b-a}\right)^2, & a\le x<\dfrac{a+b}{2}\\ 2\left(\dfrac{b-x}{b-a}\right)^2, & \dfrac{a+b}{2}\le x<b\\ 0, & x\ge b\end{cases} \quad \text{(Eq.1)}$$

The membership function in a bell shape is:

$$f(x,a,b,c)=\frac{1}{1+\left|\dfrac{x-c}{a}\right|^{2b}} \quad \text{(Eq.2)}$$

The membership function in the "S" form is presented as:

$$f(x,a,b)=\begin{cases}0, & x<a\\ 2\left(\dfrac{x-a}{b-a}\right)^2, & a\le x<\dfrac{a+b}{2}\\ 1-2\left(\dfrac{x-b}{b-a}\right)^2, & \dfrac{a+b}{2}\le x<b\\ 1, & x\ge b\end{cases} \quad \text{(Eq.3)}$$

Input variable: "Vegetation density index"

The vegetation density index (VDI) is described by the membership function considered in the range of 0 to 10, with the low, medium, and high linguistic denominations (*Fig. 3*). It could have been chosen from 0 to 1 or from 0 to 5 provided that the membership functions were allocated at intervals consistent with its name.

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The membership function with a "low" denomination is a Z-shaped function (*Eq. 1*) with $f(x,1.5,5)$. The term "medium" is described by a bell-shaped function (*Eq. 2*) with $f(x,1.5,3,5)$. Finally, the membership function described by the linguistic term "high" vegetation concentration has an S form (*Eq. 3*) with $f(x,5,8.5)$.

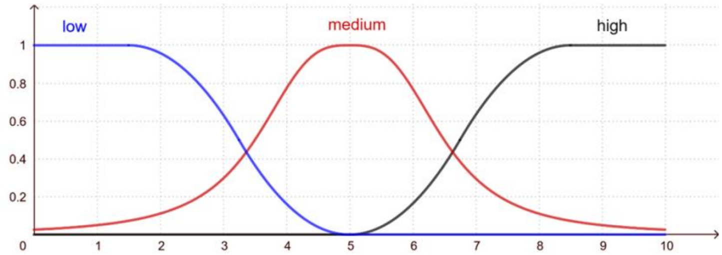

Figure 3. *Membership functions describing the vegetation concentration*

The VDI of the *i*th sampling plot is calculated as follows (*Eq. 4*):

$$VDI_i = \left[\frac{(EVI_i + NT_i)}{\max\limits_{1\le j\le n}\{EVI_j\} + \max\limits_{1\le k\le n}\{NT_k\}} \right] \cdot 10 \qquad \text{(Eq.4)}$$

where EVI_i is the result obtained by the index of vegetation between -1 and 1, which can be searched for in the software by georeferenced points; NT_i is the number of trees in the *i*th sampling plot; $\max\limits_{1\le k\le n}\{NT_k\}$ is the maximum number of trees observed among all the sampling plots (k = 1, 2, 3, …, 87); and $\max\limits_{1\le j\le n}\{EVI_j\}$ is the maximum enhanced vegetation index (EVI) observed and calculated for each point sampled in the study area (*j*=1, 2, 3, …, 87).

With regard to remotely mapping disturbances and land use/land cover (LULC), the data from the satellite-based sensors have proven useful for large-area characterization (Atzberger, 2013). The EVI will be used as input in the fuzzy system. The EVI is an "optimized" vegetation index designed to improve the vegetation signal with better sensitivity in regions of high biomass and vegetation monitoring (*Eq. 5*) (Jensen, 2009).

$$EVI = G\frac{\rho_{nir} - \rho_{red}}{(\rho_{nir} + C_1\rho_{red} + C_2\rho_{blue} + L)}, \qquad \text{(Eq.5)}$$

where, ρ_{nir} is the reflectance in the near infrared band (0.73 µm); ρ_{red} is the reflectance in the red band (0.66 µm); L is a soil adjustment factor; and C_1 and C_2 are the coefficients describing the use of the blue band for the correction of the red band for atmospheric scattering by aerosols. These coefficients were empirically determined as L = 1, C_1 = 6, C_2 = 7.5, and G (gain factor) = 2.5 (USGS, 2017). The Landsat 8 images used in this work were accessed by the United States Geological Survey (USGS) search platform in March 8, 2018.

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Input variable: "Diversity of species"

Data collection was designed to survey the variety and quantity of the plant species present on the site. The methodological guideline states that areas with the greatest number of species found should integrate the zones of greater degree of protection, such as the intangible and natural zones. For the variable "diversity of species," the membership function with a "low" qualification is a function in Z form with $f(x,1.5,5)$. The linguistic term "medium" is described by the bell-shaped function $f(x,1.5,3,5)$, while the term "high" is described by the S-shaped function with $f(x,5,8.5)$.

The diversity of tree species at the *i*th sampling plot, DTE_i was calculated using the following formula:

$$DTE_i = \left(\frac{NE_i}{\max\limits_{1\le l\le n}\{NE_l\}}\right)\cdot 10 \qquad \text{(Eq.6)}$$

where NE_i is the number of tree species observed in the *i*th sampling plot, and $\max\limits_{1\le l\le n}\{NE_l\}$ is the maximum number of tree species found among all sampling plots. The output was multiplied by 10 to scale the values between 0 and 10.

Input variable: "Regeneration index"

The input variable "regeneration index (RI)" is the variable considered in the characterization of zoning because it is a reforestation area. The classification of regeneration provides information that will be considered in the model. The linguistic denominations in the variable regeneration were low, medium, and high (*Fig. 3*).

To distinguish between terms, an area with a mean vegetation that did not enter the sample was considered as high regeneration because it did not complete the 0.30 m diameter that was considered. The term linguistic medium regeneration considered areas with a low and sparse vegetation, among others. The term "low regeneration" was used in places without vegetation in the sub-forest layer.

The membership function with the "low" qualification is a function in Z form (*Eq. 1*) with $f(x,1,5)$. The function with the "medium" qualification is expressed by the bell-shaped function with $f(x,1.5,1.5,5)$. Meanwhile, the function with the "high" qualification is expressed by the function with an S form with $f(x,5,9)$.

Output variable: "Zoning"

The output variable "zoning" has four membership functions: IZ, NZ, EUZ, and RZ (*Fig. 4*).

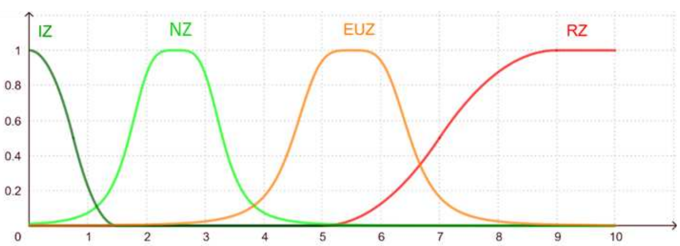

Figure 4. *Membership function of the output variable "zoning"*

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The membership function that represents IZ is a function in the Z shape with $f(x,0,1.5)$. The membership function for NZ is associated with the bell-shaped function with $f(x,0.8,2,2.5)$. The EUZ is also associated with the bell-shaped function with $f(x,1,2,5.5)$. Finally, the membership function that describes RZ is a function in the S shape with $f(x,5,9)$.

Base rule

This phase is the construction of the base rule that directly interferes with the result, which is still subjective and handmade (Rezende, 2005). Many efforts have been made to systematize or even automate the process of acquiring knowledge. In fuzzy systems, the semantics formed by the operators play a deterministic role.

The base rule is a statement of fuzzy rules in the form of if–then. A general approach would be to first quantify the linguistic statement then retransmit the quantized logical input and output ratios using the mathematical operators (Chen and Pham, 2000). In this work, the operator "and" will be used with the Mamdani controller defined as $I_c(x,\mu(x)) = \min\{x,\mu(x)\}$ (Zimmermann, 2001). The operator "and" is used with Mamdani (*Fig. 5*) when listing the input variables.

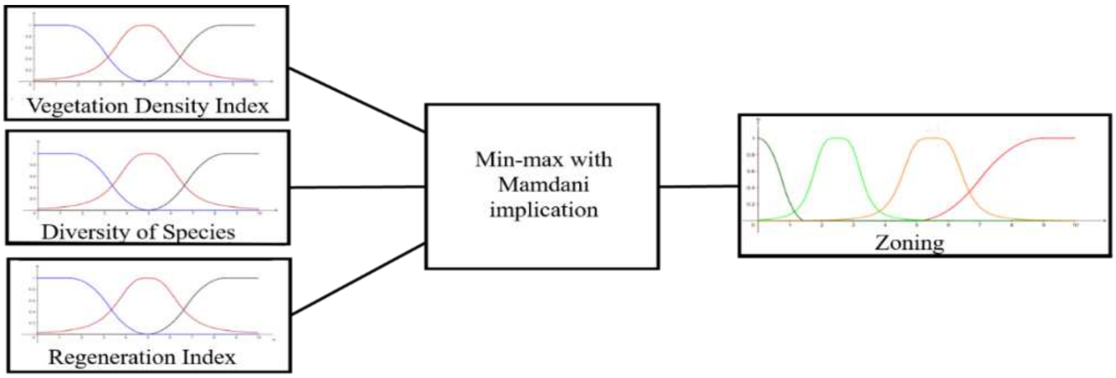

Figure 5. *Fuzzy system scheme for the proposed model*

The base rule in the proposed model will combine the input variables according to its attributes, thereby obtaining 27 rules (*Table 1*).

Table 1. *Rules for zoning delineation*

nº	*Input variables*					*Output variables*	
	If vegetation concentration	**and**	**if** species diversity	**and**	**if** regeneration	**then**	zoning
1	low	and	low	and	Low	then	RZ
2	medium	and	low	and	Low	then	RZ
…							
10	low	and	medium	and	Low	then	RZ
11	medium	and	medium	and	Low	then	EUZ
…							
26	medium	and	high	and	High	then	NZ
27	high	and	high	and	High	then	IZ

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Fuzzy inference

The information regarding the RI were observed for each sample point. VDI_i and DTE_i were also calculated according to (*Eq. 4*) and (*Eq. 6*), respectively. The input value in the fuzzy system will be a point with three coordinates, which generates a real number as a result, using the gravity center defuzzification method (GCD) (Gomide and Gudwin, 1994; Zimmermann, 2001) (*Eq. 7*):

$$GCD = \frac{\int_a^b x\mu(x)dx}{\int_a^b \mu(x)dx}. \tag{Eq.7}$$

In the GCD, [a, b] is the range of coverage of the membership functions, which in this case is [0,10]. Considering the area formed by the contribution of all the rules, (*Eq. 7*) considers the degree of pertinence (weight) of each point inserted in the model, consequently obtaining a real number as a result. This method returns the point representing the center of the area (or gravity) under the curve formed by the output membership function. The sampled point was classified according to the zone established depending on the GCD output value (*Table 2*).

Table 2. *Zoning classification according to the GCD range*

Zone	GCD range
Intangible	0 to 1.5
Natural	0 to 5.0
Extensive Use	2.0 to 9.0
Recovery	5.0 to 10.0

Each sampled point will be at the intersection of two fuzzy sets representing the zoning. The set for which the point has the highest degree of pertinence will be considered in their classification. If this point is exactly at the intersection point of the pertinence functions, this point will be classified in the most restrictive zone.

Interpolation

The inverse distance weighted (IDW) interpolation method (*Eq. 8*) (Shepard, 1968) was used to generate the thematic maps with the results of the case study applied to the model proposed in this work. Several powers were tested (i.e., 1, 2, 3, 4, 5, and 10). The one with the lowest mean error in the cross-validation was chosen.

$$X_p = \frac{\sum_{i=1}^{n} \frac{1}{d_i^p} \cdot X_i}{\sum_{i=1}^{n} \frac{1}{d_i^p}} \tag{Eq.8}$$

where, X_p is the interpolated variable; X_i is the value of the variable for the *i*th neighbor; d_i is the Euclidean distance between the *i*th neighborhood point and the sampled point; and p is the power of the distance.

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Results

The model presented an efficacy in classification, coherence between the surrounding areas, and in describing the area as observed in the fieldwork in conformity to the area documents (Management Plan, 2010). The results obtained using the model for zoning delineation in ARIE-SH allows classifying the 87 sampled points in the area in accordance to the PA defined by IBAMA (2002). The samples were classified in the range of 0.51 to 8.29 in accordance with the proposed methodology with an average of 4.70 and a CV of 0.43. The average parameters permeated the area as an "Extensive Use Zone." The IDW (power 2) interpolated thematic map (*Fig. 6a*) represents the area divided in four zones (*Table 3*). The transition areas between the zones (i.e., IZ and NZ, NZ and EUZ, and EUZ and RZ) were created by observing the coverage range (*Table 2*) of each pertinence function for the output variable (zoning) (*Fig. 6b*). The values obtained in these intervals were considered as transition regions, which may belong to zones with different degrees of pertinence (*Table 4*).

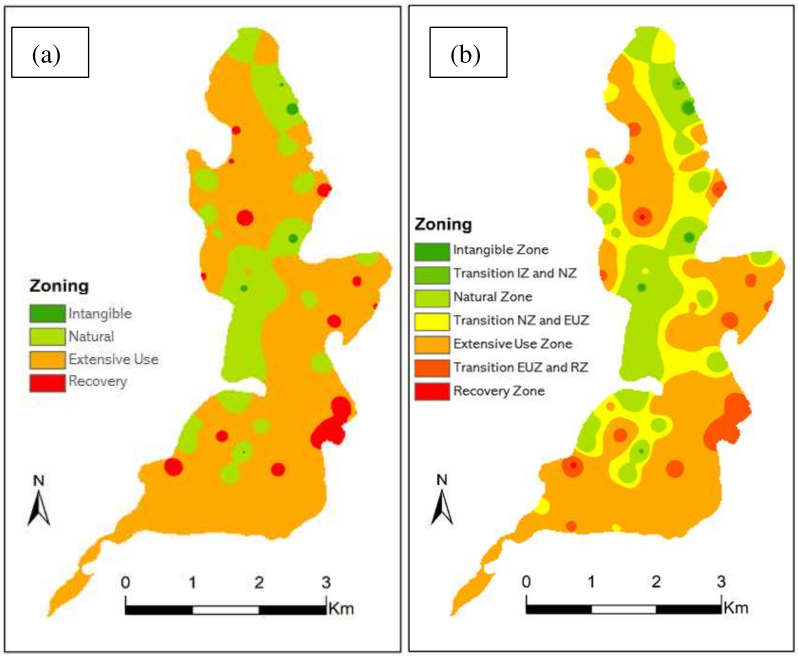

***Figure 6.** Zoning map of the area with the proposed methodology: (a) area divided in four zones and (b) area considering the regions of transition between one zone and another*

***Table 3.** Area of each zone considering the PA classification (IBAMA, 2002)*

Zone	Area (ha)	Area (%)
Intangible IZ	2.47	0.16
Natural NZ	339.63	22.41
Extensive Use EUZ	1123.2	74.09
Recovery RZ	50.60	3.34

The cross-validation performed for the PA classification produced a correlation coefficient of 0.2429, which was evaluated as poor (values under 0.5), moderate to good

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DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

(values from 0.50 to 0.75), and excellent (values above 0.75). The root mean square error (RMSE) was 1.99. The mean error (ME) was 0.0136 (*Fig. 7*).

Approximately 74.09% of the area extension belonged to the Extensive Use Zone, 0.16% to the Intangible Zone, 22.41% to the Natural Zone, and 3.34% to the Recovery Zone (*Table 3*). When zoning was distributed, including the transition areas, a more detailed understanding of the area can be achieved (*Table 4*). The natural zone that occupied 22.41% of the original map was reduced to 21.91%, but allowed the observation of a larger area (19.25%) that can be classified as a transition area between NZ and EUZ. This result indicated that this area can receive an adequate management to integrate the Natural Zone in the future.

Table 4. *Area of each zone considering the transition zone for PA*

Zone	Area (ha)	Area (%)
Intangible IZ	2.47	0.16
Transition IZ and NZ	7.55	0.5
Natural NZ	332.07	21.91
Transition NZ and EUZ	291.87	19.25
Extensive Use EUZ	831.32	54.84
Transition EUZ and RZ	50.60	3.34
Recovery RZ	0	0

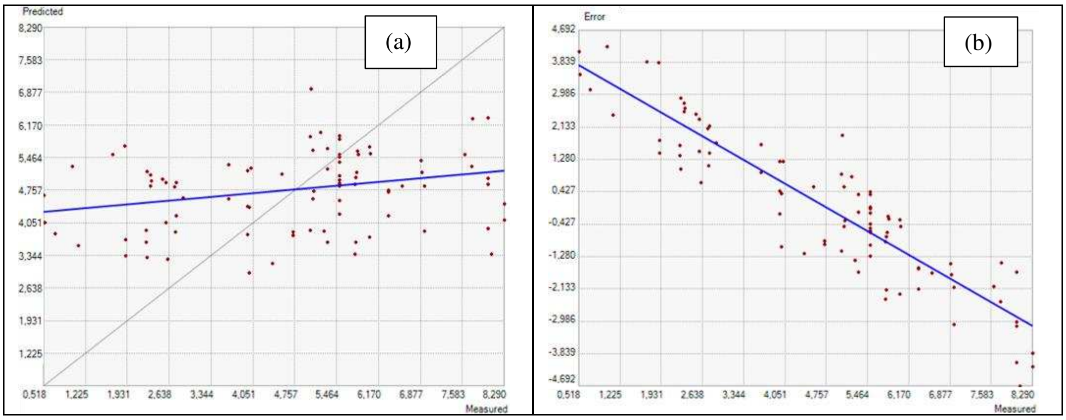

Figure 7. *Adjustment of the points evaluated in the interpolation: (a) fit for the interpolated data with a regression equation of 0.114 x + 4.23 and (b) error in the prediction with a regression equation of −0.885 x + 4.23*

Discussion

The model presents allows decision makers to classify the PA in a reasonable zoning scheme and enables it to be adjusted to any biome by observing the input variables. This classification generates reliability to the process that becomes standardized. Our model was able to describe the study area using quantitative (i.e., VDI and diversity of tree species) and qualitative (regeneration) variables because of the fuzzy logic that allows us to describe the variable flexibility; hence, it has been used to model landscapes and in different ecological and environmental studies (Sheehan and Gough, 2016).

In the case of a complex system (e.g., natural reserves), we have a model generating several zones based on measurable variables that present ease in the field work, not

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

generating great distortions according to the specialist who will reproduce the zoning. Moilanen et al. (2009) argued that a major limitation in land-use planning when made by software is the inability to consider different zone types.

Our work differed from that of Lima and Ranieri (2018) because it studied documentary based only on one type of zone with specific characteristics. Zhang et al. (2013) presented a similarity in the sense of combining methodologies (e.g., GIS and multi-criterion analysis), although they used previous knowledge, which is difficult for research in Brazil because many units do not have the minimum mandatory document that is the management plan. Vardarman et al. (2018) developed a method of zoning by observing biological factors like the invasion of exotic species resembling the variable regeneration treated in this model. It is difficult within the context of conservation to say which methodology is the best because each area has its particularity. The variables are chosen according to specificity of the area, making it difficult to compare approaches. Territorial zoning is undeniably difficult, even with its limitations, yet it reduces the external impact on the area (Bruggeman et al., 2015).

The high variation of the model classification occurred because during reforestation in the case study area, the natural distribution of the species was not observed. The area was divided in fields, where in some cases, a single species or a combination of two or three species were planted without criterion. Therefore, the interpolation method cannot understand the characteristic of the area that is extremely particular. In areas where the vegetation change is not as abrupt as natural areas, this variation tends to be very small because the neighborhood of the studied site will have similar characteristics.

The model proposed herein showed that zoning classification can be done in a standardized and simplified manner, as suggested by Lin and Li (2016), because traditional models are complex to use and interpret.

In the case of zoning, this classification may encounter different interests with different stakeholders perceiving different versions of the same problem. In this context, the implantation of an ecological park, for example, can have a double understanding. Those interested in installing the park may want to enter the natural areas, which contradicts the conservationist interests of maintaining the intangibility of a core area. In this sense, approaches that consider transition zones present more information for decision makers, and can be interpreted as a buffer for the possible damages on the intangible and natural areas, that is, work as attenuating human intervention, for example (Liu and Li, 2008).

Zoning is indispensable in decision making in a conservation area, and studies of possible reproduction and understanding are valued (Cook et al., 2012). The obtained results allowed for an adequate management of each class according to its peculiarity. When transition areas are included in the classification, the knowledge of their size and location can contribute to the management of the area, thereby allowing greater care with areas that already belong to a more restrictive level, but with a small degree of pertinence.

Many preservation areas in Brazil still do not have a management plan even with numerous documents and studies that confirm the importance of zoning. The management plan has been defined since 1979 and is a mandatory document since 2002, and an integral part of this document is zoning. The preservation areas that have this territorial order do not explain the methodology to generate the territorial classification (Ministério do Meio Ambiente, 2011).

Liu and Li (2008) argued that problems with the implementation of zoning schemes in China and elsewhere include the lack of clear regulations on how to structure the spatial arrangement of zones and the lack of guidelines that determine what factors should be

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

considered. Many variables are treated and considered in Brazilian laws that define zoning in preservation areas; however, we chose to deal with vegetation because if vegetation is not in a favorable state, the fauna will also not find survival conditions. The choice permitted the use of classic vegetation indexes in the literature, such as EVI, which made the model very practical. Lin et al. (2018) compared the performance of some strategies used to evaluate areas that should be retained. The authors pointed out that unfortunately, selecting the most appropriate model is often difficult because of limited data and knowledge.

However, one of the advantages of the methodology proposed herein is the standardization in the territorial planning of preservation areas in the Atlantic Forest, and the particularity of each study area may be adequate. If an area has potential for visitation, a human intervention variable can be included. If the study area is another type, the rules of this model can be adapted to classify and describe the reality of that place.

Conclusions

The whole area under study can be covered during sampling. Moreover, with the experience acquired, the presented results can more adequately describe the studied area. We presented herein a new methodological proposal for PA zoning. The PA classification presents a systematization to the process of territorial planning with input variables having quantitative and qualitative parameters, which allows an approximation of the reasoning of specialists obtaining a numerical result easy to classify in the zones as established in the National System of Nature Conservation Units. The model provides methods of automating decisions based on fuzzy logic. This methodology is promising because it can be used automatically and independently. It also allows adaptation to any PA because it meets the law requirements. The intersections between the fuzzy set output will be areas of special attention because being in an intermediate stage, the management of this area can be improved for insertion into a category of greater preservation.

This model introduces a tool that can be used to review and monitor the zoning of protected areas that the legislation has defined to be periodically reviewed. In practice, the model offers zoning in which the spatial division presents no rigid limits, but rather flexible boundaries to the real objective of the area, thereby providing the manager with detailed spatial classification maps that can be used to plan impediments and permissions for the proper management of the area.

Future studies can work on the insertion of new variables. For example, in open areas to visitation, the variable "human intervention" can be inserted to verify the degree of impact of human action, qualifying factors as the presence of garbage, fires, tree cuttings, and signs of hunting, among others. For example, we can compare the model performance of areas with different characteristics including characteristics other than reforestation.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

APPENDIX

Coordinates:

Coordinates in the Universal Transverse Mercator (UTM) projection zone 21S and WGS 84 (World Geodetic System 84) ellipsoid.		
Point	**longitude**	**latitude**
1	767209	7251377
2	766778	7251376
3	766162	7251118
4	766501	7250791
5	765779	7250325
6	765280	7249668
7	765747	7249377
8	766461	7249379
9	766466	7249388
10	767971	7250740
11	768107	7250908
12	768241	7251278
13	767098	7250960
14	767104	7250496
15	767327	7250269
16	767028	7250243
17	766827	7250542
18	767312	7251509
19	767369	7251962
20	767385	7252360
21	766053	7250987
22	766649	7251414
23	767101	7251257
24	767447	7252459
25	767634	7252816
26	767799	7252901
27	768540	7253207
28	768633	7253195
29	768741	7252997
30	768803	7252822
31	768494	7252703
32	768162	7252579
33	768270	7253055
34	767436	7249342
35	766973	7249448
36	766627	7249622
37	767113	7249927
38	767630	7249576
39	767917	7249943
40	767916	7250327
41	767626	7250008
42	766624	7250176
43	766810	7253571
44	766837	7254195
45	766814	7254640
46	766679	7254946
47	766290	7254799
48	766031	7254491
49	766287	7254256
50	766436	7253945
51	766251	7253683
52	766214	7253284

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

Coordinates in the Universal Transverse Mercator (UTM) projection zone 21S and WGS 84 (World Geodetic System 84) ellipsoid.		
Point	**longitude**	**latitude**
53	766639	7255076
54	766707	7255565
55	766435	7255773
56	766298	7256113
57	766440	7256570
58	766708	7256859
59	767215	7256764
60	767391	7256276
61	767554	7255888
62	767721	7255495
63	767517	7255290
64	767628	7255060
65	767764	7254750
66	768007	7254634
67	767736	7254358
68	767561	7253874
69	767596	7253409
70	766467	7249868
71	765194	7249443
72	767238	7253017
73	766884	7253348
74	766828	7253100
75	766827	7252735
76	766834	7252429
77	766834	7251943
78	767009	7251743
79	766349	7251242
80	765967	7250650
81	765462	7250222
82	768641	7253572
83	768307	7253487
84	768012	7252424
85	767999	7251938
86	767931	7251239
87	767573	7251373

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50115027

COMPARATIVE PERFORMANCE OF TWO BREAD WHEAT (*TRITICUM AESTIVUM* L.) GENOTYPES UNDER SALINITY STRESS

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(Received 18th Dec 2018; accepted 19th Feb 2019)

Abstract. The study was conducted at a greenhouse of the Graduate School of Biosphere Science in Hiroshima University, Japan under the ambient conditions to find out the effect of salinity stress on some physiological and biochemical characteristics of two bread wheat genotypes and also to elucidate the salt tolerance mechanism of these wheat genotypes. Two wheat genotypes namely 'Sakha 95' and 'Misr 2' were exposed to 50, 100 and 150 mM NaCl levels of salinity. Results showed that both genotypes were varied significantly for all traits under all levels of salt stress. Among the genotypes, growth of the genotype 'Misr 2' was found much better than the 'Sakha 95', with the maintaining a higher dry biomass. The genotype 'Misr 2' also maintained a high concentrations of soluble-sugars, proline, and various antioxidant enzymes activity such as glutathione reductase (GR), peroxidase (POD) and catalase (CAT) compared with genotype 'Sakha 95'; whereas, maintained a lower levels of lipid peroxidation represented by the malondialdehyde (MDA) concentration. Indicating that genotype 'Misr 2' has ability to survive under salinity stress than the genotype 'Sakha 95'. Similarly, salinity stress also significantly changed in Ca^{++} contents and Na^{+}/Ca^{++} ratio in both wheat genotypes. The relative reduction in Ca^{++} concentration and Na^{+}/Ca^{++} ratio was found higher in the genotype 'Sakha 95' than in 'Misr 2' and lead to showing the signs injury. Thus, the genotype 'Misr 2' would be useful to develop a salinity tolerant wheat varieties in the future breeding program.
Keywords: *antioxidant, salinity stress, wheat, physiological characteristics*

Abbreviations: APX, ascorbate peroxidase; ARC, Agriculture Research Center; CAT, catalase; MDA, malondialdehyde; POD, peroxidase; NaCl, sodium chloride; GR, glutathione reductase; SOD, superoxide dismutase; ROS, reactive oxygen species; TBA, thiobarbituric acid; RWC, relative water content; ψ_{π}, the osmotic potential

Introduction

Bread wheat (*Triticum aestivum* L.) is the leading widely grown food cereal around the globe, due to it wider adaptability as well as quality of nutritive values than other cereals. Similarly, in terms of production and acreage it is also stands first. It is as a strategic crop which has a significant role on the national economy of the third world countries (Barutcular et al., 2017; Yadav et al., 2018; Yildirim et al., 2018). Whereas, it demand is increasing day by day to meet the food security of increasing population (Hossain et al., 2012; Abdelaal et al., 2018; Jahan et al., 2019). At the same time the productivity of wheat across the globe is influenced by several abiotic stresses (i.e., heat, drought and salinity); among them soil salinity is the most important one, particularly in arid and semi-arid regions (Pitman and Läuchli, 2002; Rengasamy, 2010; Sommer et al., 2015; Out et al., 2018).

Soil salinity has considered as the foremost environmental difficulties that negatively influences the growth and development of plants by altering the physico-biochemical process (Allakhverdiev et al., 2000). Approximately a 60% crop productivity in the world is loss due to soil salinity stress (Xie et al., 2016). Study depicts that nearly 20% of the total cultivated land across the world is under salt stress (Oproi and Madosa, 2014). It is well-understood that under salt stress, plants uptake high concentrations of soluble salts that lead to limit the uptake of water through the roots system, due to higher osmotic stress. As a result, limited water in plant cells influences the turgor and also changes the membrane stability (Sairam et al., 2002), and absorbed the high concentration of ions in plant cells which compete with the uptake of essential nutrients lead to nutrient deficiency (Goudarzi and Pakniyat, 2008). The most predominant salt in saline soil such as NaCl increases the concentration of Na^+ and Cl^- level in the soil, which inhibits the uptake of nutrients like Ca^{++}, Mg^{++} and K^+ by the plants and subsequently increases the uptake of Na^+ and Cl^- in susceptible plants (Khan et al., 1999).

A group of antioxidants such as glutathione reductase (GR), peroxidase (POD) and catalase (CAT) are normally linked with the plant which tolerance to various stresses, including salinity stress (Munns and Tester, 2008). Plants which are capable to preserve a high levels of soluble-sugars, proline, and various antioxidant enzymes such as GR, POD and CAT to inhibit a lower levels of lipid peroxidation by representing a malondialdehyde (MDA) and efficient reactive oxygen species (ROS) concentration scavenger (Abogadallah, 2010; Gill and Tuteja, 2010); also can maintain a lower level of stress-induced injuries (Munns and Tester, 2008; Sharbatkhari et al., 2013). Sairam et al. (2002) revealed that the increasing level of SOD, GR and APX (ascorbate peroxidase) activity in wheat varieties under salinity stress showed the better level of tolerancey against salinity stress. They also found that antioxidants such as soluble sugars, proline, glycine betaine and abscisic acid contents were also increased of these tolerance wheat varieties under salinity stress. Other findings revealed that Na^+ and K^+ concentrations in the plants' cell, and their ratio, and dry biomass of salt-induced plants are also an appropriate indicator for the screening of wheat genotypes that are tolerant to salt stress (Goudarzi and Pakniyat, 2008). Sánchez-Rodríguez et al. (2010) reported that susceptible wheat genotypes showed a higher degree of lipid peroxidation represented by the MDA and ROS concentration than the tolerant genotypes. Therefore, a well thoughtful of wheat physiological responses under salinity stress may assistance to develop wheat varieties which will be suitable to grow under salt stress condition through improving growth

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50295041

and yield. In the context, the current study was undertaken to know the effects of salinity stress on some physiological and biochemical characteristics of wheat and also to elucidate the salt tolerance mechanism of wheat genotypes.

Materials and Methods

Location

The study was undertaken in a greenhouse of the Graduate School of Biosphere Science in Hiroshima University, Japan under the ambient conditions

Plant growth and stress treatment

To fulfil the objectives of the present study, two Egyptian wheat genotypes namely 'Sakha 95' and 'Misr 2' were selected based on their agronomic performance. These two genotypes were collected from the Agriculture Research Center (ARC) in Egypt. For surface sterilization, seeds of two wheat genotypes were immersion in a 50% sodium hypochlorite solution for 30 minutes, and then carefully washed with distilled-water before sowing. Subsequently seeds were then soaked in tap-water for 24 hours at 28°C. Then germinated seeds were transferred to a 20 L half strength Hoagland solution. Ten days after germination, all seedlings were transferred to either Hoagland solution (as a control) or solution supplemented with NaCl (salinity treatment) for 12 days. Salt concentration such as 50, 100 and 150 mM (according to treatments) was applied gradually in 2 days' intermissions to avoid osmotic shock to the plants. The nutrient solution was continuously aerated with pumps and renewed every 2 days. The pH was adjusted at 5.0-6.0 daily. All treatments were arranged with a complete randomized design and were replicated in three times.

Growth measurements

Ten plants in all treatments were collected and separated into three parts (roots, stems and leaves) after 12 days of treatment imposition. Two sets of samples were prepared: one for recording dry weights (DW) for each plant after oven drying at 70°C for 3 days, and the other set was kept at -80°C for physiological analyses.

Measurement of lipid peroxidation (malondialdehyde; MDA) concentrations

The lipid peroxidation (such as MDA) concentration was determined through the reaction of thiobarbituric acid (TBA) (according to Fu and Hung, 2001), from frozen-samples (-80ºC). The lipid peroxidation concentration (MDA) was estimated by using a coefficient of absorbance (535 nm) of 155 mM^{-1} cm^{-1}.

Measurements of Na^+, K^+ and Ca^{++} concentrations

Dry biomass of root, stem, and leaves was ground finely in a sample mill separately (Model: Labo, Miser LM-Plus; Osaka Chemical Co., LTD, Japan). The fine powder was then used to determine the Na^+ and K^+ concentrations. For this, the powder was then digested with sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) at 2:1 (v/v). The Na^+ and K^+ concentrations were measured using a flame photometer (Model: ANA 135; Tokyo Photoelectric, Tokyo, Japan). The Ca^{++} concentration was also determined by

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using an inductively coupled argon plasma method (Model: ICAP-575, Nippon Jarrel Ash, Kyoto, Japan).

Measurement of sugar concentrations

The dried ground sample of leaves was boiled with 80% (v/v) ethanol in a hot water (80ºC about 20 minutes; min). The mixture was then centrifuged at 2000 rpm for 5 min; after that the supernatant was collected, and the precipitate was exposed to two more times of the same extraction process. The sugar concentration was determined in the ethanol-soluble extract by anthrone reagent method with a spectrophotometer (Model: U-2001, Hitachi, Japan), using glucose solution as standard (according to Yemm and Willis, 1954).

Measurement of leaf water potential

The osmotic potential (ψ_π) of cell sap and osmotic potential ($\psi_{\pi(100)}$) at full turgor were measured by using a Wescor 5500 vapor pressure osmometer (Model: Wescor Inc., Logan, UT. USA), and was estimated by adjusting according to Wilson et al. (1979).

Measurement of proline

The proline was determined spectrophotometrically following the ninhydrin method as described by Bates et al. (1973), using L-proline as a standard and then determined using spectrophotometer (Model: U-3310, Hitachi, Ltd. Tokyo, Japan). For determination of proline, the dried ground samples were transferred to vials subjected to methanol extraction, and stored in the dark place at 4ºC.

Antioxidant enzyme activities

Protein Assay kit (Model: Bio-Rad DC, California, USA) and bovine serum albumin as a standard were used to estimate the activity of CAT (EC 1.11.1.6), POD and GR (EC 1.6.4.2). An amount of 0.5 g fresh sample was extracted according to the following method as described by Takagi and Yamada (2013). An aliquot of 1 ml of the CAT assay mixture was also used which was contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and enzyme extract (5%). A decline in H_2O_2 was recorded at 240 nm to know the enzyme activity which is expressed as mmol H_2O_2 consumed per minute. 1 ml assay mixture containing 100 mM phosphate buffer (pH 7.5), 0.1 mM EDTA, 0.02 mM NADPH, 0.02 mM GSSG and 10% enzyme extract were used to know the GR activity. The concentration of oxidized NADPH was determined by using the extinction coefficient (6.22 mM^{-1} cm^{-1}) and 1-unit GR activity defined as μmol NADPH oxidized min^{-1}. For the measurement of POD activity, 1 ml reaction mixture contained 15 mM guaiacol, 73 mM phosphate buffer, 10 mM H_2O_2 and 2% enzyme extract. Increase in absorbance was monitored at 470 nm for 1 min and the enzyme activity calculated using the extinction coefficient (26.6 $mM^{-1}cm^{-1}$) for tetraguaiacol (Chance and Maehly, 1955). One-unit POD activity was defined as mmol tetraguaiacol formed per min.

Statistical analysis

Data were arranged and statically observed through one-way of analysis of variance (ANOVA) (Kao and Green, 2007). Treatments mean variation under different salinity treatments were observed by using Duncan's Multiple Range Test (DMRT) at the 0.05 level of significance (Duncan, 1955).

Results and Discussion

Plant growth

Both wheat genotypes exposed to 150 mM NaCl salt concentration showed that the salt stress highly influenced the leaf, stem & root dry weight (*Fig. 1*). As compared with control, leaf, stem, and root dry weight (DW) of both the genotypes were reduced significantly under salt stress treatments. While, DW reduction of genotype ‘Misr 2' was found a minimum than ‘Sakha 95'. Considering the visible signs injury, genotype ‘Sakha 95' showed the maximum signs injury than genotype ‘Misr 2' (*Fig. 1*). In the present study, plant biomass in both genotypes were decreased with the increase in the level of salinity, and it might be due to salinity stress altered the normal physiological and biochemical activities of the salinity exposed plants, which lead to decrease the DW. The assumption of the present study related to the adverse effect of salinity on plant biomass was also confirmed by Munns et al. (2006) and Rahman et al. (2017). Similarly, Dayiragije and Lutts (2006) also revealed that under salinity stress susceptible plants persist under-developed due to a decrease in cell division, elongation and also limit the synthesis of growth hormones (auxin) which lead to decrease the total biomass of the affected plant.

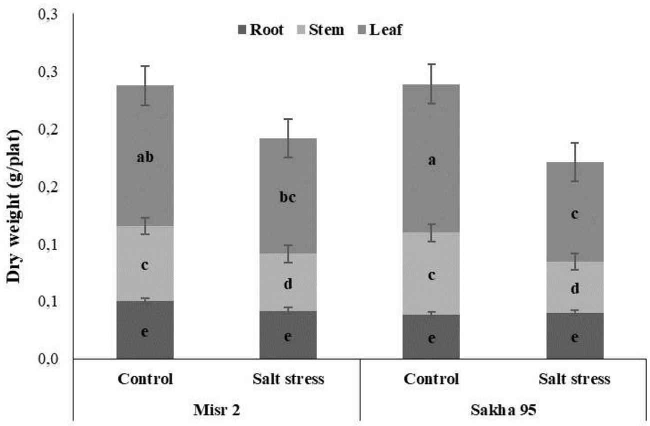

Figure 1. *Effects of salinity stress on the dry weight of the leaf, stem, and root of two wheat genotypes recorded at 12 days after germination. The values of standard error (± SE) in each bar were calculated for each treatment. The same letter indicates no significant difference (p≤0.05) between each other*

Na^+, K^+ concentrations and Na^+/K^+ ratio

Salinity treatments significantly influenced the Na^+, K^+ concentration as well as the ratio of K^+/Na^+ in all plant parts of both wheat genotypes. Under salt stress, accumulation of Na^+ increased in all of the organs of both genotypes; whereas the Na^+ concentrations significantly differed between the two genotypes (*Table 1*). As compared with control plants, the K^+ concentration in all parts of the plants was decreased significantly in both genotypes. The lower K^+ concentration was found in the genotype ‘Sakha 95' than the genotype ‘Misr 2' under stress condition. However, under the control condition, the leaf K^+ concentration was significantly higher in genotype ‘Sakha 95’ than that of genotype ‘Misr 2’. Our results revealed that the salt treatments remarkably reduced the leaf K^+ concentration in the genotype ‘Sakha 95’, whereas the

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reduction of K^+ concentration in the leaf of 'Misr 2' was minor as compared with control plants (*Fig. 1*).

Table 1. *Effects of salinity on Na and K concentration (mg g^{-1} DW) in the leaves, stems and roots of wheat. The values are the means (± S.E) of three replicates. Means followed by the same letter within each line are not significantly different ($p<0.05$)*

Salt levels (mM NaCl)	Wheat genotypes	Na^+ (mg g^{-1} DW)			K^+ (mg g^{-1} DW)			Na^+/K^+ (%)		
		Leaf	Stem	Root	Leaf	Stem	Root	Leaf	Stem	Root
0	Misr 2	5.9±0.04c	6.1±0.04c	7.7±0.03c	69.6±1.3a	90.4±1.3b	54.5±2.7b	8.5±0.2ab	6.7±0.1c	14.2±0.7c
	Sakha 95	5.8±0.01c	6.3±0.10c	6.9±0.15c	68.5±0.7ab	97.4±0.6a	67.6±3.6a	8.5±0.1a	6.4±0.1c	10.3±0.4c
150	Misr 2	29.1±0.52b	37.1±1.09b	30.2±0.58b	64.8±0.5c	81.5±0.5c	38.7±1.1c	44.9±0.9c	45.6±1.5b	78.1±1.1b
	Sakha 95	34.7±1.03a	41.5±1.18a	36.1±1.72a	66.4±0.6bc	80.2±1.8c	42.2±1.2c	52.3±1.6bc	51.9±2.5a	85.45±3.1a

It is well-documented that lower Na^+ uptake and higher K^+ uptake are the key indicators of salinity tolerance in higher plants. In the present study, the Na^+/K^+ ratio in the leaves, roots and stems of the genotype 'Misr 2' was significantly lower than the genotype 'Sakha 95'. When exposed to salinity stress, K^+ concentration in the leaves, roots and stems of both genotypes was decreased significantly, whereas, Na^+ concentration was increased significantly, ultimately causing an increase in the ratio of Na^+/K^+, resulted in a serious deterioration of the ionic homeostasis in leaves, roots and stems of affected plants. Among the genotypes, K^+ concentration was not changed, but the Na^+ concentration and the Na^+/K^+ ratio was changed and higher changed was found in genotype 'Sakha 95' than the genotype 'Misr 2' (*Table 1*). It is indicated that the genotype 'Sakha 95' is sensitive to salinity stress as a result of more accumulation of Na^+ concentration and the Na^+/K^+ ratio, causing a serious visible injury signs in the leaves, roots and stems of affected plants, whereas genotype 'Misr 2' was found a tolerant genotype as the genotype did not show any signs injury under salinity stress. The results of the present study is agreement with the results of Benderradji et al. (2011), who noticed that salt sensitive wheat genotypes could exclude a lesser amount Na^+ effectively through the transpiration stream as a result of higher concentration of Na^+ entered in to the leaf blade, consequently a higher accumulation of Na^+, and causing a cell injury. Similarly, Assaha et al. (2015), EL Sabagh et al. (2015) and Mekawy et al. (2015, 2018) revealed that the salt tolerant crops accumulated lower amount of Na^+ concentration in the shoots and leaf blades. Although, the leaf K^+ content has been suggested as a weak index of salt tolerance as compared to Na^+ content under field conditions, due to most of the susceptible genotypes do not change K^+ under salinity stress (El-Hendawi et al., 2009).

Ca^{++} concentrations and Na^+/Ca^{++} ratio

Salinity stress significantly changed in Ca^{++} concentration and Na^+/Ca^{++} ratio in the leaves, roots and stems of both wheat genotypes. While, a significant decline of Ca^{++} concentration was observed in the leaves, stems and roots of salt treated wheat plants, which leads to a significant increase of Na^+, prompting an increment of the Na^+/Ca^{++} ratio, resulting in a serious deterioration of the ionic homeostasis (*Table 2*). The relative reduction in Ca^{++} concentration in the leaves, stems and roots of wheat plants was greater in the genotype 'Sakha 95' than in the 'Misr 2'. Similarly Na^+/Ca^{++} ratio in all

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plant parts of genotype ‘Sakha 95’ was found the higher than genotype ‘Misr 2’. The results of the present study indicated that Ca^{++} concentrations and Na^{+}/Ca^{++} ratio in the all plant parts was higher and lead to showing the signs injury under stress condition. The information of the present study is also confirmed by the earlier study as reported by Islam (2001) and Yusuf (2010), who revealed that accumulation of Ca^{++} in plant parts inhibit to uptake other nutrients as well as altering the physiological and biochemical process. Similarly, Islam et al. (2011) found that higher accumulation of Ca^{++} in the leaves under salt stress significantly inhibited the growth and development of foxtail-millet, whereas the inhibition of Ca^{++} was found insignificant in poroso-millet, indicated that proso-millet is tolerant to salinity stress than foxtail-millet.

Table 2. *Effects of salinity on Ca^{++} concentration (mg g^{-1} DW) in the leaves, stems and roots of both wheat genotypes. The values are the means (± S.E) of three replicates. Means followed by the same letter within each line are not significantly different ($p<0.05$)*

Salt levels (mM NaCl)	Wheat genotypes	Ca^{++} (mg g^{-1} DW)			Na^{+}/Ca^{++} (%)		
		Leaf	Stem	Root	Leaf	Stem	Root
0	Misr 2	5.50±0.13a	3.30±0.04c	2.57±0.07d	107.02±1.82c	183.42±2.67c	298.68±6.56c
	Sakha 95	5.95±0.19a	3.16±0.08c	2.90±0.32de	98.21±3.08c	198.92±3.08c	249.07±28.86c
150	Misr 2	3.76±0.17b	2.54±0.36d	1.74±0.06e	776.88±26.19b	1,526.78±161.45b	1,736.13±34.78b
	Sakha 95	3.51±0.13b	1.89±0.06e	2.08±0.09e	990.08±29.69a	2,205.40±99.47a	1,740.98±57.26b

Effects of salinity on proline concentration in the leaf of the wheat genotypes

The increased levels of free proline in the leaf of both wheat genotypes under salinity stress conditions enhance the levels of antioxidant enzymes including proline activity which are natural responses of plants against stress. In the present study, the proline concentration in the leaves of both wheat genotypes was increased significantly under salt stress, whereas the accumulation level of proline varied remarkably between the genotypes. Among the genotypes, the genotype ‘Sakha 95’ showed a much higher level of proline accumulation than the genotype ‘Misr 2’ under salt treatment (*Fig. 2*). The results of the present study related to an accumulation of proline under salt stress also confirm by Szabados and Savouré (2010), who also noticed that the proline and total soluble carbohydrates synthesis influenced the growth and development of plant under stress conditions. To survive under stress, proline and soluble sugars could play a significant role in osmotic adjustment (Mafakheri et al., 2010). Similarly, Ueda et al. (2008) reported that proline accumulation under stress has been considered an adaptive character. They also found that higher proline synthesis during stress condition not only contribute to osmotic adjustment, but also improved the growth and development of the affected plants.

Effects of salinity on osmotic potential (ψ_π) of wheat genotypes under salinity stress

The osmotic potential (ψ_π) in both wheat genotypes was decreased significantly under saline condition (*Fig. 3*). Whereas, the genotype ‘Sakha 95’ exhibited a more negative potential than the cultivar ‘Misr 2’ under salinity treatment. Thus, the salinity-tolerant genotype ‘Misr 2’ would display a higher osmotic adjustment than the sensitive genotype ‘Sakha 95' under salinity stress conditions. The results of the present study indicated that water uptake of the plant under salinity stress was decreased due to changes in soil water potential. The information is also confirmed by Hussain et al.

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(2008), who reported that under different levels of salinity, the ionic flux in plant cell was increased that affected water potential of the plant's cell, ultimately lead to damage the plant cellular membranes. Similarly, Parida and Dus (2005) found that increasing level of osmotic potential under salt stress is due to high ion absorption and compartmentation in vacuole or the production of osmolytes is responsible for the osmotic adjustment in the cell of the sensitive plant. They also revealed that the increase of osmotic potential in salinity sensitive plants is due to the reduction of turgor pressure.

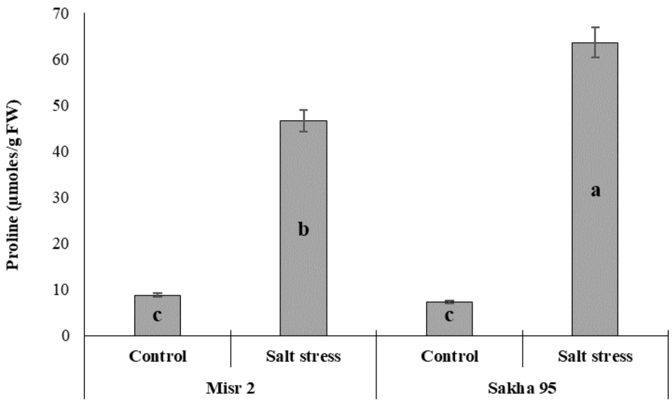

Figure 2. *Effects of salinity on proline ratio in the leaf of the wheat genotypes 'Sakha 95' and 'Misr 2' after 12 days of salinity stress (150 mM NaCl). The values of standard error (± SE) in each bar were calculated for each treatment for three replications. The same letter indicates no significant difference (p≤0.05) between each other*

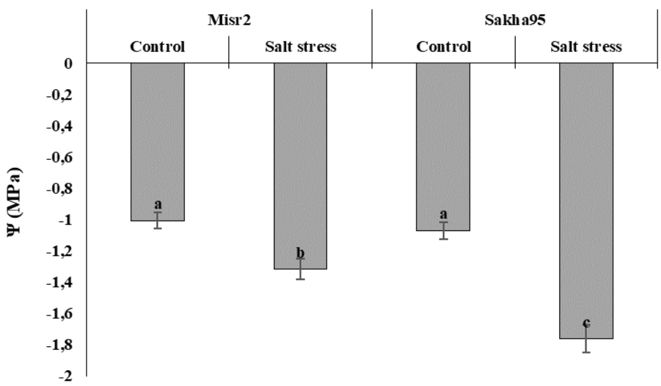

Figure 3. *Effects of soil salinity on osmotic potential (ψπ) in the leaf of the wheat genotypes 'Sakha 95' and 'Misr 2' after 12 days of salinity stress (150 mM NaCl). The values of standard error (± SE) in each bar were calculated for each treatment for three replications. The same letter indicates no significant difference (p≤0.05) between each other*

Effects of levels of salinity on Malondialdehyde concentration (MDA) of wheat genotypes

The MDA is a product of lipid peroxidation (Meloni et al., 2003), which is accumulated in plant parts under stress condition. High degree of MDA concentration in plant parts has a correlation with oxidative damage of plant cell membranes (Assaha et al., 2015). Therefore, it has been used to identify the grade of oxidative damage under

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stressful conditions including salt stress also (Gill and Tetuja, 2010). In the present study, MDA concentrations was increased significantly in the leaves of the genotype 'Sakha 95' than in 'Misr 2' under salinity treatment (*Fig. 4*). Whereas, in the roots of salinity-induced plants, the increment of MDA concentrations were insignificant in both the genotypes; however, numerically increment of MDA in genotype 'Sakha 95' was much higher than that in the roots of genotype 'Misr 2'. The results of the present study related to the oxidative damage of plant cells' membranes due to the higher production of MDA also confirmed by earlier studies (Demiral and Türkan, 2005; Koca et al., 2007), who found that a higher concentration of MDA in plant cells indicated that the plant/crop is susceptible to salinity, whereas a lower MDA concentration indicates protection of plants against oxidative stress. They also revealed that with a small concentration of MDA in plant cells is usually corresponding to an increase in antioxidant enzyme activities in plant tissues that lead to help the plant to survive under stress condition.

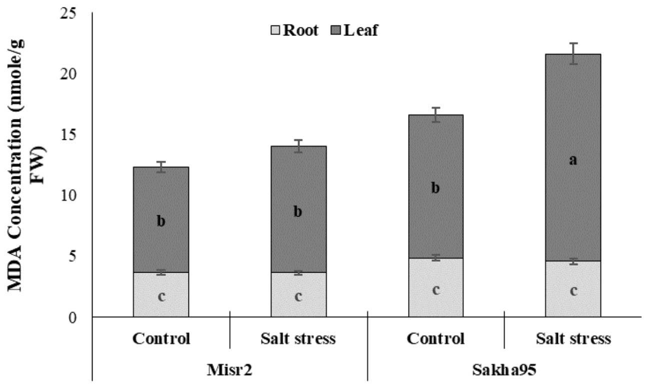

Figure 4. *Effects of soil salinity on MDA concentration in leaf and root of the wheat genotypes 'Sakha 95' and 'Misr 2' after 12 days of salinity stress (150 mM NaCl). The values of standard error (± SE) in each bar were calculated for each treatment for three replications. The same letter indicates no significant difference (p≤0.05) between each other*

Antioxidant enzymes activity under salinity stress in both genotypes

Antioxidants such as CAT, APX, POD and GR are excellent scavengers of stress-induced oxidative damage (ROS). Therefore, an increase of antioxidant enzymes activities in plant cells indicate the stress tolerant ability of the plant and have a correlation with the reduction of oxidative damage (Mittler, 2002). In our present study, the activity of all antioxidant enzymes such as CAT and POD were markedly increased in the leaf of the genotype 'Misr2' under salt stress (*Table 3*). The activities of the antioxidant enzymes CAT and POD were significantly elevated by salt treatment in the leaves of the genotype 'Misr 2', while reduced in leaves of the genotype 'Sakha 95' by salinity treatment. However, glutathione reductase (GR) activity did not alter in the leaf of 'Misr 2', while reduced in 'Sakha 95' by salinity treatment. The activities of all the enzymes (i.e., CAT, POD, and GR) were significantly reduced by salinity treatment in the roots of 'Sakha 95' than 'Misr 2' (*Table 3*). In the roots of 'Misr 2, salt stress significantly induced the activity of GR and CAT as compared to controls (*Table 3*). The results of the present study related to antioxidant enzymes activity during stress condition were also described by Apel and Hirt (2004), who noticed that there is a correlation between CAT, POD, and GR activity and salt tolerance. Mittler (2002), Meloni et al. (2003), Tammam et

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al. (2008) and Elsawy et al. (2018) reported that antioxidant enzymes such as CAT, APX, POD and GR are excellent scavengers against the oxidative damage (ROS) under stress condition including salt stress; who also revealed that an increased level of antioxidants enzyme activities in plant cells indicate the plant has the ability to reduce oxidative damage. Furthermore, during water-deficit, the enhancement of antioxidant enzymes activity is a response to the photosynthetic machinery against damage caused by ROS (Pirasteh Anosheh et al., 2012; Abdelaal et al., 2017).

Table 3. *Effects of salinity on POD (U/mg protein/min), CAT (U/mg protein/min) and GR (U/mg protein/min) in leaf and root of the wheat genotypes 'Sakha 95' and 'Misr 2' after 12 days of salinity stress (150 mM NaCl).The same letter indicates no significant difference ($p \leq 0.05$) between each other. Means followed by the same letter within each line are not significantly different ($p < 0.05$)*

Salt levels (mM NaCl)	Wheat Variety	POD (U/mg protein/min)		CAT (U/mg protein/min)		GR (U/mg protein/min)	
		Leaf	Root	Leaf	Root	Leaf	Root
0	Misr 2	0.067±0.003ab	0.040±0.004c	1.415±0.035ab	0.652±0.073cd	2.50±0.13e	3.39±0.20c
	Sakha 95	0.072±0.004a	0.030±0.004c	1.545±0.055ab	0.525±0.032de	3.02±0.19d	4.79±0.18e
150	Misr 2	0.077±0.004a	0.015±0.001d	1.655±0.042a	0.668±0.034c	2.44±0.19e	4.50±0.28ab
	Sakha 95	0.060±0.001b	0.018±0.002d	1.340±0.135b	0.503±0.019e	2.21±0.12e	3.96±0.11bc

Conclusion

From the results of the present study, it was found that both the genotypes were varied significantly for all traits under different levels of salinity, while the growth of the genotype 'Misr 2' was much better than that of 'Sakha 95', with the maintaining of higher dry biomass. The genotype 'Misr 2' also maintained a high concentrations of soluble-sugars and various antioxidant enzymes activity such as GR, POD and CAT compared with genotype 'Sakha 95'; whereas, maintained lower levels of lipid peroxidation represented by MDA concentration. Similarly, salinity stress also significantly changed in Ca^{++} concentration and Na^{+}/Ca^{++} ratio in both wheat genotypes. Among the genotypes, the relative reduction in Ca^{++} concentration and Na^{+}/Ca^{++} ratio was found the maximum in genotype 'Sakha 95' than in 'Misr 2' and lead to showing the signs injury. Thus, the genotype 'Misr 2' would be useful to develop a salinity tolerant wheat variety in the future breeding program. The above mentioned physiological and biochemical analyses may be useful as a model procedure for measuring the responses of other wheat genotypes to high-salinity in the field condition for development of salt tolerance genotypes in the future breeding program.

Conflict of interests. Authors declared no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50295041

TOXICOLOGICAL AND BIOLOGICAL EFFECT OF THREE CHEMICAL COMPOUNDS ON DIFFERENT HONEY BEE RACES (*APIS MELLIFERA* L.) (HYMENOPTERA: APIDAE)

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(Received 18th Dec 2018; accepted 20th Feb 2019)

Abstract. This study consisted of the chemical treatment on three races of forager honey bee workers in Sulaimani-Kurdistan region. The races included, *Apis mellifera carnica*, *Apis mellifera ligustica* and *Apis mellifera Native*. The TITAN 48% herbicide affected *Apis mellifera carnica* the most by ingestion, leading to 83.67% mortality after 48 h at 1.00 ppm, which increased to 100% at 10.00 ppm. *Apis mellifera Native* was most affected by the insecticide Goldti5 through feeding, which led to 50.00% mortality after 48 h at 1.0 ppm and rose to 100% at 10.00 ppm. TITAN 48% herbicide had the most severe effect on *Apis mellifera ligustica* through feeding, leading to almost 100% mortality in all concentrations after 48 h. Laboratory test indicated that fat cells showed a series of changes in the cytoplasm when exposed to the recommended dose. The granulated cytoplasm developed a few droplets while the nucleus remained unchanged.
Keywords: *honey bee, foragers, fat body, insecticides, herbicides*

Introduction

Chemical insecticides are important in crop productivity in intensive farming systems, maintaining about one and a half crop yields (Oerke and Dehne, 2004). Bohnenblust et al. (2016) found that nearly 80% of all pesticides applied to row crops are herbicides, and these applications pose potentially significant ecotoxicological risks to nontarget plants and associated pollinators. When used, pesticides can cause unintentional damage by killing useful insects, such as pollinators and natural enemies of crop pests, both within the target field (Croft, 1990) and beyond by drifting (De Jong et al., 2008). Most pesticides are used as sprays, but such herbicides and fungicides are usually used directly on the soil before planting crops. Krupke et al. (2012) demonstrated that bees are exposed to these compounds and several other agricultural pesticides in several ways throughout the foraging period. During spring, extremely high levels of clothianidin and thiamethoxam were found in planter exhaust material produced during the planting of treated maize seed. Growing concern about the impact of pesticides on pollinators is reflected in the vast literature on the subject of the last few years (Osborne, 2012). The sublethal impacts of GLY on non-target organisms such as insect pollinators have so far been poorly evaluated. In this study, we used sublethal concentrations of GLY ranging from 2.5 to 10 mg l^{-1} (Herbert et al., 2014; Thompson et al., 2014). Sol Balbuena et al. (2015) demonstrated that glyphosate (GLY) is a herbicide

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

that is widely used in agriculture for weed control. Although reports about the impact of GLY in snails, crustaceans and amphibians exist, few studies have investigated its sub-lethal effects in non-target organisms such as the honey bee *Apis mellifera*, the main pollen vector in commercial crops. Particular importance is the collapse of bee colonies (*Apis mellifera*) called Colony collapse disorder (CCD). Attempts to interpret the UNCCD have focused on biological diseases, including viruses (Cox-foster et al., 2007), nosema infection (Cameron et al., 2011), parasites such as mites (Thompson et al., 2003; Underwood and Engelsdrop, 2007) and beetles (Buczek (2009), including not only insecticides and corticosteroids but also fungicides and herbicides (Maini et al., 2010; Johnson et al., 2010).

Apart from pesticides used in agricultural production, honey bees are also exposed to the acaricides used to control varroa and other parasites. In this case, the bees interact with high residue levels on the waxy cells of the comb, which mainly affect the developing larvae, the adult honey bees and the queen (Martel et al., 2007; Zhu et al., 2014). Fat bodies are normally distributed throughout the body cavity of insects, especially in the abdomen, and appear as an irregular mass of a soft and usually white tissue composed of large loosely united cells. These cell masses are known collectively as fat body, because the cytoplasm of the cells contains small droplets of oily fat (Snodgrass and Erickson, 2003). The fat bodies can be irregularly distributed in the perivascular space of the abdomen and thorax, surrounding the organs (visceral fat body) or in the abdomen dorsal and ventral sinus, closed to the tegument (parietal fat body), in the head and even in the body appendixes (Chapman, 1978; Zanini and Caetano, 2003). Ayoub (2011) found that the average dimension of fat body cells in newly emerged workers is between 86.71-86.76 µm and in 10-days old workers the average dimension of fat body cells is between 89.15-89.95 µm while the average dimensions of fat body cells ranges between 67.33 and 69.05 µm. Fat body is the main storage agent of the metabolic device of insects and is responsible for the synthesis and supply of hemolymphs. Fat body is made up of cells of mesodermal origin, and sometimes contain epidermal cells (Oliveira and Cruz-Landim, 2003). Roma et al. (2010) demonstrated that fat body consists of a mass of cells under the epidermis, and in some insects, fat body also surrounds the digestive system and reproductive system.

The aim of this research was to shed light on the pesticides that were most commonly used in the study region to determine the sensitivity of the studied honey bee races (strains) to the lowest concentration of these pesticides, then determine the proportion of mortality and the histological properties of these strains under natural condition.

Materials and methods

Collection of forager honey bees was done at an apiary, and included three races of Honey bees: *Apis mellifera carnica*, *Apis mellifera ligustica*, *Apis mellifera* Native in April 2017. Ten foragers of each race were placed into an experimental box (30 X 30 X 30 cm) and fed with the sugar syrup that was treated with two types of insecticide and one type herbicide that are common used on the fields near the honey bee hives, with three replications. Four concentrations (1 ppm, 2.5 ppm, 5.00 ppm, 10.00 ppm) for the two types of insecticide were prepared as follows: 0.1 gm of insecticide was added to a volume of sucrose solution (10% W/V) which was then filled up to 1000 ml with the same solution to obtain 10 ppm of insecticides. Following this, the other three concentrations (5.00 ppm, 2.500 ppm and 1.00 ppm) of insecticides were directly

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DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

prepared from stock solution with sucrose solution (10% W/V) dilution. Additionally, the four concentrations of the herbicide were prepared as follows: the same procedure was used as in the preparation of the insecticide solutions. The mortality of the foragers were measured after 1, 2, 4, 6 and 24 hours, and the mortality percentage ratio has been re-evaluated using Abbott's (1925) formula.

Insecticides

In this experiment the forager honey bees were exposed to three different insecticides:

Goldti 5EC

The active ingredient is Lambda-cyhalothrin, a quick-acting pyrethroid insecticide effective after contact or ingestion (Dalaly et al., 2012). Lambda-cyhalothrin is a synthetic pyrethroid based structurally on natural pyrithrins. Lambda-cyhalothrin affects both the peripheral and central nervous system of the insect. It initially stimulates nerve cells to produce repetitive discharges and eventually cause paralysis. These sympthoms are caused by their effect on the sodium channel, a tiny hole through which sodium ions are permitted to enter the axon to cause excitation (Ware and Whitacre, 2004).

Chemical structure of Lambdacyhalothrin/chemical name: [1α(S), 3α (Z)]-($_-^+$)-cyano-(3-phenoxyphenyl) methyl-3-(2-chloro-3, 3, 3-trifluoro-1propenyl)-2, 2-dimethylcyclopropanecarboxylate.

Delta ride

The active ingredient is Acetamiprid, a systemic pesticide used to control many sucking insects, which acquired immunity against other pesticides, used on vegetables, fruit trees and other plants. The effect appears after a few minutes of spraying. Used in a concentration of (50) gm /100 litter of water, it was sprayed on and consumed by the forager honey bee workers using a hand sprayer (Dalaly et al., 2012).

Chemical structure of Acetamiprid/chemical name: (E)-N^1-[(6-chloro-3pyridyl) methyl]-N^2-cyano-N^1-methyl acetamidine.

TITAN 48%

The active ingredient is Paraquat or N, N′-dimethyl-4,4′-bipyridinium dichloride, an organic compound with the chemical formula $[(C_6H_7N)_2]Cl_2$. It is classified as a viologen, a family of redox-active heterocycles of similar structure. Paraquat was manufactured by Chevron (Dalaly and et al., 2012).

Chemical structure/Chemical name: 1, 1^1-dimethyle-4, 4-bipyridinium.

Concentration: Four different concentrations were prepared from each insecticide (Lambdacyhalothrin, Acetamiprid, Paraquat). The concentrations were chosen by preliminary range-finding tests (OECD, 1998b; Maus, et al., 2003; Tornier, et al., 2003). The concentrations used were as follows:

1. 1.00 ppm, 2.50 ppm, 5.00 ppm, and 10.00 ppm for (Lambdacyhalothrin, Paraquat) the active ingredients. These four concentration of (TITAN 48%, Goldti 5EC) were prepared as follows:

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DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

a. 0.4 ml of TITAN 48%/Goldti 5EC was added to a volume of sucrose solution (50% w/v), which was then filled up to 1000 ml by the same solution to obtain 10.0 ppm Lambdacyhalothrin/Paraquat for the ingestion experiment.

b. The three other concentration (5.00 ppm, 2.50 ppm, and 1.00 ppm of Lambdacyhalothrin and Paraquat) were directly prepared from the previously prepared solution of step (a) or (b), diluted with a sucrose solution (50% w/v) or distilled water for the ingestion and contact experiments, respectively.

2. 1.00 ppm, 2.50 ppm, 5.00 ppm, and 10.00 ppm for the Acetamiprid active ingredient were prepared as follows:

0.1 gm of Delta Ride was added to a volume of sucrose solution (10% W/V) which was then filled up to 1000 ml by the same solution to obtain 10 ppm of Acetamiprid, then the other three concentration of (5, 2.5 and 1 ppm) of acetamiprid were directly prepared from the stock solution by diluting it with sucrose solution (10% W/V).

Samples collected

Ten workers of each stage were taken for the measurements such as the dimensions of fat bodies and, using binocular dissection and a digital microscope. The ages of worker were recorded (one day, one week, two weeks and three weeks old).

Dissection

The workers were fixed on the dissecting tray after the removal of legs and wings, then immersed in physiological solution. These units were transferred to the dissecting microscope and placed upside down on the tray and fixed with pins. Workers were dissected using a sharp scalpel to create two lateral longitudinal cleavages of the abdomen after mounting the specimens on the dissecting tray with fine stainless pins (Cook and Meola, 1983; Mahmoud, 1991, 1992; Shamdin, 2003). The workers were kept in Physiological saline of 0.9% NaCl and 0.9 gm per 100 ml distilled water (Pantin, 1964). The fixed workers were dissected under 2x and 4x magnification. After cutting the cuticle and removing the muscles, the fat bodies were picked up and placed on a clean slide and were either stained with methylene blue in normal quantities, or prepared with a wet smear. Then photographs and measurements were taken with the eyepiece graticule.

Climatic information data was acquired from Sulaimani General Directorate Meteorology and Seismology.

The results were analyzed statistically using factorial RCBD design with triple replicates and performed using the XLSTA program (2016). Duncan's multiple range Test was used to determine the differences between means at P = 0.05

Results and discussion

The effects of insecticides applied orally to Apis mellifera carnica

Table 1, among others, showed the relation among insecticides, insecticide concentrations and exposure time (ABC). The lowest mortality rate was 1.00 after 1 h of treatment with a concentration of 2.50 ppm of TITAN 48% for *A. mellifera carnica*. However, the highest mortality rate was 5.333 after 1 h of treatment with a concentration of 100% of Goldti5 for *A. mellifera carnica*. While the lowest mortality rate was 2.667 after 2 h of treatment with 1.00 ppm of TITAN 48% and Goldti5 for *A.*

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mellifera carnica, the highest mortality rate was 8.667 after 2 h of treatment with 5.00 ppm of Goldti5 for adult *A. mellifera carnica*. The lowest mortality rate was 3.000 after 4 h of treatment with 1.00 ppm of Delta ride for the adult *A. mellifera carnica*. However, the highest mortality rate was 8.333 after 4 h of treatment with a concentration of 10.00 ppm of Delta ride for the adult *A. mellifera carnica*. Similarly, the lowest mortality rate of 3.333 was established after 6 h in the 1.00 ppm concentration. In addition, the highest mortality rate of 9.667 was observed in 10.00 ppm of Goldti5 after 6 h for adult *A. mellifera carnica*. The lowest mortality rate of 4.667 was established after 24 h in the 1.00 ppm for Delta ride, while the highest mortality rate of 10.00 was observed in 10.00 ppm of Delta Ride, Goldti5 and 2.50, 5.00, and 10.00 ppm concentrations of TITAN 48% after 24 h for adult *A. mellifera carnica*. As for the relation between insecticides and insecticide concentrations (AB), the lowest mortality rate was 2.466 at 1.00 ppm for Delta ride, the highest mortality rate was 8.200 at 10.00 ppm for Goldti5 in the case of adult *A. mellifera carnica*, in comparison with controls. Concerning the relation between insecticides and test periods (AC), the lowest mortality rate was 1.266 after 1 h for TITAN 48%, and the highest mortality rate was 7.000 after 24 h for TITAN 48% and Goldti5 in the case of adult *A. mellifera carnica*. As for the relation between insecticide concentrations and test periods (BC), the lowest mortality rate was 0.000 at a concentration of 1.00 ppm after 1 h, while the highest mortality rate was 10.000 at a concentration of 10.00 ppm after 24 h of testing, in comparison with controls. The relation between each insecticide (A) showed the lowest mortality rate to be 3.866 for Delta ride, while the highest mortality rate was 5.333 for Goldti5, at the significance level of $P < 0.05$ there was a significant difference among Delta ride, TITAN 48% and Goldti5. The relation between each insecticide concentration (B) showed the lowest mortality rate to be 3.511 at 1.00 ppm and the highest mortality rate was 7.510 at 10.00 ppm in the case of adult *A. mellifera carnica*, in comparison with control. As for the relation between each test period (C), the lowest mortality rate was 1.933 after 1 h, and the *A. mellifera carnica* mortality rate increased with time, up to 6.844 after 24 h.

Table 1. *Mean numbers of A. mellifera carnica mortality after being treated with various concentrations of insecticides through feeding*

Insecticide A	Concentration B	1 h	2 h	4 h	6 h	24 h	AB	A
Delta Ride	0.00	0.000 s	0.000 s	0.000 s	0.000 s	0.000 s	0.000	3.866c
	1.00 ppm	0.000 s	1.333 r	3.000 op	3.333 o	4.667 lm	2.466	
	2.50 ppm	2.333 pq	3.000 op	5.000 klm	4.667 lm	5.667 ijkl	4.133	
	5.00 ppm	3.333 o	4.667 lm	6.333 hij	5.667 ijkl	8.667 cd	5.733	
	10.00 ppm	4.667 lm	5.667 ijkl	7.333 efg	7.333 efg	10.000 a	7.000	
TITAN 48%	0.00	0.000 s	0.000 s	0.000 s	0.000 s	0.000 s	0.000	4.813b
	1.00 ppm	0.000 s	2.667 op	5.000 klm	6.000 ijk	8.667 cd	4.466	
	2.50 ppm	1.000 r	3.667 no	6.000 ijk	7.333 efg	10.000 a	5.600	
	5.00 ppm	1.667 qr	5.000 klm	7.667 ef	9.000 abc	10.000 a	6.666	
	10.00 ppm	3.667 no	5.667 ijkl	8.000 de	9.333 abc	10.000 a	7.333	

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DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

	0.00	0.000 s	0.000 s	0.000 s	0.000 s	0.000 s	0.000	
	1.00 ppm	0.000 s	2.667 op	3.667 no	5.000 klm	6.667 ghi	3.600	
Goldti 5Ec	2.50 ppm	2.667 op	5.667 ijkl	5.333 jklm	6.333 hij	8.333 de	5.666	5.013a
	5.00 ppm	4.333 mn	8.667 cd	7.000 fgh	8.000 de	10.000 a	7.600	
	10.00 ppm	5.333 jklm	7.667 ef	8.333 de	9.667 ab	10.000 a	8.200	
C		1.933	3.755	4.844	5.444	6.844	3	
	Delta Ride	2.066	2.933	4.333	4.200	5.800		
AC	TITAN 48%	1.266	3.400	5.333	6.333	7.733	B	
	Goldti 5	2.466	4.933	4.866	5.800	7.000		
	0.00	0.000	0.000	0.000	0.000	0.000	0	
	1.00 ppm	0.000	2.222	3.889	4.777	6.667	3.511	
BC	2.50 ppm	2.000	4.111	5.444	6.111	8.000	5.1332	
	5.00 ppm	3.111	6.111	7.000	7.555	9.555	6.6664	
	10.00 ppm	4.555	6.333	7.888	8.777	10.000	7.5106	

Also *Figure 1* pointed out the toxicity regression related to the *A. mellifera* carnica mortality

Effect of insecticides applied orally to Apis mellifera Native

Table 2 showed, that concerning the relation among insecticides, insecticides concentration and exposure time (ABC) the lowest mortality rate was 0.00 after 1 h of treatment with 1.00 ppm of TITAN 48% for *A. mellifera* Native. However, the highest mortality rate was 6.333 after 1 h of treatment with 10.00 ppm of Delta ride and Goldti5 insecticides on *A. mellifera* Native. The lowest mortality rate was 1.333 after 2 h of treatment with 1.00 ppm of Delta ride and TITAN 48% for the forager honey bee of *A. mellifera* Native, while the highest mortality rate was 7.333 after a 2 h treatment of 10.00 ppm of TITAN 48%. The lowest mortality rate was 2.333 after 4 h of treatment with 1.00 ppm of Delta ride and TITAN 48% insecticide for *A. mellifera* Native foragers. However, the highest mortality rate was 8.333 after 4 h of treatment with 10.00 ppm of Delta ride insecticide for the forager of *A. mellifera* Native. The lowest mortality rate was 3.333 after 6 h of treatment with 1.00 ppm of Delta ride and TITAN 48% for *A. mellifera* Native foragers. The highest mortality rate of 9.333 was found after 6 h of 10.00 ppm TITAN 48% insecticide treatment. The minimum mortality rate was 4.333 after 24 h at 1.00 ppm of Delta ride and TITAN 48% for *A. mellifera* Native foragers. However, the highest mortality rate was 10.000 after 24 h of treatment with 10.00 ppm of Delta ride, TITAN 48% and Goldti5 for foragers of *A. mellifera* Native. The relation among insecticides and insecticides concentration (AB) showed the lowest mortality rate to be 2.266 at a concentration of 1.00 ppm of TITAN 48%, and the highest mortality rate to be 7.933 at a concentration of 10.00 ppm of Delta ride for *A. mellifera* Native foragers, in comparison with controls. The relation between insecticides and tested periods (AC) showed the lowest mortality rate to be 1.6666 after 1 h of TITAN 48% insecticide treatment, while the highest mortality rate was 6.066 after 24 h of Delta ride and Goldti5 treatments for the foragers of *A. mellifera* Native. As for the relation between insecticide concentrations and test periods (BC), the lowest mortality rate was 0.444 at 1.00 ppm after 1 h, while the highest mortality rate was

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DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

10.000 at 10.00 ppm after 24 h of testing, in comparison with controls. The relation between each insecticides (A) showed the lowest mortality rate to be 3.799 for TITAN 48%, while the highest mortality rate were 4.506 for Delta ride, and at the significance level of P < 0.05 there was significant difference between Delta ride, TITAN 48% and Goldti5. The relation between each insecticide concentration (B) showed the lowest mortality rate to be 2.532 at 1.00 ppm and the highest mortality rate to be 7.799 at 10.00 ppm, for the foragers of *A. mellifera* Native, in comparison with control. The relation between each tested period (C), showed the lowest mortality rate to be 2.466 after 1 h, and that the *A. mellifera* Native mortality rate increased with time, up to 5.955 after 24 h.

Table 2. *Mean of numbers of A. mellifera hybrid mortality after being treated with different concentration of insecticides through feeding*

Insecticide A	Concentration B	1 h	2 h	4 h	6 h	24 h	AB	A
Delta Ride	0.00	0.000 x	0.000 x	0.000 x	0.000 x	0.000 x	0.000	4.506
	1.00 ppm	0.333 wx	1.333 uvw	2.333 stu	3.333 pqrs	4.333 mnop	2.333	
	2.50 ppm	3.000 qrst	4.333 mnop	5.333 jklm	6.333 ghij	7.333 defg	5.266	
	5.00 ppm	4.667 lmno	5.667 ijkl	6.667 fghi	7.667 cdef	8.667 bc	6.667	
	10.00 ppm	6.333 ghij	7.333 defg	8.333 bcd	9.333 ab	10.000 a	8.266	
TITAN 48%	0.00	0.000 x	0.000 x	0.000 x	0.000 x	0.000 x	0.000	3.799
	1.00 ppm	0.000 x	1.333 uvw	2.333 stu	3.333 pqrs	4.333 mnop	2.266	
	2.50 ppm	1.333 uvw	2.333 stu	4.000 nopq	5.000 klmn	6.000 hijk	3.733	
	5.00 ppm	2.667 rst	4.333 mnop	6.333 ghij	7.333 defg	8.333 bcd	5.799	
	10.00 ppm	4.333 mnop	6.000 hijk	7.333 defg	8.333 bcd	10.000 a	7.199	
Goldti 5Ec	0.00	0.000 x	0.000 x	0.000 x	0.000 x	0.000 x	0.000	4.453
	1.00 ppm	1.000 vwx	2.000 tuv	3.000 qrst	4.000 nopq	5.000 klmn	3.000	
	2.50 ppm	2.667 rst	3.667 opqr	4.667 lmno	5.667 ijkl	6.667 fghi	4.667	
	5.00 ppm	4.667 lmno	5.667 ijkl	6.667 fghi	7.667 cdef	8.667 bc	6.667	
	10.00 ppm	6.000 hijk	7.000 efgh	8.000 cde	8.667 bc	10.000 a	7.933	
C		2.466	3.399	4.333	5.111	5.955		
AC	Delta Ride	2.866	3.733	4.533	5.333	6.066	B	
	TITAN 48%	1.6666	2.7998	3.9998	4.799	5.733		
	Goldti 5	2.8668	3.6668	4.4668	5.200	6.066		
BC	0.00	0.000	0.000	0.000	0.000	0.000	0	
	1.00 ppm	0.444	1.555	2.555	3.555	4.555	2.532	
	2.50 ppm	2.333	3.444	4.666	5.666	6.666	4.555	
	5.00 ppm	4.000	5.222	6.555	7.555	8.555	6.377	
	10.00 ppm	5.555	6.777	7.888	8.777	10.000	7.799	

Figure 2 pointed out the toxicity regression and the mortality of *A. mellifera* Native

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The effects of insecticides applied orally to A. mellifera ligustica

In *Table 3* we can see the relation among insecticides, insecticides concentration and exposure time (ABC) among others. The minimum mortality rate was shown to be 0.000 after 1 h of treatment with 1.00 ppm of Delta ride and TITAN 48% for *A. mellifera ligustica*. However, the highest mortality rate was 8.000 after 1 h of treatment with 10.00 ppm of Goldti5 insecticides for adult *A. mellifera ligustica*. The lowest mortality rate was 0.333 after 2 h of treatment with the 1.00 ppm of Delta ride for the foragers of *A. mellifera ligustica*, while the highest mortality rate was 9.333 after a 2 h treatment with 10.00 ppm of Goldti5 insecticide. The lowest mortality rate was 1.667 after 4 h of treatment with 1.00 ppm of Delta ride for *A. mellifera ligustica* foragers. However, the highest mortality rate was 9.667 after 4 h of treatment with 10.00 ppm of Goldti5 insecticide for the foragers of *A. mellifera ligustica*. The lowest mortality rate was 3.667 after 6 h of treatment with 1.00 ppm concentration Delta ride for *A. mellifera ligustica* foragers. The highest mortality rate of 10.000 after 6 h was found in the 10.00 ppm Goldti5 treatment. The lowest mortality rate was 5.333 after 24 h at 1.00 ppm of Delta ride insecticide, as determined for the foragers of *A. mellifera ligustica*. However, the highest mortality rate was 10.000 after 24 h of treatment with 10.00 ppm of Delta ride and Goldti5, and also with 1.000, 2.500, 5.000, and 10.00 ppm of TITAN 48%, in addition to 5.000 ppm of Goldti5 for *A. mellifera ligustica* foragers. The relation between insecticides and insecticides concentration (AB) showed the lowest mortality rate to be 2.200 at 1.00 ppm of Delta ride insecticide, while the highest mortality rate of 9.400 was found under 10.00 ppm of Goldti5 for *A. mellifera ligustca* foragers, in comparison with controls. The relation between insecticides and testing time (AC) showed the lowest mortality rate to be 0.466 after 1 h of TITAN 48% insecticide, and the highest mortality rate to be 5.613 after 24 h of Goldti5 for the foragers of *A. mellifera ligustica*. As for the relation between insecticide concentrations and test periods (BC), the lowest mortality rate was 0.222 at 1.00 ppm after 1 h, while the highest mortality rate was 7.777 at 10.00 ppm after 24 h of testing, in comparison with controls. As for the relation between each insecticide (A) the lowest mortality rate appeared to be 3.706 for Delta ride, while the highest mortality rate was 5.613 for Goldti5, and at the significance level of $P < 0.05$ there was a significant difference between Delta ride, TITAN 48% and Goldti5. The relation between each insecticide concentration (B) showed the lowest mortality rate to be 3.177 at 1.00 ppm and the highest mortality rate to be 7.777 at 10.00 ppm, for *A. mellifera ligustica* foragers, in comparison with controls. As for the relation between testing times (C), the lowest mortality rate was 1.755 after 1 h, and the adult *A. mellifera ligustica* mortality rate increased with time, up to 7.377 after 24 h. The increase in exposure time and concentration of pesticide caused increase in the mortality of honeybees. The exposure of *A. mellifera* Native to Delta Ride at a concentration of 2.5 ml /a gallon of water for 24 h after feeding caused a 23.00% mortality.

Figures 1, 2 and *3* illustrate the LC50 values, and the effect comparison of TITAN 40%, Delta ride and Goldti5 on *A. mellifera* Native. TITAN 40% had an LC50 value of 1.70 ml/gallon after 24 h of exposure after feeding. While Delta ride and Goldti5 had values of 1.45 and 1.37 respectively. In *Figures 4, 5* and *6* the result showed that, based on LC50 values, a Goldti5 insecticidal deposit was 1.75 times more effective than that of Delta ride after 24 h of exposure for *A. mellifera carnica*, and that Delta Ride insecticidal deposits were 1.63 times more effective than those of TITAN 48%. *Figures 7, 8, 9* show these effects on *A. mellifera ligustica*. Delta Ride insecticidal deposits were

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1.17 times more effective than those of TITAN 48% after 24 h of exposure, while TITAN 48% insecticidal deposits were 0.50 times more effective than those of Goldti5.

Table 3. *Mean numbers of A. mellifera ligustica mortality subsequently to being treated with different concentration of insecticides orally*

Insecticide A	Concentration B	I						
		1 h	**2 h**	**4 h**	**6 h**	**24 h**	**AB**	**A**
Delta Ride	0.00	0.000 u	0.000 u	0.000 u	0.000 u	0.000 u	0.000	3.706
	1.00 ppm	0.000 u	0.333 tu	1.667 pqrstu	3.667 klmno	5.333 hijk	2.200	
	2.50 ppm	0.333 tu	2.667 mnopq	4.333 jklm	5.667 ghij	7.333 cdefg	4.066	
	5.00 ppm	1.000 rstu	3.000 mnopq	5.000 ijkl	8.000 bcdef	9.000 abc	5.200	
	10.00 ppm	3.333 lmnop	5.000 ijkl	6.667 efghi	8.333 abcde	10.000 a	6.666	
TITAN 48%	0.00	0.000 u	0.000 u	0.000 u	0.000 u	0.000 u qrstu	0.000	4.466
	1.00 ppm	0.000 u	0.667 stu	3.667 klmno	6.333 fghi	10.000 a	4.133	
	2.50 ppm	0.333 tu	2.000 opqrst	3.667 klmno	8.000 bcdef	10.000 a	4.800	
	5.00 ppm	0.667 stu	3.667 klmno	5.667 ghij	8.667 abcd	10.000 a	5.733	
	10.00 ppm	1.333 qrstu	7.000 defgh	8.000 bcdef	10.000 a	10.000 a	7.266	
Goldti 5Ec	0.00	0.000 u	0.000 u	0.000 u	0.000 u	0.000 u	0.000	5.613
	1.00 ppm	0.667 stu	0.667 stu	2.333 nopqrs	5.667 ghij	6.667 efghi	3.200	
	2.50 ppm	4.000 jklm	5.667 ghij	5.667 ghij	8.333 abcde	8.333 abcde	6.400	
	5.00 ppm	6.667 efghi	8.000 bcdef	9.000 abc	9.667 ab	10.000 a	8.666	
	10.00 ppm	8.000 bcdef	9.333 ab	9.667 ab	10.000 a	10.000 a	9.400	
C		1.755	3.428	4.422	6.222	7.377		4.640
AC	Delta Ride	0.933	2.750	3.600	5.200	6.599	B	4.612
	TITAN 48%	0.466	2.666	4.266	6.666	8.266		4.612
	Goldti 5	3.866	4.733	5.400	6.800	7.266		4.612
BC	0.00	0.000	0.000	0.000	0.000	0.000	0.000	4.612
	1.00 ppm	0.222	0.555	2.555	5.222	7.333	3.177	4.612
	2.50 ppm	1.555	3.444	4.555	7.333	8.555	5.088	4.612
	5.00 ppm	2.778	4.889	6.555	8.778	9.666	6.533	4.612
	10.00 ppm	4.222	7.111	8.111	9.444	10.000	7.777	4.612

Propit line in *Figure 3* indicated to the toxicity of the pesticide used on *A. mellifera ligustica*

Fat bodies

Generally, the first change in the perivisceral fat cells was the development of small vacuoles in the cytoplasm. This occurred in all races of honey bee workers in the control experiment (*Fig. 10*). The fat cells showed a series of changes in the cytoplasm when exposed to the recommended dose. The granulated cytoplasm developed a few droplets while the nucleus remained unchanged. In a few cells the granulated cytoplasm accumulated around the central nucleus. Increasing the dose to 10 ppm caused more alterations in cell features. The round cells developed scattered inclusions and their nucleus disintegrated. The fat bodies showed a few changes in their features with droplets forming throughout the cytoplasm. The cytoplasm became slightly more granular than normal.

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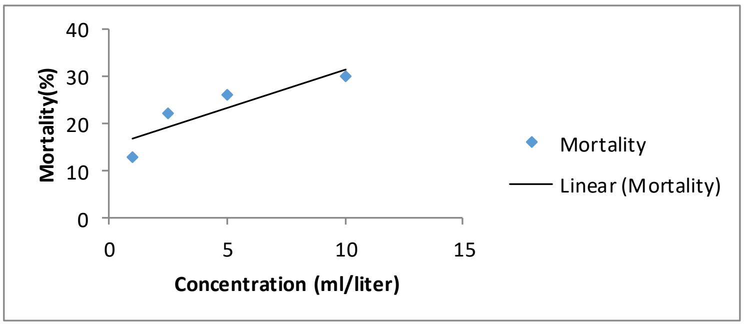

***Figure 1.** Effect of pesticides on A. mellifera native LC50 1.45 of Delta Ride feeding*

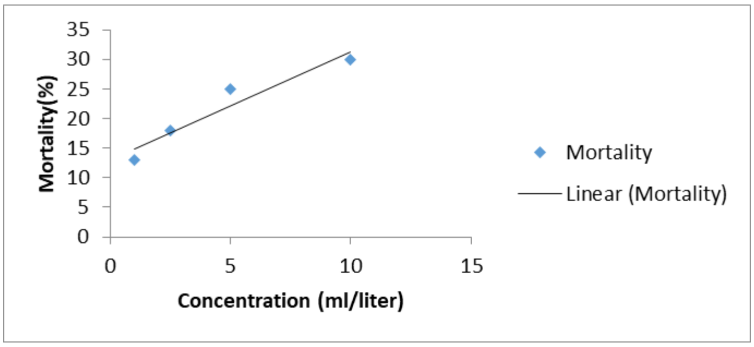

***Figure 2.** Effect of pesticides on A. mellifera native LC50 1.70 of Titan 48% feeding*

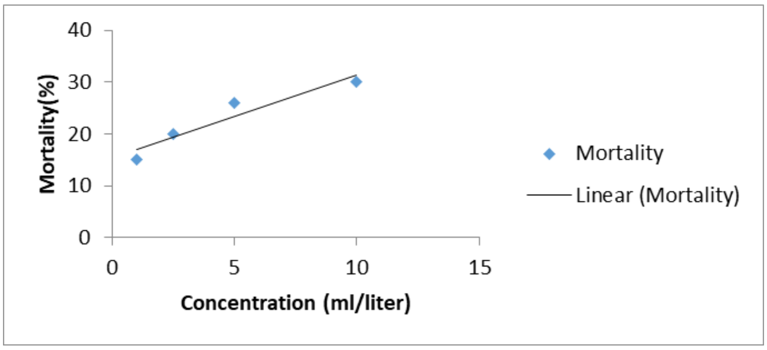

***Figure 3.** Effect of pesticides on A. mellifera native LC50 1.37 of Goldti 5 feeding*

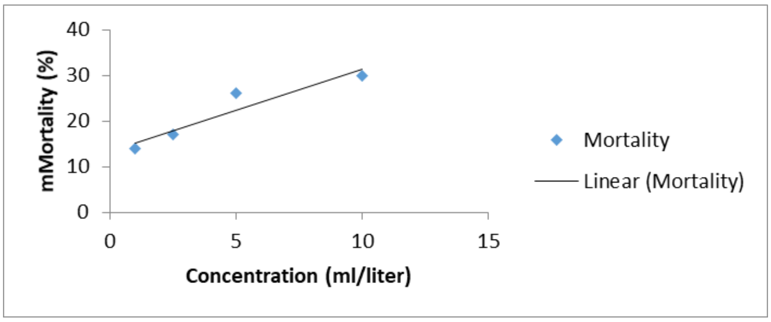

***Figure 4.** Effect of pesticides on A. mellifera carnica LC50 1.63 of Delta Ride feeding*

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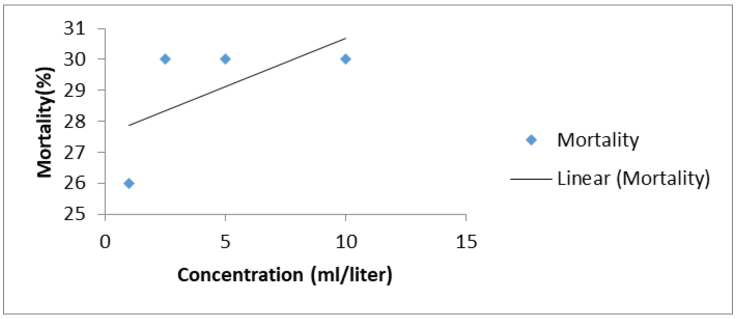

Figure 5. *Effect of pesticides on A. mellifera carnica LC50 0.483 of Titan 48% feeding*

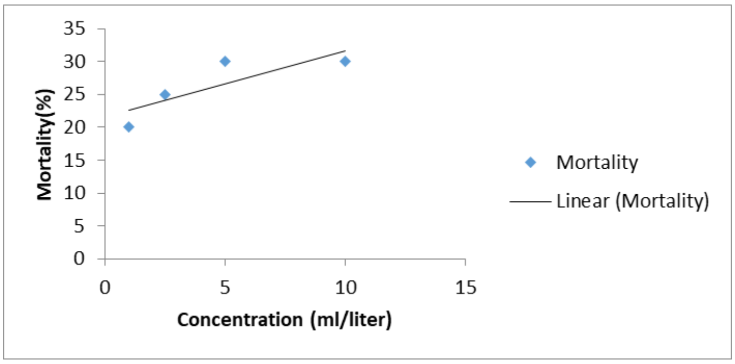

Figure 6. *Effect of pesticides on A. mellifera carnica LC50 1.75 of Goldti 5 feeding*

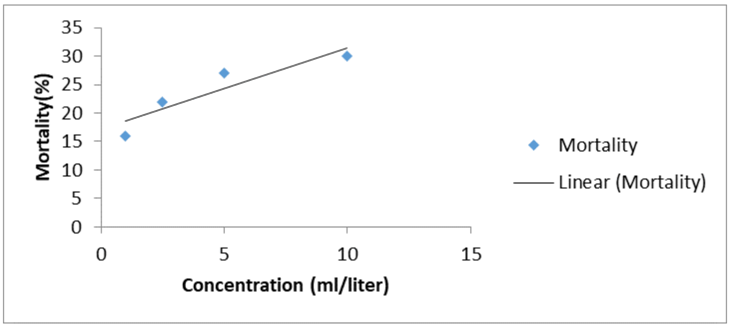

Figure 7. *Effect of pesticides on A. mellifera ligustica LC50 1.17 of Delta Ride feeding*

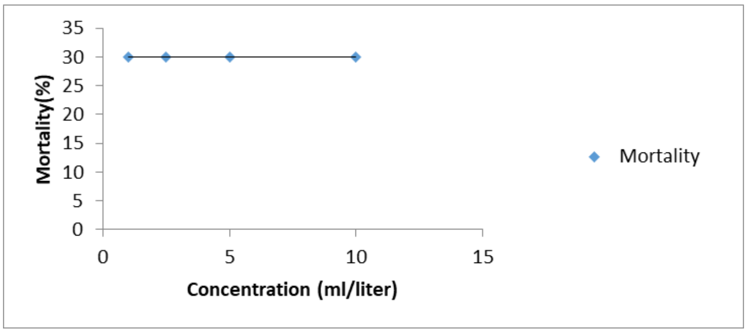

Figure 8. *Effect of pesticides on A. mellifera ligustica LC50 0.50 of Titan 48% feeding*

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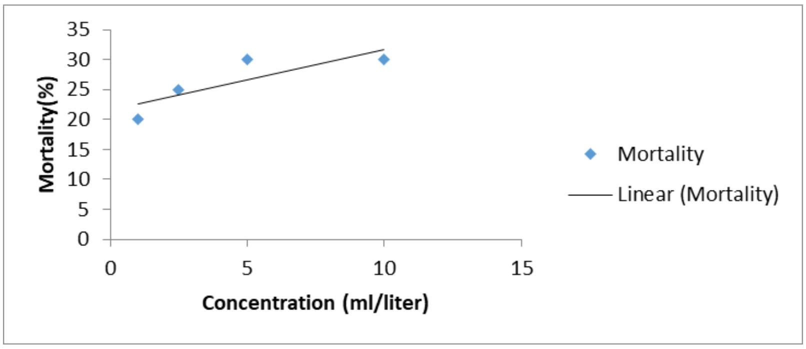

Figure 9. *Effect of pesticides on A. mellifera ligustica LC50 0.437 of Goldti 5 feeding*

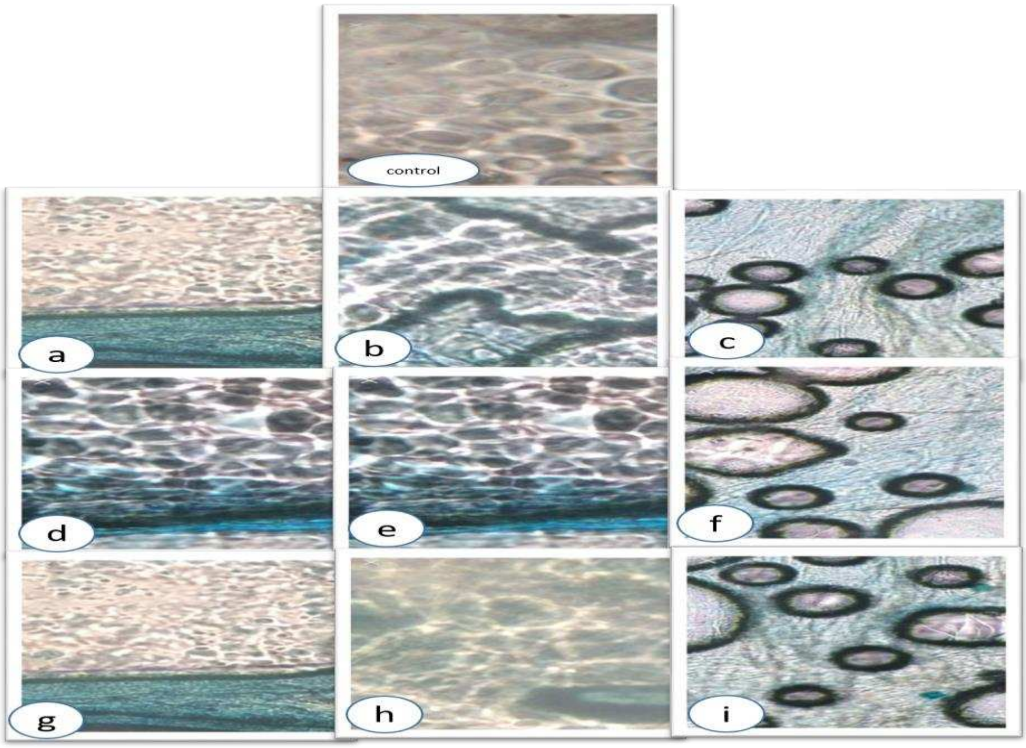

Figure 10. *Effect of pesticides on fat bodies of three races of honeybee workers: A. mellifera carnica, A. mellifera ligustica, A. mellifera Native*

The results are in agreement with Pham-Delègue et al. (2002), who found that the effect of insecticides on the direction sense of bees (reproductive ability), their communicative ability (bee dance language), and their foraging activity (olfactory learning performance) are affected by the LD50 of insecticides, and also agrees with EPPO (1993) and Thompson (2003) who found that the insecticides affect the bee larvae, division of labor, foraging, as well as the development of bee colonies while exposed to a lower lethal dose. The results are also in agreement with Bohnenblust et al. (2016), who found that nearly 80% of all pesticides applied to row crops are herbicides, and these applications pose potentially significant ecotoxicological risks to non-target plants and associated pollinators. The results similarly agree with Krupke et al. (2012), who found that bees are exposed to these compounds and several other agricultural

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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pesticides in several ways throughout the foraging period. During spring, extremely high levels of clothianidin and thiamethoxam were found in planter exhaust material produced during the planting of treated maize seeds.

Conclusion

Laboratory tests of oral ingestion application of different pesticides to susceptible honeybee foragers revealed that the most effective herbicide affecting *Apis mellifera carnica* was TITAN 48%, and that foragers are susceptible to toxicities of various pesticides (insecticides, herbicides) including pesticides not normally thought to affect them, generally having negative effects on fat bodes.

Acknowledgements. This manuscript is part of the PhD Thesis of the first author.

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ELECTRONIC APPENDICES

ANOVA tables

1. *Apis mellifera carnica*-feeding
2. *Apis mellifera ligustica*-feeding
3. *Apis mellifera* Native-feeding

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DOI: http://dx.doi.org/10.15666/aeer/1702_50435057

APPLICATION OF A GROUNDWATER MODELLING SYSTEM IN GROUNDWATER ENVIRONMENTAL IMPACT ASSESSMENT OF RIVER AND LAKE CONNECTION IN WESTERN JILIN REGION

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. Whereas groundwater numerical simulation is an essential part of groundwater environmental impact analysis, the authors propose a more accurate and suitable method for identifying and predicting the groundwater environment, laying a scientific basis for groundwater environmental impact evaluation. Targeted at the river and lake connection project in Jilin Province, this paper establishes a groundwater prediction model based on the groundwater modelling system (GMS), and considers the land salinization risk posed by the variation in groundwater level after the water diversion of the project. The results show that the depth of phreatic water in each area is greater than the upper limit of ecological water level after the implementation of the project plan, leaving a small chance for soil salinization.
Keywords: *groundwater modelling system, groundwater environmental, impact prediction, numerical simulation, soil salinization risk, water diversion project*

Introduction

The groundwater numerical simulation has been extensively applied to groundwater environmental impact analysis (Myles, 1970; Pradeep K., 2008; Li et al., 2014a). There are mainly two equivalently effective methods for numerical simulation of groundwater flow: the finite difference method (FDM) and the finite element method (FEM) (Liang, 2014). Featuring simpler method, faster speed and higher precision, the FDM, coupled with groundwater modeling system (GMS) (Environmental modelling research laboratory, 2005), was adopted to build up a model for ground water environment impact in this research.

As an enhancement of the hydraulic connection between different waterbodies, the river and lake connection system provides a strong guarantee to the smoothness of the water cycle (Li et al., 2014b). Taking the river and lake connection project in Jilin Province (Li et al., 2014c) as an example, the groundwater level was elevated to a certain extent through the diversion of groundwater to the study area. Therefore, the groundwater can be treated as an indicator in environmental impact assessment. With the aid of groundwater simulation technology, this paper predicts the variation in groundwater level before and after the leakage recharge, and analyzes the effect of the recharge on groundwater environment in light of the water level and quality, aiming to lay a scientific basis for preparation of groundwater environmental protection measures (Chao, 2008; Vishnu and Mukand, 2011).

The study area covers 9 cities and countries in western Jilin Province (*Fig. 1*). According to regional topographical and hydrogeological conditions, the western plains

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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region in the province was selected as the simulation area, and evaluated through the analytical method and analogy analysis (Martin, 1990).

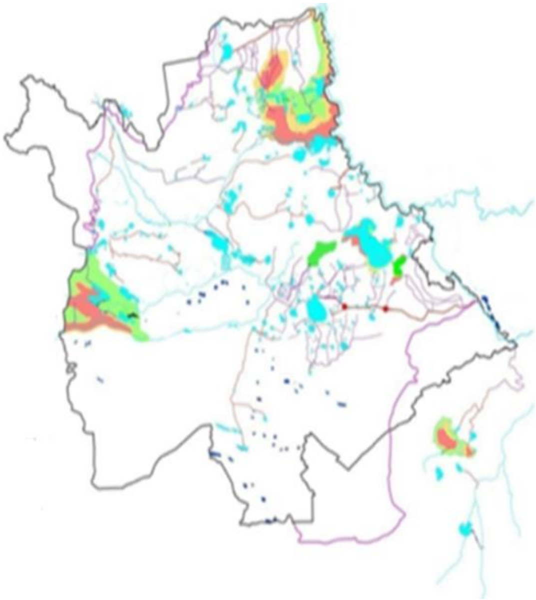

Figure 1. *Jilin Lakes connection scope of groundwater numerical simulation diagram*

Materials and methods

Conceptual hydro geological model

The lithology and thickness of the simulated area are different in the area, whether it is diving aquifer or confined aquifer. Therefore, the two computational aquifers are generalized as heterogeneous isotropic aquifers. The natural hydraulic gradient of the aquifer is very small and most of the areas are less than 1 ‰. The seepage is basically in accordance with the Darcy’s law. The groundwater flow is limited by the accuracy of the data and is treated as a planar two-dimensional flow. The elements change over time and are generalized as unsteady.

No matter the aquifer is confined or not, the lithology and thickness in the study area are subject to different degrees of changes. Hence, both confined and unconfined aquifers are generalized as heterogeneous but isotropic aquifers. In nature, the hydraulic gradient for groundwater flow in aquifer is extremely small, which is less than 1‰ in most areas. Thus, the seepage basically conforms to the Darcy’s law. Due to the limitations on data accuracy, the groundwater flow was treated as a 2D planar flow (Chao, 2011; Bai, 2006). Besides, it was treated as an unsteady flow because each element of the flow varies with time.

For the unconfined acquirer, the eastern rivers (the Nengjiang River and the Second Songhua River) were conceptualized as the river boundaries of groundwater in the simulation area. In southeastern and southern parts, the groundwater watershed of eastern upland plain was treated as the zero flow boundaries; the northwestern part of the simulation area was bordered by impermeable boundaries, for the bedrock of the surrounding hills were mainly volcanic rocks; In other sections in the western part of the simulation area, the boundaries were taken as the second flow boundaries; the roof

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was the water exchange boundary, and the floor was an impermeable boundary in single-layer aquifer area, and leakage boundary in double-layer aquifer area.

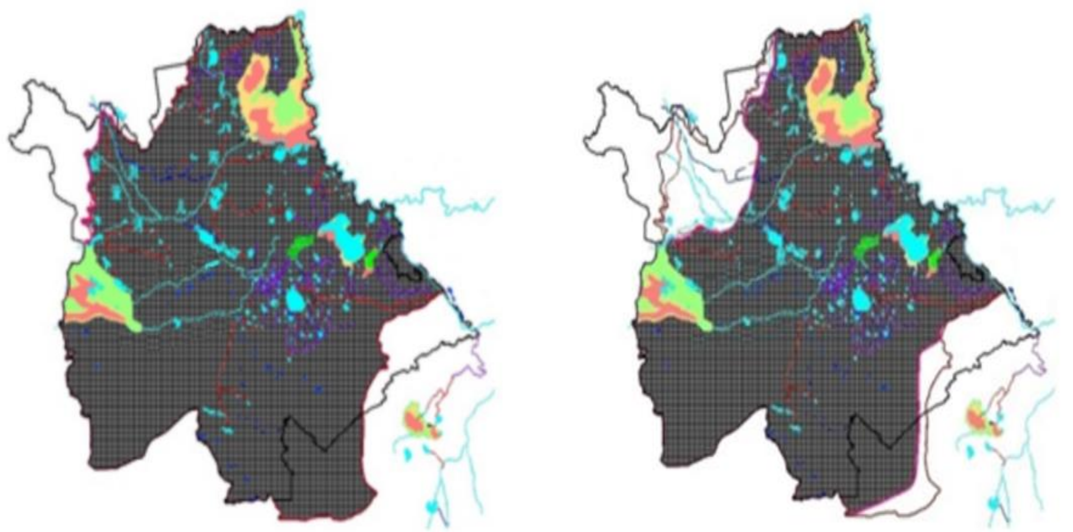
For the confined aquifer, in the southern part of the simulation area, the groundwater watershed was treated as the zero flow boundaries; the roof was a leakage boundary, as it was a layer of weak permeable soil, allowing the confined water to exchange with the upper phreatic water through the soil layer; the roof of the confined aquifer was generalized as an impermeable boundary, because the layer was formed up with extremely poor permeable tertiary mudstones.

Note: According to the characteristics of the regional groundwater flow field, zero flow boundaries refer to the boundaries perpendicular to water table contour lines.

Numerical simulation

Simulation context

The groundwater flow in the study area was simulated by a non-homogeneous, isotropic, quasi 3D model. The leakage amount was used to couple up the pore groundwater with porous confined water, upper pore-confined water and lower pore-confined water. The mathematical model was discretized by the GMS, the data interpolation was completed using the Kriging method, and the model was solved with the strongly implicit method. The 37,562 km^2 unconfined aquifer was divided into rectangles. In total, the area was split into 36,675 grid cells in 238 columns and 258 rows. Each cell was 1,000 m long, and 1,000 m wide, covering an area of 1 km^2. The meshing of the unconfined aquifer is depicted in *Figure 2a*. Total confined aquifer simulation area is 32,410 km^2, split it rectangle, it is divided into 238, 257 rows, altogether of grid cells, each cell is 1000 m long, 1000 m wide, with an area of 1 km^2. Confined aquifer model grid shown schematically in *Figure 2b*. The model was identified and verified based on the long observation data from Jan. to Dec. 2009 and from Jan. to Dec. 2012. The stress period was set to 1 month with a time step of 6 days. Consequently, the whole simulation period was divided into 36 stress periods, each of which was subdivided into 5 time periods.



a *b*

***Figure 2. a** Unconfined aquifer grid subdivision schemes. **b** Confined aquifer grid subdivision schemes*

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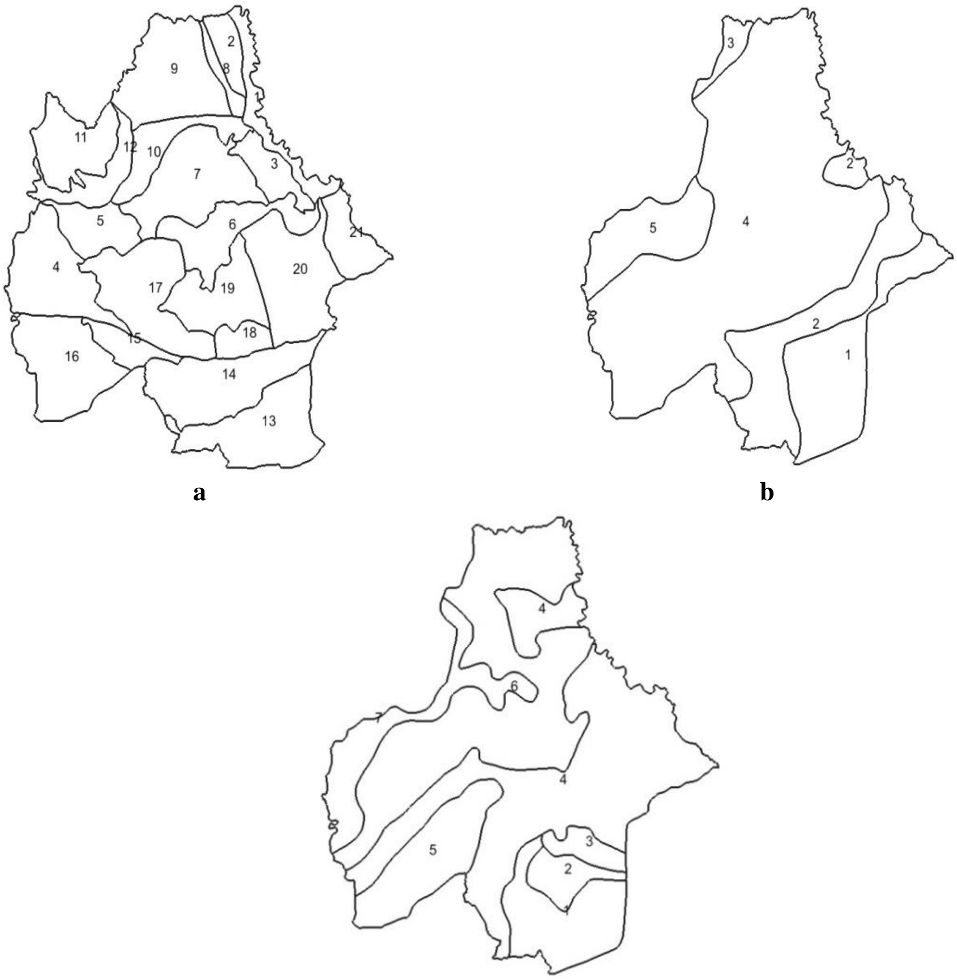
http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50595066

According to the difference in topographical and hydrogeological conditions (e.g. aquifer lithology and thickness), the unconfined aquifer was classified into 21 parameter partitions, the leakage layer was divided into 5 parameter partitions, and the confined aquifer was split into 7 parameter partitions (*Fig. 3a-c*).



a **b**

c

***Figure 3. a** Unconfined aquifer hydrogeological parameter zoning map. **b** The flow layer hydrogeological parameter zoning map. **c** The confined aquifer hydrogeological parameter zoning map*

Model identification

The model was identified with the long observation data of groundwater from Jan. to Dec. 2009, which was provided by the water conservancy department of Jilin Province. The data were selected because the source sink terms of groundwater in 2009, a dry year, have little interference in model operation. Since the project is still in the planning phase, the information of the source sink terms is not accurate enough to fit the long-time series water levels at the observation points. Hence, groundwater flow field in the wet and dry periods was employed for data fitting in the simulation. The model was deemed as satisfying the accuracy requirements when the calculated flow field was in good agreement with the measured flow field.

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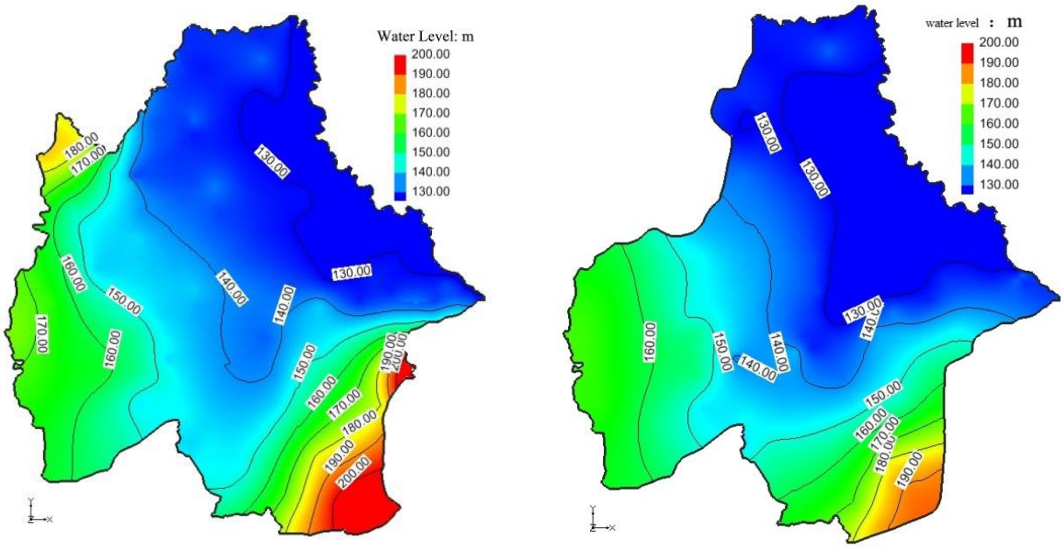
DOI: http://dx.doi.org/10.15666/aeer/1702_50595066

The source sink terms include rainfall infiltration recharge, the side run-off recharge, irrigation infiltration recharge, evaporation discharge of phreatic water, artificial exploitation amount, and river course seepage and discharge. The initial groundwater level was identified through processing these terms. According to the water levels monitored in 129 phreatic water monitoring wells and 107 confined water level monitoring wells in and around the simulation area, the initial flow fields of confined water and phreatic water in the identification period were acquired through the interpolation via the Kriging method (Mazzia and Puttii, 2002) in the GMS software (*Fig. 4a* and *b*).



a b

Figure 4. a *Identification phreatic groundwater the initial flow chart.* ***b*** *"Zero" forecast period phreatic groundwater the initial flow*

Results and discussion

After the source sink terms were inputted, the model was run to the end of the simulation period to get the calculated water level. With all things being considered, the groundwater level in the unconfined aquifer was basically stable or slightly declining under the Plan Zero, leaving little chance for soil salinization.

Based on the groundwater flow field prediction under the Plan Zero, the groundwater flow field was predicted considering the additional leakage recharge resulted from the implementation of the river and lake connection plan, and compared with the simulated results under the Plan Zero. The main source sink terms under the connection plan were consistent with those under the Plan Zero. Based on the mean annual calculated results, the additional leakage recharge was assigned to the model in light of the specific engineering plan. The simulation period lasted from Jan. to Dec. 2013. The stress period was set to 1 month with a time step of 6 days. Consequently, the whole simulation period was divided into 216 stress periods, each of which was subdivided into 3 time periods. As the model prediction plan was the same to the Plan Zero from 2013 to 2020, the authors mainly compared the variation in groundwater flow field in 2030 after the project implementation (*Fig. 5*). As shown in the prediction of groundwater flow field, the groundwater flow field under the project plan was basically the same with that under

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the Plan Zero; the piedmont in the western part and the groundwater shed in the southeastern part still had relatively high groundwater level and large hydraulic slope; the groundwater generally flew from the southwest to the northeast, mainly through the channels of the Nengjiang River and the Second Songhua River.

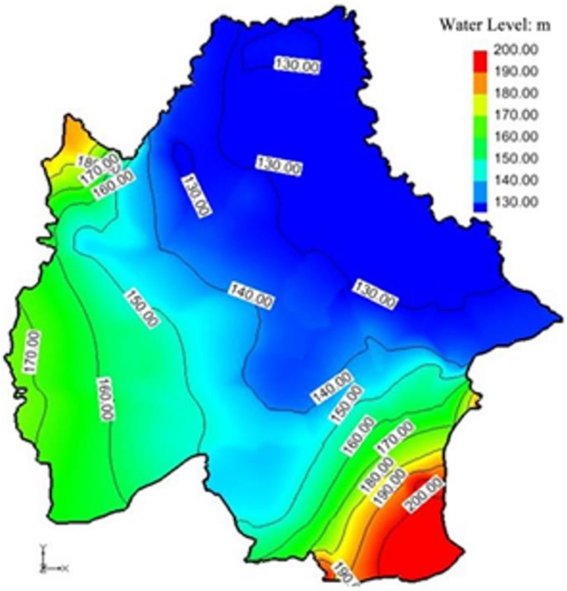

Figure 5. *Predicted flow field of unconfined groundwater*

According to the model prediction results under the connection plan, the regional groundwater level after the project implementation was higher than that in Plan Zero thanks to the increase in leakage recharge. Under the joint effect of growing groundwater exploitation and other factors, the phreatic water level continued to decline in 2013-2030 in core plate A; the groundwater level in core plate B kept falling in 2013-2020, but rebounded in 2021-2030 to a level lower than the initial level; the phreatic water level in core plate C and other edge plates remained stable in 2013-2030. *Table 1* displays the variation in phreatic water level of each area after implementing the connection plan, and compares the level with the ecological water level.

Table 1. *Plan after the implementation of the change of regional groundwater level and ecological water level of contrast*

Area	**2013-2030 Range of water level (m)**	**2013-2030 Fluctuation rate (m/a)**
A core plate (50, 136)	-1.8	-0.1
B core plate (113, 176)	-0.9	-0.05
C core plate (144, 31)	0.3	0.017
Others (211, 108)	0.1	0.006
Area	Water depth (m)	Ecological water level (m)
A core plate (50, 136)	5.8-7.8	1.88-5.17
B core plate (113, 176)	3.7-5.43	2.30-5.09
C core plate (144, 31)	2.39-3.96	2.35-4.87
Others (211, 108)	4.26-5.24	2.35-4.87

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As can be seen from the above table, the depth of phreatic water in each area is greater than the upper limit of ecological water level after the implementation of the project plan, leaving a small chance for soil salinization. However, dynamic monitoring should be applied in shallow groundwater areas to control the groundwater level within a reasonable range.

Conclusion

The river and lake connection project may bring changes to the groundwater level and quality (Mazzia and Puttii, 2007). Its influence on groundwater environment mainly attributes to the leakage recharge of the diversion water. Since the water is diverted from eastern rivers with much lower degree of mineralization than the groundwater, the leakage recharge will not increase the degree of mineralization of ground water, leaving no adverse effect on groundwater salinity variation.

With the groundwater numerical simulation technology, this paper simulates and analyzes the variation in groundwater flow field and water level of the simulation area. Only two conditions were taken into consideration: Plan Zero and the connection project plan. The prediction results show that, under the connection plan, the regional groundwater level after the project implementation was higher than that in Plan Zero thanks to the increase in leakage recharge. Under the joint effect of growing groundwater exploitation and other factors, the phreatic water level continued to decline slightly in core plates A and B, while that in core plate C and other edge plates remained stable; the depth of phreatic water in each area is greater than the upper limit of ecological water level after the implementation of the project plan, leaving a small chance for soil salinization.

Acknowledgements. This paper is funded by the High-Level Talent Introduction Plan of North China University of Water Resources and Electric Power (Grant No.: 2013038), Support Program for Young Science and Technology Innovation Talents of North China University of Water Resources and Electric Power (Grant No.: 70500), Water Conservancy Research Project of Guizhou Provincial Water Resources Department (Grant No.: KT201503) and Special Public Welfare Industry Project of Chinese Ministry of Water Resources (Grant No.: 201401041).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50595066

SYSTEM DYNAMIC ANALYSIS OF GREENHOUSE EFFECT BASED ON CARBON CYCLE AND PREDICTION OF CARBON EMISSIONS

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. With the intensification of greenhouse effect, the global average temperature gradually increases, and the atmospheric CO_2 concentration has exceeded 400 ppm. It has been a global consensus to reduce carbon emissions and the impact of the greenhouse effect. However, researches are rare on how to develop a reasonable emission reduction target and distribute funds in maintaining sustainable development. Based on the system dynamics method in the environmental system, we took a macro approach to simulate the trends of CO_2 amount, CO_2 concentration and temperature change in the next 5 decades. Through sensitivity analysis, we found anthropogenic CO_2 emissions as the major factor influencing the above trends. The iteration result at the step size of -0.4% showed that the -3.2% CO_2 emission reduction rate can achieve the Paris Agreement goal. On this foundation, we calculated the future amount of global CO_2 emission reduction and its investment and depict the latter, which provides reference for relevant sectors to formulate CO_2 emission reduction policies.
Keywords: *global warming, CO_2, emission reduction, investment path, policies*

Introduction

Reducing carbon emissions and slowing down the greenhouse effect has become the consensus across the globe, as countries around the world have signed several climate agreements to mitigate the greenhouse effect. As early as 1992 (Tu, 2005), the United Nations Conference on Environment Development formulated a convention on climate change in Rio de Janeiro, Brazil. In 1997, 149 countries adopted the Kyoto Protocol, which set the goal of limiting the average temperature rise below 2 °C by the end of the 20th century (Meinshausen et al., 2009). The year of 2015 witnessed the signing of the Paris Agreement among representatives from more than 200 countries. According to it, the rise in global temperature should not exceed 2 °C or if it can be achieved 1.5 °C compared to the value before industrialization (Li, 2017; Ogle et al., 2018); the countries and regions around the world should achieve the peak carbon emissions as soon as possible and meet the goal of zero CO_2 emission in the second half of the 21st century (Chen and Chen, 2016; Marino et al., 2017).

To ensure that the temperature rise is not higher than 2 °C, the equivalent of anthropogenic CO_2 should not exceed 450 ppm (which used to be 280 ppm before industrialization) (Elzen and Höhne, 2008). IPCC experts concluded that the 2020 emission volume of developed countries should be reduced by 25-40% compared to the 1990 one, which is 15-30% for developing countries (Elzen and Höhne, 2010). The results of a variety of studies show with a higher than 66% probability, that if we want

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the total anthropogenic temperature rise at the end of the century not to exceed the temperature of the 1861-1880 period by more than 2 °C, countries should keep the amount of CO2 emissions accumulated since 1870 below 2,900 Gt, and yet it was already 1,900 Gt by 2011 (Magazzino, 2016). In June 30, 2015, our country submitted the document of Intended Nationally Determined Contribution (INDC), committing to reach the peak carbon emissions roughly by 2030, drop the carbon intensity by 60%-65% than 2005, and occupy around 20% accumulated carbon emissions quota of the total according to the equity requirements in the effort-sharing scheme (Cui et al., 2016). Most of the current researches are concentrated on total amount control, focusing little on the annual amount and allocation of investments on carbon dioxide emission reduction. The fragmented CO_2 emission goal-settings among countries are disadvantageous to unifying CO_2 emission reduction paths (Sikharulidze et al., 2016; Huang et al., 2016; Gotovsky et al., 2018). In this study, the system dynamics model was used to predict the temperature change trend and carbon dioxide emission trend in the next 50 years. By analyzing the influence of different emission strategies on the greenhouse effect, the optimal emission strategy was finally determined. The research can provide a reference for the country to formulate a reasonable carbon emissions policy.

Material and methods

Carbon cycle model and greenhouse effect

The land and sea on the surface of the earth will absorb the short-wave radiation from the sun and convert it into heat which returns to the outer space in the form of long-wave radiation. This circulation helps balance the terrestrial temperature. However, CO_2, CH_4 and other greenhouse gases are active in reflecting long-wave radiation off the atmosphere, causing the "greenhouse effect" as the temperature on the earth surface rises (Frolking et al., 2006; Köhler et al., 2017). Greenhouse effect is a natural phenomenon caused by the emission of large amounts of greenhouse gas due to the rapid economic development in recent years. In this scenario, the atmospheric and marine temperatures will ultimately increase, and the glacial sheet in the Polar Regions will melt down, causing the rise in sea levels and the change of climate patterns (Cloy, 2018; Perry et al., 2012; Shao et al., 2016).

The main measure to control the greenhouse effect are to control carbon emissions, mainly due to the recent high contribution of CO_2 84% to atmospheric radiation (Levin, 2012). Therefore, it is necessary to study on the global carbon cycle model. In doing so, there are mainly two approaches: dynamic simulation and statistics (Bayer et al., 2015). The latter method is simple but physically blurred. With the analysis of historical data, this method can directly establish the model of carbon cycle law to show the past and future development trend. However, it fails to illustrate the system dynamics behaviors of carbon cycle to climate change, and the source and integrity of known data limits the precision of the model. The statistical method can be combined with the dynamics model to achieve good effect (Isacs et al., 2016). The system dynamics method includes the radiation convection mode, energy balance mode, and atmospheric circulation mode (Li and Tan, 2000). They will be analyzed in the following part, so that we can choose the most suitable mode for this study.

Radiation convection: By studying the effect of greenhouse gas on the solar radiant energy and the terrestrial and atmospheric radiation, we can use this method to calculate

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DOI: http://dx.doi.org/10.15666/aeer/1702_50675080

the potential contributions of different green gases to global warming and the atmospheric contours when the radiations reach a balanced state. Also, by considering the atmosphere as a column, this mode allows the establishment of a greenhouse effect model with clear physical meanings and simple calculations (Inamdar and Ramanathan, 1994). Nevertheless, as the influence of atmospheric circulation is excluded, this method is insufficient for the biosphere analysis and the carbon cycle effect research in a large scale.

Atmospheric circulation: through the study of carbon cycle law of the ocean, the atmosphere and the terrestrial biosphere under global warming, this mode includes the marine factors of water content, heat and chemical effect and the terrestrial factors of plant photosynthesis and respiration in analyzing carbon cycle (Babič, 2017; Chen et al., 2015; Xu and Shang, 2016; Zhang et a., 2018). As the description of the physical process of carbon cycle in the atmosphere, this mode fits well for long-lasting, large-scale issues with highly precise outcomes. However, the error of research results can be high due to the large data pool and the complicated calculation procedures.

Energy balance: this mode is mainly based on the energy balance model established under the law of conservation of energy. The energy balance equation is *Equation 1*:

$$C\frac{dT}{dt} = R\downarrow - R\uparrow \qquad \text{(Eq.1)}$$

where:
C: the thermal inertia of land, ocean and the atmosphere,
R↓: incoming radiation,
R↑: outgoing radiation.
Equation 1 can be converted into *Equation 2*:

$$C\frac{\partial T}{\partial t} = Q(1-\alpha) - \Delta I - dvi(\mathrm{F}) \qquad \text{(Eq.2)}$$

where:
Q: solar radiation,
α: reflectivity,
ΔI: outgoing long-wave radiation,
dvi (F): net energy flux along the circle of latitude.

This method takes into account the carbon cycle system and the influence of biosphere on atmospheric temperature increased. It can reflect the physical processes and the sensitivity of different indicators to climate change in a clear and simple way (Pugh et al., 2016). In this study, the energy balance model will be used in line with the data obtained from the atmospheric circulation mode and the radiation convective mode to establish a system dynamics model of the greenhouse effect.

Relationship between CO_2 concentration and temperature increase

According to the present study, the atmospheric CO_2 concentration is correlated with the increase in atmospheric temperature. By collating the average terrestrial temperature data statistics from the NASA Goddard Institute for Space Research (*Fig. 1*), we find that the average temperature of the earth has increased annually since 1958, rising by 1 °C in 2015. Furthermore, we fit the equation of temperature increase (ΔT) at the R^2

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value of 0.8875, as shown in *Equation 3*. The reliability of these data should be high as they conform to the statistical principle.

$$\Delta T = 0.0001Y^2 + 0.0086Y - 0.0914 \quad \text{(Eq.3)}$$

where Y: the difference of temperature between the calculation year and the year of 1958.

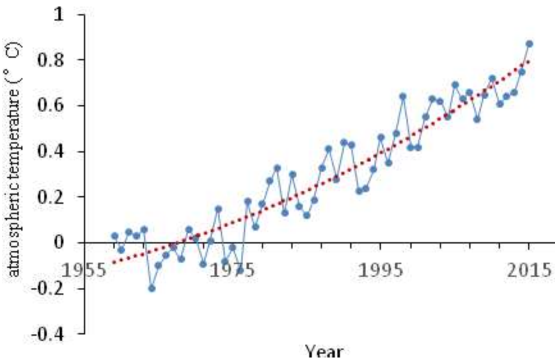

Figure 1. *The broken line of annual temperature increase since 1958*

According to *Equation 3*, it can be calculated that the temperature increase at the end of this century will reach 3.15 °C without considering the increase in CO_2 emissions from social development or human intervention. This value is far higher than the ideal value of the Kyoto Protocol. Actually, due to social development and economic boom, the CO_2 emissions will inevitably increase.

Figure 2 is the environmental monitoring data from the American Earth System Research Lab. The atmospheric CO_2 concentration has increased from 315 ppm in 1958 to the current 406 ppm, which is only 44 ppm lower than the threshold of 450 ppm. With these data, the CO_2 concentration equation can be obtained, as shown in *Equation 4*:

$$C_{CO2} = 0.0123Y^2 + 0.8119Y + 314.47 \quad \text{(Eq.4)}$$

where Y: the difference of CO_2 concentration between the calculation year and the year of 1958.

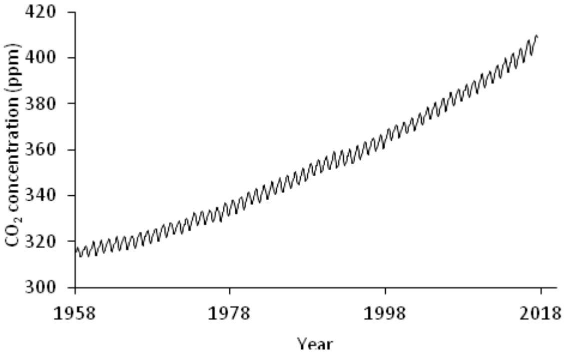

Figure 2. *Changes in atmospheric CO_2 concentration since 1958*

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According to the average annual data, we obtain the average annual growth line of atmospheric CO_2 concentration after 1958. It can be seen from *Figure 3* that the average annual growth rate of CO_2 concentration increases from 0.75 ppm in 1958 to the current value of 2.98 ppm, indicating a fast speed of growth.

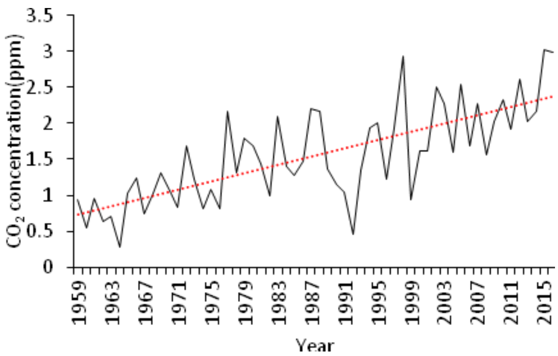

Figure 3. *The average annual growth line of CO_2 concentration since 1958*

The CO_2 concentration (C_{CO2}) and atmospheric temperature increase (ΔT) are fitted into a relationship line, as in *Figure 4*. With another fitting, we obtain the relationship formula between CO_2 concentration and atmospheric temperature increase (ΔT), as shown in *Equation 5*:

$$\Delta T = 8\times10^{-5} C_{CO2}{}^2 - 0.0472 C_{CO2} + 6.777 \quad \text{(Eq.5)}$$

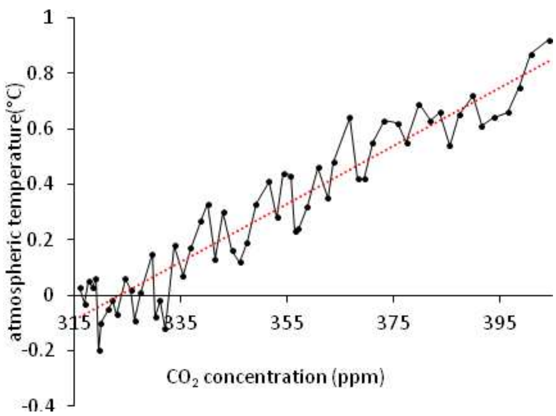

Figure 4. *The relationship line of CO_2 concentration and atmospheric temperature growth trend*

Establishment of the system dynamics model of greenhouse effect

Greenhouse effects in the global carbon cycle

The main source of greenhouse gases in the atmosphere are anthropogenic emissions, the respiration of plants and animals, and the release of dissolved permafrost. Greenhouse gases are adsorbed mainly by dissolving in ocean and plant photosynthesis (Albergel, et al., 2010; Stark et al., 2018), as shown in *Figure 5*.

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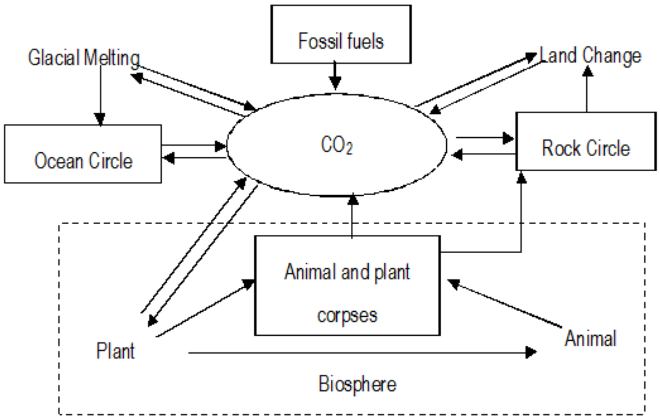

Figure 5. *Global carbon cycle flow char*

As can be seen from *Figure 5,* human production life needs to consume fossil fuels. The resultant large amount of CO_2 is discharged into the atmosphere and, through atmospheric circulation, partly dissolved into sea water and accumulated in the form of carbonate while partly absorbed by plants through photosynthesis. If plants are eaten by animals, some of the carbon will stay in the animal body and not released in the form of CO_2 until the animal is dead and the dead body is under microbial decomposition; the other carbons will exist in soil layers and turn into the carbon component of rock after a long time. In this process, those that cannot be fixed will circulate in the atmosphere and generate the greenhouse effect. As a result, the atmospheric temperature will rise, melting glaciers and thawing permafrosts. Consequently, the CO_2 that used to accumulate in glaciers and permafrosts will be released to the air, which intensifies the greenhouse effect. The atmospheric CO_2 equilibrium equation is as follows (*Eq. 6*) (Shi and Guo, 1997):

$$\frac{dn_a}{dt} = p_{fos} + p_{bio} + k_{ma}(\mathrm{N_m} + \xi_{\mathrm{nm}}) - \mathrm{k_{am}}(\mathrm{N_a} + \mathrm{n_a}) + \mathrm{F_{bi,a}} + \mathrm{F_{a,bi}} + \mathrm{F_{h,a}} \quad \text{(Eq.6)}$$

where:
P_{fos}: CO_2 release rate of fossil fuels,
P_{bio}: the rate of CO_2 release due to land use change,
K_{ma}: ocean atmosphere exchange coefficient,
K_{am}: atmosphere ocean exchange coefficient,
N_m: total carbon in the ocean,
N_a: total carbon in the atmosphere,
n_a: atmospheric carbon increment,
ξ_{nm}: ocean buffer factor,
$F_{bi,a}$: CO_2 exchange flux from land to the atmosphere,
$F_{a,bi}$: CO_2 exchange flux from the atmosphere to land,
$F_{h,a}$: CO_2 exchange flux from soil humus to the atmosphere.

System dynamics principles

System Dynamics (short for "SD") is an approach to understanding the dynamic behaviour of complex systems over time created by Professor Jay W. Forrester of the

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Massachusetts Institute of Technology (Alirezaei et al., 2017). It is based on the theory of feedback control and computer simulation technology in studying the relationship between the structure, function and dynamic behavior of complex systems. The system dynamics emphasizes the system as a whole, understand the composition of the system and the interaction of system components, and can carry on the dynamic system simulation experiment to examine the system dynamic change behavior and trend when the different parameters or strategic factors are inputted. In this way, decision-makers can take different measures and observe the simulation results in different scenarios.

The system dynamics model is a causal mechanism model. It emphasizes the decisive role of system inherent mechanism played in system behaviors, performing well in addressing long-term and periodic issues. In the case of insufficient data and unquantized parameters, feedback loops are also usable in some researches. The system dynamics model is good at handling high-order, non-linear, time-varying complex problems. Because of the unparalleled advantages of system dynamics in the study of complex nonlinear systems, it has been widely used in many fields such as society, economy, management, resources and environment.

System dynamics model in the global carbon cycle

The greenhouse effect model was established in Vensim according to the principle of the carbon cycle and the equations summarized above, as shown in *Figure 6*. Based on the 2015 report of the United Nations Environment Program, the CO_2 concentration reaches 400.21 ppm, the atmospheric CO_2 content is 1,965 Gt, and the global anthropogenic CO_2 emission is 36.3 Gt with the total atmospheric CO_2 amount as the variable of integration. By measuring World Bank statistics, the annual CO_2 emission in recent years grows at the rate of 0.82%. The CO_2 produced by the change in land used is about 9.47% of anthropogenic emission (Bergamaschi et al, 2013), while the CO_2 equivalent released annually from the global permafrost is about 1.84 GT. The ocean absorbs about 35% of anthropogenic CO_2 per year, and the proportion is 20% to the biosphere.

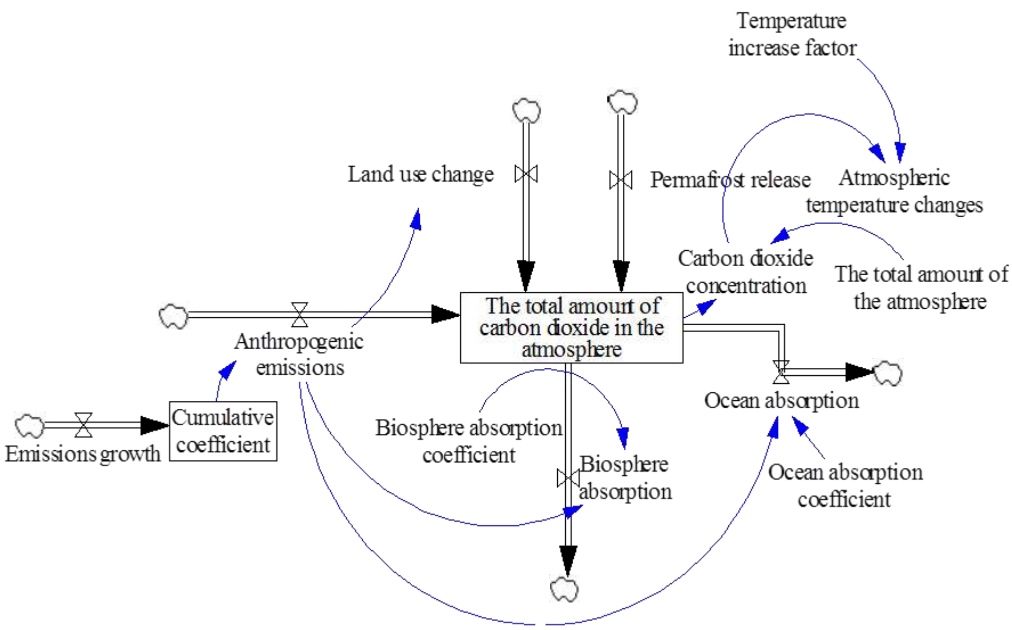

Figure 6. *Global carbon cycle and greenhouse effect model*

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Results and discussion

With 50 years as the data boundary, these data are inputted into the model for simulation analysis without taking any carbon reduction measures.

The total amount of CO_2 in the atmosphere is analyzed and shown in *Figure 7*. The figure shows the constant increase in total atmospheric CO_2 amount from the 1,964 Gt in 2015 to the final 3,581.85 Gt, rising by 82.38%. The initial CO_2 concentration of 404 ppm grows to 736.92 ppm by the fifth decade, which far exceeds the warning line of 450 ppm.

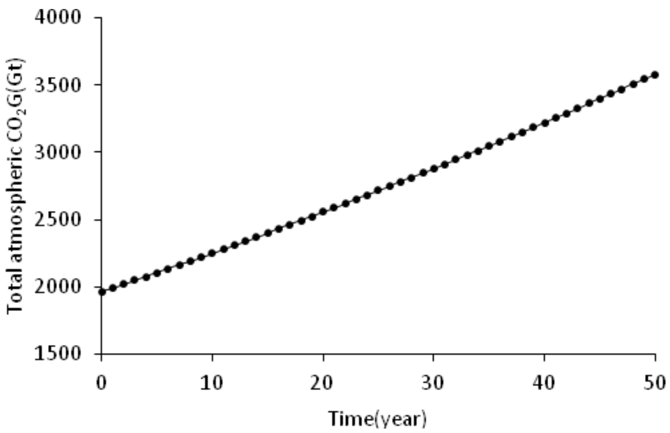

Figure 7. *Total atmospheric CO_2 amount*

The impact of anthropogenic emissions growth rate on key indicators

The anthropogenic emissions are mainly determined by the emission growth rate (Van den Bergh and Botzen, 2015). By adjusting the emission growth rate, the impact of the emission growth rate on the atmospheric index can be found, providing an intuitive way for determining the reasonable emission amount on which basis the goals of the upper limit and reduction amount of emitted CO_2 can be formulated.

The current annual emission growth rate is 0.82%, and we set the decline in growth rate to 0.4%, as shown in *Figure 8*. With the decrease of the CO_2 emission growth rate, the anthropogenic CO_2 emission line begins to tilt down. When the emission growth rate drops to -2%, the anthropogenic emissions will become zero in 50 years. As the emission growth rate continues to decrease, the time required for reaching zero carbon emissions will be shortened gradually. When the emission growth rate is -3.2%, the zero carbon emissions will be achieved in 28 years. However, the lower the emission growth rate is, the more challenges the technologies will be posed to. After a comprehensive thought, we will discuss what the reasonable decline amount of growth rate should be below.

To obtain the reasonable decline amount in growth rate, we re-simulate the model in terms of the CO_2 concentration in the atmosphere at different emission rates, as shown in *Figure 9*. The figure shows that the CO_2 concentration line becomes inverse parabolas with peaks. The peak value gradually decreases as the decline in growth rate is enlarged. When the growth rate becomes -3.2%, the sole peak will emerge in the 22th year at the value of 453.99 ppm. The calculated result complies with the result of INDC.

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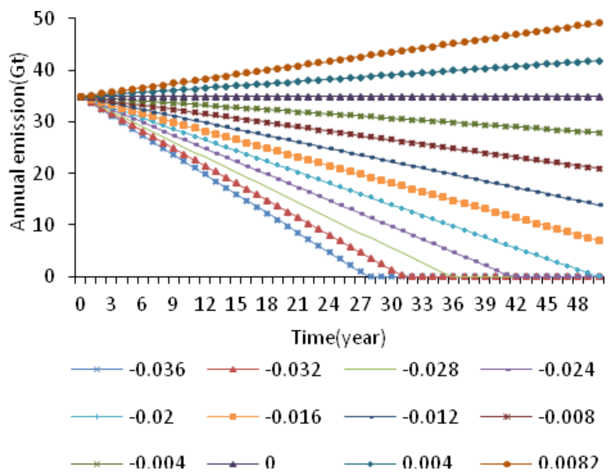

Figure 8. *Annual emission amounts at varying emission growth rates*

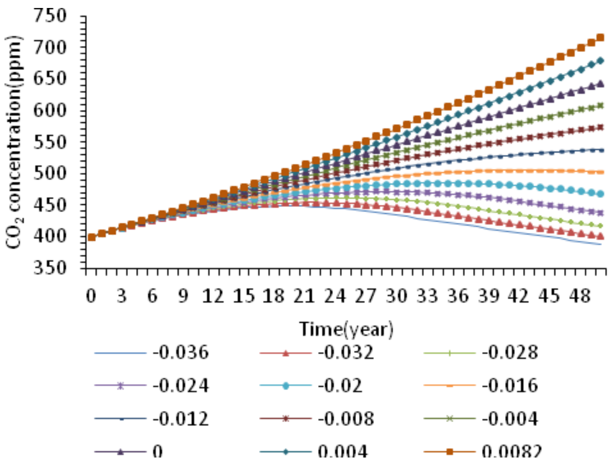

Figure 9. *CO_2 concentration in the atmosphere at different emission rates*

We continue to simulate the model to analyze the average atmospheric temperature changing with the CO_2 emission growth rate, as shown in *Figure 10*. As can be seen from the figure, if the emission growth rate remains unchanged, the atmospheric temperature change curve will go up exponentially, which will bring disastrous consequences to the environment. When the CO_2 emission growth rate declines to -0.8%, the average atmospheric heating curve will present the form of inverse parabola. When the CO_2 emission growth rate declines to -2.8%, the peak temperature becomes 2.029 °C. When the CO_2 emission growth rate declines to -3.2%, the peak temperature becomes 1.836 °C, which meets the requirements of CO_2 concentration. Therefore, -3.2% is the reasonable decline value of CO_2 emission growth rate in order to address the greenhouse effect.

Emission reductions and emission reduction investments

The emission reduction amounts are calculated according to the emission growth rate, and then we draw them in lines in *Figure 11*. It can be seen that there is an

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inflection point in the 31th year, indicating the realization of zero-emission. This result can be basically equated with the Paris Agreement goal of realizing zero-emission in the second half century. In the next 5 decades, the global carbon emissions reduction amount should increase from the initial 1.4 GT p.a. to the final value of 49.16 GT p.a., and the total emission reduction amount is 1,580 GT, China needs to bear 20% of the total emission reduction amount, which is about 316 GT.

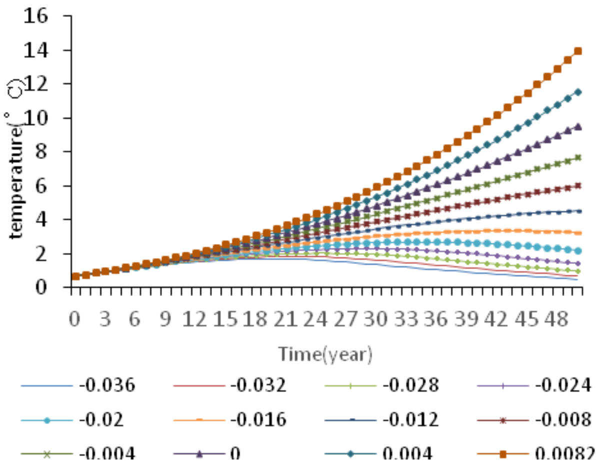

Figure 10. *The average atmospheric heating curves at different emission rates*

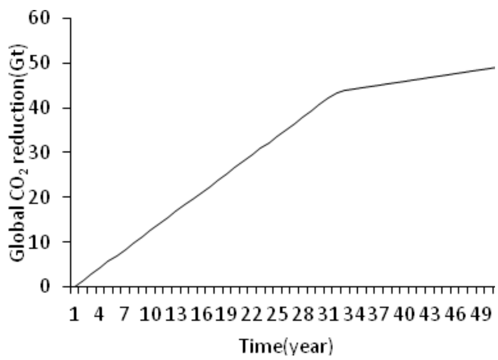

Figure 11. *Global CO_2 reduction line in the next 50 years*

Some scholars have measured the macroeconomic cost of CO_2 emission reduction, which ranges between US $456/t and US $592/t (Fan et al., 2010). We take the average of US $524/t to calculate the future global investment on carbon emissions (see *Fig. 12*). The figure shows that it is necessary to constantly increase investments on carbon emissions in the future 50 years until the annual investment value rise to US $2.57 trillion. The total sum of investments should reach US $82.81 trillion across the globe.

Taking China as an example, in formulating investment policies, the investment on CO_2 emission reduction in the next 50 years can be calculated on the premise that China bears the 20% of global carbon emissions reduction amount. And the related data is shown in *Figure 13*. To address climate change, China should continuously increase

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investments on carbon emissions reduction in the future 5 decades until the annual investment amount reaches US $0.514 trillion. The total sum of Chinese investments on carbon emissions reduction should be US $16.56 trillion and the total carbon emissions reduction amount should be 316 GT.

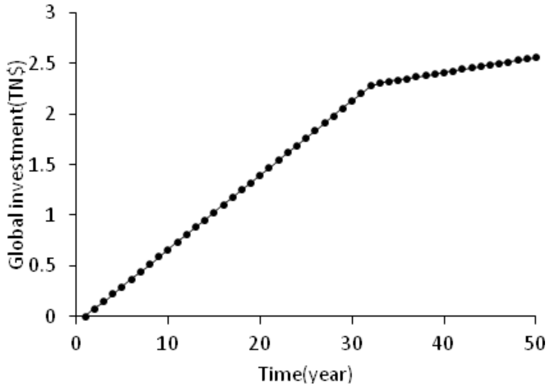

Figure 12. *Global investment line on CO_2 emission reduction in the next 50 years*

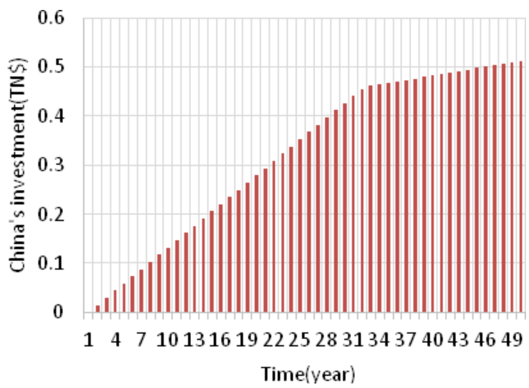

Figure 13. *China's investment on CO_2 emission reduction in the next 50 years*

Conclusion

The greenhouse effect is an environmental phenomenon that triggers global climate change and has attracted the attention of all countries in the world. Although countries are committed to reducing carbon emissions, related goals can only be realized by them setting emission reduction targets according to scientific calculations and investing a reasonable amount of funds on the targets. Reduce carbon emissions, and gradually weaken the impact of greenhouse effect. Through the macro-data based system dynamics analysis, we can see that the task of global CO_2 emission reduction is still arduous. Only by settling the CO_2 emission reduction funds can a country achieves the objectives specified in the Paris Agreement. With certain responsibilities, countries and regions need to make reasonable investments to help reduce global CO_2 concentrations, so as to avoid the unpredicted and uncontrollable consequences brought by the otherwise intensified greenhouse effects. In future research, it is necessary to strengthen the accumulation of basic data, study the relationship between different industrial development and carbon emissions, improve the accuracy of carbon emissions

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DOI: http://dx.doi.org/10.15666/aeer/1702_50675080

prediction model, and timely adjust the carbon emissions strategy through more accurate model.

Acknowledgements. This work was supported by National Key R & D Program of China (Grant No. 2016YFC0700201) and supported by National Natural Science Foundation of China (Grant No. 51708394).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50675080

SAFETY EVALUATION OF RURAL DRINKING WATER SOURCES IN NANG COUNTY, TIBET AUTONOMOUS REGION OF CHINA

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(Received 5th Feb 2019; accepted 6th Mar 2019)

Abstract. This paper evaluates the quality of drinking water sources of 9 villages in Nang County, Tibet Autonomous Region (TAR) of China using the water quality index (WQI) model of the Canadian Council of Ministers of the Environment (CCME), and verifies the evaluation results by the comprehensive pollutant index (CPI) method. The results show that CCME WQI and CPI have outputted similar rankings, and the results of the two methods have high Spearman's rank correlation coefficient. This means CCME WQI is a feasible way to evaluate the safety of rural drinking water sources. CCME WQI evaluation shows that the water qualities in the 9 villages satisfy the requirements on drinking water.
Keywords: *comprehensive pollution index (CPI), the CCME water quality index (WQI), drinking water safety, water quality protection, Spearman's rank correlation coefficient*

Introduction

The hygiene and safety of drinking water directly bears on the health of people. To ensure drinking water safety, it is necessary to implement a whole process control before, during and after consumption. This control strategy relies heavily on the safety evaluation of drinking water. To date, the safety of rural drinking water has been mainly evaluated by analytic hierarchy process (AHP) (Guo et al., 2018; Xu et al., 2016), fuzzy evaluation (Mohamed et al., 2018; Wang et al., 2018), entropy weight method (Li et al., 2018), principal component analysis (PCA) (Naz et al., 2016), Comprehensive Pollution Index (CPI) (Savio et al., 2018), etc. In most studies, only one of these methods is adopted to evaluate the safety of drinking water. However, the evaluation result on the same object may vary from method to method. This problem has not been fully recognized or rationally solved in the existing studies on safety evaluation of rural drinking water. Moreover, there is no report on the safety features or regional differences of drinking water (Hood et al., 2014).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

Currently, Canadian Council of Ministers of the Environment (CCME) water quality index (WQI) (Hurley et al., 2012) is mainly applied in the management of surface water environment, while the comprehensive pollution index (CPI) (Sun et al., 2013) is mainly used to evaluate the drinking water sources. Targeting the quality evaluation of drinking water sources in 9 villages of Nang County, China's Tibet Autonomous Region (TAR), this paper explores the correlation and differences between CCME WQI and CPI, with the aim to assess the applicability of CCME WQI in the evaluation of drinking water sources. Specifically, CCME WQI was adopted to evaluate the safety of drinking water in the 9 villages, and results were verified by CPI method. The evaluation of drinking water safety in these villages can lay a solid basis for decisions on selecting and protecting drinking water sources.

Materials and methods

Based on the field monitoring data of drinking water in 9 villages of Nang County, TAR, two evaluation models were constructed by CCME WQI and CPI, respectively.

Overview of the study area

Nang County (28°40'~29°29'N; 92°28'~93°31'E) is a border county southwest of Nyingtri City and southeast of TAR. It covers an area of more than 4,200 km^2 on the northern foothills of the Himalayas and in the middle and lower reaches of the Yarlung Tsangpo River. With jurisdiction over 6 townships, the county is home to over 18,000 people living in 51 administrative villages and 1 neighborhood community. Most administrative villages are scarcely populated and scattered in remote areas, making it difficult to implement centralized water supply. Currently, the villagers mainly drink natural surface water, which fluctuates greatly by season due to the low vegetation coverage, and the drinking water sources are far from abundant (Tian et al., 2015). To rationalize the selection of rural drinking water sources, it is imperative to carry out targeted safety evaluation of rural drinking water and adopt rational remediation measures.

Data sources

As shown in *Figure 1,* a total of 9 monitoring points were arranged in light of the status of the drinking water sources. The monitoring was carried out in August, 2018. Four sampling points were selected at each monitoring point to measure 16 items, namely, zinc, copper, iron, manganese, total hardness, dissolved oxygen (DO), chemical oxygen demand (COD), total dissolved solids, arsenic, hexavalent chromium, fluoride, anionic synthetic detergent, cyanide, volatile phenols, lead and selenium. These items were selected according to relevant literature (Chang et al., 2017), and analyzed according to the *Environmental Quality Standards for Surface Water* (GB3838-2002) and the *Water and Wastewater Analysis Method*.

Analysis methods

In this paper, CCME WQI is employed to evaluate the drinking water sources at the 9 monitoring points, and CPI was selected to verify the evaluation results of CCME WQI.

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DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

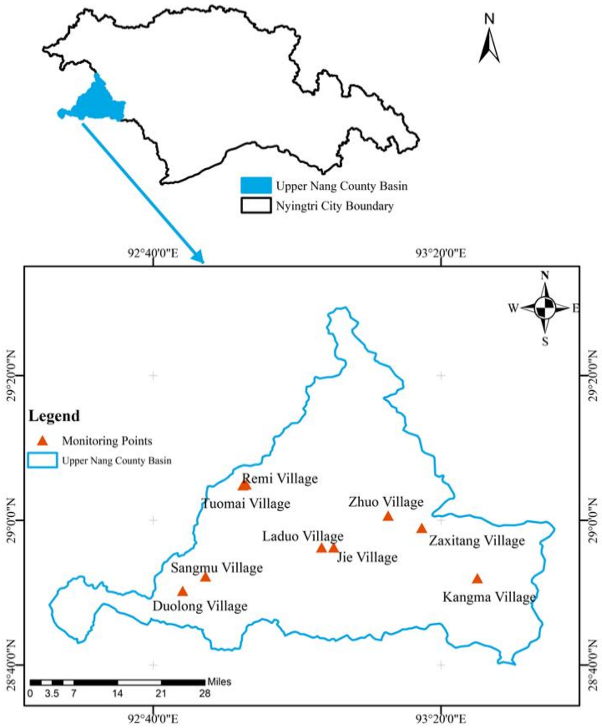

Figure 1. *Location map of monitoring points*

CPI

CPI (Meng et al., 2014), an indicator of the degree of water pollution, is the sum of the relative pollution indices of different pollution items. This method can determine the pollution degree of the water bodies in our research. CPI value can be derived from single pollution indices by the following equations:

$$P_{ij} = \frac{c_{ij} - c_{jok}}{c_{jok+1} - c_{jok}} + P_{jok} \quad \text{(Eq.1)}$$

where P_{ij} is the single pollution index of the j-th item of the i-th water sample (the serial number of water sample i = 1, 2, …, n; the serial number of pollution item j = 1, 2,…, m); c_{ij} is the measured content of the j-th item of the i-th water sample; c_{jok} is the standard content of level k of the j-th item; c_{jok+1} is the standard content of level k + 1 of the j-th item; P_{jok} is the standard index value of level k of the j-th item. The c_{ij} falls between c_{jok} and c_{jok+1}.

$$P_i = \frac{1}{m} \sum_{i=1}^{m} P_{ij} \quad \text{(Eq.2)}$$

where P_i is CPI of the i-th water sample; m is the total number of pollution items.

CCME WQI

CCME WQI is a water quality management tool that evaluates the water quality of the target water body against three aspects: the number of terms whose objectives are

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not met (Scope), the frequency with which the objectives are not met (Frequency) and the amount by which the objectives are not met (Amplitude). This method has been highly lauded for its flexibility in parameter selection and study area, intuitiveness in computing process and evaluation results, and tolerance of the missing values.

(1) Scope F_1

The percentage of items that do not meet their objectives during the evaluation period, relative to the total number of evaluation items of the i-th sampling point F_{1i} can be expressed as:

$$F_{1i} = \frac{m}{M} \times 100$$

where M is the total number of evaluation items of each sampling point in the evaluation period; N is the number of sampling points; m is the number of items failing to meet the objectives of water quality.

(2) Frequency F_2

The percentage of individual monitoring of the i-th sampling point that does not meet objectives F_{2i} can be expressed as:

$$F_{2i} = \frac{q}{M \times p} \times 100$$

where p is the number of times by which each item of a sampling point is monitored; q is the number of times by which a sampling point fails to meet the objectives of water quality.

(3) Amplitude F_3

Let C_j be the objective of water quality for the j-th item and a_{ij} be the monitored value of the j-th item of the i-th sampling point. Then, the amount by which failed items do not meet their objectives e_{ij} can be calculated as follows:

For items that must exceed the objective (e.g. DO), the amplitude e_{ij} can be calculated as:

$$e_{ij} = \begin{cases} \frac{C_j}{a_{ij}} - 1 & a_{ij} < C_j \\ 0 & a_{ij} \geq C_j \end{cases}$$

For items that must not exceed the objective (e.g. total hardness and COD), the amplitude e_{ij} can be calculated as:

$$e_{ij} = \begin{cases} \frac{a_{ij}}{C_j} - 1 & a_{ij} > C_j \\ 0 & a_{ij} \leq C_j \end{cases}$$

(4) Level classification

CCME WQI is defined in the interval [0, 100]. The greater the value of CCME WQI, the better the evaluation result. CCME WQI can be calculated as:

$$\text{CCME WQI} = 100 - \frac{\sqrt{F_1^2 + F_2^2 + F_3^2}}{1.732}$$

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DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

According to the value of CCME WQI, the quality of the target water bodies was divided into 5 levels (Alazawii et al., 2018; Feng et al., 2018) (*Table 1*).

Table 1. *Five levels of water quality*

Level	The value of the CCME WQI
Excellent	[94,100]
Good	[79,94)
Fair	[64,79)
Marginal	[44,64)
Poor	[0,44)

Results and discussion

Monitoring data and water quality objectives

As mentioned before, 16 water quality items, namely, zinc, copper, iron, manganese, total hardness, DO, COD, total dissolved solids, arsenic, hexavalent chromium, fluoride, anionic synthetic detergent, cyanide, volatile phenols, lead and selenium, were measured at the four sampling points of each monitoring point. The measured value of each item is recorded in *Table 2*.

Table 2. *Measured value of each item*

Item	Station	Tuomai Village	Zhuo Village	Zaxitang Village	Remi Village	Sangmu Village	Duolong Village	Laduo Village	Jie Village	Kangma Village
Zinc (mg/L)	1	0.13	0.09	0.12	0.06	0.07	0.09	0.06	0.08	0.09
	2	0.14	0.11	0.13	0.07	0.08	0.11	0.09	0.09	0.1
	3	0.15	0.1	0.13	0.06	0.09	0.12	0.11	0.09	0.11
	4	0.08	0.08	0.13	0.07	0.08	0.07	0.07	0.08	0.08
Copper (mg/L)	1	0.008	0.007	0.15	0.009	0.011	0.012	0.012	0.014	0.01
	2	0.01	0.009	0.17	0.011	0.013	0.014	0.012	0.015	0.012
	3	0.009	0.007	0.13	0.013	0.014	0.014	0.014	0.013	0.016
	4	0.007	0.007	0.11	0.007	0.008	0.006	0.006	0.008	0.008
Iron (mg/L)	1	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
	2	0.019	0.02	0.019	0.019	0.019	0.018	0.019	0.02	0.018
	3	0.02	0.021	0.017	0.019	0.021	0.016	0.016	0.021	0.019
	4	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Manganese (mg/L)	1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
	2	0.006	0.007	0.007	0.007	0.005	0.007	0.006	0.008	0.005
	3	0.007	0.008	0.008	0.008	0.006	0.008	0.007	0.009	0.006
	4	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Ca^{2+} (mg/L)	1	87	176	165	179	254	287	247	219	143
	2	489	470	562	570	555	586	544	511	441
	3	501	486	558	582	598	592	536	523	445
	4	125	189	199	198	264	278	248	236	148
DO (mg/L)	1	7.96	8.03	8.06	8.11	8.12	7.85	8.06	8.02	8.05
	2	7.67	8.16	8.25	8.02	8.13	7.95	8.22	8.35	8.65
	3	7.89	8.21	8.26	8.12	8.28	8.25	8.65	8.37	8.45
	4	7.85	8.09	8	8.25	8.36	7.68	8.15	8.26	8.16
COD (mg/L)	1	1.35	1.36	1.41	1.25	1.75	1.95	2.02	1.58	1.61
	2	3.46	5.62	4.54	5.64	5.1	5.01	5.12	4.55	4.88
	3	6.23	6.25	5.23	6.25	5.28	5.38	5.31	5.26	5.14
	4	3.25	2.36	3.15	2.25	4.21	2.01	2.68	3.68	3.26

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Solid solubility (mg/L)	1	227	178	223	185	265	198	213	231	176
	2	745	741	358	536	654	464	456	485	458
	3	689	685	485	562	689	498	495	489	487
	4	326	215	256	167	245	138	235	312	259
Arsenic (mg/L)	1	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
	2	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
	3	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
	4	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Hexavalent chromium (mg/L)	1	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
	2	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
	3	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
	4	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
Fluoride (mg/L)	1	0.5	0.45	0.3	0.7	0.6	0.5	0.5	0.3	0.4
	2	0.8	0.9	0.8	1	1.1	0.8	0.8	0.8	0.8
	3	0.9	0.8	0.9	1.1	1.1	0.9	0.8	0.9	0.8
	4	0.6	0.5	0.6	0.6	0.5	0.6	0.5	0.4	0.4
Anionic synthetic detergent (mg/L)	1	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	2	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	3	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
	4	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Cyanide (mg/L)	1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	2	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	4	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Volatile phenols (mg/L)	1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	2	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	4	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Lead (mg/L)	1	0.0034	0.0054	0.0066	0.0068	0.0065	0.0058	0.0054	0.0067	0.0064
	2	0.0036	0.0056	0.0078	0.0048	0.0078	0.0069	0.0075	0.0075	0.0069
	3	0.0042	0.0054	0.0067	0.0065	0.0068	0.0078	0.0067	0.0078	0.0072
	4	0.0045	0.005	0.0056	0.0052	0.0065	0.0048	0.0054	0.0067	0.0053
Selenium (mg/L)	1	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
	2	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
	3	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
	4	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025

According to the *Environmental Quality Standards for Surface Water* and the *Sanitary Standard for Drinking Water Quality*, the objectives of the 16 water quality items were determined (*Table 3*).

Table 3. *Objectives of the 16 water quality items*

Item	Objective	Unit	Item	Objective	Unit
Zinc	1	mg/L	Fluoride	1	mg/L
Copper	1	mg/L	Anionic synthetic detergent	0.2	mg/L
Iron	0.3	mg/L	Cyanide	0.05	mg/L
Manganese	0.1	mg/L	Volatile phenols	0.002	mg/L
Arsenic	0.05	mg/L	Selenium	0.01	mg/L
Lead	0.01	mg/L	DO	5	mg/L
Total hardness*($CaCO_3$)	450	mg/L	COD	4	mg/L
Hexavalent chromium	0.05	mg/L	Total dissolved solids*	1000	mg/L

*No objective is specified for the corresponding item in the *Environmental Quality Standards for Surface Water*. The objective was thus extracted from the *Sanitary Standard for Drinking Water Quality*

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CPI evaluation results

According to the *Technical Rules for the Evaluation of the Safety Status of Drinking Water Sources in Urban Areas* (2005), two aspects of the water quality of river water sources should be evaluated, namely, the level of general pollutants and the level of toxic pollutants. Specifically, the water quality status index of river and underground water sources = 0.3 × the level of general pollutants + 0.7 × the level of toxic pollutants. The level of general pollutants measures by eight items, including zinc, copper, iron, manganese, total hardness, DO, COD and total dissolved solids (the total hardness and total dissolved solids, which are water quality evaluation items for underground water sources, are treated as those for surface water sources), while the level of toxic pollutants is measured by another eight items, including arsenic, hexavalent chromium, fluoride, anionic synthetic detergent, cyanide, volatile phenols, lead and selenium (the anionic synthetic detergent, which is a water quality evaluation item for underground water source, is treated as one for surface water sources). The specific calculated results are shown in *Table 4*.

Table 4. *CPI evaluation results*

Monitoring points	Level of general pollutants	Level of toxic pollutants	CPI	Ranking
Tuomai Village	1.193	2.700	2.248	5
Zhuo Village	1.173	2.700	2.242	3
Zaxitang Village	1.229	2.700	2.259	7
Remi Village	1.181	3.400	2.734	8
Sangmu Village	1.280	3.400	2.764	9
Duolong Village	1.209	2.700	2.253	6
Laduo Village	1.184	2.400	2.035	2
Jie Village	1.187	2.700	2.246	4
Kangma Village	1.109	2.400	2.013	1

In light of CPI evaluation results in *Table 4,* the villages in the study area were ranked in descending order by the quality of drinking water source: Kangma Village, Lado Village, Zhuo Village, Jie Village, Tuomai Village, Duolong Village, Zhaxitang Village, Remi Village and Sangmu Village. The water qualities in all villages belong to Level III. On the level of general pollutants, the water quality belongs to Level II; On the elvel of toxic pollutants, the water quality belongs to Level III at 9 monitoring points and Level IV at 2 monitoring points. CPI evaluation results demonstrate that the drinking water sources at 9 monitoring points meet the requirements on Level II water source protection zone for centralized supply of drinking water (Sun et al., 2014; Wang et al., 2017), and can provide drinking water to the local areas. These water sources mainly face high content of toxic pollutants, which should be controlled in future water treatment.

CCME WQI evaluation

CCME WQI evaluation results of the 9 villages in Nang County were computed according to the equations in the section “Analysis methods – CCME WQI” and recorded in *Table 5*.

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Table 5. *CCME WQI evaluation results*

Item	Tuomai Village	Zhuo Village	Zaxitang Village	Remi Village	Sangmu Village	Duolong Village	Laduo Village	Jie Village	Kangma Village
F_1(%)	12.5	12.5	12.5	18.75	18.75	12.5	12.5	12.5	6.25
F_2(%)	3.125	6.25	7.8125	9.375	10.9375	6.25	6.25	7.8125	4.6875
F_3(%)	2.4836	3.0841	2.3596	3.5515	3.6032	2.7043	2.5397	2.5265	2.1806
CCME WQI	91.3967	91.7369	91.3809	87.7241	87.2956	91.7814	91.7989	91.3652	95.3169
Ranking	5	4	6	8	9	3	2	7	1

According to CCME WQI evaluation results, the villages can be ranked in descending order of drinking water source quality as: Kangma Village, Laduo Village, Duolong Village, Zhuo Village, Tuomai Village, Zhaxitang Village, Jie Village, Remi Village and Sangmu Village. Comparing CCME WQIs with *Table 1,* it can be seen that only one monitoring point belongs to the excellent level, while all the other points belong to the good level. All monitoring points are under limited or no threat, and low or extremely low risk. To sum up, the monitoring points can serve as reliable and safe water sources for centralized supply of drinking water (Venkatramanan et al., 2016).

Results and analysis

The evaluation results of CPI were compared with those of CCME WQI to find their correlations (*Fig. 2*).

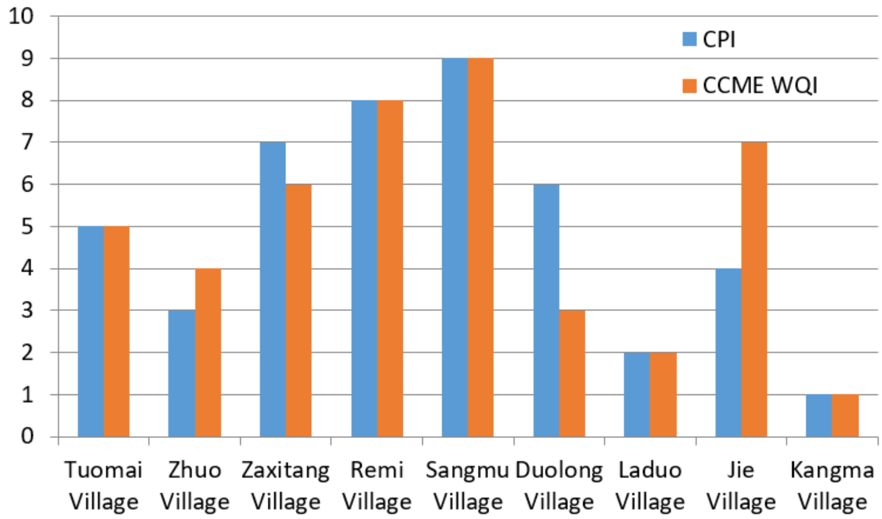

Figure 2. *Comparison between evaluation results of CPI and those of CCME WQI*

As shown in *Figure 2,* the water quality ranking of the villages of CCME WQI differs from that of CPI in the following areas: the ranking of two monitoring points (Duolong Village and Jie Village) change by three places; the ranking of no monitoring point change by two places; the ranking of two monitoring points (Zhuo Village and Zhaxitang Village) change by one place; the ranking of the other five monitoring points remains the same. To verify the reliability of CMME WQI method, the research results were tested by the Spearman's rank correlation coefficient.

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DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

The Spearman rank correlation coefficient (Dikbas, 2018) is a nonparametric measure of statistical dependence between two variables. Here, this measure is adopted to check the consistency between the evaluation results of CPI and those of CCME WQI. The calculation formula is as follows:

$$r = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n(n^2-1)}$$

Where d_i is the rank difference of each item; n is the number of evaluation items. The Spearman's rank correlation coefficient falls in the interval of [0, 1]. The closer the value is to 1, the better the correlation.

The Spearman's rank correlation coefficient between the evaluation results of CPI and those of CCME WQI is 0.8333, revealing a close positive correlation. In other words, the two evaluation methods have outputted highly consistent evaluation results.

The comparison between *Tables 4* and *5* shows that CPI and CCME WQI have different emphases in calculation. CPI consists of the level of general pollutants and that of toxic pollutants. The former is about the mean value, while the latter only considers the peak value (without considering the data size or data volume). As a result, the level of toxic pollutants may be exaggerated when a few toxic pollutant items exceed the objectives and differ in the amount by which the objectives are not met, which pushes up the CPI value. The opposite situation will occur when many toxic pollutant items exceed the objectives but agree in the amount by which the objectives are not met.

CCME WQI is made up of three parts: Scope, Frequency and Amplitude. Among them, only the Amplitude is related to the mean value, while the Scope and Frequency are about the number of terms whose objectives are not met and the frequency with which the objectives are not met, respectively. When the mean value does not change much, CCME WQI is negatively correlated with the Scope and Frequency. The opposite situation will occur if only a few terms exceed the objectives and differ in the amount by which the objectives are not met. In summary, CPI and CCME WQI both increase with the growth in the amount by which the objectives are not met and with the reduction of the number of terms failing to meet the objectives. However, the results of the two methods exhibit as the deterioration and improvement of water quality, respectively (Jie Village vs. Zhuo Village). The inverse is also true, as evidenced by Zhaxitang Village vs. Duolong Village.

Conclusions

This paper evaluates the quality of drinking water sources of 9 villages in the Yarlung Tsangpo River Basin using CCME WQI model, and verifies the evaluation results by CPI method. The subjective weighting of the CPI method may affect the evaluation results, while CCME WQI, free of subjective factors, can output relatively stable results. To ensure the reliability of CCME WQI, the evaluation results were subjected to reliability test by the Spearman's rank correlation coefficient. The following conclusions were drawn from the drinking water source qualities evaluated by CCME WQI.

(1) According to the evaluation results and ranking of monitoring points, CCME WQI and CPI have outputted similar rankings, and the results of the two methods have high Spearman's rank correlation coefficient. This means CCME WQI is a feasible way

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DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

to evaluate the safety of rural drinking water sources. However, CCME WQI also has certain limitations as CPI.

(2) Through CCME WQI evaluation, the 9 villages can be ranked in descending order of drinking water source safety as Kangma Village, Laduo Village, Duolong Village, Zhuo Village, Tuomai Village, Zhaxitang Village, Jie Village, Remi Village and Sangmu Village. Besides, the water qualities at these monitoring points satisfy the requirements on drinking water.

In the future, CCME WQI can be applied to rural drinking water sources after the operation is mature and simplified. And then the safety evaluation of rural drinking water sources will be completed quickly.

Acknowledgements. This work was supported by the Natural Science Foundation of Tibet (NO.XZ 2018 ZR G-20), National Natural Science Foundation of China (NO.51868069,51769034）, the Program for Scientific Research Innovation Team in Colleges and Universities of Tibet Autonomous Region.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50815091

PREPARATION OF SILVICULTURAL PLANS IN *PINUS SYLVESTRIS* L. FORESTS: CASE STUDY OF OLTU PLANNING UNIT

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(Received 10th Jan 2019; accepted 8th Mar 2019)

Abstract. Forest resources in Turkey, according to the principles of ecosystem-based, multiple-use management approach, forest management chief/planning units are governed by forest management plans. Forest management planning process involves taking inventory, preparing the database with Geographic Information Systems (GIS), determining the forest management units and silvicultural treatments, and the preparation phase. After its completion begins the process of preparing silvicultural plans. First, in the regeneration areas, ecological conditions are evaluated and tree seeds are measured. Then regeneration methods are applied in this area and a detailed spatial and temporal planning is performed. Three different silvicultural plan tables are prepared by the forestry legislation. Beside the preparation of silvicultural plans, its enforcement has put an additional workload on forest management chief officers. Selected as a pilot region, the preparing of a silvicultural plan and monitoring its application with geographic database was undertaken at Oltu Forest Planning Unit. First of all, afforestation, regeneration and tending areas according to forest management plan were measured and evaluated. Then, the existing geographic database was updated to prepare the silvicultural plan. Then, in order to prepare and perform silvicultural prescriptions, spatial and temporal arrangements were added to the geodatabase. In the next step, the allowable cut was calculated. Finally, silvicultural treatments were applied at regeneration and tending areas, and illustrated with tables, graphs and maps. The paper is a case study for Oltu Forest Planning Unit, consisting mostly of *Pinus sylvestris*, while other tree species should be supported by a similar study in Turkey, particularly for mixed stands.
Keywords: *silvicultural recipe, Pinus sylvestris L., geographic information systems, forest management plan, allowable cut*

Introduction

Forest areas around the world are either decreasing or degrading structurally due to clearings for agriculture and residential development, road and energy line constructions, illegal logging and harmful forestry practices. Numerous plant and animal species living in these areas either become extinct or are under risk of extinction. Human population and demands are continuously increasing and becoming more varied. According to the Food and Agricultural Organization (FAO, 2015) data, global forest areas are 3.99 billion hectares (ha) in size and make up 30.6% of terrestrial lands. There has been a decrease of approximately six times the total forest area of Turkey (129 million ha) in global forest areas since 1990. As a result of the aforementioned reasons, utilization of forest resources is handled internationally and assessed globally with many conventions, primarily the Convention on Biodiversity. Sustainable management of forest resources takes form according to the criteria and indicators determined by the forestry philosophy of each country. Every country has made the required legal legislations and the technical and administrative infrastructure according to its own culture and economic structure (Yolasığmaz, 2013). 148 countries have

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

policies that support sustainable forest management and 145 of them prepared forestry legislations based on these policies. 39% of forest areas (2.1 billion ha) are managed by forest management plans (FAO, 2010; FAO, 2015; GDF, 2014a).

In Turkey, forest areas are administrated by the Ministry of Agriculture and Forestry. Forests are treated in two categories as protected and managed. Protected areas like national parks, nature reserve areas and nature parks are managed by Nature Conservation and National Parks General Directorate using long-term development plans and management plans. Management forest are managed by the General Directorate of Forestry at the scale of the administration chief offices/forest planning units, which are the smallest planning and administration units, using forest management plans prepared according to the principles of the ecosystem based multiple use forest management. Planning process consists of a) the inventory of forest ecosystem, b) setup of a geographic database using geographic information systems (GIS) technology, c) the generation of associated digital and baseline maps (stand map, forest function map, forest management units map etc.), d) the determination of management goals and conservation targets with a participatory approach by preparing forest management units map, e) the arrangement of utilization (i.e., determination of the location, time, quantity and the silvicultural treatments for the allowable cut), f) the presentation in plan format, (i.e., the preparation of cutting map and the associated tables, which are the final outputs) (GDF, 2017; Asan, 1999; Yolasığmaz, 2013; Başkent et al., 2008a, b, 2005; Asan, 1999).

The forestry philosophy in Turkey, in particular the planning approach and the basic principles need to be explained briefly to understand the topic better. 300 m × 300 m plots are established within the planning unit using a systematic random sampling approach during forest inventory, which is the first phase of forest management plan preparation. The main goal of sampling is the generation of the baseline stand map. This map contains stands, compartments and sub-compartments. Compartments are those areas defined by natural lines like streams and artificial lines like roads and whose borders do not change. Sub-compartments are the sections of stands enclosed by compartments. Stand is a forest fragment that is greater than one hectare in size and differs from the adjacent forest areas by species, mixture, canopy closure and development stage. The main goal of forest area inventory and tree volume and increment inventory is to decide on stand symbols and to determine the stand borders and parameters. A stand symbol contains information like tree species, mixture, stand development stage and closure. Tree species are displayed by symbols. For example, species found at the study area including *Pinus sylvestris*, oak, poplar and juniper are displayed by the symbols Çs, M, Kv and Ar, respectively. The number of trees in the stand and stand volume are considered together to determine the tree species mixture type. The species that exceeds 10% of the stand values for these parameters are included in the stand symbol. The species that has the greatest number or volume by proportion is written first in the stand symbol. Stand development stages are symbolized by the letters *a* (young stands: < 8 cm dbh), *b* (pole pine stands: 8 -19.9 cm dbh), *c* (pre-mature stands: 20-35.9 cm dbh), *d* (mature stands: 36-51.9 cm dbh) and *e* (old stands: >52 cm dbh). Crown closure, or canopy closure (B (degraded); 1-10% crown cover, 1; 11-40% crown cover, 2; 41-70% crown cover, 3; 71-100% crown cover) is defined as the sheltering or shadowing of the ground by tree crowns (GDF, 2017). Development stages and crown closure are two important parameters because the former one is used to determine the method of silvicultural treatment to be applied in the stand and the latter is used to decide on the quantity and the intensity of the allowable cut.

Silvicultural treatments are grouped under four headings including afforestation, tending, regeneration and rehabilitation (Saatçioğlu, 1971; Genc, 2017; GDF, 2017, 2014b). The type and method of the silvicultural treatment and the allowable cut are decided based on factors like ecological and growth conditions of the forest area, biological requirements of tree species, road network, transport and management opportunities, technical capacities of forest enterprises, management goals, conservation targets, forest types and stand parameters. While the silvicultural treatment is applied to the entire sub-compartment, the sub-compartment is/can be partitioned at fields where only afforestation is carried out (Eraslan, 1982). Afforestation areas are the gaps in the forest regime that are to be planted with trees within the plan period. Regeneration areas are those fields which are either done or about to be finished with their management periods and either will be regenerated or their regeneration has begun in the previous period and is still going on. Tending areas are the forested areas except afforestation and regeneration areas. These fields undergo silvicultural treatments in accordance with management goals and conservation targets according to primarily species and then stand canopy closure, development stage, and whether it's a pure or mixed stand. Tending treatments including weeding, release cutting and weeding and thinning are proposed for young, pole pine and pre-mature and old stands, respectively. The silvicultural method used in thinning is low cutting and it is considered heavy and moderate based on the dose or the amount of the treatment. In regeneration areas, final yield allowable cut is taken while in tending areas, intermediate yield allowable cut is taken. Afforestation, regeneration and tending areas are shown by different colors according to the pertinent legislation in harvest maps, which are the final output of forest management plans (Saatçioğlu, 1971; Genç, 2001; GDF, 2017, 2014b; Yolasığmaz and Güner, 2016).

The dominant species at the study is the *Pinus sylvestris*, which is a light tree. It is not damaged by frost. It has tap root system and grows fast in youth stage. Therefore, the weeding period in *Pinus sylvestris* forests is either one or two years. Pole pine stand stage lasts about eight to ten years. The initial thinnings in premature stand stage are heavy low cuttings. The subsequent thinnings that take place every ten years are moderate low cuttings. Tending treatments like the weak low thinnings are performed towards the end of management period past 70 years of stand age. Regeneration is carried out in closed stands with gaps where there are no sufficient seed trees based on slope. Uniform clear cutting method is performed in areas where there is no danger of erosion whereas planting under the forest canopy method is employed in the areas where there is risk of erosion. Uniform shelterwood method, which is based on natural regeneration, is preferred in *Pinus sylvestris* stands where there is sufficient quantity of seed trees. Preparation cuts generally are not needed in the regeneration practices performed using the uniform shelterwood method. Abundant seed years occur every two to four years. Difficulty in determining the abundant seed year of *Pinus sylvestris* is not encountered because its seeds mature over three years. A light cutting after two to three years following the seed cutting done in the abundant seed year and a subsequent removal cutting two to three years later must be performed as the seedlings grow fast. Weeding must be carried out at least once after the removal cutting to reduce the competition of seedlings with the dense live cover present at the areas.

In Turkey, preparation of silvicultural plans, which are the application bases of forest management plans, has been incorporated in the planning process as a legal necessity within the last decade. However, standards for the preparation and application stages of the silvicultural plans could not be established completely. Moreover, silvicultural plans are prepared by conducting a series of field and office work in addition to the ones done for forest management plans with the technical support of administration of chief officers and other administrators.

The type of the silvicultural treatments, the decided functional allowable cut and the sub-compartments in which the silvicultural treatment will take place are identified in two different tables for the regeneration/afforestation and tending areas in the forest management plans. The silvicultural treatment times to be carried out in these areas involve ten or twenty year periods depending on the plan execution schedule. However, the exact location of treatment in each year and the types of regeneration and tending methods to be applied are not clearly stated (GDF, 2017, 2014b).

Three additional tables are prepared as part of the silvicultural plan preparation according to the pertinent legal notice. The first of these tables indicates the field studies that need to be carried out during the preparation of the silvicultural plan, the second one identifies the treatment methods to be applied to the regeneration and tending areas and the third one provides information on the years of the silvicultural treatments and the allowable cut (GDF, 2014b).

Besides, spatial databases generated using GIS technology are designed to prepare forest management plans and maps and thus they are not suited for preparing silvicultural plans and associated maps. The monitoring of structural changes in the forest also is not suited to the monitoring and control of the silvicultural treatments (Yolasığmaz and Keleş, 2009).

The main aim of this study is to prepare a silviculture plan of Oltu Forest Planning Unit dominated by Pinus sylvestris. In addition, the database prepared using GIS is to measure the success of silvicultural treatments and to monitor forests.

Materials and methods

Materials

The main materials used in the study are the forest management plan attribute and digital data of the Oltu Forest Planning Unit and the geographic database. The measurements and examinations in the study area especially were focused on afforestation and regeneration areas where soil analyses data, ecological conditions, current stand structures and abundant seed years were assessed and determined.

Study area

The study area is enclosed by 714000-777000 (30° 41’ 08” 42” – 40° 39’ 45”) east longitudes and 4470000-4510000 (40° 32’ 46” – 40° 46’ 51”) north latitudes in zone 37 according to UTM coordinate system and ED 50 datum (*Fig. 1* and *2*). The planning unit is bordered by Narman Forest Planning Unit to the southeast, Tortum Forest Planning Unit to the southwest, Hisar Forest Planning Unit to the northeast Kılıçkaya Forest Forest Planning Unit in the Yusufeli Forest Enterprise to the northwest. Oltu Forest Planning Unit is within the boundary of Oltu Forest Enterprise with a distance of 100 to 180 km to Erzurum province. Elevation ranges from 1105 to 3045 m. Average

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

temperature is 9.8 °C and the annual precipitation is 393.3 mm according to meteorological data. High slope topography and harsh winter conditions render living conditions difficult resulting in accelerating migration from the area to urban regions (GDF, 2015a).

Figure 1. *Images of the study area*

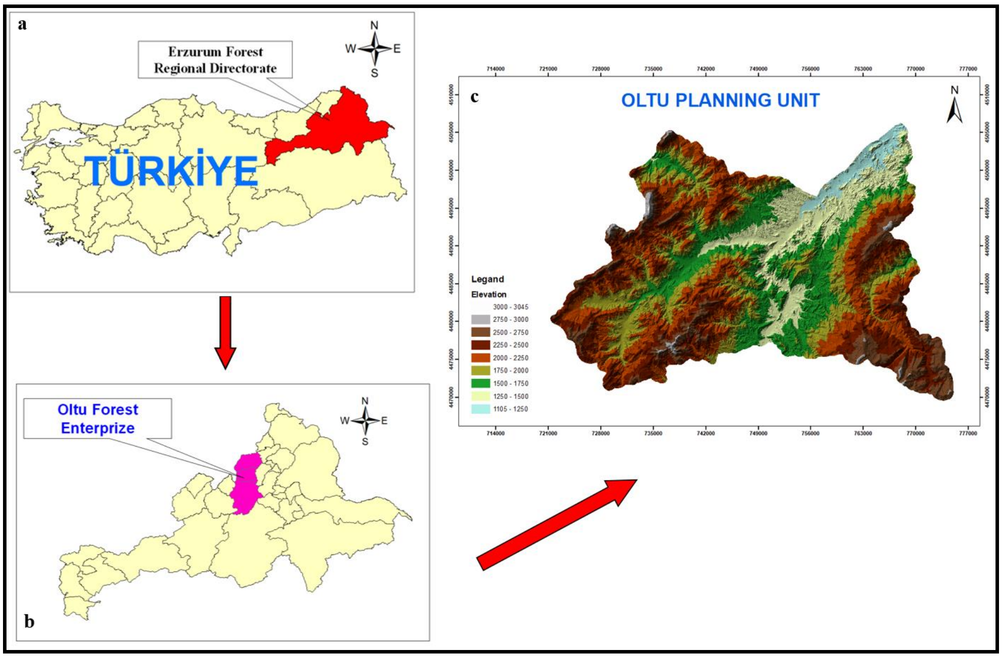

Figure 2. *Location of the study area according to the administrative organization of Turkish forests. a) Location of Erzurum Forest Regional Directorate within Turkey, b) location of Oltu Forest Enterprise within Erzurum Forest Regional Directorate, and c) elevation map of Oltu Forest Planning Unit*

The total area of the study area is 105108.707 ha of which 28788.303 ha is forested and 13755.785 ha is non-forest area. The planning unit is comprised of 586 compartments and 2679 sub-compartments with 43 different stand types identified (GDF, 2015a).

The dominant species of the flora include *Pinus sylvestris* (L.), *Juniperus* sp., *Qercus* sp. and *Populus tremula* (L.). Even though a comprehensive plant sociology study is not conducted at the study area, four different tree species (*Platanus orientalis*, *Acer* sp., *Pyruselae agnifoliave*, *Sorbus torminalis*) that do not form stands by themselves but are part of the mixture either individually or in groups were recorded in the forest management plan. Additionally, four different small trees and shrub forms and nine plant species were identified and also recorded in the forest management plan. The management units used for wood production are dominated by *Pinus sylvestris* (L.). There are also poplar (*Populus tremula* (L.)) stands with normal canopy closure. Forest areas where *Pinus sylvestris*, juniper and poplar codominated and formed mixtures together were identified in degraded areas.

Oltu Forest Planning Unit is grouped into eight different forest management units according to the forest management plan (*Table 1*). 17.455% of the study area is planned to be utilized for economic purposes while 53.061% is planned for ecological use and the remaining 29.485% is planned for socio-cultural use.

Methods

Field measurements and assessments

Investigations were done in afforestation and regeneration areas in the summer months of 2015. Data related to ecological conditions (slope, aspect, elevation, soil

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

type, soil depth and stoniness, etc.) were collected and current stand structure was inspected in area. Abundant seed years and presence or absence of seed trees were determined in order to decide on the regeneration method.

Table 1. *Area distribution of the forest management unit in Oltu Planning Unit (GDF, 2015a)*

Forest management units	Area (ha)	%
A-Wood Production	18346.336	17.455
B-Nature Conservation	37767.906	35.932
C-Wildlife Development Areas	5353.919	5.094
D- High Mountain Forest Ecosystem	11244.117	10.698
E- Forest Ecosystem Monitoring Areas	43.090	0.041
F- Erosion Control - Soil Conservation	1362.304	1.296
G- Conservation of Water Sources	30748.068	29.254
H- Recreation	242.969	0.231
Total	**105108.707**	**100.000**

Design and configuration of database

The baseline map used for the preparation of silvicultural plans is the forest management plan geographic database. The primary dataset within the database associated with compartments, sub-compartments, stand parameters like stand type information, age class and site quality class and forest management units and harvest map were used directly. Data including the volume of trees per hectare (m^3/ha), annual tree volume increment (m^3/ha/year), types of silvicultural treatments as defined in the plan (afforestation, regeneration and tending) and the decided functional allowable cuts/tending allowable cuts (m^3) were entered in the geographic database of the silvicultural plan in addition to the forest management plan data. Also, data titles displaying the fundamental components of a silvicultural plan like the silvicultural treatment method, five different types of silvicultural treatments, five different silvicultural treatment times, the quantity of the allowable cuts decided for each treatment, the number of treatments and the realized allowable cut were added as columns to the database.

Preparation of silvicultural recipes

Afforestation areas are gaps in the forest and degraded forest areas that are suitable for afforestation. Silvicultural recipe to be applied to the gaps and degraded forest areas (*Tables 2* and *3*).

Table 2. *The silvicultural recipe to be applied to gaps*

Silvicultural treatment number	Silvicultural recipe
Silvicultural Treatment_1	Planting
Silvicultural Treatment _2	Weeding (1 year after planting)
Silvicultural Treatment _3	Weeding (2 years after planting)
Silvicultural Treatment _4	Release cutting (10 years after second weeding)

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Table 3. *The silvicultural recipe to be applied to degraded forest areas*

Silvicultural treatment number	Silvicultural recipe
Silvicultural Treatment_1	Clear cutting+Planting
Silvicultural Treatment _2	Weeding (1 year after planting)
Silvicultural Treatment _3	Weeding (2 years after planting)
Silvicultural Treatment _4	Release cutting (10 years after second weeding)

Regeneration areas are those stands in which regeneration treatments started in the previous plan period and are ongoing or they either completed their management plan cycle or are in the process of completion. Factors like the technical capacity of the forest enterprise, workforce and road condition beside growth environment characteristics like elevation, slope, soil properties (soil type, stoniness, soil depth, etc.) and stand parameters such as the biological requirements of the tree species, canopy closure and mixture patterns were considered during the silvicultural treatments to be applied to these areas. Regeneration areas were divided into three groups as follows "areas currently undergoing regeneration", "*Pinus sylvestris* stands with canopy closure level 1" and "*Pinus sylvestris* stands with canopy closure levels 2 and 3" (*Tables 4, 5* and *6*).

Table 4. *Silvicultural recipe to be applied to stands that currently are undergoing regeneration (Çsd1/Çsa0)*

Silvicultural treatment number	Silvicultural recipe
Silvicultural Treatment_1	Removal cutting (in 2015 or 2016 years)
Silvicultural Treatment_2	Weeding (1 year after removal cutting)
Silvicultural Treatment_3	Release cutting (10 years after second weeding)

Table 5. *Silvicultural recipe to be applied to Pinus sylvestris stands with canopy closure level 1 (Çsd1)*

Silvicultural treatment number	Silvicultural recipe
Silvicultural Treatment_1	Clear cutting + planting or planting under the forest canopy
Silvicultural Treatment_2	Removal cutting (3 years after the planting)
Silvicultural Treatment_3	Weeding (1 year after removal cutting)
Silvicultural Treatment_4	Release cutting (10 years after second weeding)

Table 6. *Silvicultural recipe to be applied to Pinus sylvestris stands with canopy closure levels 2 and 3 (Çsd2/ Çsd3)*

Silvicultural treatment number	Silvicultural recipe
Silvicultural Treatment_1	Seed cutting (abundant seed year)
Silvicultural Treatment _2	Light cutting (3 years after seed cutting)
Silvicultural Treatment _3	Removal cutting (2 or 3 years after light cutting)
Silvicultural Treatment _4	Weeding (1 year after removal cutting)
Silvicultural Treatment _5	Release cutting (10 years after second weeding)

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DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

Tending is proposed to all forest areas except afforestation and regeneration areas. Factors like the technical capacity of the forest enterprise, sociocultural and socioeconomic structure of the villages within and adjacent to the forest, workforce potentials of these villages, social problems and disputes and road condition were considered during the spatial arrangement of the tending areas. Tending cuts every ten years are proposed during a management period of twenty years due to the biological requirements of *Pinus sylvestris* species. Low cutting method is used in *Pinus sylvestris* forests primarily even though the silvicultural treatment method varies based on the development stages of stands. Allowable cut during tending is calculated following the inventory conducted in the field and it is determined based on the forest function that the area provides, management goals and conservation targets. The current characteristics of the stands, (i.e., stand parameters including canopy closure and stand development stage) affect the dose and method of the silvicultural treatment to be applied to these areas. The silvicultural treatment methods to be applied to each stand type in tending areas are shown in *Table 7*.

Table 7. *Silvicultural treatment methods to be applied to tending areas*

<table>
<tr><th>Stand symbol</th><th>Silvicultural treatment -1</th><th>Stand symbol</th><th>Silvicultural treatment -2</th></tr>
<tr><td>Çsa0, ÇsYaa0, CvBma0</td><td>Weeding</td><td>Çsa0, ÇsYaa0, CvBma0</td><td>Release cutting (Heavy low thinning)</td></tr>
<tr><td>Çsa</td><td>Weeding</td><td>Çsa</td><td>Release cutting (Heavy low thinning)</td></tr>
<tr><td>Çsab1, Çsab2</td><td>Release cutting (Heavy low thinning)</td><td rowspan="3">Çsab2, Çsb3, Çsbc2, Çsbc3, Çsc2, Çsc3, Çscd2, Çscd3, Çsd3, ÇsKvbc2, ÇsKvbc3, Kvb2, Kvbc2, Kvbc3, KvÇsbc2, KvÇsbc3</td><td rowspan="3">Moderate low thinning</td></tr>
<tr><td>Çsb3, Çsbc3, ÇsMbc3, Kvb3, Kvbc3, KvÇsbc3</td><td>Heavy low thinning</td></tr>
<tr><td>Çsab2, Çsbc2, Çsc2, Çsc3, Çscd2, Çscd3, Çsd3, ÇsKvbc2, ÇsKvbc3, Kvb2, Kvbc2, KvÇsbc2</td><td>Moderate low thinning</td></tr>
<tr><td>Çsbc1, Çsc1, Çscd1, Kvb1, Kvbc1</td><td>Weak low thinning</td><td>Çsab1, Çsbc1, Çsc1, Çscd1, Çsd1, Kvb1, Kvbc1</td><td>Weak low thinning</td></tr>
</table>

Tending areas are divided into ten tending blocks in traditional planning. Here, each tending block was further grouped into ten separate sub-tending blocks, which is different from the traditional approach. Since the legislation requires that the harvest jobs be given to the local residents in the nearest settlement, spatial planning was carried out to provide employment to each village every year.

Calculation of the allowable cuts

Intermediate yield allowable cut (the allowable cut taken from tending areas)

Ten separate tending blocks were established and each tending block was further grouped into ten separate sub-tending blocks and numbered. The treatment years for the sub-blocks within each tending block were determined according to their ordinal hierarchy such that the first set of treatments would be completed between 2015 and

2024 and the second set of treatments would be completed between 2025 and 2034 across the tending blocks.

Final yield allowable cut (the allowable cut taken from regeneration areas)

The entire volume within regeneration areas will be harvested during the 20 year plan period but in different years. Once the youth is established healthily, the trees in the canopy will be harvested by removal cutting. The remaining trees will increase their volume during the period between seed cutting and removal cutting. Therefore, half of the tree volume increment was added to the current tree volume in calculations of the allowable cut in regeneration areas. The allowable cuts that will take place in these areas based on the silvicultural treatment method and the cutting technique were calculated/determined as follows:

- In degraded *Pinus sylvestris* stands (BÇs), the entire volume will be harvested by clear cutting in the first treatment and seedlings will be planted.
- In *Pinus sylvestris* stands with level 1 canopy closure (Çscd1) where planting under the forest canopy will take place, it is assumed that 20% of the current tree volume will be harvested during the initial treatment, 50% of the remaining tree volume will be harvested during the second treatment, i.e., the light cutting, and that 97% of the remaining tree volume will be harvested by the next treatment, which is the removal cutting.
- In *Pinus sylvestris* stands with level 1 canopy closure (Çscd1) where uniform clear cutting will take place, it is assumed that 97% of the tree volume will be clear cut during the initial treatment and the area will be replaced by seedlings.
- In *Pinus sylvestris* stands with level 2 canopy closure (Çscd2) where uniform shelterwood method will be applied, it is proposed that 20% of the current tree volume will be harvested during seed cutting, which is the initial treatment, 50% of the remaining tree volume will be taken during light cutting, which is the second treatment and that 96% of the remaining tree volume will be taken during removal cutting.
- In *Pinus sylvestris* stands with level 2 canopy closure (Çscd2) where uniform clear cutting will take place, it is assumed that 97% of the tree volume will be clear cut during the initial treatment and the area will be replaced by seedlings.
- In *Pinus sylvestris* stands with level 2 canopy closure (Çscd2) where planting under the forest canopy will take place, it is assumed that 20% of the current tree volume will be harvested during the initial treatment, 50% of the remaining tree volume will be harvested during the second treatment, i.e., the light cutting, and that 97% of the remaining tree volume will be harvested by the next treatment, which is the removal cutting.
- In *Pinus sylvestris* stands with level 3 canopy closure (Çscd3), it is assumed that uniform shelterwood method will be applied and 40% of the current tree volume will be harvested during initial treatment, 50% of the remaining tree volume will be taken during light cutting, which is the second treatment and that 96% of the remaining tree volume will be taken during removal cutting.
- In stands currently undergoing regeneration where light cutting is recommended, it is proposed that 50% of the current tree volume will be harvested and that 96% of the remaining tree volume will be taken during removal cutting. If light cutting is not recommended at these areas, than it is

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DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

proposed that 96% of the tree volume at the area will be harvested by removal cutting.
- It is proposed that 3-4% of tree volume will be left at regeneration stands as value trees for ecological and biological balance.

Results and discussion

According to the forest management plan data, the total tree volume at the planning unit is 1415343.798 m^3, annual tree volume increment is 44214.787 m^3/year and the amount of agreed allowable cut during the plan period of 20 years is 1121891.449 m^3 (*Table 8; Fig. 3*).

Table 8. *The distribution area of the silvicultural treatments by type and other areas according to the forest management plan*

Silvicultural treatment	Area (ha)	%
Afforestation	1.981	0.002
Regeneration	121.933	0.116
Tending	11646.306	11.080
No treatment	79514.118	75.649
Other forest areas	74.439	0.071
Agricultural area	12673.448	12.057
Residential areas	1076.484	1.024
Total	**105108.707**	**100.000**

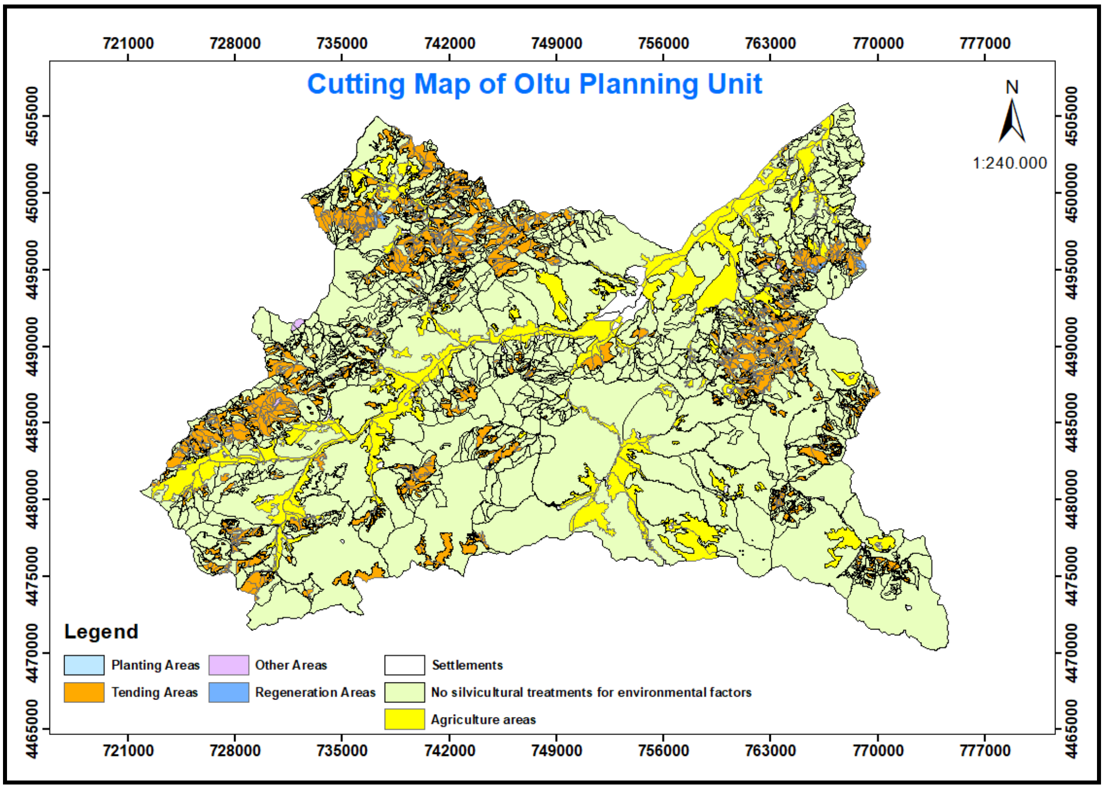

Figure 3. *Cutting map of Oltu Planning Unit according to forest management plan*

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In the silvicultural plan, thinning and release cutting are proposed as the first treatments at tending areas. Thinning and release cutting are planned for 81.262% and 17.685% of the tending areas, respectively (*Table 9; Fig. 4*).

Table 9. *The distribution of the area and allowable cut by silvicultural treatment method in silvicultural plan*

Silvicultural method	Area (ha)	%	Allowable cut (m^3)	%
Afforestation areas	**1.981**	0.017	**-**	-
Planting	1.981	0.017	-	-
Tending areas	**11646.306**	98.947	**97974.418**	87.671
Thinning	9564.720	81.262	97974.418	87.671
Release	2081.586	17.685	-	-
Regeneration areas	**121.933**	1.036	**13777.483**	12.329
Natural regeneration_Uniform shelterwood	35.275	0.300	4114.895	3.682
Natural regeneration_Uniform clear cutting	12.149	0.103	97.190	0.087
Artificial regeneration_Planting under the forest canopy	74.509	0.633	9565.397	8.559
Sum	**11770.219**	100.000	**111751.901**	100.000

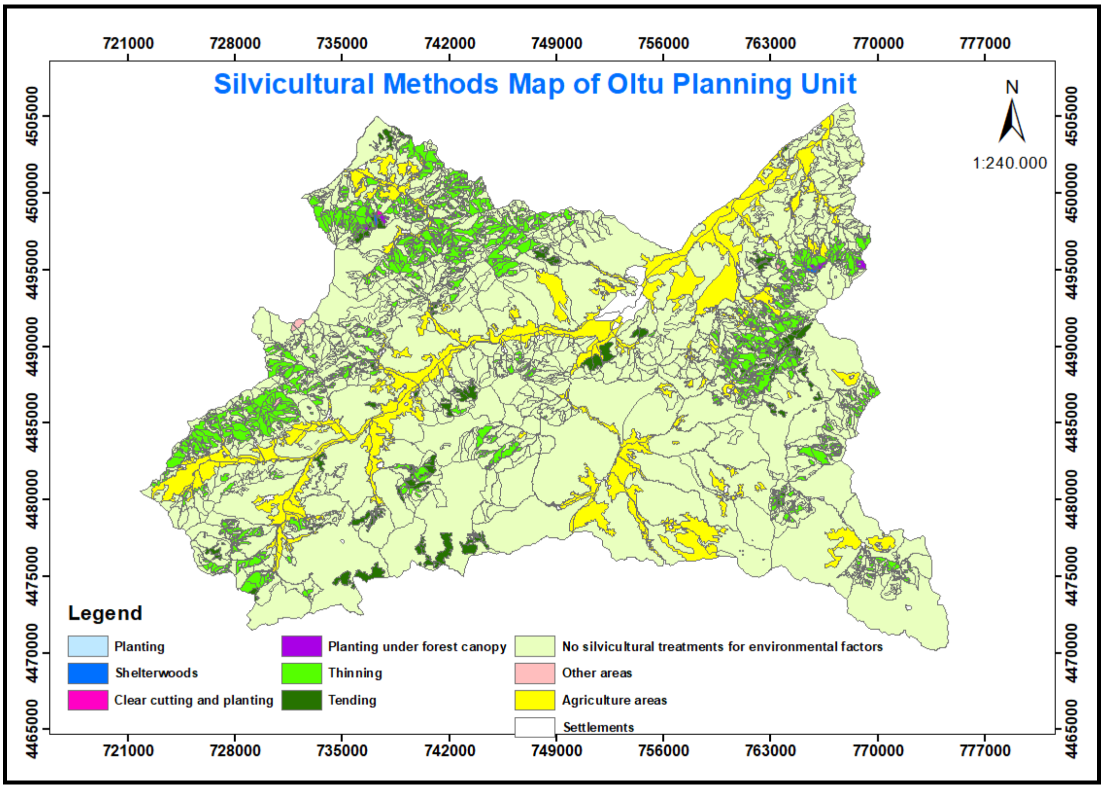

Figure 4. *Silvicultural methods map of Oltu Planning Unit*

112189.449 m^3 of allowable cut is proposed according to the forest management plan while 111751.901 m^3 is proposed in the silvicultural plan. 437.547 m^3 of allowable cut

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was abandoned, which consisted of the value trees left at the area to allow for the sustenance of wildlife.

Abundant seed year occurs every two years and the first abundant seed year was 2015. Therefore, regeneration started in the abundant seed year of 2017. The focus was on completing the ongoing regeneration practices in the planning unit.

We tried to keep the size of the tending areas even across the years, where the average annual size and the average tending allowable cut quantity were 1186.702 ha/year and 4898.721 m^3/year, respectively. The total quantity of the allowable cut of the tending areas is 97974.418 m^3 and makes up 87.671% of the total allowable cut whereas the allowable cut that will be harvested from the regeneration areas constitutes 12.329% of the total allowable cut. We aimed to keep the annual treatment area size and the quantity of the annual allowable cut equal during the treatments to be applied to the regeneration areas.

The average annual treatment area size and the average quantity of the annual allowable cut to be conducted in the regeneration areas were planned as 21.774 ha/year and 688.874 m^3/year, respectively. The average annual treatment area size and the average annual allowable cut quantity were 1186.702 ha and 5587.595 m^3/year, respectively when the allowable cut and treatment area size were examined across the years and regeneration and afforestation areas were combined (*Table 10; Fig. 5*).

Table 10. *Silvicultural treatments areas and the allowable cut distributions according to the treatments years*

Treatment year	Afforestation	Tending		Regeneration		Total treatment area (ha)	Total allowable cut (m^3)
	Area (ha)	Area (ha)	Allowable cut (m^3)	Area (ha)	Allowable cut (m^3)		
2015	-	1150.529	5958.670	-	-	1150.529	5958.670
2016	-	1194.489	4921.596	-	-	1194.489	4921.596
2017	-	1154.303	4083.296	17.591	368.882	1171.894	4452.178
2018	-	1167.978	4946.626	19.462	1572.548	1187.440	6519.174
2019	-	1147.563	3898.569	62.432	859.504	1209.995	4758.073
2020	-	1177.190	5072.762	47.712	1360.985	1224.902	6433.747
2021	-	1178.904	6264.392	5.209	-	1184.113	6264.392
2022	-	1162.920	4371.161	19.045	2343.676	1181.965	6714.837
2023	-	1108.471	5129.365	53.203	3507.587	1161.674	8636.952
2024	-	1203.958	4340.771	34.158	-	1238.116	4340.771
2025	1.981	1150.529	5958.670	27.368	-	1179.877	5958.670
2026	1.981	1194.489	4921.596	-	-	1196.470	4921.596
2027	-	1154.303	4083.296	-	-	1154.303	4083.296
2028	-	1167.978	4946.626	28.480	3764.301	1196.458	8710.927
2029	1.981	1147.563	3898.569	45.718	-	1195.262	3898.569
2030	-	1177.190	5072.762	16.691	-	1193.881	5072.762
2031	-	1178.904	6264.392	5.209	-	1184.113	6264.392
2032	-	1162.920	4371.161	-	-	1162.920	4371.161
2033	-	1108.471	5129.365	19.045	-	1127.516	5129.365
2034	-	1203.958	4340.771	34.158	-	1238.116	4340.771
Sum	5.942	-	97974.418	-	13777.483	-	111751.901

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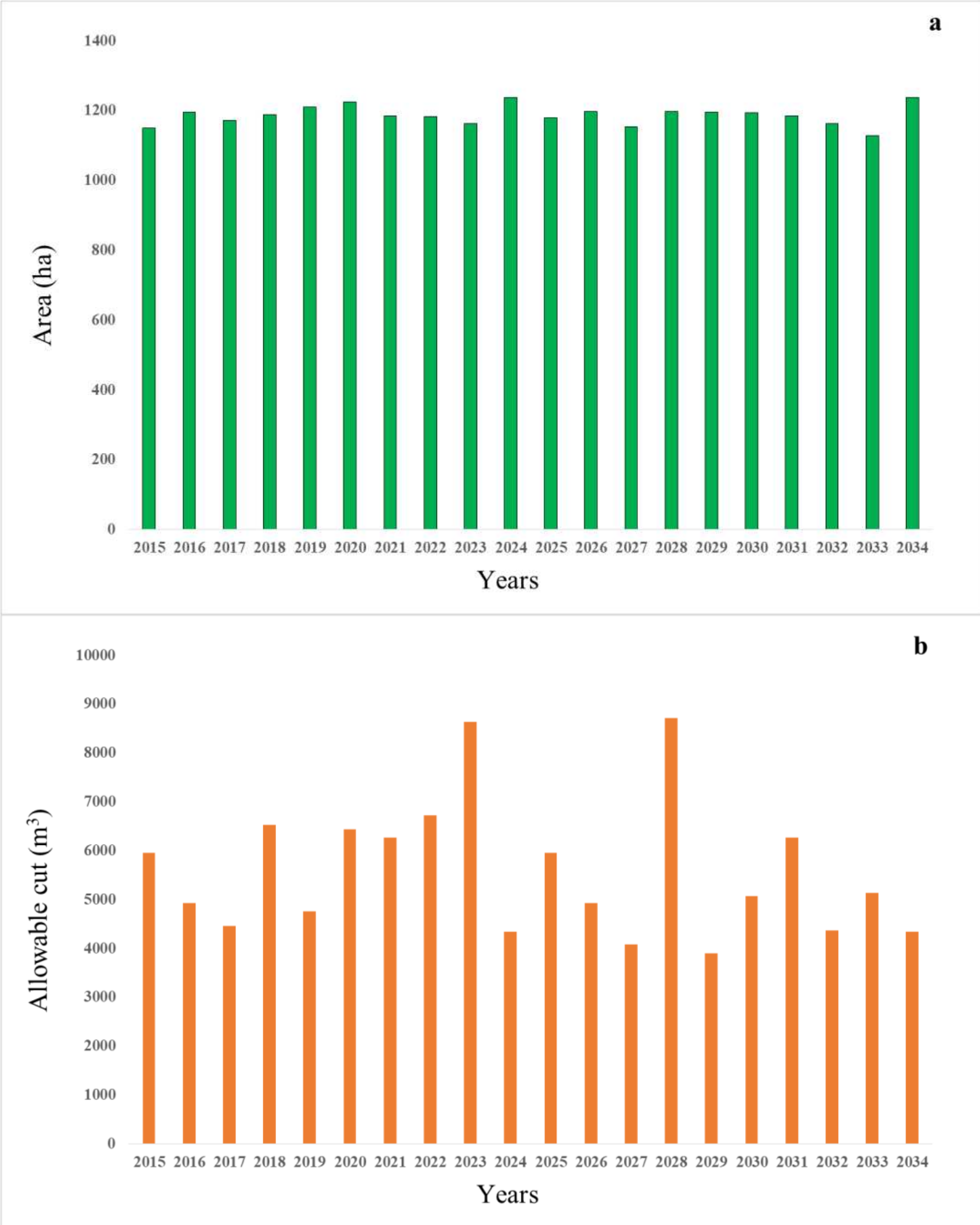

Figure 5. *The distribution of total treatment area (a) and allowable cut (b) according to treatment years*

Conclusion

The silvicultural plan of Oltu Forest Planning Unit was prepared using the joint approaches of forest management and silviculture disciplines. A new spatial database was designed in the silvicultural plan by using the treatment areas in the forest management plan and the associated geographic database as the base. Silvicultural recipes were prepared according to the silvicultural treatment type (afforestation, regeneration and tending) for each sub-compartment. Final outputs in the form of harvest maps or in other words, silvicultural plan map was produced and provided to the users.

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General assessments like the silvicultural treatment methods to be applied at the study area, their distribution across the years and the number of treatments were performed and provided as outputs of this study using the analysis, query and presentation capabilities of GIS. In addition to these outputs, other outputs including detailed silvicultural plan tables, numerous analyses on stand parameters like development stage, age and site quality classes as well as the treatment areas and the application years for the five separate treatment types can be generated and provided.

Overall, 1.98 ha area was agreed to be afforested, 11646.306 ha was decided to undergo tending cuts and 121.933 ha area was decided to be regenerated in the forest management plan of Oltu Forest Planning Unit. The forest management plan was adhered to in the silvicultural plan and thus there was no change in these areas. Total of 111751.901 m^3 allowable cut was planned at the study area during the 20 year plan period, comprised of 97974.418 m^3 intermediate yield allowable cut and 13777.483 m^3 final yield allowable cut.

The annual tree volume increment is 44214.786 m^3/year and approximately 12.64% of the annual increment will be harvested as the allowable cut every year. This is much lower than the national average annual allowable cut to annual tree volume increment ratio of 39.87% (GDF, 2015b). As stated earlier quantitatively, large portion of Oltu Forest Planning Unit is utilized for ecologic and social goals. Similarly, the demand for wood production in the US decreased, as opposed to increasing demand for ecological and social services (D'Amato et al., 2017). As a result, silvicultural practices and planning approaches in Turkey are developing within the framework of ecological and social objectives.

The silvicultural plan was prepared for Oltu region forests, which are composed of pure *Pinus sylvestris* stands generally dominated by one species and specifically for Oltu Forest Planning Unit. Different tree species, different species compositions and different forestry problems exist in different regions of Turkey because of the geographic diversity of the country. The number of silvicultural treatments, their years and the quantity of the allowable cut also will differ in these regions. Therefore, similar studies need to be conducted in different forest regions of Turkey, in which silvicultural plans and digital baselines should be generated for each forest planning unit.

Acknowledgements. This study was carried out as part of the project 2016. F10.02.02 supported by the Scientific Research Projects Coordinatorship of Artvin Çoruh University (AÇÜBAP). Foremost, we thank (AÇÜBAP) for the support they provided. Also, we extend our thanks to the Anatolia Environment Forestry Mapping Inc. Company (ANÇEO), the General Directorate of Forestry and the Faculty of Forestry of Artvin Çoruh University for all the support and help they provided during the preparation of the silvicultural plan and the forest management plan and for the acquisition of the data.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_50935108

WHAT'S GOING ON AT THE UNIVERSITIES? HOW MUCH HAS THE RESEARCH REVEALED UNIVERSITY STUDENTS' ATTITUDES TOWARDS THE ENVIRONMENT? A CASE STUDY OF BURSA, TURKEY

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(Received 11th Jan 2019; accepted 20th Feb 2019)

Abstract. The aim of this research is to reveal the environmental consciousness levels of university students determine how they evaluate the society and the University at this level of consciousness and analyse the attitudes and behaviour they exert. For this purpose, a structured survey was conducted at nine faculty students at Bursa Uludag University in Turkey. Students' attitudes and behaviour were examined through their gender, family income level, parental education and professions and the field of the study variables. In this research, the questionnaire consisting of open and close–end questions was used; SPSS 23 program was used to evaluate the data. The research revealed that variables other than gender and the fathers education have a significant effect on the environmental awareness of the students. Furthermore, the students stated that universities did not take an active role in environmental protection. They attributed the lack of environmental awareness to the lack of adequate environmental protection laws and penalties. They wanted society to be more active in protecting the environment and universities to include students in environmental decisions and practices. The results show that students have inadequate environmental knowledge, therefore, display lower levels of environmental attitude and do not exert highly favourable behaviour.
Keywords: *policy-making, higher education, knowledge, participation, green behaviour*

Introduction

The environment can be defined as the surroundings or conditions in which a person, animal, or plant coexists and interacts. The environment is in a state of integrity and equilibrium with its living and non-living beings and is an ordered system capable of tolerating its own adversities within a certain degree. It can also be defined as all the factors that affect the lives of people and society in all aspects physically, psychologically and culturally (Baki and Cengiz, 2002). *Environmental awareness* is the understanding of the consequences of environmental damage and its sustainable level of use (Kollmuss and Agyeman, 2002). Environmental awareness requires a willingness to take positive actions against environmental problems.

Problems encountered in the natural and cultural environment in forms of population growth, urbanisation and improper land use have soared tremendously as a result of industrialisation and technological developments (Gayford, 2002; Mazi and Demirci, 2004; Palabiyik and Altunbas, 2004; Withgott and Brennan, 2007). As the environmental problems expanded and penetrated, the unrestrained nature of the problems has been realised. Thus, national and international efforts have been intercalated to solve them (Najam and Cleveland, 2003). Since destroying natural and cultural resources in the world is not a new phenomenon, the measures taken to protect nature are not new either.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

Nonetheless, the emergence of legal measures, the increase in environmental awareness, the systematic conservation of nature that requires the segregation of protected areas and the emergence of international organisations coincide in the nineteenth and twentieth centuries. Aforementioned developments accelerated, especially after the 1960s.

Individuals with sufficient environmental sensitivity are vital to solve environmental problems permanently. The intensity of societal perception about nature and the environment's known and unknown problems, the response and the discomfort displayed by individuals and society are the indicators of environmental awareness. The level of awareness may vary depending on a number of factors such as one's life experiences, beliefs, socioeconomic level, interactions with the natural environment and educational levels (Aydin and Cepni, 2010).

The major cause of many environmental problems is the irresponsible behaviour of the individuals and along with negative attitudes towards the environment. Therefore, it is necessary to re-establish those behavioural patterns and attitudes to eliminate the existing environmental problems, to prevent new problems and to achieve a more sustainable future (Lehman and Geller, 2004).

Environmental attitudes are the sum of all the positive and negative attitudes and thoughts of individuals, who exhibit environmentally beneficial behaviour, environmental value judgments and readiness to solve them. *Environmentally friendly* behaviour refers to a behaviour that harms the environment as little as possible and is influenced by various factors. The most important of these factors is environmental attitude.

While environmental attitudes are seen as strong determinants of environment-friendly behaviour, there is a subtle difference between the two. From primary to university, students' attitudes and behaviour about the environment have been frequently examined in the literature, especially in developing countries.

Bowonder (1987) argues that environmental problems in developing countries are growing and becoming more difficult to resolve as a consequence of the combined effect of population growth, urbanisation, poverty and industrialisation. Progress is slower than developed countries (OECD Green Growth Indicators, 2017) and enforcement are mostly driven by pressure from more developed countries and international institutions such as United Nations Environmental Programme (UNEP) Global Environment Facility (GEP), Food and Agriculture Organisation of the United Nations (FAO), United Nations Educational, Scientific and Cultural Organisation (UNESCO), United Nations Industrial Development Organisation (UNIDO) and World Health Organisation (WHO) Intergovernmental Panel on Climate Change (IPCC) (Stephan and Zelli, 2009).

The population in developing countries is younger; hence young people need to shoulder the responsibility to solve them. Turkey's number of students exceeded the population of 143 countries. In the 2018-2019 academic year in Turkey, 17 749 876 students receive primary and secondary education in state and private schools affiliated by the Ministry of National Education. There are 7 560 000 students in higher education and a total of 25 309 876 students in Turkey (National Education Statistics, 2018). Compared to Finland and Denmark with a population of 5.5 million, Bulgaria with a population of 7 million, Azerbaijan with a population of 10 million, the Netherlands with a population of 17 million, Australia with a population of 25 million, Chile with a population of 18 million and Cameroon with a population of 25 million are behind the student population in Turkey (World Population Prospects, 2017). This explains the importance of research on students at varying levels of educational stages (Anonymous, 2018).

Universities bear certain responsibilities in increasing knowledge, awareness and inventing technology. They educate young generations to perform important social roles effectively; thus they have a critical role in enhancing human behaviour and promote pro-environmental behaviour (Corcoran and Wals, 2004; Frank and Meyer, 2007). In consequence, university students are regarded as decision makers, opinion shapers and the community leaders of the future in a given society (Lee, 2008; Lozano, 2006; Waas et al., 2010; Wright, 2007; Zilahy and Huisingh, 2009).

Environmental awareness, environmental conservation and sustainability have become frequently discussed issues in universities in the last twenty years. University students are equipped with more diversified and exclusive resources such as several means of media, research projects, conferences, and workshops to learn about the environment (Morigi and Krebs, 2012; Silveira and Cruz, 2012). Despite this rich resources, whether they exert sufficient environmental knowledge and awareness or reflect this knowledge to their behaviour are still questionable (Hartmann and Apaolaza–Ibazez, 2012; Levine and Strube, 2012; Markowitz et al., 2012).

Berberoglu and Tosunoglu (1995) and Oguz et al. (2010) reported that university students did not have sufficient awareness and participation in environmental issues. Arunkumar (2012) reported an average level of environmental awareness. Many studies have shown that the majority of university students are aware of environmental problems and believe sustainability is an important goal to be achieved. Some research confirms that students environmental knowledge influence their environmental behaviour (Huang and Shih, 2009; Thapa et al., 2005), remaining research is inconclusive. Environmental awareness and knowledge may well influence the individual's environmental behaviour. Environmental sensitivity could intensify environmental behaviour and increase participation (Sivek and Hungerford, 1990). Environmental knowledge affects behavioural intentions positively (Wang et al., 2014). As Abbas and Singh (2014) reported, a higher proportion of university students possess a high level of environmental knowledge but a low level of participation. They show interest in global problems such as climate change, acknowledge that environmental problems are serious and take a positive attitude towards it; however, the students inclined to exert minimum environmentally friendly behaviour in their everyday activities (Elder, 2003; Heyl et al., 2013; Kollmuss and Agyeman, 2002; Kormos and Gifford, 2014; Muderrisoglu and Altanlar, 2011; Schultz et al., 2005; Sharma, 2014). Hadlock and Beckwith (2002) in their study found that people did not try to understand the reasons or were not willing to participate in environmental issues that were not directly related to them. In addition, the availability of options and infrastructure, the degree of sacrifice students have to make (Arbuthnott, 2009; Kagawa, 2007; Stern, 2000) determines their environmental behaviour. Consequently, habitat or natural species become trivial to them unless there is a situation directly related or harmful. In short, there is always no linear relationship between environmental attitudes and environmentally friendly behaviour.

As stated earlier university students are more likely to access the information about the environment and achieve positions to take meaningful action and to build a more sustainable world (Sharon and Wright, 2006). For this reason, it is crucial to identify the factors affecting students' environmental behaviour. Undeniable number of research focuses on students from various countries e.g. United States (Levine and Strube, 2012; Meyer, 2016), China (Chuanhui and Hanwei, 2011), Taiwan (Chen and Tsai, 2015; Pan

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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et al., 2018), Philippines (Ejem and Bello, 2013), Rwanda (Kabera, 2017), Malaysia (Elsawahli and Mohit, 2010; Hassan et al., 2011), Mexico, Brazil, Portuguese (Córtes et al., 2016), Spain (Vicente–Molina et al., 2013), India (Panth et al., 2015), Israel (Yavetz et al., 2009), Bangladesh (Ullah et al., 2013), United Arab Emirates (UAE) (AlMenhali et al., 2018), Yemen (Abdulrab, 2015), Poland (Demeskhant, 2013), Serbia (Major et al., 2017), Romania (Crumpei et al., 2014), Hungary (Zsóka et al., 2013), Ireland (Nicolaou and Conlon, 2013) aimed at determining the environmental attitudes, awareness and behaviour of university students.

Researchers claim that students' behaviour is affected and formed by their existing environment (Asunta, 2004; Lukman et al., 2013); some of which are namely: family, friends, neighbours, and cultural values. Different research revealed contradictive results for above-mentioned factors. Also, other variables such as educational background, the field of study, family income, parental education, parental occupations and where they live revealed a similar contradiction. Following this, some of this research specifically concentrated on demographic factors (Meyer, 2016; Sharma, 2014; Vicente–Molina et al., 2018), others focused on cultural factors (Córtes et al., 2016; Kaplowitz and Levine, 2005; Kilbourne and Polonsky, 2005; Major et al., 2017; Vicente–Molina et al., 2013). Researchers also wanted to reveal whether the major student study could be an influencing factor (Budak et al., 2005; Chen and Tsai, 2016; Demeskhant, 2013; Duman–Yuksel and Ozkazanc, 2015; Kiper, 2014; Nicolaou and Conlon, 2013; Pan et al., 2018). While the majority of the research was conducted on university students in general, there is substantial research on the teacher candidate students (Goldman et al., 2006; Ozden, 2008; Pe'er et al., 2007; Tuncer et al., 2009; Yavetz et al., 2009).

The purpose of this study is to explore undergraduate students' awareness and behavioural patterns towards the environmental problems at Bursa Uludag University in Turkey. The city of Bursa is founded in one of the most fertile plains of Turkey (Eisenlohr et al., 1995). It is believed that this research in Bursa with three million residents (TurkStat, 2017) and about 75 thousand students (Bursa Uludag University, 2018) will provide significant results for the field. It will allow researchers to compare the results of the current research and shed light on the issues raised in previous researchers.

Materials and methods

The universe and the sampling

This study was carried out at Bursa Uludag University, Turkey. Bursa Uludag University is the seventh largest university in Turkey (Higher Education Council, 2018). It accommodates 74 822 students 42 917 of whom undergraduate, in 14 faculties, 4 institutes, and 15 vocational schools. 10 of the 14 faculties are located on the central campus, Gorukle. The number of students here was 47 138 in the 2017-2018 academic year (Bursa Uludag University, 2018). Other faculties and vocational high schools are distributed in various districts of the province. However, this research is limited to Gorukle campus and 9 faculties, taking the student distribution rates of the faculties into consideration and the transportation restrictions of the campuses in the districts.

For this research, a structured questionnaire was prepared by carefully scanning the existing literature and reviewing the previous work. At the beginning of the lecture, the students were asked to fill in this questionnaire under the supervision of the trained interviewers with the permission of the lecturer. The age of the students varies between

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18 and 24 in 4-year faculties, while the rate of the Medical School varies between 18-26, 18-21 in vocational schools, and 18-26 in Art School. The research was carried out from March to May 2018.

The sample size was determined by estimating a proportion formula used by Thompson (2012). To obtain an estimator p having probability at least 1 - α of being no farther than d from the population proportion, the sample size formula based on the normal approximation gives

$$n = \frac{Np(1-p)}{(N-1)\left(\frac{d^2}{z^2}\right) + p(1-p)} \quad \text{(Eq.1)}$$

Hence, the minimum sample size was found to be 680 (32 questionnaires were discarded because they were incomplete, resulting in a valid sample of 648 individuals).

Data collection

The survey questionnaire consisted of 32 questions in three parts: The first part contained items to measure the socio-demographic and economic characteristics of the students such as gender, family income; parental education and parental occupation (see *Appendix*). In the second part, students were asked to describe 'environment' and 'environmental pollution' with their own words in two open-ended questions. Open-ended questions allow participants to express their views without being affected by the researcher (Foddy, 1993). However, open-ended questions also have drawbacks in comparison to close-ended questions. They require extensive coding and often result in significantly larger nonresponse rate (including 'Don't know', 'Prefer not to answer' and blanks) (Dohrenwend, 1965). Further, four multiple choice and one 'yes' and 'no' questions were asked. The third part included 14 items to measure the students' environmental behaviour and attitude. They were asked to indicate what extent they participate in the statements in the questionnaire on a 5 point Likert type scale. (1 = Strongly Disagree; 2 = Disagree; 3 = No opinion, 4 = Agree, 5 = Strongly Agree).

SPSS 23.00 package was used to analyse the data. The Independent Sample T-test and the One-way ANOVA were performed to reveal the relationships between the variables.

The Cronbach Alpha reliability coefficient of the survey was found to be $\alpha = 0.731$. A Shapiro-Wilk test was used to test for normality assumption. The analysis showed that [D (648) $p < 0.001$] the data does not show normal distribution. Therefore, the skewness and kurtosis values are further used and skewness of 0.132 (SE = 0.096) and kurtosis of -0.329 (SE = 0.192) was found. As the Skewness and kurtosis values are between +1.5 and -1.5, recommended by Tabachnick and Fidell (2013), the normality assumption is accepted.

Results and discussion

General characteristics of participants

Of the 648 respondents, 47.2% were female and 52.8% were male. Around 33.3% were from the Faculty of Economic and Administrative Sciences, 12.42% were from the Faculty of Education. Detailed respondent distribution of faculties is given in *Table 1*.

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DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

***Table 1.** Respondent distribution of faculties*

	Frequency	Valid %	Cumulative %
Education	96	14.8	14.8
Arts and Sciences	66	10.2	25.0
Science and Life Sciences	66	10.2	35.2
Economic and Administrative Sciences	216	33.3	68.5
Engineering and Architecture*	66	10.2	78.7
Medicine	36	5.6	84.3
Veterinary	48	7.4	91.7
Agriculture	54	8.3	100.0
TOTAL	648	100.0	

*The results of the Faculty of Architecture and Engineering are combined

30.6% of participants' family income fell between $588–640. This means both parents work in the family but they earn minimum wage. Almost a quarter of students family income (24.1%) is around legal salary, meaning only one of the parents contribute to the family budget at the minimum wage level. 22.2% of the students reported to have a family income less then minimum level meaning none of the parents actively work, they are either unemployed or retired. Half of the students' mothers had only compulsory basic education (50.9%) while 21.3% of their fathers had a university degree. As the income of the family and education of the household reflects, a large majority (80%) of the participants' mothers are housewives. *Table 2* shows that only 16.7% of the mothers found to be working and providing income for the family. 21.3% of fathers was retired and 3.7% were unemployed, therefore, a quarter of participants' fathers were actively employed.

Demographic findings

Gender

In order to assess the gender effect on student environmental awareness and attitude, an independent samples *t*-test was conducted. The result indicated that there was *no significant difference* in gender effect between males and females, $t(646) = -0.528$, $p = 0.598$ (*Table 3*).

Many studies examining the effects of gender on environmental attitudes and behaviour stated that there is no meaningful and consistent relationship between gender and environmental attitudes (Isildar and Yildirim, 2008; Kanbak, 2015; McDaniel and Alley, 2005; Robinson and Crowther, 2001; Sadik and Sadik, 2014; Stern et al., 1995; Zelezny et al., 2000). Artun et al. (2013) and Timur and Yilmaz (2011) show that gender is not influential at the levels of environmental literacy of teacher candidates. The participants' gender has not been prevalent in their environmental judgments either (Ozdemir et al., 2004; Sever and Yalcinkaya, 2012; Sungur, 2017). Genc and Genc (2013) and Gercek (2016) concluded that students' perception of environmental ethics did not differ significantly according to their gender. Stated examples underline the outcome of this research.

***Table 2.** Summary of demographic variables*

	n	%	M	SD		n	%	M	SD
Gender					Family income $*				
Female	306	47.2	0.48	0.48	0–294	156	24.1	2.06	0.43
Male	342	52.8	0.48	0.48	295–587	144	22.2	2.41	0.58
Total	648	100	2.31	0.48	588–640	198	30.6	2.37	0.42
					641–900	78	12	2.4	0.49
					901 +	72	11.1	2.38	0.34
					Total	648	100	2.31	0.48
Mother's education					Father's education				
Literate	72	11.1	2.05	0.45	Literate	30	4.6	2.43	0.78
Primary education	330	50.9	2.32	0.46	Primary education	264	40.7	2.29	0.46
Secondary education	192	29.6	2.45	0.49	Secondary education	216	33.3	2.27	0.5
University education	54	8.3	2.11	0.4	University education	138	21.3	2.39	0.4
Total	648	100	2.31	0.48	Total	648	100	2.31	0.48
Mother's occupation					Father's occupation				
Housewife	516	79.6	2.31	0.47	Self employed	156	24.1	2.42	0.44
White collar	36	5.6	2.60	0.54	White collar	78	12	2.40	0.22
Blue collar	54	8.3	2.22	0.45	Farmer	30	4.6	2.03	0.29
Self employed	18	2.8	2.45	0.42	Blue collar	138	34.3	2.4	0.44
Other	24	3.7	1.88	0.43	Retired	222	21.3	2.2	0.57
Total	648	100	2.31	0.48	Unemployed	24	3.7	2.09	0.48
					Total	648	100	2.31	0.48

*Converted to $ on the exchange rate Central Bank of the Republic of Turkey on 14.11.2018. Min legal salary 2018 Gross (Monthly) 2.029,50 TL. Min legal salary 2018 Net (Monthly): 1.603,12 TL. Exchange rate Dollar buy: 1$= 5.4625 TL, Dollar sell: 1$= 5.4724 TL

***Table 3.** An independent sample t-test outcome for gender variable*

Gender	N	$\bar{X}$	Sd	Df	t	p
Female	306	2.30	0.479	646	-0.528	0.598
Male	342	2.32	0.482			

Family income

Many research reviled middle and lower middle–class families display lower level attitudes toward the environment than more affluent families (Altin et al., 2002; Sama, 2003; Tekin and Gunes, 2018; Yilmaz and Erkal, 2016). de la Vega (2006) in his study on students and parents in Southwest Florida, showed parental income did not make any significant difference for awareness or knowledge. However, he observed a significant difference in their attitudes. Parent with an annual income of >$15,000 scored considerably lower in attitude compared to parent with an income of $15,000-29,000.

A one–way ANOVA was conducted to reveal whether family income affects students' environmental attitudes (*Table 4*). An analysis of variance showed that family income on the level of their environmental attitude was *significant* [$F(4,643) = 15.09$, $p < 0.001$]. In order to find out which income levels differ on the environmental attitude, post hoc comparisons using the Tamhane's T2 test was performed. Results indicate that only children of families with the lowest income group (2.06 ± 0.43) differ in terms of their environmental attitudes from children of families with all other income groups (please refer to *Table 2* for means scores).

Table 4. *Analysis of variance (ANOVA) between demographic variables and university students' environmental attitude*

	df	*MS*	*F*	*p*
Family income	4	3.21	15.09	0.000*
Fathers education	3	3.52	16.34	0.000*
Mothers education	3	0.59	2.57	0.053
Mothers occupation	4	2.06	9.40	0.000*
Fathers occupation	5	1.96	9.01	0.000*
Field of study	7	1.877	8.811	0.000*

*p < 0.001

University students belong to the lowest income level ($ < 294; 2.06 ± 0.43) differ from all other income levels in their awareness levels. Students from other income levels do not exhibit any statistically meaningful difference in their awareness. This result partially supports to the findings of Altin et al. (2002), Sama (2003), Tekin and Gunes (2018) and Yilmaz and Erkal (2016) that lowest level income groups displayed lower levels of environmental awareness means. On the other hand, students from families of all other income levels scoring very close means, suggest that parental income only significant at a point but not as a whole. This fact is in line with the findings of de la Vega (2006). It suggests that parental income is only of the utmost importance in the lowest income group and not meaningful for all other income groups

Parental educational and occupational analysis

Whether parents' educational levels and their occupation affect students' attitude and behaviour has been investigated thoroughly in the literature. Children learn by observing and imitating their parents, therefore, what goes on within the family may affect the formation of children's' environmental attitude and consequently their behaviour. Students raised by highly educated parents especially by educated mothers are expected to demonstrate a higher level of environmental awareness and attitude. The substantial number of research found a positive significant relation between parents' occupation and educational level and students' behaviour. Especially 'mothers' levels of education claimed to have a more positive effect on students' behaviour (Altin et al,. 2002; Bozoglu et al., 2016; Erol and Gezer, 2006; Goldman et al., 2006; Kanbak, 2015; Kayali, 2010; Kose et al., 2011; Ozden, 2008; Ozmen et al., 2005; Pe'er et al., 2007; Sam et al., 2010; Sama, 2003; Tekin and Gunes, 2018; Timur and Yilmaz, 2011; Yilmaz and Erkal, 2016; Vaizoglu et al., 2005; Zuzovsky, 2001).

A one–way ANOVA was conducted to reveal whether parental educational level affects students' environmental attitudes (*Table 4*). Statistically significant difference was found between mothers' educational level and students' environmental attitude [F (3, 644) = 16.34, p < 0.001]. Tukey post-hoc comparison reveals that children with

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

literate mothers (2.05 ± 0.45), and mothers with primary (2.32 ± 0.46) and secondary (2.45 ± 0.40) education differ significantly between their environmental attitudes. Interestingly, no statistically significance found between the children of literate mothers and those having mothers with university education (2.11 ± 0.40).

Result confirms that as mothers' education levels increases, the environmental awareness of their children increases.

A one-way ANOVA was conducted to reveal whether mothers' occupation affects students' environmental attitudes. A statistically significant difference found between mothers' occupation and students' environmental attitude [F (4,643) = 9.40, $p < 0.001$]. Tukey post–hoc comparison reveals that children's of white collar (2.60 ± 0.54) mothers significantly differ from the ones with blue-collar (2.25 ± 0.45) and housewife mothers (2.31 ± 0.47). No statistical significance observed between housewife and blue-collar mothers children environmental attitude. Therefore, it can be said that mothers's occupation has a positive impact on their children's environmental attitudes.

Another one–way ANOVA analysis was performed to analyse whether fathers' education affects their children's environmental attitudes. No statistically significant difference observed between fathers education and students' environmental attitude [F (3,644) = 2.57, $p = 0.053$].

Despite the fact that the education level of fathers does not significantly affect the environmental awareness of children, their professional status found to be highly influential [F (5,642)= 9.01, $p < 0.001$]. This effect is much more significant than the mothers' professional status. Post hoc comparisons using the Tamhane's T2 test indicated the children of working fathers (white-collar (2.40 ± 0.44), self-employed (2.42 ± 0.44) and blue-collar (2.40 ± 0.44) have a higher environmental awareness. There was no statistically significant difference between the non-working (retired (2.20 ± 0.57); unemployed (2.09 ± 0.48)) and farmer (2.03 ± 0.29) fathers' children. Participants who have farmers father have shown the least environmental awareness. It is thought that the fact that farmers work under very difficult conditions and have very little income has an important effect on this finding.

Field of study

The study field and environmental attitudes that students exhibited was one of the variables included in almost every research conducted in the field. The aim is to see whether the environmental attitudes and behaviour of the students have an impact on the selection of the subject they attend at the university and to examine whether the subject they choose and faculty they attend have impacted on their environmental attitudes and behaviour.

Research on the universities reveals many contradictions. For example, a study by Ozmen et al. (2005) found that the Medical School and Health Science Vocational School Students 'Environmental Attitude Scale' results are higher 'Health Services Vocational School.' Similarly, Kolomuc et al. (2013), Oguz et al. (2011) and Talay et al. (2004) found that Heath Sciences students were more knowledgeable in subjects such as air pollution, organic farming and environmental problems. In contrast, Tekin and Gunes (2018) found the students of the Faculty of Dentistry scored the lowest level of environmental behaviour. Kaplowitz and Levine (2005) stated the five highest scoring colleges are the Colleges of Osteopathic Medicine, Human Medicine, Agriculture and Natural Resources, Veterinary Medicine and Natural Science. Tekin and Gunes (2018), Kose et al. (2011), Ek et al. (2009) and Ozmen et al. (2005) found that Economic and Administrative Sciences students displayed more positive environmental attitudes than Engineering students. Yazici and Babalik (2016) found students of social sciences consider recycling important more than others. In contrast, Benton and Funkhouser (1994) and Benton (1994) consistently found business students lag behind their peers.

Goldman et al. (2006) reveal that students who study in environmentally affiliated subject achieved a higher overall mean score for environmental behaviour than non-environmentally affiliated fields. In the same line, Tikka et al. (2000) found biology and forestry student displayed the highest levels of environmentally related activities. Vicente–Molina (2013, 2018) in the US and Spain found that science and engineering students are more likely to behave pro-environmentally than social science students.

One-way ANOVA using post hoc Tamhane's T2 test was undertaken to see if the field of study affects students' environmental attitudes. Statistically significant difference observed between the students' field of study and their environmental attitude [F (7, 640) = 8.81, $p < 0.001$]. Medical School students (2.48 ± 0.34) displayed the highest level of environmental awareness followed by Business School (2.41 ± 0.49) and Veterinary School (2.40 ± 0.53). In contrast to Kaplowitz and Levine (2005), agriculture students achieved the lowest mean scores (2.05 ± 0.36, 95%) along with the Science and Life Sciences (2.07 ± 0.51) students.

This research outcome confirms the findings of Oguz et al. (2011), Ozmen et al. (2005) and Talay et al. (2004). Kolomuc et al. (2013) underline Medical students have the highest environmental awareness (M = 2.48). Benton (1994) and Benton and Funkhouser (1994) consistently confirmed that business students lagged behind their peers. In contrast, this research revealed the opposite. While this research did not analyse particular branches, the School of Education where prospect teachers are educated achieved a medium level of awareness (2.39 ± 0.45). Students' scoring average to lower level in the science faculties can be interpreted as not a failure of the curriculum but of the poor performance of the university itself. Students of agriculture may be expected to score the much higher because they are supposed to be more knowledgeable about the causes and consequences of environmental problems. This is particularly important for Turkish content because while Turkey has been making efforts to expand its industry, agriculture still maintains its importance.

Faculty distribution of the environmental problems perceived by students may reveal a more fruitful picture (*Table 5*).

Table 5. *Faculty distribution of the most important problems perceived by students*

Field of study	%							
	Air	**Water**	**Soil**	**Noise**	**CC/GW**	**UU**	**Other**	**Total**
Education	18.8	18.8	0.0	18.8	**25.0**	6.3	12.5	100.0
Arts and Sciences	18.2	**36.4**	0.0	0.0	18.2	27.3	0.0	100.0
Science and Life Sciences	9.1	9.1	9.1	**27.3**	9.1	36.4	0.0	100.0
Business School	22.2	13.9	8.3	2.8	13.9	33.3	5.6	100.0
Engineering and Architecture	9.1	18.2	9.1	18.2	18.2	27.3	0.0	100.0
Medical School	**50.0**	0.0	0.0	0.0	16.7	33.3	0.0	100.0
Veterinary	0.0	25.0	0.0	12.5	12.5	**37.5**	12.5	100.0
Agriculture	22.2	0.0	**33.3**	0.0	11.1	33.3	0.0	100.0
TOTAL	18.5	15.7	7.4	9.3	15.7	28.7	4.6	100.0

CC: climate change, GW: global warming, UU: unplanned urbanisation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

The medical student stated air pollution as a serious environmental problem by 50%. This may be that respiratory illnesses once were frequent in winter times are spreading over all seasons and increasing its intensity. Therefore, air pollution-related illnesses are becoming more visible. On the other hand, arts and science students thought water pollution is an environmental threat (36.4%). As expected, third of agriculture students were concerned with soil pollution. On the other hand, climate change, global warming and urbanisation were a common concern across faculties. Veterinary schools concern with urbanisation is striking (37.5%). Perhaps unlike agricultural student whom urbanisation obliterates their work field, urbanisation creates additional problems to a veterinary specialist such as strayed and starved animals.

Fundamentals of environmental awareness

The second part of the questionnaire aimed to analyse students' environmental knowledge. They were given two open-ended questions and asked to describe 'environment' and 'pollution' with their own words in 'one sentence'. Two–thirds of students left these two questions blank and other students only written unrelated single words. Stated words did not provide sufficient data to make a meaningful content analysis which is often the problem with open-ended questions. Students also did not give reasoning when they have chosen 'other' option on the 'multiple choice' questions.

When students were asked to state 'the most important environmental problem of Turkey', almost a third of them declared this as 'unplanned urbanisations'. Turkey is a developing country. 23.4% of citizens are between 15–29 years old (19 183 261; TurkStat, 2018a). Unemployment rate is 10.9% and non–agricultural unemployment rate is 13.0% (TurkStat, 2018b). Domestic immigration is over 20% in some cities (Tekirdag 20.8%, Yalova 20.2%). This figure is 14.7% in another industrial city Kocaeli, 7.2% in Bursa. High young population and unemployment rate lead to a high labour migration, which lays the ground for unplanned urbanisation. In fact, this cause new environmental problems such as traffic, air, water and noise pollution, loss of fertile land and deterioration of the natural environment and also accelerate existing problems. Therefore, for students in Bursa, the fourth most populous and seventh largest industrial city of Turkey, this finding is not surprising. Air pollution ranked second and water pollution ranked third equality with climate change and global warming. Interestingly soil pollution ranked the least important problem.

There found no significant differences between women and men in the answers given according to gender. In males, unplanned urbanisation (35.1%) and air pollution (19.3%) were higher than females, whereas, in females, water pollution and global warming (19.6%) were higher than males (*Table 6*).

Ullah et al. (2013) in their study in Bangladesh cited unplanned urbanisation (32%) as the major problem followed by air pollution (17%) and soil pollution and loss of farmland (13.5%). Sadik and Sadik (2014) research on the teacher candidate students of social sciences in Adana revealed similar result: urbanisation was found the most serious problem (28.5%) and population growth (10.8%) ranked second. Kulozu (2016) in Ataturk University also identified unplanned urbanisation (27.3%) highest followed by air pollution (20.9%) and environmental pollution (18.5%). In the same line, male students in Hatay ranked urbanisation as the most serious problem (Bozdogan et al., 2016). Gulgun et al. (2008) research similarly ranked the very same problem second (18.5%) after air pollution (24.22%).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

Table 6. *What is the most important environmental problem in Turkey?*

	Female F %		Male F %		TOTAL F %	
Unplanned urbanisation	66	21.6	120	35.1	186	28.7
Air pollution	54	17.6	66	19.3	120	18.5
Water pollution	60	19.6	42	12.3	102	15.7
Climate change/global warming	60	19.6	42	12.3	102	15.7
Noise pollution	30	9.8	30	8.8	60	9.3
Soil pollution	18	5.9	30	8.8	48	7.4
Other	18	5.9	12	3.5	30	4.6
TOTAL	306	100	345	100	648	100

Other research highlighted Ozdemir et al. (2004) stated that the problems of the Faculty of Medicine students in the first three ranks were air pollution with 37.5%, waste disposal with 36.2% and deforestation with 30.6%. The most important problems stated by the students of the Faculty of Health Education of Ankara University were air pollution with 49.5%, environmental pollution with 26.6% and wastes with 25.7% (Celen et al., 2002). As for students of Landscape Architecture, Environmental Engineering and City and Regional Planning in Ankara, the most important environmental problem in Turkey is air pollution (17.1%). It follows water pollution (14.9%), urbanisation (14.3%) and depletion of natural resources (14.3%) (Oguz et al., 2011). Erdal et al. (2013) reveal similar findings: students consider air pollution (73.91%) Turkey's most important environmental problem, followed by water (60.87%), soil (33.99%) and noise pollution (54.15%). Kanbak (2015) reveals air pollution as first, water second and solid waste third-ranked problem for Physical Sciences at Kocaeli University. For Health and Social Sciences, air pollution comes first, forest destruction lands come second and solid waste problem comes third. According to students at Suleyman Demirel University, the most important environmental problems in Turkey are air pollution, forest loss and solid waste treatment (Yazici and Babalik, 2016). Bozdogan et al. (2016) cited air pollution is the most worried problems in females. Ozen and Ozen (2017) state the ranking of the Faculty of Veterinary Faculty of Firat University as air pollution, waste and water pollution.

Artun et al. (2013) indicated that 54% of social studies teacher candidates chose global warming, 33% air pollution, 10% greenhouse gas, 10% distorted urbanisation, and 4% ozone layer perforation. Yadav and Pathak (2013) research of female university students in Jharkhand India, reviled that 33% of respondents consider global warming as the main problem, 41.67% consider pollution followed by 33.33% air pollution, 33.33% ozone depletion, 25% water pollution, and 25% soil pollution. Ermolaeva (2010) in Fort Collins, Colorado illustrates that students are most worried about the destruction of wilderness and forests (9.4%), air pollution (9.3%), people's consumption habits (8.9%), water pollution (8.8%), growing waste/not enough recycling (8.7%) and urban problems (8.2%). Amongst the various environmental problems of China, students ranked water pollution as the most urgent (Wong, 2003). They ranked deforestation and soil erosion problems as the second and urban air pollution was as the third most urgent environmental problem. In the study conducted on medical students in Finland, more than half of the participants stated that the most important environmental risk was water pollution (Kasma–Ronkainen and Virokannas, 1996).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The different answers given by the above studies show that the geographic and socio-economic situation of the country and or the region affected the students' responses and the issues they prioritised changed. For example, surveys, where unplanned urbanisation is the highest, are usually developing countries like India and Turkey. Students who indicate priority problems as air and water pollution are seen to live in regions or countries they are overpopulated or naturally depleted of such resources.

Students were asked whether their university sensitive to environmental problems. An overwhelming number of students have disagreed (*Table 7*).

Table 7. *Are universities susceptible to environmental problems?*

	Frequency	%	Cumulative %
Yes	162	25.0	25.0
No	486	75.0	100.0
TOTAL	648	100.0	

This finding is in line with Erdal et al. (2013) who revealed very similar result in Gazi Osman Pasa University in Tokat where 75.49% of students declared no and 24.1% declared yes. Oguz et al. (2011) also examined Landscape Architecture (Ankara University, Bilkent University), Environmental Engineering (Middle East Technical University) and City and Regional Planning (Middle East Technical University, Gazi University) in Ankara. Their score was 69% 'no' and 31% 'yes' to the same question. Similar results were revealed in Yazici and Babalik (2016) research at Suleyman Demirel University.

Students have given a unanimous reply to this statement. Such high numbers indicate that universities fail to achieve their part in protecting the environment. Since universities should be the forerunners of environmental protection, the reason why students have such low perceptions of their universities environmental sensitivity should be urgently and thoroughly analysed.

When students were asked the reason why there is no sensitivity to environmental problems in society (*Table 8*), the most striking answer was the lack of deterrent sanctions against environmental offenders (40.7%). In the second statement, respondents admitted that although they were aware of environmental problems, they were reluctant to reflect this behaviour to their lives (26.9%) and the consequences of environmental problems were not obvious to people (18.2%). Only 4.6% claimed that institutions and organisations that are in charge of protecting the environment are not effective and 2.8% highlighted the need for training and education. On the other hand, Oguz et al. (2011) indicated highest ranked outcome with 32.9% that people are aware of the problems but do not act to protect it while Erdal et al. (2013) research achieved 21.99% to the very same statement. Being indifferent to the consequences of environmental problems scored 20.7% in Erdal et al. (2013) and 12.9% in Oguz et al.'s (2011) research. Oppositely, inadequate education and not to witness implementation are regarded main reasons for lacking environmental sensitivity (Oguz et al., 2011, 31.4%; Erdal et al., 2013, 24.3%).

Another striking result of the survey was that non-governmental organisations (NGOs) (47.2%) stood out in the evaluation of the organisation that made the most important contribution in the fight against environmental problems. Despite this finding, Korkmaz (2018) points out that NGO's activities in the environment in particular

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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climate change are inadequate despite raising activities of civil society organisations. Universities (2.8%) were considered the least contributing organisations (*Table 9*). The ministries (19.4%) and public service announcements (18.5%) ranked lower than NOGs. This research is in the same line with Erdal et al.'s (2013) research. There, student ranked NGOs work first with 38.74% but universities scored 16.21% unlike 2.8% that Bursa Uludag University scored. Related ministries scored 20.95% and charitable foundations achieved 24.11%.

Table 8. *What's the reason for not having sensitivity towards environmental problems?*

	Frequency	%	Cumulative %
The consequences of environmental problems are not known.	120	18.5	18.5
Deterrent punishment/sanctions not issued.	264	40.7	59.3
People are aware of environmental problems but they do not act to protect it.	174	26.9	86.1
Not enough education and implementation.	18	2.8	88.9
Shortage of relevant institutions.	30	4.6	93.5
Other	42	6.5	100.0
TOTAL	648	100.0	

Table 9. *Which organisation makes the most important contribution to prevent environmental problems?*

	F	%	Cumulative %
Activities of non–governmental organisations	306	47.2	47.2
Public service announcements	120	18.5	65.7
Research and educational activities of universities	18	2.8	68.5
Works of the Ministry of Environment and Urbanism	126	19.4	88.0
Works of the municipalities	78	12.0	100.0
TOTAL	648	100.0	

The students of the Faculty of Health Education stated that they rely on voluntary organisations at the highest rate (92.5%) in solving environmental problems (Celen et al., 2002). 54.9% of the students in the Faculty of Education at the Hacettepe University stated that the written and visual media were the most important agenda determinants and the least contributing group was the politicians (Yilmaz et al., 2002).

When students were asked the role of society in protecting the environment they stressed that people should take an active part in protecting the environment (36.1%) (*Table 10*). As earlier question already pointed out, students believe that people are aware of the problems but they did not reflect this awareness into their behaviour. Despite students' statement of the most important environmental problem as urbanisation in the earlier question, preventing unplanned urbanisation was given the least importance (3.7%). This finding must be investigated further. It had already been revealed that the work of related ministries and municipalities were considered unsatisfactory. Preparing city plans and deciding the way in which the city will expand is their work but citizens also play a pivotal role. It will not be realistic to expect from ministries or municipalities to prevent urbanisation problems or at least alleviate them. Society must be proactive preventing urbanisation problems.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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34% of the students of Hacettepe University responded cared not to pollute the environment. This rate was 27.8% for Bursa Uludag University students. Moreover, 20% of the students in Hacettepe stated that people should use 'recycled materials' (Yilmaz et al., 2002). The same statement is marked by 17.6% in Uludag University.

Table 10. *What is the role of society in protecting the environment?*

	F	**%**	**Cumulative %**
To take care not to pollute the environment	180	27.8	27.8
To prevent unplanned urbanisation	24	3.7	31.5
To use environmentally friendly products	114	17.6	49.1
To actively take part in preserving the environment	234	36.1	85.2
Other	96	14.8	100.0
TOTAL	648	100.0	

Bursa Uludag University students strongly emphasized (47.2%), wanting to involve in the decision-making process. They also want to be able to monitor and evaluate the outcome of those decisions (*Table 11*). They stressed that environmental education should be compulsory throughout the university education. Only a handful of departments include environment related lectures in the curriculum. In fact, the majority of departments of social sciences, medicine and economics do not cover environmental issues in any way.

Table 11. *What is the role of universities in protecting the environment?*

	F	**%**	**Cumulative %**
Environmental education should be compulsory	90	13.9	13.9
Students should take part in the decision making and application process	306	47.2	61.1
More Research and Development activities should be organized	90	13.9	75.0
Should work in cooperation with the industry	72	11.1	86.1
Experimental waste should not be randomly disposed	78	12.0	98.1
Other	12	1.9	100.0
TOTAL	648	100.0	

Various research highlights that they are in favour of mandatory environmental education (Ek et al., 2009, 84.1%; Oguz et al., 2010, 93.4%). Some stated that compulsory courses should be included in the curriculum at all educational stages (Ek et al., 2009, 33%; Oguz et al., 2011, 74.4%). Various researchers have specifically emphasized that a course on the environment at the university must be compulsory (Erdal et al., 2013, 65%; Grodzinska–Jurczak and Trabula, 2001; Ozdemir et al., 2004, 61.7%; Ozmen et al., 2005, 44.9%).

The equal number of students also stressed that more research and development activities should be organized by the university. The substantial number of science students have witnessed the random disposal of experimental waste and raised their concern. Last but not the least, students expressed the need for more research and development but ranked working with the industry least important.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

University students' attitudes and behavioural patterns towards environmental problems

In the third part of the questionnaire, statements aimed to understand the students' awareness levels about environmental issues and to determine their attitudes at this awareness level. It is hoped that the answers to these questions will show the extent to which university students reflect their environmental attitudes to their behaviour. The questions and answers given in this section are given in *Table 12*.

Table 12. *Questionnaire statements and responses (n = 648)*

	1	2	3	4	5	M	SD
I have taken enough education on the environment at school before the University.	30.6%	26.9%	14.8%	23.1%	4.6%	3.56	1.27
I donate money or become a member of environment-related organisations.	13.9%	27.8%	24.1%	30.6%	3.7%	3.18	1.12
When purchase. I prefer products with a recycle sign.	17.6%	12.0%	33.3%	26.9%	10.2%	3.00	1.23
I prefer to read in a digital environment instead of printing to avoid paper waste.	11.1%	29.6%	14.8%	31.5%	13.0%	2.94	1.25
I take an active part in protecting the environment.	7.4%	17.6%	36.1%	32.4%	6.5%	2.87	1.02
I take care not to use consumer goods that can damage the ozone layer.	3.7%	18.5%	30.6%	32.4%	14.8%	2.64	1.06
When I see someone polluting the environment. I don't remain unresponsive and I warn them.	5.6%	10.2%	34.3%	31.5%	18.5%	2.53	1.08
I dispose of materials such as plastic, glass, paper, battery etc. separately and help them to be recycled.	2.8%	7.4%	18.5%	39.8%	31.5%	2.10	1.02
When I need to use paper. I use them double-sided.	0.9%	9.3%	11.1%	42.6%	36.1%	1.96	0.96
I prefer to use public transport when I'm travelling.	3.7%	6.5%	10.2%	31.5%	48.1%	1.86	1.08
I try to use resources such as electricity, water etc. as efficient as possible.	0.9%	3.7%	7.4%	41.7%	46.3%	1.71	0.83
I make sure that my rubbish goes into the garbage bin.	0.9%	1.9%	6.5%	13.9%	76.9%	1.36	0.76
Our sensitivity to the environment is also our responsibility to society.	0.0%	0.9%	5.6%	17.6%	75.9%	1.31	0.62
I'm aware that environmental pollution is a problem for future generations.	1.9%	0.0%	3.7%	14.8%	79.6%	1.30	0.71

1: strongly disagree, 2: disagree, 3: no opinion, 4: agree, 5: strongly agree

Attitudes towards environmental problems

Researchers in the field agree that students have not been able to convey the level of consciousness to their behaviour. According to Ullah et al. (2013) in Bangladesh, only 12% of students took part in environmental activism and only 35% of them are interested in joining any environmental organisation. Hassan et al. (2011) found that UKM students in Malaysia had a high level of knowledge, awareness and attitudes about the environment, but low prevalence of participation in environmental activities. This view is supported by Wahida et al. (2004) (cited in Hassan et al., 2011). They say that awareness towards environmental issues and awareness about the need to protect the environment has increased among society, but the level of individual participation in protection activities still remains low. Azizan's (2008) (cited in Hassan et al., 2011) findings also support Wahida et al. (2004) (cited in Hassan et al., 2011). He underlines

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DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

that the students have a good awareness of environmental problems but have not yet taken this awareness into action.

In support of the above argument, the result of this research indicates that the students who are actively involved in protecting the environment remain at 38.9%. Comparison to other research, Oguz et al. (2011) revealed similar results: 39% of the students actively involved in activities related to environmental issues. Gulgun et al. (2008) showed a more optimistic figure: more than half of the students (56.4%) actively involved in these activities. Kiper (2014) found 72.1% of the students were 'sometimes' active. The most worrying response came from Ozmen et al. (2005); 84.9% of the students did not participate in any environmental activities.

It is not surprising that the ratio of students who actively participate in the events related to environmental issues, and the ratio of those who are members or donate to the environment–related organisations are close (34.3%). The findings of the previous research similarly reveal low participation rates. Wong (2003) found that 6% of the students, Budak et al. (2005), 12.9% and Timur and Yilmaz (2011), 17.4% of student were members of the mentioned organisations. Wong (2003) in China further showed only 1.7% of the students was active members of such organisations.

It may be assumed that the income level of the students participating in the study is at the lower and middle class; hence affects their ability to pay their membership fees or to donate to such institutions. However, the general finding in the literature is that students do not participate in studies and activities on environmental issues regardless of income level. For example, Cabuk and Karacaoglu (2003) asked students whether they participate in volunteer organisations working on the environment and the reply was 52.2% 'sometimes', and 38.7% 'never.' Isildar and Yildirim (2008) showed a more negative result: 21.7% of the students said 'sometimes', 65.2% said 'never'. In the Kanbak (2015) survey, 62.6% of the respondents said that they did not participate in the activities of any environmental organisation.

What is important here is that although more than half of the students (57.5%) state that they have not received any education about the environment before the university, they are still reluctant to participate in such events and improve their knowledge of the environment. One of the outcomes of this low educational level is the inability to define the concepts of *environment* and *pollution* in their own words in the previous section of the questionnaire. The previous research in the field gives contradictory results to this statement as well. For example, Ozdemir et al. (2004) reported 94.4% of the students received environmental education at any time during their education. 75.9% of the participants in Ozmen et al. (2005), 44% of the participants in Erdal et al. (2013), and 47.2% of the participants in Kiper (2014)'s research reported receiving environmental education before the university. Paksoy and Paksoy (2015) stated that 35% of the students received environmental education at primary and 23% at the secondary education.

Despite the inadequate education, knowledge base and lack of willingness to participate in environmental problems, an overwhelming percentage of the students (94.4%) agree that environmental pollution will pose a problem for future generations. They also realise that sensitivity towards environmental problems means also a responsibility to society (93.5%). Students are once again in contradiction with themselves, while they are reluctant to join environmental organisations and participate in their activities, they are almost unanimous in agreeing that it is a social duty to protect the environment.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

The other contradiction posed by the students is that, although they think it is a social duty to protect the environment, when it comes to 'warning' someone polluting the environment they remain 'indifferent.' Only 10.2% 'agreed' that they warn people and 5.6% strongly agreed, 34.3% was 'unresponsive' and 31.5% 'disagreed.' In fact, this ratio coincides with the rates of participation in environmental activities and membership to related organisations.

These results bring the following question to mind. If a student really believes that environmental protection is a social duty and pollution is a threat to the future, why do they not play an active role in protecting the environment? The fact that students expect others to display 'behaviour' that they do not do 'themselves' can be interpreted in two ways: One of them is social desirability bias. Social desirability is the tendency to respond to question in a manner that will be viewed favourably by others. They do this to exhibit a positive image of themselves to avoid receiving an unfavourable opinion. This could lead to either over-reporting 'right' behaviour' or under-reporting 'bad' or 'undesirable' behaviour (Fisher, 1993). Or they really feel the need to protect the environment and warn those who do not fulfil their environmental responsibilities. However, they are unable to put their intentions into action for various reasons such as being condemned by people, judged as disrespectful and not knowing the legal sanctions.

Although other researchers in the field have reported higher figures, they have shown that students, in general, are reluctant to warn the polluters. Ullah et al. (2013) in Bangladesh reported 56.5% of those to warn them. This ratio is 47% in Paksoy and Paksoy's (2015) and 44.2% in Kiper's (2014) work.

Purchasing and disposing behaviour

In the introduction section of the article, it was stated that having knowledge about the environment affects the attitudes of the individual in a positive way (Sivek and Hungerford, 1990) and that the positive attitude leads to positive behaviour (Wang et al., 2014).

The statements that measured the students' attitudes did not display a very revealing picture. It was observed that the students were not knowledgeable about the environment and did not have a positive attitude towards the environmental problems.

The survey included eight statements aimed at determining students' environmental behaviour. The extent to which students' responses reflect their attitudes to their behaviour is examined below.

When the university students behavioural patterns were examined, the lowest mean ($M = 1.30$) was obtained from the statement indicating that 'they make sure their rubbish goes into the garbage bin'. 90.8% of the participants state that they pay attention. It is expected that young people with university education will reflect this sensitivity. To see the actual behavioural patterns, it is necessary to evaluate other statements.

When examined in into more depth, it is observed that the ratio of the students who sorted their garbage as plastic, glass, paper, battery and disposed of separately, decreased to 71.3%. It is also noteworthy that the ratio of those who say they have 'no opinion' is close to 20%. Kiper's (2014) research has also yielded more concerning results; while 24.4% of those who say they always sort household waste out, 19.8% said that they never do it.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Recyclable packaging has been on the market for more than two decades. The benefit of recycling to the environment and the country's economy is often emphasized in the written and visual media, and publications that attempt to raise public awareness about the use of recyclable products are widely available. Perhaps, due to this emphasis, young people may have had the illusion that all products are sold in recycled packages or that each product is recyclable. Likewise, when purchasing a product, the ratio of those agreed that they make sure that the product is in recycled packaging, dropped to 37.1%. The ratio of those who said they absolutely agreed with the statement, further dropped to 20.2%. The ratio of those who strongly disagreed being 17.6%, is concerning. Present results despite not being promising, are not far from other studies undertaken. Kiper (2014) studied the environmental attitudes of Landscape Architecture students and he observed that 40.7% of those claimed 'always' paid attention. Erdal et al.'s (2013) study showed a similar outcome with the current research results. In his study, those who absolutely agreed were 26.48% partially agreed were 28.46%.

On the other hand, the rate of those who participated in the statement that they chose products that would not harm the ozone layer increased to 47.2%. The findings of the present research are consistent with previous research. Paksoy and Paksoy (2015) research states 43% of the participants, Gulgun et al.'s (2008) research states 39.23% of them do not use the products that damaged the ozone layer. However, it is striking that in both statements, a third of the students said "they had no idea".

As in the previous statement, the students indicating that they use natural resources such as electricity, water and natural gas sparingly reached to 90%. Especially in developing countries, utility bills amount higher than in developed countries and constitute a significant portion of the household budget. Therefore, the careful use of these resources may reflect the environmental consciousness of the students and or may have acquired as a result of a necessity imposed by their parents because participating students are usually from families belonging to the lower and middle-income groups

Above findings may be interpreted as the fact that the participant students are generally in low and middle-income families, therefore they rather pay more attention to the price of the product rather than its eco-friendly properties.

As a result of technological developments in recent years, people especially young people use digital environments increasingly. Consequently, various studies are carried out to monitor their digital literacy development. Many businesses have adopted paperless office principles for over a decade. Bookstores have extended their e-book range, and universities have started to broadcast lectures online and moved lecture material to the virtual environment. Students were asked a special question about whether they read in digital media to save paper. Research findings showed that students do not prefer to read in digital media, contrary to common assumption. Only half of the students (44.5%) read on screen. 40.7% still prints the lecture notes and they do seem to concern with paper saving.

Despite not reading from the screen, students were sensitive about paper use. 48.1% was 'strongly agreed' and 35.1% 'agreed'. 78.7% of the respondent stated that they used both sides of papers. Nevertheless, the rate of those who said that they would definitely use double-sided paper is behind the findings of other studies. Cabuk and Karacaoglu (2003) came up with the figures of 'always' 63.8%, and 'sometimes' 30.1%. Whereas Erdal et al. (2013) found those who 'absolutely agree' with were 51.38%, and those who 'agree with' were 33.20%

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Car ownership is regarded as a symbol of economic welfare and social status in many countries. Nonetheless, the public transport usage rate depends on the availability of alternatives. Bicycle usage is a new phenomenon in Turkey. Urban metro is also available only in a few major cities and suburban train network is almost negligent. In addition, universities can develop a variety of approaches depending on whether they are in rural or urban areas. In small cities, students can have easier access by foot or by bicycle. This present research reveals 79.6% of the students' prefer public transport. Bursa Uludag University's distance from the city centre makes it difficult to reach the campus by bicycle. Furthermore, the starting of the city metro line from campus helps to explain the height of this number. It should also be remembered that the majority of the participants come from the middle and lower-middle income families. There is often one car in these families and the cost of driving can reach the amounts that students cannot afford with their allowance. The ratio of the students who indicate their preferred transportation as public transport varies from 64% to 87% (Isildar and Yildirim, 2008; Muderrisoglu and Altanlar, 2011; Oguz et al., 2011) in the literature.

The statements of the questionnaire that examined the students' behavioural patterns shown that their environmental behaviour was changed depending on the situation they are in.; Their economic situation and the existing opportunities could also affect how they behave. It is possible to claim that their overall behaviour is on the positive side considering the level of knowledge they have and the attitudes they maintain.

Conclusions

Increasing environmental problems have resulted in an increase in the research carried out on the subject matter. The aim of the research was to identify the causes of these problems and to solve them. Literature is dominated by the studies that examine students' attitudes and behaviour towards environmental issues and environmental problems at almost every level, ranging from primary school to university.

While research in developed countries focuses on how to solve them and how to prevent future problems, research in developing countries is more about understanding the causes of problems. Especially, studies toward university students have a significant share in the current research. This interest emphasizes that university students are leaders who will manage the future. Studies examining the environmental attitudes and behaviour of university students have exposed many contradictions and have laid the foundation for new research. This research, while analysing survey questions, attempted to include findings and contrasts revealed in the previous research in the context of Bursa Uludag University.

It has been observed that gender is not significant on students' environmental awareness. The research found that students belong to the lowest income level ($<294) differ from all other income levels in their awareness and display lower level environmental awareness. Other income levels did not appear to be a predictive factor of environmental behaviour. As the level of education of the mother increased, the students' environmental awareness increased, but when the mother's education reached the university level, the difference was not significant. Mother occupation too was significant on the student's environmental attitude; students whose mothers were a white collar and self–employed showed higher environmental awareness. In contrast, students who had blue–collars and self–employed fathers achieved higher environmental awareness. Students with farmer fathers scored the lowest awareness.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Fathers' education not reveals any significance. Finally, Medical and Business School students displayed higher levels of awareness while the School of Agriculture students scored the lowest means.

Most of the students in the research were unable to describe the environment and environmental pollution. This may be the result of them not receiving adequate environmental education up until the university. The students stated that the country's biggest problem was urbanisation. This was followed by air and water pollution. Faculty of Agriculture students do not consider soil pollution as an important problem is one of the remarkable findings of the research.

Another important finding was that the students find a lack of punishment and sanction the most important reason for the lack of sensitivity to the environment in society. On the one hand, it is striking that education about the environment students receive before they come to university is not sufficient. While on the other hand, the ratio of those who stated that the cause of environmental insensitivity in society is lack of education remains at 2.8%. Although students emphasize the lack of knowledge, they want to increase penal sanctions instead of making environmental education more accessible and effective.

As in other studies, Bursa Uludag University students stated that those who make the most contributions to the environment are voluntary organisations. The 2.8% share of 'education and research of universities' in the environment protection rate is directly proportional to the 75% 'no' answer to the statement of whether universities are doing their part about the environment. These findings should be very concern rising for the university. Students indicate that they want to take part in the environmental decision–making mechanisms of the University. They claim that society should play a more effective role in environmental protection. Another important finding of the study is that students did not see the problem of urbanisation as one of the tasks of society although they ranked this as the most important problem for the country.

In the section that examines the attitudes and behaviours' of the students. it was seen that students adopted the highest behaviour in the society, such as preferring public transportation, using the papers in two sides, separating the garbage on disposal. However, it was observed that one of every three students abstained in behaviour such as using recycled packaging, buying products that do not damage the ozone layer, taking active duties as members of the related institutions and warning the environment polluters. Therefore, the answers that are given suggest that students are inclined to social desirability bias. Or as this result has been underlined by other researchers, it can be interpreted that students do not reflect this to their behaviour even though they have a positive attitude towards the environment.

Acknowledgements. We would like to thank Emeritus Professor Derek J. Clements-Croome for his proofreading, suggestions and feedback during the preparation of the manuscript.

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APPENDIX

Questionnaire

Gender: Female ( ) Male ( )
Faculty: Education ( ) Arts &Science ( ) Science & Life Sciences ( ) Business School ( ) Engineering ( ) Architecture ( ) Medical School ( ) Veterinary ( ) Agriculture ( )
Family Income: $0-294 ( ) $295–587 ( ) $588–640 ( ) 641–900 ( ) 901 + ( )
Mothers Education: Literate ( ) Primary Education ( ) Secondary Education ( ) University ( )
Fathers Education: Literate ( ) Primary Education ( ) Secondary Education ( ) University ( )
Mothers Occupation: White-collar ( ) Blue-collar ( ) Self Employed ( ) Housewife ( ) Other ( )
Fathers Occupation: White-collar ( ) Blue-collar ( ) Self Employed ( ) Farmer ( ) Retired ( ) Other ( )

Please describe the environment in one sentence.
....
What pollution means to you?
....
What is the most important environmental problem in Turkey?
Air pollution ( ) Water Pollution ( ) Soil Pollution ( ) Noise Pollution ( )
Climate Change /Global Warming ( ) Urbanisation ( ) Other: ....

Are universities susceptible to environmental problems?
Yes ( ) No ( )
What's the reason for not having sensitivity towards environmental problems?
( ) The consequences of environmental problems are not known.
( ) Deterrent punishment/sanctions not issued.
( ) People are aware of environmental problems but they do not act to protect it.
( ) Not enough education and implementation.
( ) Shortage of relevant institutions.

Which organization makes the most important contribution to prevent environmental problems?
( ) Activities of non–governmental organizations. ( ) Works of the municipalities.
( ) Works of the Ministry of Environment and Urbanism. ( ) Public Service Announcements.
( ) Research and educational activities of universities.
What is the role of society in protecting the environment?
( ) To take care not to pollute the environment ( ) To prevent unplanned urbanization
( ) To use environmentally friendly products ( ) To actively take part in preserving the environment
Other:...
What is the role of universities in protecting the environment?
( ) Environmental education should be compulsory
( ) Students should take part in decision making and application process
( ) More R & D and training activities should be organized
( ) Should work in cooperation with the industry
( ) Experimental waste should not be randomly disposed
( ) Other:...

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DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

	1	2	3	4	5
I have taken enough education on the environment at school before the University.					
I donate money or become a member of the environment–related organisations.					
When purchase. I prefer products with a recycle sign.					
I prefer to read in a digital environment instead of printing to avoid paper waste.					
I take an active part in protecting the environment.					
I take care not to use consumer goods that can damage the ozone layer.					
When I see someone polluting the environment I do not remain unresponsive and I warn them.					
I dispose of materials such as plastic, glass, paper, battery etc. separately and help them to be recycled.					
When I need to use paper. I use them double–sided.					
I prefer to use public transport when I'm traveling.					
I try to use resources such as electricity, water etc. as efficient as possible.					
I make sure that my rubbish goes into the garbage bin.					
Our sensitivity to the environment is also our responsibility to society.					
I'm aware that environmental pollution is a problem for future generations.					

1: Strongly Disagree 2: Disagree 3: No opinion 4: Agree 5: Strongly Agree

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DOI: http://dx.doi.org/10.15666/aeer/1702_51095138

CHLORPYRIFOS INDUCED CHANGES ON THE PHYSIOLOGY OF COMMON CARP (*CYPRINUS CARPIO* LINNAEUS, 1785): A LABORATORY EXPOSURE STUDY

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(Received 14th Jan 2019; accepted 26th Feb 2019)

Abstract. The present study was designed to study the effects of the pesticide chlorpyrifos (CPF) on the physiology of the economically important fish species common carp (*Cyprinus carpio* Linnaeus, 1785) by applying a biomarker approach. This pesticide is considered as a priority substance in surface waters according to Directive 2013/39/EU of the European Parliament and of the Council. The fish were treated with decreasing concentrations of CPF for 72 hours (acute exposure) and the histological structure of gills and respiration rate were examined. In sum, we found pronounced alterations in the gill structure and changes in the respiration rate index, regardless of the applied pesticide concentrations which indicates its negative effects on non-target aquatic species such as common carp. The results from such studies could be incorporated in the legislation to prevent water contamination in areas with intensive agricultural practices by applying biomarkers, and an update could also be initiated on the maximum permissible concentrations of CPF in surface waters.
Keywords: *biomarkers, fish, gills, histology, pesticides*

Introduction

The increasing use of pesticides in agricultural activities for controlling pests and diseases attacking agricultural crops is considered a major problem worldwide. However, after their application, these compounds migrate to the aquatic environment mainly by drift and transportation via air, and runoff from agricultural set ups (Marino and Ronco, 2005). Currently, pesticides are also commonly used in several other areas such as livestock and households (Fevery et al., 2016). In addition, according to Katsumata et al. (2006) in recent years, more than 10,000 chemicals have been used for industrial and agricultural purposes. After prohibiting the use of organochlorine (OCs) insecticides, the organophosphorus insecticides (OPs) have become the most commonly used compounds worldwide.

Chlorpyrifos (O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate; CPF) is a moderately toxic broad-spectrum lipophilic OP insecticide (Kow of 4.7) (Mugni et al., 2016) and one of the several OP compounds developed in the 1960s to replace persistent OC pesticides (Kumar et al., 2017). Today it is the most intensively used OP pesticide in agriculture. CPF is commonly used to control foliage and soil-borne insect pests on a variety of crops including rice, cotton, fruit, vegetable crops, lawns and ornamental plants, as well as subterranean termites (Rao et al., 2005; Fang et al., 2008). The urban uses of CPF include landscape maintenance, structural pest control,

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DOI: http://dx.doi.org/10.15666/aeer/1702_51395157

dormant sprays and pet products (Solomon et al., 2014). However, according to Humphrey et al. (2004) its usage is restricted in inhabited areas. The highest amount of CPF produced globally (3.64–4.99 million kg) was recorded in 2007 (Grube et al., 2011). In India, it was reported that CPF was the second most used agricultural insecticide (9540 tons) in 2013–2014 (Kumar et al., 2017). In addition, this pesticide is one of the most widely used OPs in Argentina (Asselborn et al., 2015). As other OP insecticides, the primary site of action of CPF in animals is the acetylcholinesterase enzyme which is inactivated by phosphorylation, thereby interfering with normal cholinergic nerve transmissions (USEPA, 1986). Moreover, acetylcholinesterase plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate (Kwong, 2002).

Aquatic species can be exposed to various OP pesticide concentrations which range from lethal to sublethal (Streit and Kuhn, 1994). Few reports are available on the effects of CPF on non-target aquatic organisms and some authors demonstrated its adverse effects on aquatic invertebrates and fish. Varó et al. (1998) pointed out EC_{50} values ranging from 0.95 to 18 mg l^{-1} of CPF in several *Artemia* species. Earlier reports also revealed fish kill incidents in association with CPF in water reaching several hundred parts per billion (Abdel-Halim et al., 2006). According to Varó et al. (1998) CPF passes via air-drift or surface runoff into natural waters, where it is accumulated in different aquatic organisms, especially in fish, making it vulnerable to several discernible effects. Thus, it is found that CPF is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in Zebra fish (Yu et al., 2015); it affects the sex steroid production and thyroid follicular development in adult and larval lake sturgeon (Brandt et al., 2015); as well as the acetylcholinesterase activity in climbing perch and Zebra fish (Nguyen et al., 2015) and the activity of glucose-6-phosphate dehydrogenase in rainbow trout (Topal et al., 2014); it causes oxidative stress in common carp and Zebra fish (Chen et al., 2015); it impacts the protein synthesis in Zebra fish (Liu et al., 2015) and DNA and RNA synthesis in common carp (Wang et al., 2014), and causes histopathological alterations in common carp (Xing et al., 2012). Moreover, CPF is known to change the lysosomal membrane stability in other aquatic species such as Zebra mussel (Yancheva et al., 2017a,b).

A biomarker is known to be a change in a biological response, either at molecular, cellular, histological, physiological or behavioral level that can be related with the exposure to toxic environmental elements (Colin et al., 2016). One advantage of biomarkers relative to bioindicators is that the former allow examining specific target organs and cells, including gills, kidney and liver which are affected by exposure to environmental chemicals and are responsible for vital functions such as respiration, excretion and the accumulation and biotransformation of xenobiotics (Van der Oost et al., 2003).

According to Varó (1998) the histopathological effects produced in different tissues of non-target organisms are usually used to biomonitor polluted areas and environmental conditions in aquaculture systems, and the use of histopathological biomarkers in biomonitoring has many advantages. As stated by Hinton and Laurén (1990) the histological effects are consequences of biochemical mechanics that provide interpretative power about damage effects to individuals done at the molecular level. These biomarkers can be used as an early warning system for potential effects at the level of the individual. In addition, the histopathological lesions are easily recognizable alterations that ideally indicate alone a pathological condition,

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DOI: http://dx.doi.org/10.15666/aeer/1702_51395157

also reflecting the severity and duration of pollution events. Although they cannot reveal toxic identity, the histological biomarkers can identify the pollution type through its mode of action, further supporting the use of histopathological biomarkers as faithful indicators of environmental pollution (Colin et al., 2016).

The gills of aquatic animals are usually used to assess pollution effects, as they are the first organ which comes in contact with the aquatic pollution. They are also highly vulnerable to toxic chemicals, mainly because their large surface area facilitates the interaction and their absorption. The toxicant absorption through gills is rapid and therefore, the toxic response in gills is also rapid (Pandey et al., 2008; Topal et al., 2015). Moreover, the histological lesions of gills are frequently the consequence of exposure to various contaminants (Fonseca et al., 2016) with the severity being dependent on the pollutant concentration and exposure time span (Tchounwou et al., 2012). Last but not least, the gill histopathological changes were previously recognized as valuable biomarkers of water ecosystem stressors (Pereira et al., 2013; Cruz et al., 2015).

According to Chen and Yang (2007), Dobreva et al. (2008), and Nishida (2011) the toxic effects of different pollutants may be linked to changes in the respiration rate and may also be used as an indicator of polluted waters. These changes are associated with insufficient amount of oxygen in the water, causing stress to the aquatic organisms. In addition, the rate of respiration reflects the metabolic activities of organisms and the responses due to changes in the surrounding environment could also serve as an indicator of adjustment capacity (Kumar et al., 2012).

The fish communities are key elements to evaluate the ecological condition of different water bodies based on biomarkers (Hermoso and Clavero, 2013), while histopathology is the standard method for assessing both short- and long-term xenobiotic effects on fish (Hinton and Lauren, 1990). What is more, to face environmental problems Water Framework Directive (WFD, Directive 2000/60/EC) has driven the need to assess the ecological status of water bodies through an integrative ecosystem approach using elements of biotic quality such as bioindicators. Along with diatoms, macroinvertebrates and macrophytes, the fish are key elements to assess the ecological status of water aquatic ecosystems (Fonseca et al., 2017).

Since 2000, aquaculture has been growing 6% per year and now has become a major food industry in the world market, as well as an important source of meat in the human diet (FAO, 2016). Common carp (*Cyprinus carpio*, Cyprinidae) which belongs to the omnivorous fish is one of the most important commercial species in aquaculture with a successful spread all over the world, and it is also highly valued in many different areas of biology (Kong et al., 2018). According to Zoral et al. (2017) common carp is a major cultured fish species, accounting for 10% of freshwater aquaculture production worldwide. Furthermore, common carp is introduced as one of the most suitable fish models for toxicological studies (OECD, 1992). The dominance of common carp in the aquatic systems and the fact that it has a better capacity for resistance against pollutants rather than other laboratory fish, such as Zebra fish and Japanese medaka are common reasons for choosing this exact species in toxicological tests (Lee et al., 2012; Pirsaheb et al., 2019).

Based on the above, in terms of aquatic toxicology it is essential to study the effects of CPF which is considered as a priority substance in surface waters according to Directive 2013/39/EC of the European parliament and the Council on non-target organisms, such as fish. Hence, the main objective on the present work was to study

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DOI: http://dx.doi.org/10.15666/aeer/1702_51395157

the possible negative effects of a short-term exposure (72 hours) of decreasing and environmentally relevant concentrations of CPF on the physiology of common carp by applying a biomarker approach as we hypothesized that we would also observe changes in the fish at lower concentrations than the permitted annual average concentrations in surface waters. Thus, we aimed to semi-quantitatively assess the gill histology through a histology-based fish health protocol and to measure the respiration rate.

Materials and methods

Experimental set up

Common carps with no external lesions were obtained from the Institute of Fisheries and Aquaculture, located in the city of Plovdiv, Bulgaria where fish are reared under strict and controlled conditions. They were weighed (g; total weight including viscera) and the total length (cm) were recorded (22.5 g ± 3.5; 13.5 cm ± 2.5). The fish were transported in plastic tanks equipped with air pumps to the laboratory of Faculty of Biology, Plovdiv University, Bulgaria. After transportation they were moved in glass tanks with 100 l chlorine free tap water (by evaporation) to acclimatize for a week. All tanks were equipped with air pumps for permanent aeration and the water was kept oxygen saturated. The fish were kept under a natural light/dark cycle (12:12) and they were not fed prior to the experiment. The fish were divided into four test groups (n=15), including the control. Thereafter, they were treated with different and decreasing (environmentally relevant) soluble concentrations of CPF, dissolved in cyclohexane for 72 hours (acute, short-term experiment). According to the national and EU legislation the annual average concentrations (AA-EQS) of CPF in inland surface waters and other surface waters is 0.03 µg l^{-1}. The tested concentrations were prepared as 50 and 30% of the AA-EQS. The reason for this selection is that the natural waters are usually contaminated with organic substances in low concentrations which subsequently lead to chronic exposure effects for the aquatic organisms. Therefore, in the current experiment for 100 l tanks, 3 µg (AA-EQS), 100%, 0.03 µg l^{-1}), 1.5 µg (50% from AA-EQS, 0.015 µg l^{-1}) and 0.9 µg (30% from AA-EQS, 0.009 µg l^{-1}) were applied. No fish mortality was observed. The basic physical characteristics of the water such as: pH, temperature, oxygen level and conductivity were followed strictly three times on the 24^{th}, 48^{th} and 72^{nd} hour according to a standard procedure (APHA, 2005) with a combined field-meter (WTW, Germany). All experiments were conducted in accordance with the national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes according to Directive 2010/63/EU.

Histological techniques

Fish dissection was performed according to the international standard procedures given in the EMERGE Protocol (Rosseland et al., 2003). All histological were prepared according to a standard procedure for light microscopy analysis (Gautier, 2011). For each fish, the gills were sampled and preserved in 10% neutrally buffered formaldehyde for 24 hours. The preserved samples were washed in tap water, dehydrated in a series of increasing ethanol concentrations (70% – 80% – 85% – 96% – 100%), cleared in xylene, infiltrated with liquid paraffin with a melting point of 54–56°C and finally embedded in paraffin wax. They were then sectioned (5 µm thick) on a rotary

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microtome (Leica RM2125 RTS, Germany) according to Humason (1962). The sections were stained with hematoxylin-eosin (H&E) and observed with a light microscope (Leica DM 500, Germany). The glass slides were labelled and were examined "blind". Firstly, the histological lesions were assessed according to the semi-quantitative system proposed by Bernet et al. (1999). Briefly, according to this method the histological lesions were classified into three reaction patterns – circulatory, regressive and progressive changes. Each reaction pattern includes several alterations which concern either functional units of the organ or the entire organ (see Bernet et al., 1999). As we did not find any inflammatory or neoplastic (tumor) alterations, these reaction patterns were excluded. Secondly, a six degree (0–6) severity gradation scale (SGS) which represents the severity of each alteration was defined according to Saraiva et al. (2015). Each grade represents specific histological characteristics and is categorized as follows: (0) – no histological alterations which represented normal gill histological structure; (1) – mild histological alterations; (2) – moderate histological alterations; (3) – pronounced histological alterations; (4) – severe histological alterations and (5) – very severe histological alterations. The relevance of a lesion depends on its pathological importance, i.e. how it affects the organ function and the ability of the fish to survive. Therefore, the pathological extent of each lesion was defined with an importance factor according to Bernet et al. (1999) as follows: (1) – minimal pathological importance, the lesion is easily reversible as toxicant exposure ends; (2) – moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralized and (3) – marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. The final value for each alteration results from the multiplication of the score value with the importance factor. Summing up these final values for one reaction pattern or organ gives the index for the respective reaction pattern i.e. index for circulatory disturbance (IC), index for regressive changes (IR), index for progressive changes (IP) for the gills or organ index (OI). Organ index values were used to classify the severity of histological response using classes based in the scoring scheme proposed by Zimmerli et al. (2007): Class I (index ≤ 10) – normal tissue structure with slight histological alterations; Class II (index 11–20) – normal tissue structure with moderate histological alterations; Class III (index 21–30) – moderate modifications of normal tissue; Class IV (index 31–40) – pronounced histological alterations of the organ; Class V (index N 40) – severe histological alterations of the organ. The sum of the multiplied score values and importance factors of all diagnosed changes for each specimen resulting in different indices were statistically analyzed. The results were presented as averages. Besides the indices calculated by extent (score value) and pathological importance (importance factor) of lesions, a further point of interest to us was the prevalence of histological changes. Histological lesions were observed in each individual from the tested concentrations (15 individuals in concentration, 3 tested concentrations). From each individual 10 slides were prepared (10 slides x 15 individuals = 150 slides per concentration). From each slide approximately 100 lamellae were observed and the specific lesions were recorded. The prevalence was presented as average for all studied specimens for each concentration (n=15). We proposed the following classification: (10-30%) – alterations which occurred rarely; (30-50%) – alterations which occurred frequently; (50-70%) – alterations which occurred more frequently, and (>70%) – alterations which prevailed in the gill histological structure.

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Respiration rate measurements

The respiration rate was measured along with the histological analysis on the 72nd hour. At the given time 5 fish were transferred in 30 l glass aquaria filled with water from the test tanks. The oxygen levels were measured using an oximeter (WTW, Germany). The tanks were filled with water until the edge and covered with plastic foil in order to prevent oxygen access from the air. The tanks were left for one hour and thereafter, the oxygen level was again measured. The respiration rate was determined by calculating the difference in the dissolved oxygen levels before and after one hour following Tsekov (1989): $I = Q_2/G$ where I – respiration rate index; G – weight of the fish, Q_2 – oxygen consumed by the fish between the two measurements (the difference between the oxygen levels before and after one hour Q_2=Q-Q_1 hour). Q was calculated by the following formula: $Q = V \times q$, where: Q – total oxygen level in the tank; V – volume of the water in the tank; q – level of dissolved oxygen in one liter of water (mg l^{-1}).

Statistical analysis

For the statistical processing of the data the software package Graph Pad Prism 7 for Windows (USA) was used, as well as the software package Past v. 3.0 (Hammer et al., 2001). The histological index results were presented as average values per tested group. The number of fish affected by a specific histological lesion was presented as percentage prevalence. The normal distribution was tested with the D'Agostino-Pearson normality test. T-test was applied to see if there is a significant difference between the histological alterations in the exposed groups and the control, as well as between the different lesions. Pearson’s correlation index (r) was applied, using normalized data (square root transformation) when the data were not normally distributed (Fowler et al., 1998) in order to determine if there is correlation between the respiration rate and the pesticide concentrations. The index of determination (r^2) was also given. A level of probability $p < 0.05$ was considered as statistically significant.

Results

Water quality

The basic physical water quality parameters measured at the three tested concentrations are presented in *Table 1*. They did not differ significantly ($p > 0.05$) compared to the control and were also within the acceptable water quality guideline ranges for aquatic ecosystems (DWAF, 1996).

Table 1. *Basic water physical properties measured in the three CPF test tanks, including control*

Test tank	pH	T (°C)	Conductivity (µS cm-1)	Dissolved oxygen (mg L-1)
0.9 µg CPF	8.3±0.5	16.5±1.5	370 ±5.5	8.5±1.5
1.5 µg CPF	8±0.5	17.3±2	365 ±3.5	8.3±0.3
3 µg CPF	7.5±1.5	18.5±0.5	410 ±0.5	7±0.5
Control	8.5±0.5	19±0.5	353.3±0.3	9.1±1

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Gill histology

No external abnormalities were identified on any of the common carps, both control and tested fish. The general gill structure of control carps was typical for teleostean (Evans et al., 2005). In addition, our results for the control carps corresponded to Rajeshkumar et al. (2017). The gills of the control group were characterized with normal morphology including primary and secondary lamellae. The gills had four gill arches on each side of the buccal cavity. Each arch was composed of numerous gill filaments which had two rows of secondary lamellae which ran perpendicular to each filament. Each lamellae was made up of two sheets of epithelium delimited by many pillar cells which were contractile and separate the capillary channels. The single-cell thick lamellar epithelia contained goblet and chloride cells. One to two erythrocytes were usually recognized within each capillary lumen. Therefore, based on the proposed scale we determined the gill histology of control common carp as relatively normal (0) (*Fig. 1, Tables 2 and 3*). However, the macroscopic examination of the common carp gills exposed to different CPF concentrations for 72 hours showed alterations which differed in type, severity and prevalence. These lesions are presented in *Tables 2 and 3*, and respectively in *Figures 2, 3 and 4*.

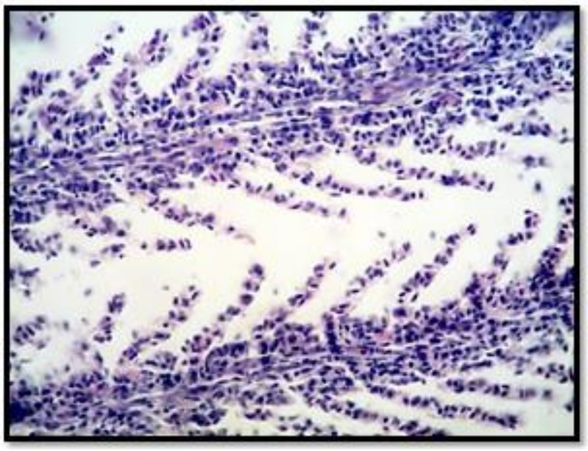

Figure 1. *Histological structure of common carp gills, control, x 200, H&E*

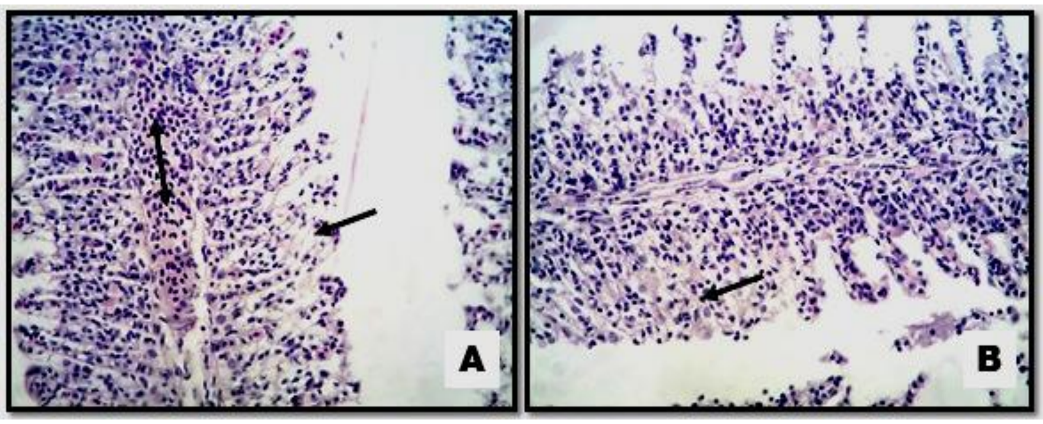

Figure 2. *Histological alterations in common carp gills after exposure to 0.9 µg CPF, x 400, H&E:* ***A*** *– Vasodilatation in the primary lamellae and lamellar lifting;* ***B*** *– Proliferation of gill epithelium and partial fusion*

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Table 2. *Histological assessment tool for common carp gills exposed to different CPF concentrations for 72 hours (average results; n = 15 fish in each test tank)*

Gills reaction pattern	**Functional unit of the tissue**	**Alteration**	**Importance factor**	**Score value**				**Index control**	**Index 0.9 µg**	**Index 1.5 µg**	**Index 3 µg**
				control	**0.9 µg**	**1.5 µg**	**3 µg**				
Circulatory disturbances	Blood vessels of secondary lamellae	Vasodilatation along the length of the blood vessel	$W_{GC1}=1$	0	1	2	2	$I_{GC}=0$	$I_{GC}=4$	$I_{GC}=9$	$I_{GC}=13$
		Vasodilatation at the basal part of the blood vessel	$W_{GC2}=1$	0	1	2	2				
		Vasodilatation at the apical part of the blood vessel	$W_{GC3}=1$	0	0	1	1				
	Blood vessels of primary lamellae	Vasodilatation	$W_{GC4}=2$	0	1	2	4				
Regressive lesions	Epithelium	Degeneration (necrosis)	$W_{GR1}=3$	0	0	1	2	$I_{GR}=0$	$I_{GR}=0$	$I_{GR}=3$	$I_{GR}=6$
Progressive lesions	Epithelium (secondary lamellae)	Lamellar lifting	$W_{GP1}=1$	1	2	4	4	$I_{GP}=1$	$I_{GP}=11$	$I_{GP}=34$	$I_{GP}=25$
		Proliferation	$W_{GP2}=2$	0	1	4	4				
	Epithelium (primary lamellae)	Edema	$W_{GP3}=1$	0	1	2	4				
		Proliferation of stratified epithelium	$W_{GP4}=1$	0	2	4	2				
		Proliferation of glandular cells	$W_{GP5}=1$	0	1	4	4				
		Fusion	$W_{GP6}=3$	0	1	4	1				
Gill Index I_G								**$I_G=1$**	**$I_G=15$**	**$I_G=46$**	**$I_G=44$**

Table 3. *Percentage prevalence of histological alterations in the common carp gills exposed to different CPF concentrations for 72 hours*

Histological alterations		**Prevalence, %**			
		Control	**0.9 µg**	**1.5 µg**	**3 µg**
Circulatory disturbances	Vasodilatation along the length of the blood vessel in the secondary lamellae	0	16	31	39
	Vasodilatation at the basal part of the blood vessel in the secondary lamellae	0	13	29	35
	Vasodilatation at the apical part of the blood vessel in the secondary lamellae	0	0	19	23
	Vasodilatation of central venous sinus	0	23	33	63
Average, %		**0**	**13**	**28**	**40**
Regressive lesions	Degeneration (necrosis)	0	0	16	53
Average, %		**0**	**0**	**16**	**53**
Progressive lesions	Lamellar lifting	16	75	83	86
	Proliferation of epithelium covering secondary lamellae	0	25	56	61
	Edema	0	28	46	59
	Proliferation of stratified epithelium	0	48	85	56
	Proliferation of glandular cells	0	15	78	84
	Fusion	0	21	69	25
Average, %		**2.7**	**35.3**	**69.5**	**61.8**

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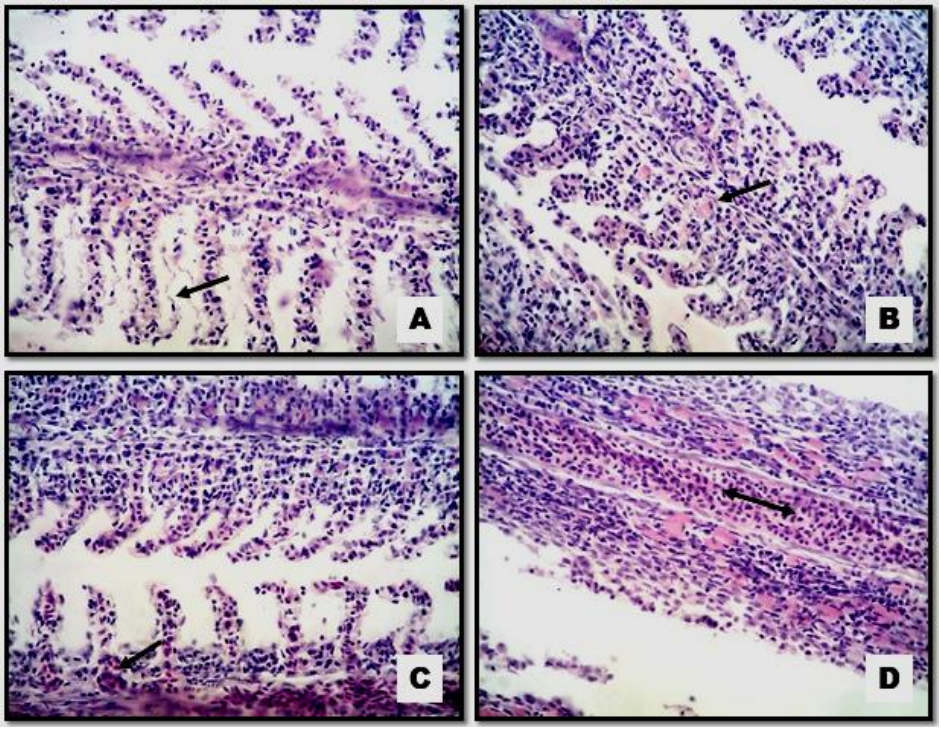

Figure 3. *Histological alterations in common carp gills after exposure to 1.5 μg CPF, x 400, H&E:* ***A*** *– Lamellar lifting;* ***B*** *– Proliferation of the glandular cells in the gill epithelium of the main lamellae;* ***C*** *– Vasodilatation at the basal part of the blood vessel in the secondary lamellae;* ***D*** *– Vasodilatation of the primary lamellae*

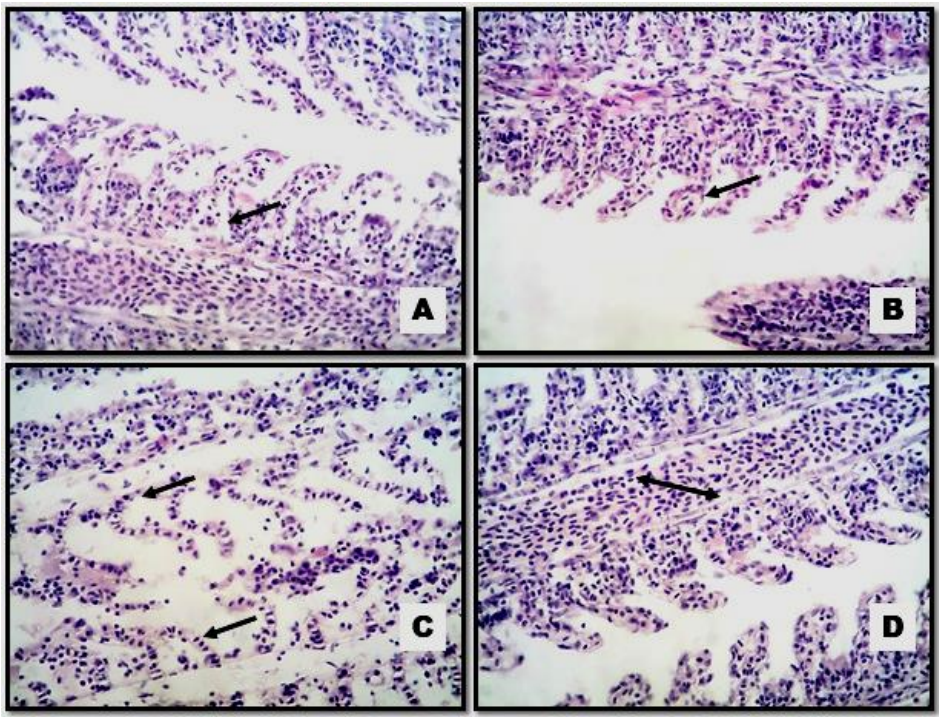

Figure 4. *Histological alterations in common carp gills after exposure to 3 μg CPF, x 400, H&E:* ***A*** *– Edema;* ***B*** *– Proliferation of the gill epithelium in the secondary lamellae;* ***C*** *– Degeneration of lamellar epithelium;* ***D*** *– Vasodilatation of the primary lamellae*

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Overall, from the conducted study on the negative effects of CPF on the histological structure of common carp gills alterations which we categorized overall in three groups – proliferative (progressive) changes, degenerative (regressive) changes in the gill epithelium and changes in the circulatory system (circulatory disturbances) were observed. We found that the proliferative alterations among the three groups were with the most severe degree of expression at all three tested concentrations, and these were lamellar lifting, edema, epithelial proliferation covering the primary and secondary lamellae, glandular cell proliferation (*Table 2*). Furthermore, these alterations showed an increase in the degree of expression in line with the increase in the pesticide concentration, except the proliferation of filament epithelium and fusion. These changes were expressed in a higher degree at the CPF concentration of 1.5 µg. We observed the most severe proliferative alteration, fusion, in the area of the secondary lamellae which completely altered the structure of the studied organ. Proliferation of epithelial tissue covering the filament reached the tip of the lamellae, whereby the individual secondary lamellae were not distinguishable. In general, fusion was presented at all tested concentrations, but its most severe degree of expression was detected at the CPF concentration of 1.5 µg (*Table 2*). This in turn, probably demonstrates the inclusion of active cell division processes associated with compensatory-adaptive mechanisms against the entry of the toxicant at this concentration.

The observed degenerative changes also showed an increase in the degree of expression along with the increase in the CPF concentration. However, degeneration of epithelial cells in the filament and secondary lamellae was not found at the lowest applied concentration (0.9 µg). On the other hand, at the same CPF concentration we observed a more active manifestation of proliferative changes in the gill epithelium which was also confirmed by the presence of fusion expressed in a mild degree of expression. At a CPF concentration of 1.5 µg the proliferative changes prevailed over the degenerative ones, with a severe degree of fusion which covered the majority of the secondary lamellae. In contrast, the degenerative changes were found to a mild extent (*Table 2*). This in turn, also shows a more active inclusion of compensatory-adaptive responses expressed through cell division processes compared to the lesser expression of necrotic processes in the common carp gills. At the highest applied concentration which equals AA-EQS of CPF in surface waters (3 µg), unlike the previous ones, we found predominant degenerative changes associated with more active involvement of necrotic processes in the gill epithelium. There was also a gradual substitution of the degenerative over the proliferative lesions associated with necrotic processes which showed a more severe degree of damage at the highest concentration, most likely due to the higher toxicity of CPF.

The changes found in the gill circulatory system consisted of vasodilatation in the main sinus and the blood vessels in the secondary lamellae. Similarly to the proliferative and degenerative changes in the gill epithelium these alterations also showed a certain tendency to increase the degree of expression with increasing the CPF concentration. At the lower applied concentrations (0.9 µg) vasodilatation of the secondary lamellae was found in their basal part, with a mild degree of expression (*Table 2*). Basal sinus vasodilation was observed in a severe degree of expression at the highest CPF concentration, probably indicating an increase in the internal blood vessel volume associated with red blood cell influx.

Overall, the occurred changes limited to the severe degree of expression which we linked with the particular exposure time of 72 hours. As for the very severe degree of

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histological alterations, we consider that a longer exposure period is required which in turn is associated with further thorough studies.

The statistical analysis showed a significant difference ($p < 0.05$) between the score value for the control and the three groups of alterations (circulatory disturbances, progressive and regressive lesions) observed in the fish gills exposed to the highest CPF concentration. No significant differences were found for the reaction patterns between the different tested CPF concentrations.

The mean gill index (I_G) showed variation comparing the different CPF concentrations (*Table 2*). It was highest ($I_G = 46$) for the common carps exposed to 1.5 µg CPF, followed by the one calculated for the fish treated with 3 µg CPF ($I_G = 44$), but they were not significantly different ($p > 0.05$). Significantly different were the gill indexes (I_G) for the common carps exposed to the control and the lowest CPF concentrations compared to the other two ($p < 0.05$). Evaluating these results in terms of the severity of the histological response, the gill index (I_G) results of common carp were classified as Class II – normal tissue structure with moderate histological alterations for the lowest concentration and Class IV – pronounced histological alterations of the organ for the mean and highest concentration, respectively.

As for the percentage prevalence the gills were most affected in terms of the progressive alterations at all tested CPF concentrations (respectively 0.9, 1.5 and 3 µg) compared to the regressive and circulatory lesions (*Table 3*). In addition, the t-test proved significant differences ($p < 0.05$) between the percentage prevalence of the progressive lesions compared to the other two gill histological patterns at all applied CPF concentrations. Significant differences were also found between the circulatory disturbances and progressive lesions at 0.9, 1.5 and 3 µg. In the control group we found only lamellar lifting which was presented in 16% of the observed samples.

Respiration rate measurements

The results from the respiration rate measurements are presented in *Table 4*.

Table 4. *Index of respiration rate of common carp exposed to CPF concentrations at the 72nd hour (n=5 fish for each 30 l tank)*

CPF concentrations	Fish weight. g (G)	Oxygen content (mg l^{-1})					Index of respiration rate (I)
		Start		End		Total	
		q	Q	q_{1h}	Q_{1h}	(Q_2)	
72 hour							
Control	71.3	6.4	192.00	6.1	183.00	9.00	0.126
0.9 µg	64.7	6.2	186.00	5.9	177.00	9.00	0.139
1.5 µg	54.5	5.9	177.00	5.6	168.00	9.00	0.165
3 µg	59.5	5.9	177.00	5.5	165.00	12.00	0.202

Overall, we determined that the fish increased its respiration rate most likely because CPF disrupted the gill histological structure and thus, destabilized the respiration process. At the end of the acute experiment (72^{nd} hour) we recorded a 1.6 times increase in the respiration rate of the fish exposed to the highest CPF concentration, compared to the control. We also registered a strong correlation between the pesticide concentrations and the respiration rate index, although it was statistically insignificant ($r = 0.92$, $p = 0.08$). Furthermore, the index of determination showed that 84.64% of the observed correlation was probably caused by the CPF concentration.

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Discussion

Water quality

The results on the basic physical water properties showed a good agreement with the control. Therefore, we consider that the lesions in the gill histological structure and respiration rate measurements were not due to changes in the abiotic factors.

Gill histology

As described by Bernet et al. (1999) in some publications lesions were described only morphologically and in these studies the effects of water pollution correlated with the abundance of lesions found in the studied organ. However, other studies concentrated on a few alterations in the organ and assessed their extent by using a scale. With the quantification of the alterations, statistical evaluation becomes practicable. The corresponding indices can be compared more easily than the morphological descriptions of pathological changes. We agree with Bernet et al. (1999) that histology is a descriptive science and the assessment of morphological changes will always depend on the investigator's experience, but such a tool that we also used allows more reliable interpretation of the results and comparisons with others. Furthermore, to properly interpret results from histopathological evaluations, it is essential to realize that a healthy control condition is not characterized by the complete absence of any histological lesions, but may display moderate alterations such as minor structural disorders or mild inflammatory reactions (Bernet et al., 2004). In our study we only observed lamellar lifting in the gills of control common carp which was categorized as a mild lesion. We also share the opinion of Zimmerli et al. (2007) that a technical disadvantage of histology is its qualitative nature. For this reason, in the present study we applied the approach of Bernet et al. (1999) and Zimmerli et al. (2007) that developed an evaluation scheme to transform qualitative histological observations into a semi-quantitative index.

The gills are among the most delicate structures of the teleost body and are very sensitive to environmental conditions (Saraiva et al., 2015). We support the statement of Camargo and Martinez (2007) that by assessing the degree of expression of gill alterations, the extent of exposure to toxicants can be traced, given that the gills are involved in respiration, osmoregulation and excretion. They are an effective tool in biomonitoring studies also because of their large surface area that comes into contact with water, as well as their permeability (Evans et al., 2005; Vigliano et al., 2006). Therefore, the importance of gills in the respiration and ionic regulation of fish has led to many studies on the changes in this organ under the exposure of various environmental pollutants, including pesticides.

In the present experiment the degree of expression of lamellar lifting and edema was found to be mainly severe in the common carp gills. In addition, there was a clear proportional relationship found between the CPF concentrations and the degree of expression of these changes, and at the highest concentration they were severely expressed. Similarly to Jiang et al. (2011) we also consider that the most commonly found changes in gills under the action of toxicants are edema and lifting of the lamellar epithelium. However, they can serve as a protective mechanism because the separation of the epithelium from the lamellae increases the distance through which the pollutants in the water have to pass before they reach the bloodstream (Arellano et al., 2004). Lifting of the lamellar epithelium and edema were also found by other authors

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such as Girija et al. (2014) Babatunde et al. (2014) and Saraiva et al. (2015). Furthermore, Schmidt et al. (1999) and Bernet et al. (2004) also consider alterations such as branching of the secondary lamellae as an adaptation to low oxygen concentrations as it increased the surface area in brown trout after exposure to untreated sewage.

Proliferation of the epithelial tissue in the filament and in the secondary lamellae, as well as the glandular cells showed a difference in the degree of expression. We agree with Mallatt (1985) according to who epithelial tissue proliferation is one of the major alterations observed in fish gills in contaminated aquatic ecosystems. Similarly to Hassan (2011) we also consider that epithelial cell proliferation, partial fusion of secondary lamellae and lamellar lifting are protective mechanisms which increase the distance between the external environment and the blood, and this serves as a barrier to the entry of toxicants. Similar lesions in freshwater fish after pesticide contamination were found by Gabet et al. (2014). As for the most severe proliferative alteration, along with Butchiram et al. (2009) and Karthigayani et al. (2014), we also observed fusion as a result of activating epithelial cell division processes and hyperplasia of epithelial cells after exposure to pesticides leading to significant proliferative changes. We agree with Bentivegna et al. (2015) and Fonseca et al. (2017) that cell proliferation probably occurs as defense mechanisms, leading not only to increased epithelial thickness, but also in, extreme cases, to lamellar fusion. Indeed, the increased thickness of epithelium prevents further toxicant absorption, but on the other hand, this causes the adverse effect of compromising gas and ion exchange mechanisms (Fonseca et al., 2017).

In addition, along with hyperplastic epithelial areas reaching fusion, we also found such with thinning of the lamellae due to degenerative processes. The identified higher degree of observed degenerative changes at higher tested concentration confirmed the reduction and probably subsequent exclusion of compensatory-adaptive processes. However, we consider that the toxicant could act by simultaneously involving two processes – active cell division and degenerative-necrotic destruction. It is noteworthy that there was an inverse relationship between these lesions i.e when fusion was expressed in a more severe degree; the degenerative changes were in a lower one and vice versa. Furthermore, at the highest CPF concentration (0.03 μg = AA-EQS) the degenerative processes were intensified and gradually shifted the proliferating ones. We consider that this would eventually affect the functioning of the organ, and could therefore inevitably cause oxidative stress and destabilize the health of the overallfish health. Our results confirmed the results of Theurkar et al. (2014) who found similar histological lesions, such as degeneration and necrosis of epithelial cells covering the filament, but at sublethal concentrations of insecticide monocrotophos. Together with the activation of compensatory-adaptive mechanisms associated with proliferative changes, we also found changes in the blood vessels of the gills of the experimental fish. At the lower concentrations, the histological changes were mild and with the increasing concentration, they shifted to moderate and severe.

The observed alterations, such as vasodilatation in the secondary lamellae and the main sinus were presented in varying degrees of expression, but we found a clear relationship between the impact and CPF concentration. The changes were found more frequently in the area of the venous sinus than in the secondary lamellae. Our results corresponded to those of Shubat et al. (1982) according to who vasodilation is observed mostly in the main sinus. In addition, we agree with Schwaiger et al. (2004) who suggested that the circulatory system alterations lead to a higher degree of

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stress-induced damage in the organism. On the other hand, according to McHugh et al. (2011) the circulatory disturbances and regressive alterations are generally regarded as reversible when the toxicant exposure stops.

Respiration rate

In terms of the other biomarker which we measured, the fish reacted by increasing their respiration rate with the increase of the pesticide concentrations after the 72nd hour of exposure. We agree with Kumar et al. (2012) who suggested that in most cases the respiration rate increases with the increase of the pollutant concentration and level of toxicity. As stated by Stoyanova et al. (2017) and Yancheva et al. (2017b) the reason for this is that the organism tries to deliver more oxygen to all tissues and organs triggered by the oxidative stress due to the toxic exposure. Overall, our respiration rate measurements were in line with the histological lesions in the gills which confirmed that CPF affects negatively the respiration process and thus, physiology of common carp.

Conclusions

In sum, we can point out that the present study is a contribution in the field of pesticide contamination, especially for the short-term exposure effects of CPF on the physiology of commercially important fish species, such as common carp. Based on our results we can conclude that the gill changes which were identified were CPF related and CPF also affected the histology of carp gills by activating compensatory-adaptive and degenerative mechanisms leading to pathological alterations. In addition, the extent and prevalence of the proliferative alterations were predominant over the degenerative alterations, and those in the circulatory system. These changes subsequently changed the physiology of common carp by disrupting the gill function. Regardless of the applied concentrations the negative effects of CPF were also confirmed by the respiration intensity measurements. Moreover, the negative effect of CPF was observed at all three decreasing concentrations based on the permitted annual average concentrations (AA-EQS) in Directive 2013/39/EU which confirmed its toxicity on common carp. Overall, the results from the present study enhanced the importance of multi-biomarker approach in biomonitoring programs to assess the impact of organophosphorus pesticides on freshwater ecosystems. Thus, we strongly suggest that further studies in this particular area are continued.

Acknowledgements. The National program "Young Researches and Postdocs, 2018" financed by the Ministry of Education and Science, Bulgaria is highly appreciated. The authors also thank the Ministry for Education and Science, Bulgaria and The Scientific Research Fund for the financial support of project M26/6 (Scientific Fundamental Research for Young Scientists and Postdocs). We also thank the experts at the Regional Accredited Laboratory, Executive Environment Agency, Ministry of Environment and Water, Plovdiv, Bulgaria for providing the toxicant.

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DOI: http://dx.doi.org/10.15666/aeer/1702_51395157

CONVERSION FROM GRASSLAND TO CROPLAND AND LENGTH OF CROPPING HISTORY DRIVING SOIL METHANE UPTAKE IN CHINA

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(Received 17th Jan 2019; accepted 27th Feb 2019)

Abstract. The change of land use from grassland to cropland in Northern China has raised serious concern about regional carbon (C) cycle and greenhouse gas balance. We measured soil methane (CH_4) uptake using manual static chambers in grassland and cropland soils in the agro-pastoral ecotone of Inner Mongolia over three growing seasons (2010-2012). The primary aims were to assess the effect of undisturbed grassland and croplands from converted grassland with different land use histories on gas fluxes and systematically compare the site-specific CH_4 uptake factor. We found a significant difference ($P < 0.001$) in CH_4 uptake between grassland and croplands from reclaimed grassland for 5, 10 and 50 years old, and cropland soils in 5 and 10 years old were a significant sink of CH_4. Compared with cropland soils, the grassland had the lowest cumulative CH_4 uptake, with 141.4, 210.0 and 236.0 mg/m^2 during growing seasons of 2010, 2011 and 2012, respectively. Over the 3 growing seasons, the cumulative CH_4 uptake of croplands aged 5, 10 and 50 was 544.5, 361.7 and 266.1 mg/m^2. With the increase of farming time, the methane accumulation and absorption of C5, C10 and C50 decreased. Differences in CH_4 uptake of grassland and cropland with different length of cropping history can be explained by the amount of soil ammonium nitrogen (NH_4^+-N) and soil moisture. We conclude that (i) croplands for 5 and 10 years old from reclaimed grassland are the best approach considered here for optimizing the land use as a sink for atmospheric CH_4, and (ii) the practice that croplands from grassland reclaimed for more than 10 years old should be planted into grasslands is recommended for managing CH_4 uptake and soil carbon sink in the agro-pastoral ecotone of Inner Mongolia, China.
Keywords: *land-use change, greenhouse gases, soil physical and chemical properties, carbon sink*

Introduction

Methane (CH_4) is the second most important greenhouse gas in the atmosphere after carbon dioxide (CO_2). The atmospheric concentration of CH_4 has been increasing by 0.3%/yr (Liu et al., 2007). Although the main sink for atmospheric CH_4 is its oxidation in the troposphere by hydroxyl(-OH) (Khalil, 2000), aerobic soils are the only biological sinks for atmospheric CH_4 with an estimated global sink of 20-45 Tg CH_4/yr^2 (IPCC, 2007). Methane uptake is influenced by several factors including land use change, temperature, precipitation, N input and soil properties (e.g., moisture, temperature, texture, pH and C/N ratio) (Liu et al., 2009; Mou et al., 2014). Among these factors, land use change and soil properties are considered to be important drivers of the magnitude of methane uptake (Dörr et al., 1993; Ojima et al., 1993; Steudler et al., 1995). Changes in land use or intensification of land management directly affect the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51595172

CH_4 uptake and the atmospheric CH_4 budget (Smith et al., 2000; Verchot et al., 2000; Merino et al., 2004).

Inner Mongolia steppes account for approximately 80% of grassland in China. Land use conversions from grassland to cropland have occurred in the arid and semi-arid lands of Asia (ASAL) during the 20th century. The agro-pastoral ecotone of Inner Mongolia is included in the ASAL of Asia. The transition from livestock grazing to farming causes changes in land use practice. The grassland in Inner Mongolia is a typical *Leymus chinensis* temperate steppe, where land use types are often diverse with frequent changes. Wang et al. (2005) and Liu et al. (2007) found that CH_4 uptake rates and soil moisture were negatively correlated, while other soil properties were not discussed in these studies (Wang et al., 2005; Liu et al., 2007). How these changes have altered or will alter the CH_4 uptake remains unknown. Moreover, although much is known regarding CH_4 uptake, few data specific to soils of agro-pastoral ecotone is available, and few studies have been conducted on the effect of length of cropping history or soil properties on CH_4 uptake in this region. The effects of the conversion of grassland to cropland on CH_4 uptake are uncertain. The determining factors that mediate the influence of land use change on CH_4 uptake have not been elucidated.

Therefore, we conducted a study in the agro-pastoral ecotone of Inner Mongolia to investigate the effects of land use change on CH_4 uptake. The objectives were to understand the impact of cropping history and soil properties on CH_4 uptake from the conversion of grassland to cropland. This study investigated CH_4 uptake throughout 3 years in an agro-pastoral ecotone in Inner Mongolia, China. We compared cropland soils with different length of cropping history and adjacent grassland that were derived from the same parent material of soil under the same climate.

Materials and methods

Description of the study site

The study was conducted in Taipusi County, Inner Mongolia (China), located 41º49′52″north latitude and 115°13′26″east longitude, at an elevation of 1400 m above sea level (*Fig. 1*).

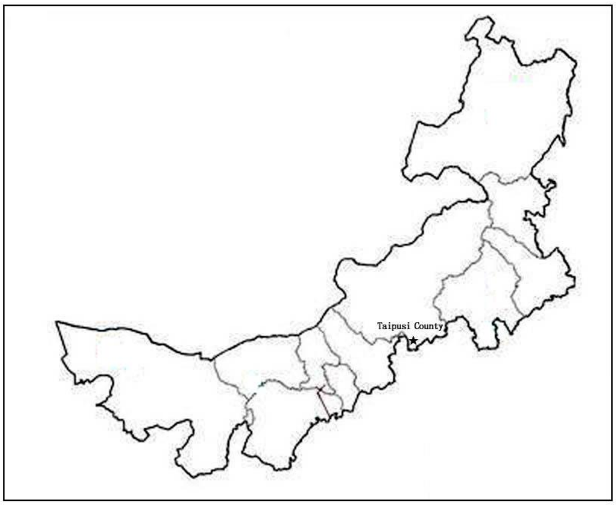

Figure 1. *Location of study area in Inner Mongolia, China*

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DOI: http://dx.doi.org/10.15666/aeer/1702_51595172

Located in the south of the Xilin River and characterized by scattered farms in the steppe, the study area is very representative of both the Eurasian temperate grasslands (Wang et al., 2007) and the steppe region of Inner Mongolia (Tong et al., 2004). The grassland is dominated by *L. chinensis* which is the typical vegetation of the region's grassland (Geng et al., 2010). The grassland in the study site was a natural clearing of native vegetation, without grazing or additional treatments such as fertilization or grass seeding. This region falls in the semi-arid temperate climatic zone. The mean annual temperature is approximately 1.6ºC, ranging from a mean monthly temperature of -17.6ºC in January and 17.8ºC in July. The region receives an average of 400 mm of precipitation annually. The growing season is from late April to early October.

Four sites were selected for the study, including an area of grassland (reference site) and croplands (farmer-managed fields) for 5, 10, and 50 years old from reclaimed grassland (*Table 1*). The length of cropping history was determined according to a field inventory and Taipusi County records. Hereafter, the four sites are referred to G (grassland), C5 (5 y old cropland), C10 (10 y old cropland) and C50 (50 y old cropland). The 4 sites presented similar slope and soil type within the adjacent ecosystems including flat areas of grassland soil and cropland soil. N, P and K compound fertilizers were applied before planting. Agriculture management in C5, C10 and C50 soil after conversion was carried out similarly.

Table 1. *Site characteristics of the agro-pastoral ecotone on Taipusi County, Inner Mongolia*

Site†	**Coordinates**		**Land use history**
	Latitude N	Longitude E	
G	41º49′52″	115°13′26″	Natural grassland
C5	41º50′29″	115°13′19″	Cropland reclaimed for 5 years old
C10	41º50′49″	115°13′43″	Cropland reclaimed for 10 years old
C50	41º50′53″	115°13′23″	Cropland reclaimed for 50 years old

There were three replicates plots for every natural grassland (G) and cropland from reclaimed grassland with different length of cropping history (C5, C10, and C50), respectively, and the area of each plot is 100 m × 100 m. Grassland vegetation was *L. chinensis* and *Stipa capillata.* Potatoes (*KexinNO.1*) and oats (*Avena sativa L.*) were grown in the three cropland sites in 2010, 2011 and 2012. Potatoes and Oats were alternatively planted in 2010, 2011 and 2012, respectively. Cropland was plowed once a year before planting. The plowing depth was 30-35 cm via machine plow. N, P and K compound fertilizer ($N:P_2O_5:K_2O$ = 15:10:10)(450 kg/ha) was incorporated into the soil before planting once a year. The crops were planted in May and were harvested in September, and the soil was bare from October to the following April. The croplands were irrigated in spring and autumn.

Measurement of methane flux

Field measurements of CH_4 flux were conducted in the four soils from April 2010 to October 2012. Measurements were taken every 15 days between June and August and once a month in April, May, September and October.

Three square chambers (n = 3) were randomly placed in each plot replicate and were in the same location for the duration of the measurements. All the chambers were fixed to the ground, reaching approximately 10 cm down into the soil. Aluminum flux collars

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were permanently installed to ensure reproducible placement of the gas collection chambers for successive CH_4 flux measurements throughout the growing season. The top edge of the collar had a groove that could be filled with water to seal the rim of the chamber. The chamber was equipped with a circulating fan to ensure complete gas mixing and was wrapped with a layer of sponge and aluminum foil to minimize air temperature changes inside the chamber during the period of the measurement. Cross-section of the chamber was 0.25 m^2 (0.5 m × 0.5 m). The height of chamber (or H) was 0.5 m. Samples were collected over a 30-min period (0, 10, 20 and 30 min) after closing the chambers at 8:00-11:00 am. Gas samples were taken using 25 mL gas-tight plastic syringes which were closed with stopcocks. The air temperature inside the chamber was recorded by thermometer for each measurement. Soil temperature (0-5 cm depth) was continuously recorded using temperature sensors interfaced with data loggers (HSY-TL881). During the CH_4 flux measurement period, soil water content was monitored using a TDR instrument (MP-406 Kits for moisture measurement). Five temperatures and five soil moisture data were measured on each plot. The aboveground biomass of the potatoes and oats under the sampling chamber (0.25 m^2) were determined by harvesting followed by oven-drying at 105ºC to constant weight.

Air samples were analyzed for CH_4 using a modified gas chromatograph (Agilent 6820D, Agilent Corporation) equipped with a flame ionization detector (FID) (Wang and Wang, 2003). Nitrogen was used as the carrier gas (30 mL/min). The oven was operated at 55ºC and the FID was operated at 200ºC. Flux was determined from the slope of the mixing ratio change in four samples taken 0, 10, 20 and 30 min after chamber closure. The gas samples were rejected if they did not yield an R^2 greater than 0.90 over the 30 min period. If the detected mixing ratio in the samples is decreased with the prolonging of time, it is a negative value, representing uptake. Carbon sinks into soil. Otherwise, it represents emissions.

Flux calculation

The CH_4 flux was calculated with *Eq. 1* (Yang et al., 2018):

$$F = 1000 \cdot S \cdot H \cdot M \cdot \left(Pa/Ps \right) \cdot \left(Ts/Ta \right) / 22.4 \qquad \text{(Eq.1)}$$

where F refers to CH_4 flux (μg/(m^2 min)), S is the linear slope of the concentration change with time over the measurement period (μL/(L min)), H is the valid height of the sampling chamber (m) and M is the molar mass of CH_4 (16 g/mol). Pa and Ta are the actual measured atmospheric pressure and temperature inside the chamber, respectively. Ps and Ts are the standard conditions (760 mm Hg, 273.15K). The standard molar volume of CH_4 (L/mol) is 22.4. Seasonal (7 month values) amounts of CH_4 uptakes were sequentially accumulated from the uptakes between every two adjacent intervals of the measurements.

Soil property measurements

Soil samples were collected for chemical analysis during the CH_4 flux measurements. The measurements were taken once every 15 days from June to August and once a month in April, May, September and October. Three replicates were collected from each site (G, C5, C10 and C50), with 10 sampling locations for each composite replicate. The samples were air-dried and sifted through 2 mm for

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physicochemical analysis. The sample of soil bulk density was collected using a steel cylinder (5 cm of diameter, 5 cm of height). Soil organic carbon (SOC), total nitrogen (TN), NH_4^+-N and NO_3^--N were determined following procedures. SOC was determined using a TOC (Total Organic Carbon) analyzer (Sievers 5310 C, GE Analytical Instruments, USA) (Lim and Choi, 2014), and TN was measured via the dry combustion method using a C/N Analyzer (Vario Macro, Elementar, Germany) (Yan et al., 2012). Soil ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were measured using a micro-Kjeldahl procedure (Aulakh et al., 2000). Soil microbial biomass C (MBC) and microbial biomass N (MBN) were determined using the chloroform ($CHCl_3$) fumigation–incubation method (Nunan et al., 1997). The pH value was measured in 1:2.5 soil/H_2O (w/w) suspension with a Titrino pH meter (Metrohm Ltd. CH.-901, Herisau, Switzerland) fitted with a glass electrode (Godsey et al., 2007).

Data analysis

Data were analyzed using ANOVAs and General Linear Mixed Model to assess the effect of the study site. Pearson's correlation analysis was performed to investigate the relationship between CH_4 uptake and soil properties (i.e., soil temperature, soil moisture and soil NH_4^+-N content etc.). The R^2 (square of Pearson correlation coefficient) values were used to determine the fitness of regression functions. For all analysis, statistical significance was determined at $P < 0.05$. All statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Seasonal change on CH_4 uptake flux from the soils of grassland and croplands with different ages reclaimed

The CH_4 uptake flux in the grassland, C5, C10, and C50 soils showed obvious seasonal variability from 2010 to 2012 in the study (*Fig. 2*). During the first month of the growing season, CH_4 uptake increased rapidly in C5 soil. However, the seasonal uptake of CH_4 in G and C50 soils increased moderately, and there was no significant uptake peak. Throughout the testing stage, CH_4 uptake was lower in grassland and C50 soil than that in C5 and C10 soils. The maximum uptake flux of CH_4 in the grassland soil was 0.06, 0.12 and 0.11 mg/(m^2 hr), and was 0.09, 0.13 and 0.20 mg/(m^2 hr) in the C50 soil during the growing seasons in 2010, 2011 and 2012, respectively (*Fig. 2*). CH_4 uptake flux was the highest in C5 and C10 soils during the experimental period. The peak of CH_4 uptake flux in C5 and C10 soils was detected between June and July. The maximum CH_4 uptake flux of C5 soil was 0.27, 0.23 and 0.29 mg/(m^2 hr), and was 0.22, 0.20 and 0.29 mg/(m^2 hr) in C10 soil in 2010, 2011 and 2012, respectively. CH_4 uptake declined in all of the study sites at the end of the growing season (*Fig. 2*).

Cumulative methane uptake in the soils of grassland and croplands with different land use history

There were significant differences for cumulative CH_4 uptake in the grassland soil, C5, C10, and C50 cropland soils in 2010 (F=273.7, $P < 0.0001$), 2011 (F=264.8, $P < 0.0001$) and 2012 (F= 362.4, $P < 0.0001$). The land use conversion in more recently-established croplands (5 and 10 years old) from grassland to cropland promoted greater atmospheric CH_4 uptake compared to C50 and grassland type.

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The grassland and C50 soils exhibited lower cumulative CH_4 uptake with 141.4 and 224.0 mg/m^2 than that of the C10 and C50 soils in 2010, 2011 and 2012, respectively (*Fig. 3*). Cumulative CH_4 uptake during the growing season was 544(±103) and 361(±47) mg/m^2 in C5 and C10 soils, respectively. The cumulative uptake flux in C5, C10, and C50 soils was 190%, 90% and 38% higher than that of the grassland soil. As the cropping history increased, the cumulative CH_4 uptake decreased in C5, C10, and C50 soils.

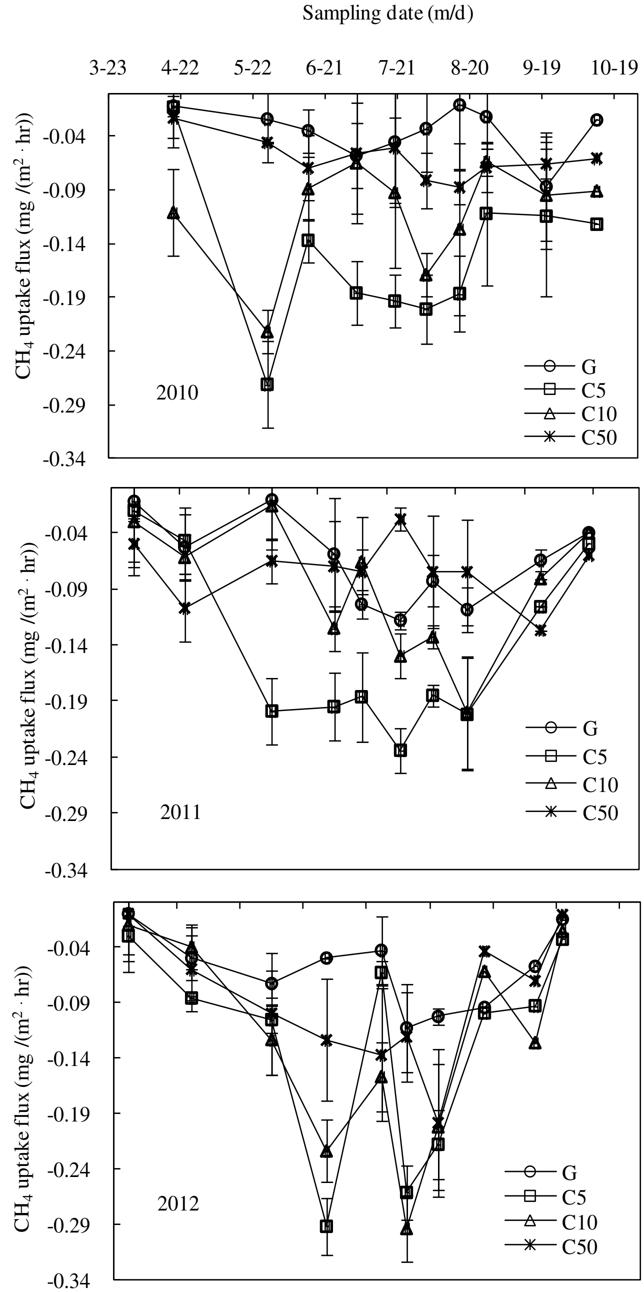

Figure 2. *Seasonal CH_4 uptake flux from the grassland and cropland with different length of cropping history during the growing season in 2010, 2011, and 2012; G: natural grassland; C5, C10, and C50: cropland for 5,10, and 50 years old from reclaimed grassland, respectively; The vertical bars represent standard error*

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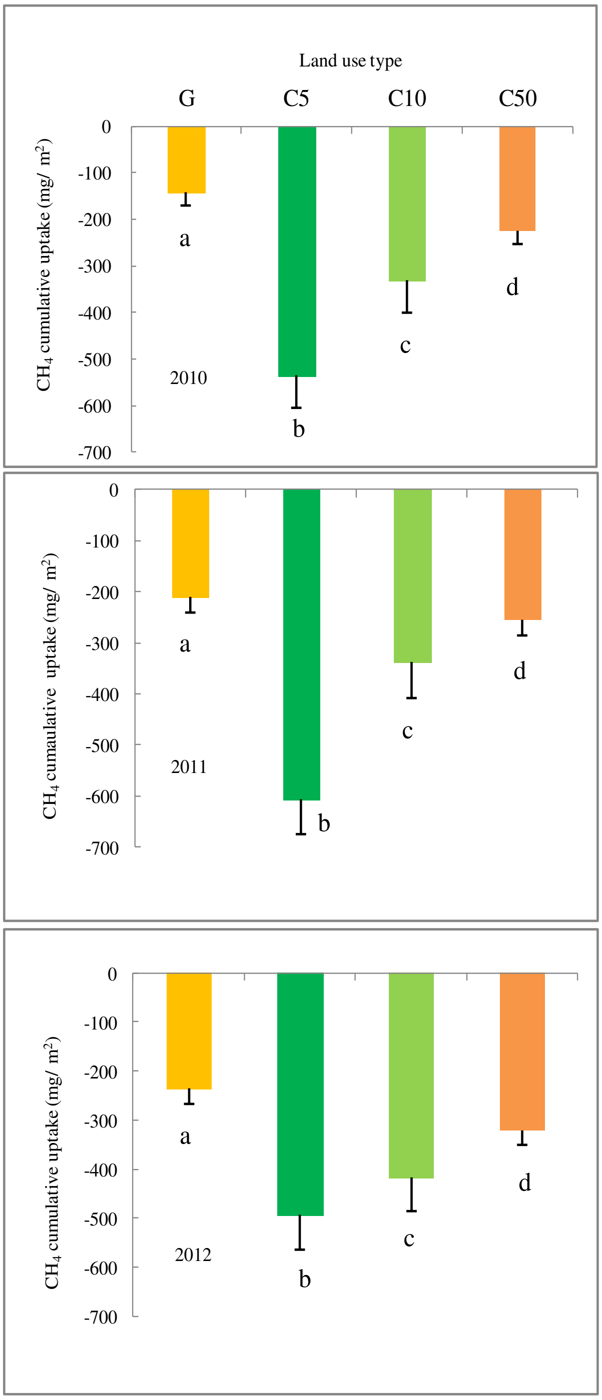

Figure 3. *Cumulative CH_4 uptake from the grassland and cropland soils during the growing seasons in 2010, 2011, and 2012; G: natural grassland; C5, C10, and C50: cropland for 5,10, and 50 years old from reclaimed grassland, respectively; The vertical bars represent standard error; different letters represent significant differences between the treatments*

The changing relationship between CH_4 uptake and moisture or temperature of soil from different land use types

A significantly negative correlation in grassland, C5, C10, and C50 soils (r = 0.52, 0.75, 0.63, 0.62; P < 0.01) was detected between CH_4 uptake flux and soil volumetric

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moisture (% v/v) when soil temperature was above 5ºC (*Fig. 4a-d*). Soil moisture was a major factor affecting CH_4 uptake flux in the soils. The correlation between temperature and CH_4 uptake flux was not observed in the soils ($P > 0.05$) (*Fig. 5*). Soil moisture was the important controlling factor for CH_4 uptake flux when the temperature was not the limiting factor.

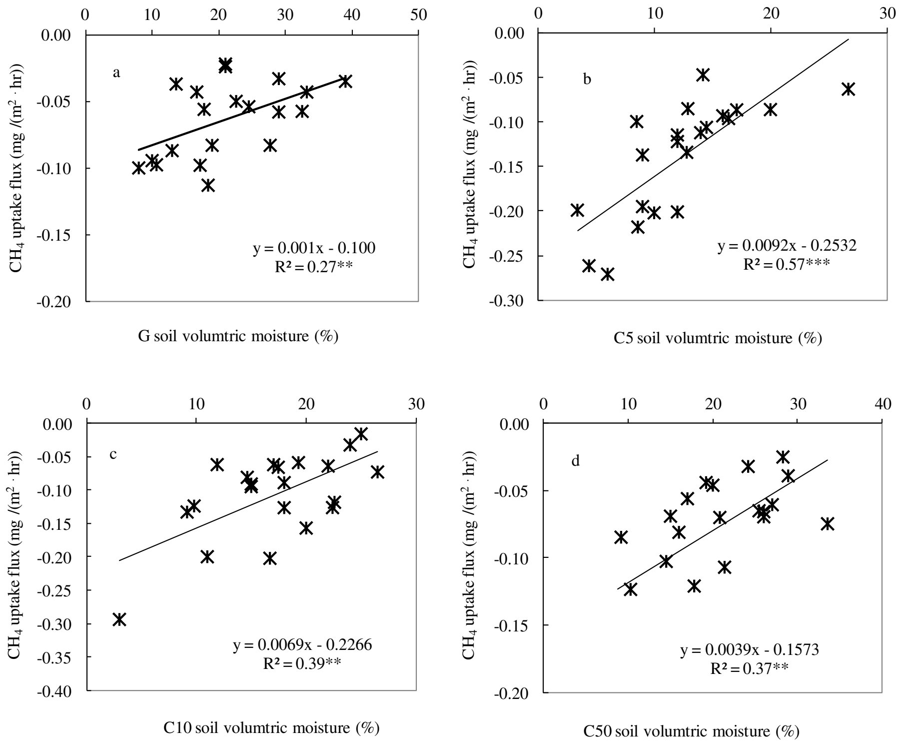

***Figure 4.** Correlation between CH_4 uptake flux and soil moisture from grassland and cropland soils; The soil volumetric moisture was simultaneously measured when gas sample were collected from 2010 to 2012; C5, C10, and C50: cropland for 5, 10, and 50 years old from reclaimed grassland, respectively*

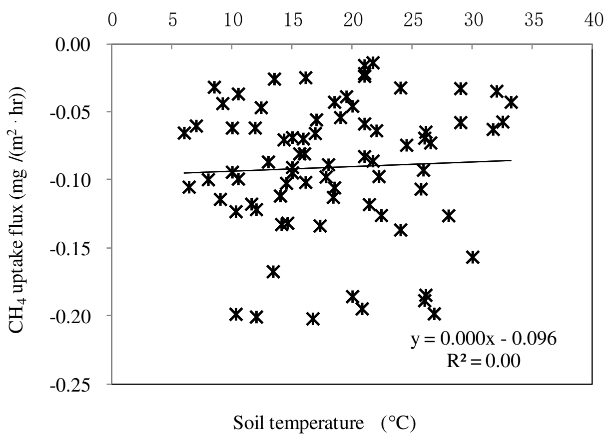

***Figure 5.** Correlation between CH_4 uptake flux and soil temperature in grassland and cropland soils; Soil temperature was simultaneously measured when gas sample were collected from 2010 to 2012*

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Potential links between cumulative methane uptake and soil NH_4^+-N

A negative relationship (r = -0.86; P < 0.01) was found between cumulative CH_4 uptake and soil NH_4^+-N in the soils (*Fig. 6*). Soil NH_4^+-N and NO_3^-–N were greater in the cropland soils than that in the grassland soil in our study (*Table 2*). The soils with a high NH_4^+-N content showed lower cumulative CH_4 uptake. However, no correlation was observed between cumulative CH_4 uptake and soil NO_3^-–N in the growing season. The regression analysis indicated that a linear combination of soil NH_4^+-N explained more than 73% of the variability in the cumulative CH_4 uptake, and may account for the difference of the cumulative CH_4 uptake in the soils (*Fig. 6*).

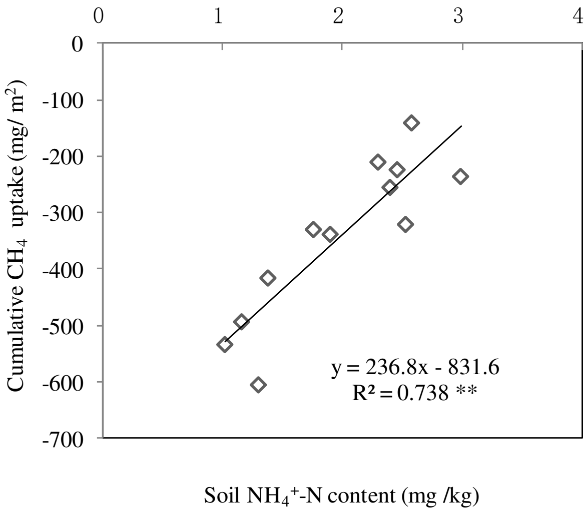

Figure 6. *Correlation between cumulative CH_4 uptake and soil NH_4^+-N in grassland and cropland soils; NH_4^+-N content was seasonal mean value from 2010 to 2012*

Table 2. *Physical and chemical properties of grassland and cropland soils with different length of ropping history (means±SD)*

Years	Soil code	‡SOC %	TN g kg^{-1}	Soil bulk density g cm^{-3}	pH	‡MBC mg kg^{-1}	MBN mg kg^{-1}	NH_4^+-N mg kg^{-1}	NO_3^-N mg kg^{-1}
2010	†G	2.46±0.01	0.25±0.01	1.42±0.18	8.0±0.05	271±33.3	105±28.0	1.02±0.35	7.08±9.78
	C5	1.58±0.05	0.17±0.01	1.32±0.08	7.6±0.08	976±146.2	66±4.6	2.58±0.45	10.36±7.73
	C10	1.30±0.02	0.13±0.01	1.20±0.02	7.6±0.01	454±42.1	51±13.3	2.46±0.22	20.10±14.89
	C50	0.79±0.13	0.09±0.01	1.40±0.06	7.6±0.03	442±46.2	36±10.5	1.76±0.32	24.89±12.42
2011	G					232±47.4	49±9.7	1.30±0.30	8.00±0.84
	C5					857±89.1	48±20.8	2.50±0.20	13.60±1.21
	C10					426±72.5	25±9.2	2.40±0.10	20.00±3.84
	C50					272±106.3	22±5.3	1.90±0.13	32.00±2.09
2012	G					183±38.5	48±9.8	1.16±0.37	7.30±1.60
	C5					885±36.2	37±12.0	2.99±0.36	10.60±1.12
	C10					365±23.6	30±11.2	2.53±0.38	11.00±1.04
	C50					233±28.3	15±6.4	1.38±0.75	23.60±3.03

† G: natural grassland; C5, C10, and C50: cropland for 5, 10, and 50 years old from reclaimed grassland
‡ SOC: soil organic carbon; BD: bulk density; TN: soil total nitrogen
‡ MBC: microbial biomass C; MBN: microbial biomass N

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Discussion

Cropland soils from reclaimed grassland increasing methane uptake

The CH_4 uptake flux in the grassland soil was 0.001-0.120 mg/(m^2 hr) from 2010 to 2012 in our study. This falls in the range of a study conducted by Wang et al. (2015) (0.017-0.162 mg/(m^2 hr)) in a typical steppe dominated by *Leymus chinensis* (Trin.) Tzvel at Guyuan State Key Monitoring and Research Station of Grassland Ecosystem (Wang et al., 2015). In our study, the cropland soil after conversion from grassland to cropland increased the CH_4 uptake from 2010 to 2012 (141-235 mg/m^2 in G, 534-737 mg/m^2 in C5, 210-605 mg/m^2 in C10, and 235-493 mg/m^2 in C50). We found that the croplands from reclaimed grassland accelerated the CH_4 uptake. Compared to natural grasslands, agricultural soils from reclaimed grassland experience more physical disturbance due to fertilizer application as well as plowing, planting and harvesting with heavy agricultural equipment, which increases the aeration and porosity of the soils, affects mineralizable carbon and the other biochemical attributes (Rong et al., 2015), and creates favorable conditions for CH_4 oxidation. The oxidation of atmospheric CH_4 in upland soils is a biological process governed by CH_4-oxidizing bacteria and is dependent on the availability of both CH_4 and oxygen (O_2) in the soil profile (Yang et al., 2018). Boeckx et al. (1997) also reported that grassland had a lower methane uptake capacity than arable land in a study conducted in Belgium.

Decreasing methane uptake in cropland soils with the increase of cropping history from reclaimed grassland

CH_4 uptake rule on a temporal trend of cropping history was discovered in the agro-pastoral ecotone of Inner Mongolia. CH_4 uptake in cropland soil decreased with the increase of cropping history from 5 to 50 years after the grassland was converted into cropland (*Fig. 3*). Higher CH_4 uptake in the cropland soils from C5 and C10 was found in more recently-established croplands. The CH_4 uptake of C50 soil is close to that of grassland soil. Changes in soil carbon and soil properties after long-term fertilizer application affect methane oxidative bacteria community structure and soil methane oxidation rate (Hütsch et al., 1993). To reduce CH_4 uptake was proved on a period (7 years) of inorganic nitrogen application (Mosier et al., 1998).This study showed that enhancing or reducing CH_4 uptake by soil cultivation depends partly on the time elapsed from the conversion of grassland to cropland. In the field experiment, none of the CH_4 uptake was correlated with potatoes and oats biomass production ($P > 0.05$).

Methane uptake driven by the soil physicochemical properties of different land use type

Our study showed that the conversion of natural grassland to cultivated land can temporarily increase the uptake of CH_4 from the atmosphere depending on soil moisture level and soil NH_4^+-N. Soil moisture ranged from 6%-26% in C5, 3%-25% in C10, and 9%-34% in C50. Soil moisture in grassland is the highest (8%-39%) from 2010 to 2012. CH_4 uptake decreased as soil moisture level increased, which is consistent with previous studies (Price et al., 2004). There was negative correlation between CH_4 uptake and soil moisture in a semi-arid steppe. Moisture content is an important factor on regulating the transport process of methane from soil macro pores to methane oxidizing bacteria and the diffusion of methane into the soil (Wang et al., 2005; IPCC, 2007). Quantity and activity of methanotrophs significantly reduce on higher moisture levels in the soil

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(Castro et al., 1992; Sitaula et al., 1995). As soil moisture increases, the soil microbial community is covered by a thick water film which reduces the microorganisms activity and hinders the spread of CH_4 oxidation. CH_4 in soil water diffuses at a slower rate compared to soil air.

The effect of temperature on CH_4 uptake capacity in our study was not pronounced between 10ºC and 26ºC in grassland soil and cropland soils (*Fig. 5*). Castro et al. (1995) found that soil temperature was not an important controller of CH_4 uptake when temperature ranged between 10°C and 20°C (Castro et al., 1995). Warming (4.5°C) increased the average seasonal CH_4 uptake by 65.9% in the permafrost region of an alpine meadow (Chen et al., 2017). Soil moisture on CH_4 uptake was more important than temperature when the temperature was not the limiting factor. Irrigation and management in cropland create an optimum temperature for soil methanotrophs. The seasonality of CH_4 uptake exhibited a strong dependency on the seasonal variation of soil moisture in a typical semi-arid steppe in Inner Mongolia (Chen et al., 2010).

The negative correlation between CH_4 uptake and soil NH_4^+-N is detected over the whole testing stage (r=0.86, n=12). This result was consistent with previous findings in an incubation experiment under ambient CH_4 levels (Chan and Parkin, 2001). Soil NH_4^+-N is a key determinant on CH_4 oxidation capacity due to the significant competitive inhibition of CH_4 uptake by NH_4^+-N in recently cultivated soils (Jacinthe and Lal, 2005).

There were no significant correlations between CH_4 uptake and soil properties such as SOC content, MBN concentration, MBC, TN, soil sand content, pH, soil clay content and soil bulk density in all of the soils ($P > 0.05$) (*Table 2*, *Fig. 3*). As there was no significant difference for SOC, TN, soil bulk density and pH between the four types of land use in 2010. In addition, they change more slowly than other physicochemical properties such as NH_4^+-N, NO_3^-N, MBN, and MBC. Therefore, these data were not measured in 2011 and 2012. Variation in soil moisture and soil NH_4^+-N may have concealed the changes in soil bulk density, SOC, TN, soil MBN content, MBC, soil clay content and soil pH from land use change, although CH_4 uptake is affected by various factors. In our study, soil moisture (*Fig. 4*) and soil NH_4^+-N content (*Fig. 6*) were the major factors determining the difference on CH_4 uptake between grassland and cultivated land.

This study was designed to address how land use change affects CH_4 uptake and what soil parameters are the most important for assessing CH_4 uptake from the conversion of grassland to cropland in the agro-pastoral ecotone of Inner Mongolia. These efforts will improve our understanding of land use change on the CH_4 uptake in the agro-pastoral ecotone. The observed effects of physical and chemical properties of soil on CH_4 uptake in this study will support to estimate CH_4 fluxes based on soil properties from different land use types using model and simulation method. However, in our study, we examined the effects of land conversion on soil methane uptake in Inner Mongolia. The effects of nitrogen fertilizer application on methane uptake in croplands and grassland need to be further studied in the future.

Conclusions

The four land-use types (G, C5, C10 and C50) that we studied in the semi-arid steppe of the agro-pastoral region of northern China were sinks for atmospheric CH_4 with an average uptake flux of 0.06-0.29 mg m^{-2} hr^{-1}. Cropping history of the conversion from

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grassland to cropland affects CH_4 uptake. Land-use types exhibited different soil CH_4 fluxes with the maximum CH_4 uptake occurring in the C5 land-use type, and CH_4 uptake was higher for C10 compared to C50 type. The CH_4 uptake of C50 soil was approximately equal to that of grassland soil. Soil moisture and soil NH_4^+-N content are the key driving factor on CH_4 uptake of the observed differences between grassland and arable lands with different reclaimed history and may provide a possible approach for estimating soil CH_4 fluxes. Our results contribute to understanding soil uptake levels of atmospheric CH_4 in four important land-use types in northern China. The cropland reclaimed more than 10 years old should be planted into grasslands to facilitate CH_4 uptake and soil carbon sequestration in the agro-pastoral ecotone of Inner Mongolia. However, how long will the cultivated grassland be grazed and reclaimed into cropland be conducive to carbon sink? These tasks need to be studied in the future.

Acknowledgements. We sincerely appreciate the anonymous reviewers for their critical and valuable comments to help improve this manuscript. We thank colleagues of Chemistry and Environmental Sciences College, Inner Mongolia Normal University, for their help in laboratory analyses. postgraduate Jianghong Zhao and Jianhua Hou for their assistance in situ observation and measurement, undergraduate Lei Ma and Zhiwei Liu. The project was funded under the auspices of National Natural Science Foundation of China (No. 41565009, 41675140, 41865010), The National Key Research and Development Program of China, grant number (No. 2016YFC0400105), Inner Mongolia Youth Innovative Talent Training Program of Prairie Excellence Project 2016.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51595172

THE EFFECT OF PHYSICO-CHEMICAL VARIATIONS ON PHYTOPLANKTON STATUS IN THE MARGIN OF CHOGHAKHOR WETLAND, IRAN

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(Received 17th Oct 2018; accepted 2nd Jan 2019)

Abstract. Many human activities in the margin of Choghakhor wetland, (Iran) causes evaluation of the phytoplankton status in relation to the physico-chemical parameters in different seasons of 2011 in 5 marginal stations of the wetland. Water samples prepared in triplicate and temperature, pH, dissolved oxygen, nitrate and phosphate concentration were measured using APHA methods. The phytoplankton identified, counted and Shannon diversity index (H′). Phytoplankton community included 59 genera, 43 families and 6 divisions, among which Bacillariophyta was the most common (46.6%) followed by Chlorophyta (32.0%), Cyanophyta (13.3%), Dinophyta (4.4%), Euglenophyta (3.0%) and Chrysophyta (0.8%). Phytoplankton density, nitrate, and phosphate concentration were different in different stations and seasons ($P < 0.05$). The highest density was recorded for *Nitzschia* followed by *Cosmarium*, *Microcystis*, *Peridinium*, *Euglenaria* and *Dinobryon*. Also, *Microcystis* density was maximum in station 5 [S5]. There were significant correlations between the phytoplankton density and nitrate concentration ($r = 0.6$, $P < 0.01$), Chlorophyta and phosphate concentration ($r = 0.3$, $P < 0.01$), Cyanophyta and phosphate concentration ($r = 0.2$, $P < 0.05$). Although wetland water was still clean ($H' > 3$), high density of *Nitzschia* and *Microcystis* as eutrophication bio-indicators showed starting of eutrophication. *Microcystis* as the most abundant genus of *Cyanophyta* must be seriously controlled to avoid blooms in the wetland.
Keywords: *phytoplankton composition, physico-chemical parameters, Choghakhor wetland, nitrate, phosphate*

Introduction

The enrichment and pollution due to human activities are one of the most important environmental problems in wetlands that cause serious risks and may damage their fauna and flora (Rosińska et al., 2017). Land use and human activities lead to entry of chemicals from various resources including residential and industrial wastewater as well as agricultural runoffs to aquatic environments, especially wetlands (Chang, 1995; Khan and Ansari, 2005; Bressler and Paul, 2018). These compounds mainly contain high concentrations of nitrogen and phosphorus (Fonge et al., 2012) and could affect

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

physicochemical characteristics and quality of water as well as the bio-communities of the wetlands. These effects vary in different ecosystems. For example, different studies have shown that increase of agricultural and residential wastewater inputs to wetlands causes changing water quality, variation in growth of phytoplankton communities, decrease water dissolved oxygen and ultimately an eutrophication status and elimination of the wetlands (Paludan et al., 2002; Sakset and Chankaew, 2013; Stević et al., 2018; Zhang, 2018). Hence, it is necessary to evaluate the growth of phytoplankton bio-communities in the wetlands exposed to agricultural and residential pollutants to control the effects of the pollutants and to preserve and maintain their life (Khan and Ansari, 2005).

Phytoplankton as photosynthetic organisms are the major producer especially in deep water as well as are the base of food chain in aquatic ecosystems (Muhammad et al., 2005). Other aquatic organisms are dependent on the phytoplankton directly and indirectly (Kelly, 1998; Moss, 2009; Jones et al., 1996). The production levels of phytoplankton maximizes when the water physicochemical characteristics are at optimum levels (Sinha and Srivastava, 1991; Muhammad et al., 2005; Sahu et al., 2012). Therefore, the composition of phytoplankton communities is a bio-indicator for the quality of lake water (Peerapornpisal et al., 2004; Wu et al., 2014; Sakset and Chankaew, 2013; Bellinger and Sigee, 2015).

The communities of the phytoplankton in freshwater ecosystems such as wetlands and lakes are mainly composed of Chlorophyta (green algae), Cyanophyta (blue-green algae), Bacillariophyta (diatoms) and Dinophyta (Rosén, 1981; Eloranta, 1986; Sorayya et al., 2011). Also, growth, ecological distribution, presence and abundance of the phytoplanktonic communities are affected by numerous environmental factors especially changes in water quality or mineral nutrients (Stević et al., 2018; Zhang, 2018). Algal blooms, especially in Cyanophyta and Dinophyta, is the main problems due to the excessive inputs of organic and inorganic compounds -such as agricultural fertilizers and pesticides- to aquatic environments (Rosińska et al., 2017). It causes to unfavorable taste and odor of water as well as destruction of organisms due to toxic substances produced by this phytoplankton (Fonge et al., 2012).

International wetland of Choghakhor is one of the most important water bodies in Iran, have different capabilities as valuable habitat for birds as written by Ramsar convention (Ebrahimi and Moshari, 2006). Nowadays, this wetland is affected by human activities especially agriculture runoffs and residential wastewaters (Nadushan and Fatemi, 2008; Samadi, 2016). Due to the obvious effects of human activity on the excessive phytoplankton abundance and its deleterious effects on biology of aquatic ecosystems (Chang, 1995; Khan and Ansari, 2005; Bressler and Paul, 2018), in this study the phytoplankton status involving density, composition, diversity as well as spatial / temporal distribution in relation to physico-chemical changes were evaluated in different seasons of 2011 as well as different stations designated in the margin of Choghakhor wetland.

Materials and methods

Description of study area

Choghakhor wetland is located on the Gondman district, Boroojen city in the province of Chahar-Mahal-Bakhtiari, Iran. This province is located on the southwest part of Iran which is near Zagros Mountains. This area extended 1500 ha between 31°

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

54′ 32″ N to 31° 56′ 32″ N and 50° 53′ 58″ E to 50° 56′ 09″ E, with an average altitude of 2400 m above sea level (Ebrahimi and Moshari, 2006). Choghakhor is a permanent shallow lake by an average depth of 2 m. Rainfall occurs at spring (mid-March) and fall (mid-October) with the mean annual rainfall of 380 mm.

In the southern part of the wetland, the villages of Sangchin, Avorgan, Sibak, Dastgerd, Khaniabad, Sakiabad, Khedrabad and Galugerd are located from east to west, respectively. Recent human activities in the vicinity of the Choghakhor wetland have caused to increase agricultural runoffs and residential wastewaters, especially at its southern and western parts (Samadi, 2016).

Determination of sampling stations

The location of sampling stations was selected on topographic map considering the aim of the study in the margin of the wetland at different coordinates (*Table 1; Fig. 1*). Although Chaghakhor is a uniform lake with a steady slope (Ebrahimi and Moshari, 2006), entire environment of the lake was evaluated during the sampling period and 5 stations were selected, eventually. It was assumed that the selected station represented the surrounding area (*Table 1*).

Table 1. *Coordinates of sampling stations (S1 - S5) in Choghakhor wetland*

Station	Coordinate (degree -minute -second)
S1	50° 52′ 51.49″ E, 31° 55′ 49.27″ N
S2	50° 54′ 12.20″ E, 31° 56′ 11.14″ N
S3	50° 52′ 58.36″ E, 31° 45′ 51.88″ N
S4	50° 55′ 54.52″ E, 31° 55′ 07.84″ N
S5	50° 56′ 05.69″ E, 31° 55′ 17.24″ N

Sample collection

Sampling was done in 1 liter Teflon bottles at a depth of 50 cm at five stations with 3 biological replicates in spring (May), summer (August), fall (October) and winter (December) seasons in 2011.

First, the sampling containers were washed with the wetland water (three times) to accurate measurement of physicochemical properties of water. Then, the one set of the collected samples were transferred to the laboratory under standard conditions to evaluate some physicochemical properties of water using American Public Health Association [APHA] standard methods (Amy et al., 1992) (*Fig. 2*). The water temperature (thermometer; HANNA, PHep 4 HI98127, USA), pH (pH-meter; JENWAY3330, Canada), dissolved oxygen (DO-meter; Pro20 Dissolved Oxygen Instrument, Xylem, Japan) as well as the nitrate and phosphate concentration (spectrophotometer; Hach- DR 2400, USA) were measured based on the instruments manual.

Other set of the collected samples were used for phytoplankton assessment. These samples were fixed with three drops of Lugol's solution (0.007 v/v) in polyethylene containers (250 ml) to easy identification and then were transferred to laboratory in a cooler packed with ice blocks.

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

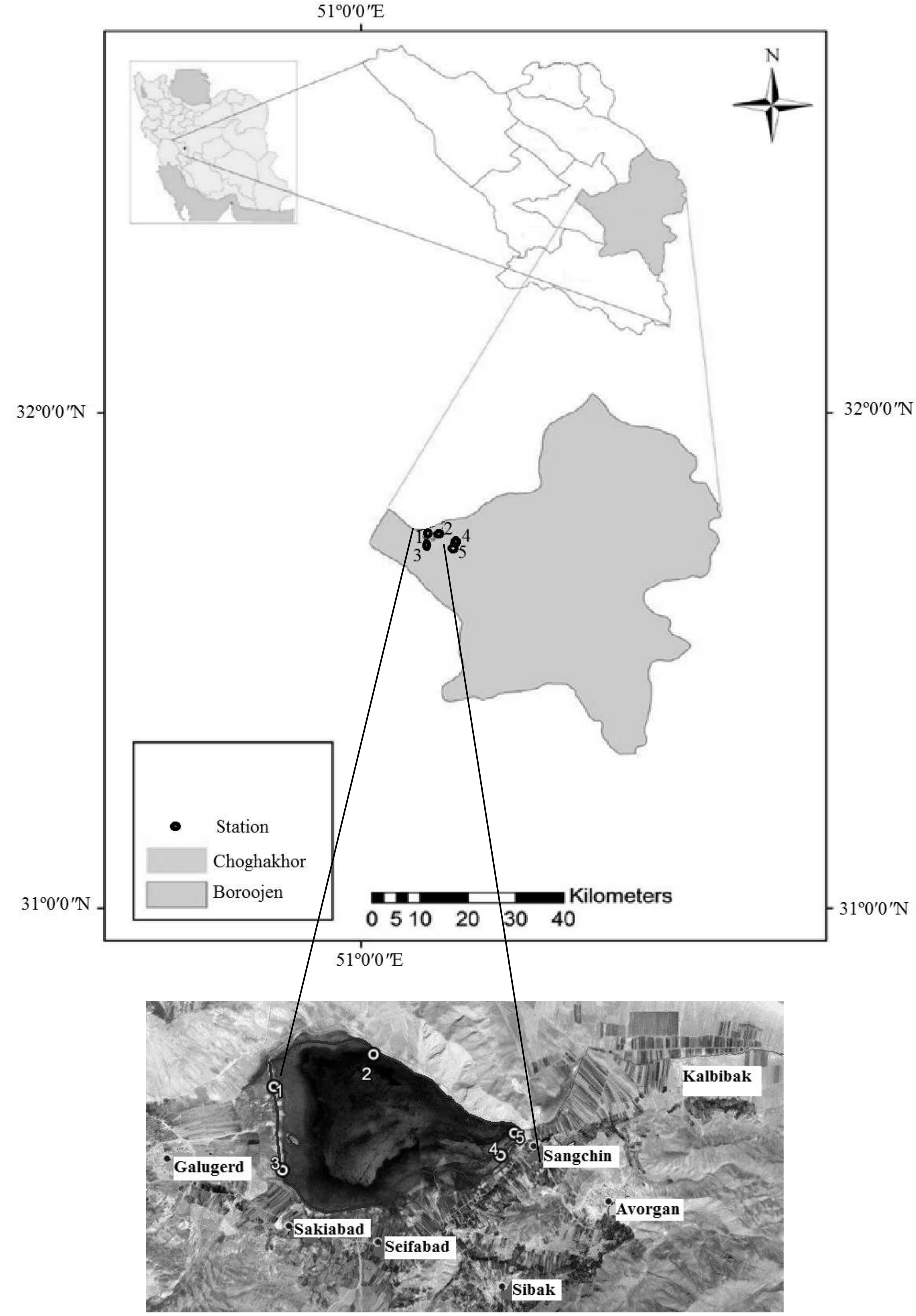

Figure 1. *Location of sampling stations (S1 – S5) in Choghakhor wetland (ArcMap 10.1). (N: north, E: east)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

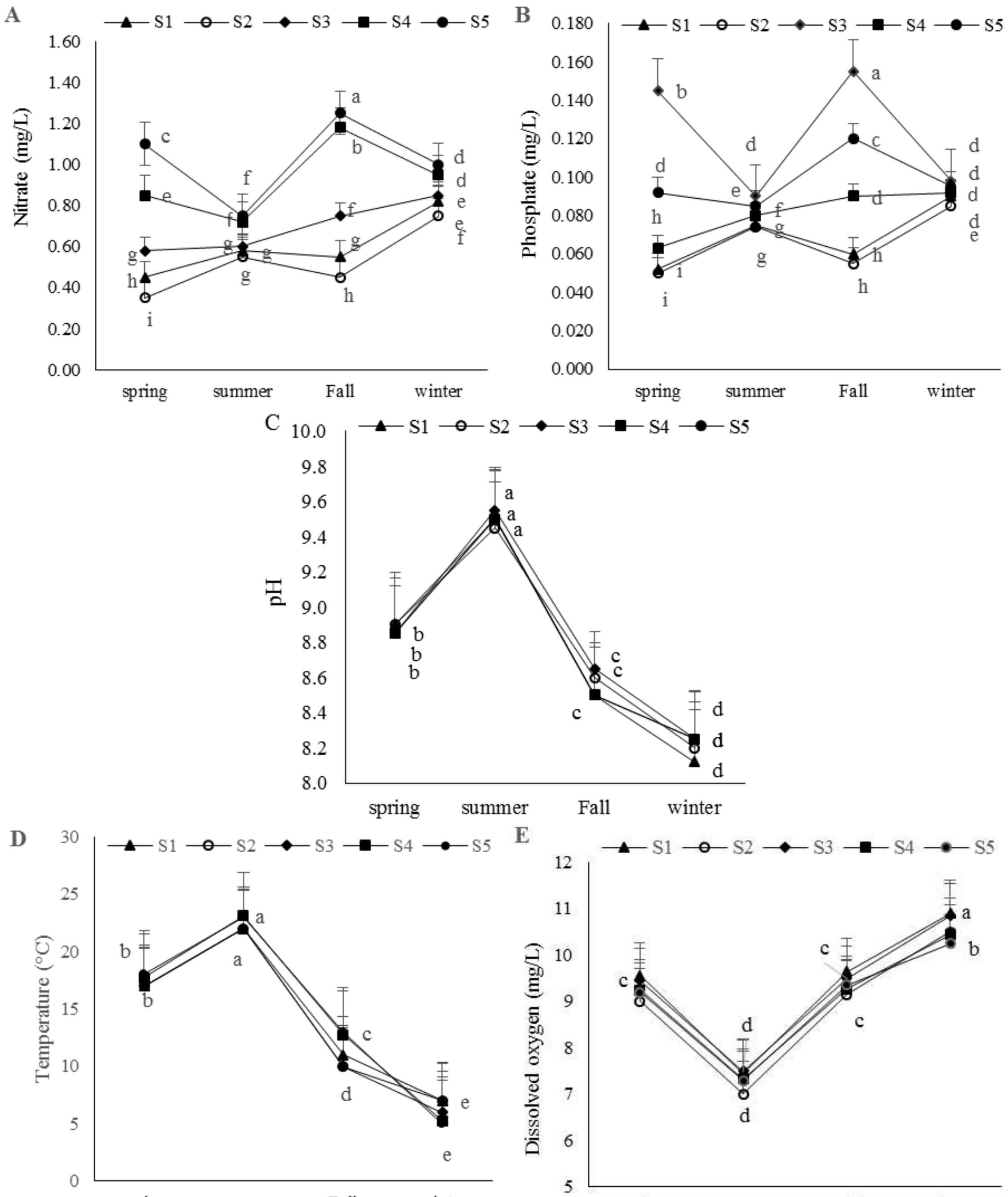

Figure 2. *Physico-chemical parameters of water in different seasons and stations (S1 – S5) in Choghakhor wetland. Data represent the means values ± SE (n = 3). Different letters in the mean values indicate difference between the means based on Duncan's multiple range tests, P < 0.05*

Identification, count and diversity of phytoplankton

The genera of phytoplankton were identified using binocular optical microscope (Olympus CH20i, Japan) with a magnification of 1000x (with emersion oil) and the

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

related text books and papers (Krammer and Lange-Bertalot, 1986; Bellinger and Sigee, 2015). To count and measure the phytoplankton density, 1 ml from each sample were evaluated using Sedgwick rafter slides (Wildco 1801-A10, JA Whitlock & Co, Australia) with three technical replicates.

The Shannon diversity index (H′) was calculated (Eq. 1):

$$H' = \sum P_i * \ln P_i \qquad \text{(Eq.1)}$$

Where P_i is proportion of total sample belonging to i^{th} genus and i is the number of genera (Magurran, 1988).

Statistical analysis

Data analysis were done using One-way ANOVA (SPSS21) and the mean values were compared using Duncan῾s multiple range tests at 95% confidence interval ($P < 0.05$). Pearson correlation coefficient was used to evaluate the correlations between physicochemical parameters of water and phytoplankton density and diversity.

Results

Physico-chemical parameters

Physicochemical characteristics of Choghakhor wetland are given in *Figure 2*.

Nitrate

In average, nitrate concentration (mg/L) were different ($P < 0.05$) in different seasons and stations (*Fig. 2A*). High amount of nitrate was observed in S5 and then in S4, S3, S1 and S2 respectively. Nitrate concentration in S2 and S1 decreased from the spring to winter. However, maximum concentration of nitrate was measured in S4 and S5 in the fall as well as the minimum in S2 in the spring (*Fig. 2A*).

Phosphate

Phosphate concentrations (mg/L) average in different season and stations were significantly different ($P < 0.05$) (*Fig. 2B*). Phosphate in different stations varied as: S3 > S5 > S4 > S2 ≈ S1. In S3, phosphate was highest in the fall and then varies as: spring > winter ≈ summer. In S5, phosphate was respectively highest in the fall followed by the spring or winter and lastly summer. Also, phosphate in S2 was maximum in the summer, afterward it was higher in the winter, fall and spring, respectively. The maximum concentration of phosphate was measured in S3 in the spring and fall (0.155 and 0.145, respectively) and the minimum ones (0.050) in S2 in the spring (*Fig. 2B*).

pH

Different stations had the same pH but, the seasons showed different pH ($P < 0.05$). pH was 9.55 in the summer and 8.12 in the winter by an average pH of 8.78. The wetland water is completely alkaline throughout the year (*Fig. 2C*).

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

Water temperature

The results showed that the annual water temperature was 14 °C in average. Water temperature in different season was significantly different (P < 0.05) but there were no significant difference between the studied stations. The maximum and minimum temperatures were measured in the summer and winter, respectively (*Fig. 2D*).

Dissolved oxygen

The concentration of water dissolved oxygen varies from 7 to 10.9 mg/L. Only, dissolved oxygen was different in different stations in the winter, as S1, S2 and S3 were different from S4 and S5. Also, different seasons had different dissolved oxygen (except for spring and fall) as it was maximum in the winter and minimum in the summer. There were observed a reverse trend for temperature and dissolved oxygen variations (-0.7, P < 0.01) (*Fig. 2E*).

Identification of the phytoplankton

In this study, 59 genera belonging to 43 families and 6 divisions were identified (*Table 2*). Bacillariophyta was the most common phylum (46.6%) with the most abundant number of genera followed by Chlorophyta (32.0%), Cyanophyta (13.3%), Dinophyta (4.4%), Euglenophyta (3.0%) and Chrysophyta (0.8%) (*Table 2*). The most abundant genus was *Nitzschia* (14.8%) belonging to Bacillariaceae family and Bacillariophyta phylum. The density and abundance of the genera in each division are given in *Tables 3-6*.

Table 2. *The genera identified in the Choghakhor wetland*

Genus	Family	Phylum	P	Genus	Family	Phylum	P
Ankistrodesmus	Selenastraceae	Chlorophyta	1.2	*Amphora*	Catenulaceae	Bacillariophyta	0.9
Closterium	Closteriaceae		2.4	*Asterionella*	Tabellariaceae		1.1
Cosmarium	Desmidiaceae		7.0	*Cocconeis*	Cocconeidaceae		1.4
Desmodesmus	Scenedesmaceae		3.5	*Cyclotella*	Stephanodiscaceae		6.2
Mougeotia	Zygnemataceae		2.4	*Cymatopleura*	Surirellaceae		1.1
Oedogonium	Oedogoniaceae		1.2	*Cymbella*	Cymbellaceae		0.6
Pediasterum	Hydrodictyaceae		1.2	*Denticula*	Bacillariaceae		1.3
Staurastrum	Desmidiaceae		1.2	*Diatoma*	Tabellariaceae		1.1
Scenedesmus	Scenedesmaceae		1.1	*Diploneis*	Diploneidaceae		0.7
Spirogyra	Zygnemataceae		3.5	*Delicata*	Gomphonemataceae		1.1
Staurodesmus	Desmidiaceae		1.2	*Epithemia*	Rhopalodiaceae		0.6
Tetraedron	Chlorococcaceae		1.2	*Frustulia*	Amphipleuraceae		1.0
Volvox	Volvocaceae		4.6	*Fragilaria*	Fragilariaceae		1.4
Zygnema	Zygnemataceae		0.4	*Gomphonema*	Gomphonemataceae		1.5
Total:			**32.0**				
Anabaena	Nostaceae	Cyanophyta		*Gyrosigma*	Naviculaceae		0.8
Anacystis	Cyanophyceae		2.5	*Martyana*	Fragilariaceae		0.9
Anathece	Synechococcaceae		2.3	*Melosira*	Melosiraceae		1.2
Cyanoptyche	Gloeochaetales familia incertae sedis		0.4	*Navicula*	Naviculaceae		0.8
Merismopedia	Merismopediaceae		0.3	***Nitzschia***	Bacillariaceae		14.8
Microcystis	Microcystaceae		0.4	*Pinnularia*	Pinnulariaceae		1.8
Nostoc	Nostocaceae		4.8	*Planothidium*	Achnanthidiaceae		1.0
Oscillatoria	Oscillatoriaceae		0.4	*Tetracyclus*	Tabellariaceae		0.9
Pseudanabaena	Pseudanabaenaceae		0.5	*Surirella*	Surirellaceae		1.4

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

Phormidium	Oscillatoriaceae		0.2	*Synedra*	Fragilariaceae		1.3
Planktolyngbya	Leptolyngbyaceae		0.3	*Ulnaria*	Ulnariaceae		1.7
Rhabdogloea	Synechococcaceae		0.4	**Total:**			**46.6**
Spirulina	Spirulinaceae		0.3	*Ceratium*	Ceratiaceae	Dinophyta	0.6
Total:			**13.3**	*Glenodinium*	Peridiniales incertae sedis		0.6
Euglenaria	Euglenaceae	Euglenophyta	1.8	***Peridinium***	Peridiniaceae		3.2
Lepocinclis	Phacaceae		0.2	**Total:**			**4.4**
Trachelomonas	Euglenaceae		1.0	***Dinobryon***	Dinobryaceae	Chrysophyta	**0.8**
Total:			**3**	**59**	**43**	**6**	**N**

P represents the abundance (%)

Table 3. *The density and abundance of identified genera belonging to Chlorophyta in the Choghakhor wetland. Data represent the means values + SE (n = 3)*

No.	*Genus*	Density	Abundance (%)
1	*Ankistrodesmus*	486 ± 315	4
2	*Closterium*	969 ± 635	7
3	***Cosmarium***	2869 ± 1855	**22**
4	*Desmodesmus*	1435 ± 951	11
5	*Mougeotia*	972 ± 640	7
6	*Oedogonium*	493 ± 318	4
7	*Pediasterum*	478 ± 315	4
8	*Staurastrum*	509 ± 328	4
9	*Scenedesmus*	435 ± 280	3
10	*Spirogyra*	1434 ± 936	11
11	*Staurodesmus*	481 ± 308	4
12	*Tetraedron*	476 ± 307	4
13	*Volvox*	1866 ± 1221	14
14	*Zygnema*	168 ± 116	1

Table 4. *The density and abundance of identified genera belonging to Cyanophyta in the Choghakhor wetland. Data represent the means values + SE (n = 3)*

No.	*Genus*	Density	Abundance (%)
1	*Anabaena*	1021 ± 363	19
2	*Anacystis*	925 ± 405	17
3	*Anathece*	162 ± 61	3
4	*Cyanoptyche*	142 ± 77	3
5	*Merismopedia*	159 ± 60	3
6	***Microcystis***	1969 ± 714	**36**
7	*Nostoc*	177 ± 59	3
8	*Oscillatoria*	220 ± 97	4
9	*Pseudanabaena*	97 ± 42	2
10	*Phormidium*	122 ± 51	2
11	*Planktolyngbya*	160 ± 105	3
12	*Rhabdogloea*	127 ± 94	2
13	*Spirulina*	142 ± 71	3

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

The genus *Nitzschia* with the density of 6054 ± 1915 cell/L (32% abundance) was the most common in Bacillariophyta (*Table 5*). Also, *Cosmarium* (2869 ± 1855 cell/L, 22%), *Microcystis* (1969 ± 714 cell/L, 36%), *Peridinium* (1293 ± 1310 cell/L, 73%), *Euglenaria* (717 ± 605 cell/L, 59%) were the most common genera in Chlorophyta, Cyanophyta, Dinophyta and Euglenophyta, respectively (*Tables 3, 4* and *6*). It was found only one genus from Chrysophyta named as *Dinobryon* with the density of 314 ± 337 cell/L (*Table 6*).

Table 5. *The density and abundance of identified genera belonging to Bacillariophyta in the Choghakhor wetland. Data represent the means values + SE (n = 3)*

No.	*Genus*	Density	Abundance (%)
1	*Amphora*	360 ± 115	2
2	*Asterionella*	432 ± 138	2
3	*Cocconeis*	557 ± 178	3
4	*Cyclotella*	2544 ± 967	13
5	*Cymatopleura*	439 ± 622	2
6	*Cymbella*	240 ± 78	1
7	*Denticula*	517 ± 165	3
8	*Diatoma*	439 ± 141	2
9	*Diploneis*	301 ± 97	2
10	*Delicata*	459 ± 147	2
11	*Epithemia*	262 ± 85	1
12	*Frustulia*	428 ± 137	2
13	*Fragilaria*	573 ± 183	3
14	*Gomphonema*	622 ± 199	3
15	*Gyrosigma*	325 ± 104	2
16	*Martyana*	367 ± 118	2
17	*Melosira*	477 ± 153	3
18	*Navicula*	317 ± 102	2
19	***Nitzschia***	6054 ± 1915	32
20	*Pinnularia*	743 ± 237	4
21	*Planothidium*	401 ± 128	2
22	*Tetracyclus*	379 ± 122	2
23	*Surirella*	557 ± 178	3
24	*Synedra*	520 ± 166	3
25	*Ulnaria*	701 ± 224	4

Table 6. *The density and abundance of identified genera belonging to Dinophyta, Euglenophyta and Chrysophyta in the Choghakhor wetland. Data represent the mean values + SE (n = 3)*

No.	*Genus*	Phylum	Density	Abundance (%)
1	*Ceratium*	Dinophyta	246 ± 242	14
2	*Glenodinium*		243 ± 245	14
3	***Peridinium***		1293 ± 1310	73
1	***Euglenaria***	Euglenophyta	717 ± 605	59
2	*Lepocinclis*		99 ± 85	9
3	*Trachelomonas*		389 ± 325	32
1	*Dinobryon*	Chrysophyta	314 ± 337	100

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Phytoplankton density

The total phytoplankton density was seasonally and spatially different (P < 0.05) (*Fig. 3A*). The density was highest in S5 in the fall (55563 cell/L) followed by spring (53788 cell/L) while it was the lowest in S2 in the spring (31922 cell/L) followed by S1 in the fall (31952 cell/L).

The phytoplankton community showed highest divergence in S4 and S5 in the summer and winter as well as in S4 in the spring and fall. The less diversity of the phytoplankton community was observed in the spring in S1 (*Fig. 3B*).

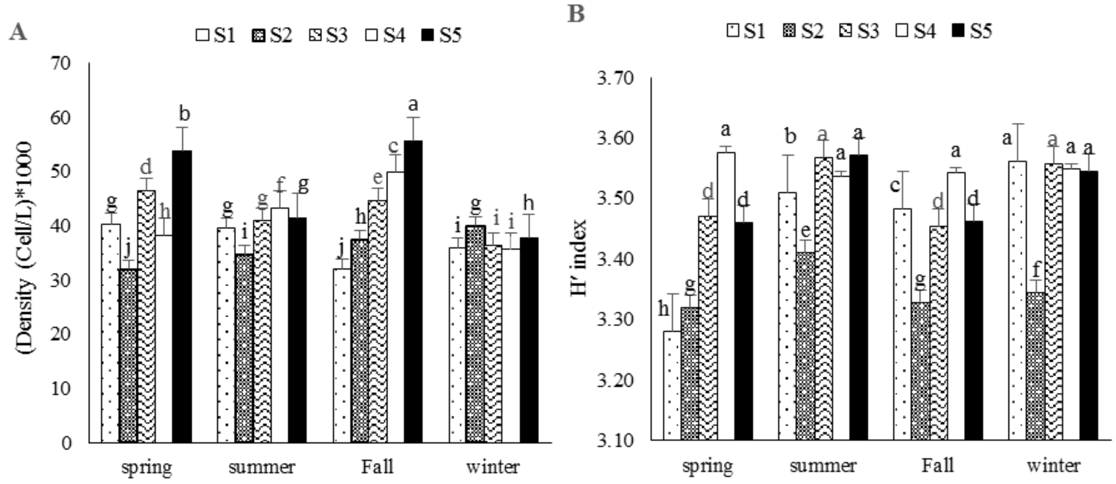

Figure 3. *Total phytoplankton density (A) and diversity (H' index: Shannon diversity index) (B) in different seasons and stations (S1-S5) in the Choghakhor wetland. Different letters in the mean values indicated difference between the means based on Duncan's multiple range tests, P < 0.05*

The density of Chlorophyta, Cyanophyta, Bacillariophyta, Dinophyta, Euglenophyta and Chrysophyta were different in different seasons and stations (S1 - S5) in the wetland (P < 0.05) (*Fig. 4*). The density of Chlorophyta in S5 was highest (7756 Cell/L) followed by S3, S4, S1 and S2 (5655 Cell/L), in the fall. Also, the density of Chlorophyta in S2 in the winter was highest followed by the summer, fall and spring while in S5 it was highest in the fall followed by spring, winter and lastly in the summer. The highest density of Cyanophyta was observed in S5 followed by S4, S3, S1 and S2, in the fall. In S5, Cyanophyta showed the maximum density in the fall and afterwards in the spring. Cyanophyta density in S2 in the fall was maximum and different from other seasons. Bacillariophyta density in S2 was more than the other stations (S2 > S1 > S3 ≈ S4 > S5) and in it was highest in the spring (P < 0.05). In S2, Bacillariophyta had the highest density in the fall and winter afterwards in the spring and summer. Bacillariophyta density in S3 and S5, in the summer and spring was greater than winter and fall.

In average, the highest and lowest density of Dinophyta was observed in S5 and S3, respectively. Dinophyta in the summer were most frequent than other seasons and they were not found in the winter in any stations. In the summer, the density of Dinophyta in S4 was highest and in S2 was at least. In the spring and fall, Dionophyta was found at maximum density in S5 and greater than S2 (*Fig. 4*). Euglenophyta and Chrysophyta in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

S5 and in the winter were more abundant than other stations (*Fig. 4*). The lowest density of Euglenophyta was observed in S2 and did not change during the year. Also, Chrysophyta in S2 and S1 were not found in any of the seasons.

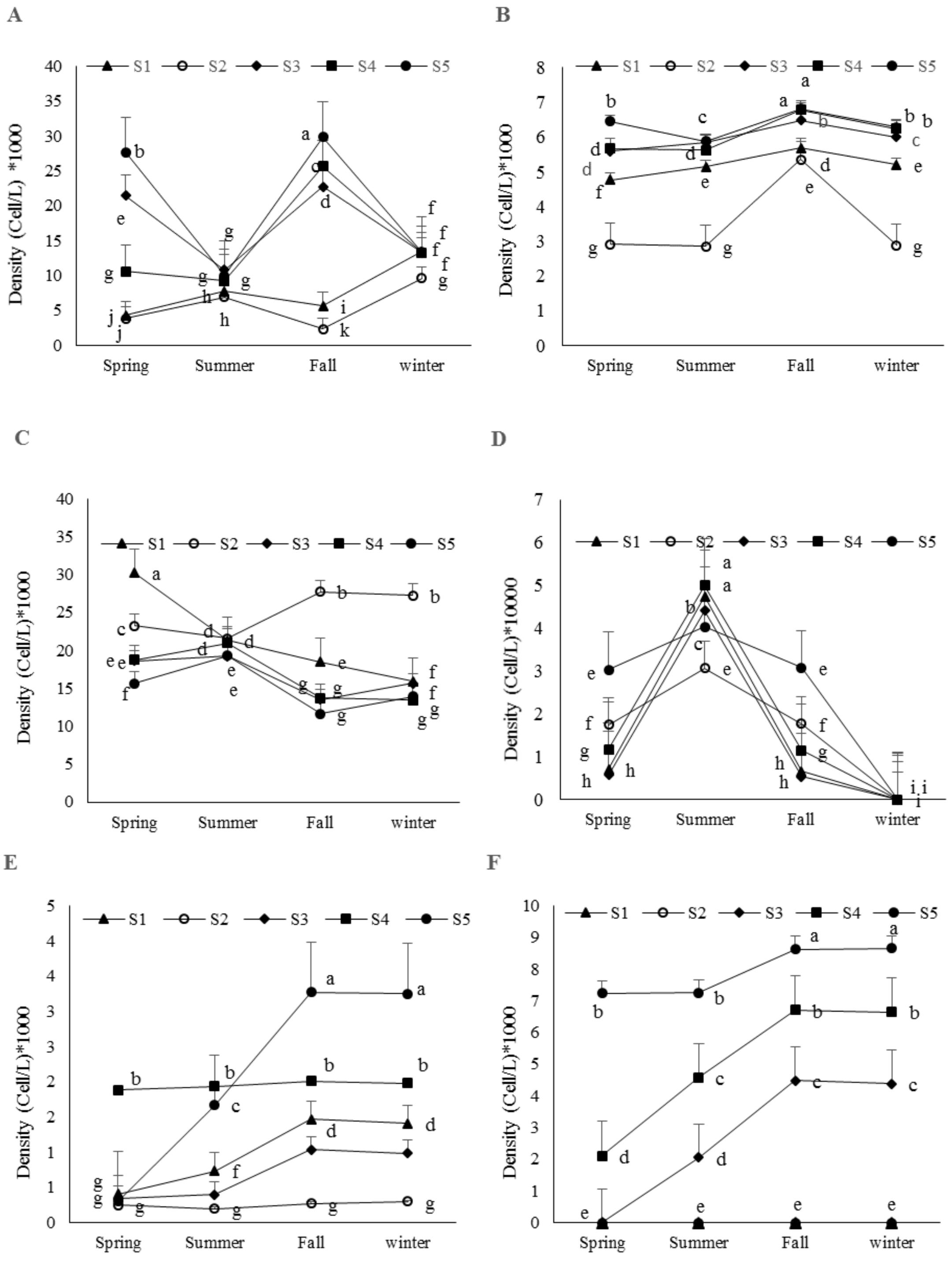

***Figure 4.** Density of different divisions: Chlorophyta (A), Cyanophyta (B), Bacillariophyta (C), Dinophyta (D), Euglenophyta (E) and Chrysophyta (F) in different seasons and stations (S1 - S5) in the Choghakhor wetland. Data represent the means values + SE (n = 3). Different letters in the mean values indicated difference between the means based on Duncan's multiple range tests, P < 0.05*

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

Phytoplankton diversity

The phytoplankton diversity index (H′) in different stations and seasons was different and was more than 3 ($P < 0.05$) (*Fig. 3*). S4, S3 and S5 had highest diversity (3.55, 3.51 and 3.50, respectively). The diversity index of S1 was 3.46 while in S2 was least (3.35). Also, phytoplankton diversity was highest in the summer (3.52) followed by winter (3.51), fall (3.45) and spring (3.414).

In S5, S3 and S2, H′ was changed similarly as it was highest in the summer followed by winter, fall and spring. In S1, H′ in the winter was maximum followed by the summer, fall and spring. H′ in S4 did not change during the year.

Pearson correlation coefficient was shown in *Table 7* to compare the correlations between physicochemical parameters of water and phytoplankton density and diversity.

Table 7. *Pearson correlation coefficient between the total density and diversity (H') of phytoplankton, density of Chlorophyta (Chl.), Cyanophyta (Cya.), Bacillariophyta (Bac.), Dinophyta (Din.), Euglenophyta (Eug.) and Chrysophyta (Chr.) with physico-chemical parameters (T: temperature, DO: dissolved oxygen, pH, N: nitrate concentration and P: phosphate concentration) of water in the Choghakhor wetland*

	Density	**H′**	**T**	**DO**	**pH**	**N**	**P**	**Chl.**	**Cya.**	**Bac.**	**Din.**	**Eug.**	**Chr.**
Density	1												
H′	-0.2	1											
T	0.2	-0.7**	1										
DO	0.2	0.5**	-0.7**	1									
pH	0.1	-0.7**	-0.9**	-0.7**	1								
N	0.6**	0.2	-0.3*	0.7**	-0.3*	1							
P	0.2	0.4**	-0.5**	0.9**	-0.4**	0.6**	1						
Chl.	0.9**	0.3*	-0.2	0. 4**	-0.2*	0.7**	0.3**	1					
Cya.	0.6**	0.3**	-0.2	0.4**	-0.2*	0.6**	0.2*	0.6**	1				
Bac.	-0.4**	-0.5**	0.3*	-0.2*	0.3**	-0.5**	-0.1	-0.7**	-0.7**	1			
Din.	0.3*	-0.8**	0.8**	-0.7*	0.8**	-0.2	-0.7**	-0.1	0.02	0.1	1		
Eug.	0.2*	0.4**	-0.3*	0.2	-0.2*	0.4**	-0.02	0.4**	0.6**	-0.6**	-0.1	1	
Chr.	0.6**	0.1	-0.2	0.1	-0.1	0.4**	-0.1	0.6**	0.7**	-0.7**	0.1	0.7**	1

*Correlation is significant at the 0.05 level
**Correlation is significant at the 0.01 level

Discussion

Variations in the physico-chemical parameters of water

The results showed that S5 had highest nitrate concentration followed by S4, S3, S1 and S2 (*Fig. 2A*). Other studies on Choghakhor wetland has been shown that this wetland is affected by human activities including agricultural runoffs containing chemical fertilizers and residential wastewaters mostly in the southern and western parts (Ebrahimi and Moshari, 2006; Nadushan and Fatemi, 2008; Samadi, 2016). The residential wastewaters containing high nitrate concentration and phosphate (Khan and Ansari, 2005) has been caused to Choghakhor wetland pollution in south east and North West parts (Ebrahimi and Moshari, 2006; Nadushan and Fatemi, 2008; Samadi, 2016). It seems that the higher nitrate concentration in S5 and S4 may be due to the residential wastewaters leakage from Avorgan (28387 m^3) and Sibak (23433 m^3) villages into the stations. S3 and S1, are also received residential wastewaters from adjacent villages

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especially from Galugerd with the lower volume (13208 m^3). S2 is located in the north of the wetland in the vicinity of the unutilized lands and so received minimum residential wastewater or agricultural runoff (Samadi, 2016). In fact, S2 can be considered as a control site to compare the effect of vicinity of the stations to residential and agricultural land uses on water quality and phytoplankton status. However, nitrate concentration in S2 was at least. Also, nitrate concentration in S5 and S4 in the fall and spring were more than the winter and summer. It may be due to the start of rainfall in the spring as well as fall, which causes leakage of residential wastewater flow from the adjacent regions to the wetland. The vicinity of S2 to unutilized lands was the reason for its lowest nitrate concentration than other stations in the spring and fall, in spite of rainfall. Accumulation of nitrate in the winter in S2, is likely related to less biochemical activities of macrophytes and less consumption of nitrate by them (Moss and Balls, 1989; Rana et al., 1995). Also, nitrate concentration was high in S2 in the summer due to less rainfall, rise of temperature which enhances decomposition followed by evaporation (Santhanam and Perumal, 2003). However, maximum concentration of nitrate was measured in S5 in the fall as well as the minimum in S2 in the spring (*Fig. 2A*).

Phosphate concentration was the highest in S3 followed by S5 and S4 and it was the lowest in S1 and S2. There are evidences supports that the Choghakhor wetland has been polluted by chemical farming fertilizers mainly containing high amounts of phosphate and to some extent nitrate in southwest (Ebrahimi and Moshari, 2006; Nadushan and Fatemi, 2008; Samadi, 2016) similar to other wetlands (Nassar and Gharib, 2014), especially. The vicinity of S3 to the farming lands leads to entry of agricultural runoffs containing much phosphate. Although S5 and S4 may receive less agricultural runoffs than S3, their phosphate content was remarkable (*Fig. 2B*). In S3 and S5, phosphate was highest in the fall and spring than other seasons due to more rainfall in theses seasons and consequently more leakage of the runoffs to this stations. Also in S1 and S2, maximum phosphate was measured in the summer followed by the winter as well as it was minimum in the spring followed by fall (*Fig. 2B*). It seems that raining in the fall and spring caused to dilution of the wetland water and low nutrient status. Also, highest phosphate in the summer in S2 may related to less rainfall, increase in temperature, decomposition enhancement followed by evaporation (Santhanam and Perumal, 2003). Also, phosphate concentration in S2 in the winter likely is because of less growth of macrophytes and less consumption of the phosphate due to higher share of macrophytes for phosphate absorption than phytoplankton (Rana et al., 1995; Moss, 2009).

The wetland water is completely alkaline throughout the year (pH ~ 8.78) as its pH was 9.55 in the summer and 8.12 in the winter and it was the same in different stations (*Fig. 2C*). Water temperature in different stations was similar and it was maximum in the summer and minimum in the winter (*Fig. 2D*). Dissolved oxygen of S4 and S5 was more than S1, S2 and S3 only in the winter. Also, dissolved oxygen was maximum in the winter and minimum in the summer (Watson et al., 1997). In fact, there were significant correlations between water physicochemical parameters (*Table 7*) (Peerapornpisal et al., 2004). There was a negative correlation between dissolved oxygen and temperature (-0.7, $P < 0.01$) (Moss and Balls, 1989). Similarly, there was a negative correlation between pH and temperature (-0.9, $P < 0.01$). Nitrate and phosphate concentration were negatively correlated with the pH (-0.3, $P < 0.05$ and -0.4, $P < 0.01$, respectively).

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Phytoplankton density

Although different concentrations of nitrate and phosphate were measured in different stations (*Fig. 2A, B*), the total density of phytoplankton seems to be affected by variations in nitrate concentration, alone (*Fig. 2A*) because total phytoplankton density was positively correlated with nitrate concentration (0.6, $P < 0.01$) and not correlated with phosphate concentration (*Table 7*) and also there was the highest total density in S5 in the fall or spring and the lowest in S2 in spring and in S1 in fall (*Fig. 3*). As previously mentioned, S2 was considered as control because of its more distance to residential and farming land uses as well as any leakage of the polluted runoffs, unlike the S5. In fact, the vicinity of S5 by the residential regions caused to increase nitrate content especially in rainfall seasons and consequently to increased total density of the phytoplankton.

The result also showed that the Chlorophyta density (*Fig. 4*), nitrate and phosphate concentration (*Fig. 2A* and *B*) in S2 was less than S5. Also, there was correlations between Chlorophyta density and dissolved oxygen ($r = 0.4$, $P < 0.01$), pH ($r = -0.2$, $P < 0.05$), nitrate ($r = 0.7$, $P < 0.01$) and phosphate concentration ($r = 0.3$, $P < 0.01$). Perhaps, high phosphate and nitrate concentrations in S5 may be the reason for high density of Chlorophyta in this station. Similarly, other studies has been shown that physico-chemical factors such as phosphate, nitrate, temperature and dissolved oxygen support the growth of Chlorophyta (Rana and Nirmal Kumar, 1992; Hegde and Sujata, 1997; Nirmal Kumar et al., 2005).

Also, the density of Chlorophyta, in S2 - as control station- in the winter and summer was higher than fall and spring while in S3, S4 and S5 - as polluted stations by nitrate and phosphate- it was higher in the fall and spring than winter and summer (*Fig. 4*). This difference may be related to the accumulation of the nutrients in S2, in the winter due to less biochemical activity and in the summer due to evaporation and less rainfall in these seasons. These results were similar to a previous work (Kumar and Oommen, 2011).

Accumulation of nitrate and phosphate in S5 in the fall and spring was due to rainfall in these seasons and leakage of the nutrients from adjacent polluted lands to these stations which caused to increase the density of Chlorophyta. In this study, an increase in the frequency of Chlorophyta at station 5 corresponds to the results obtained by Zębek and Szymańska (Zębek and Szymańska, 2017)

According to the correlation coefficients were observed between Cyanophyta density and dissolved oxygen content ($r = 0.4$, $P < 0.01$), pH ($r = -0.2$, $P < 0.05$), nitrate ($r = 0.6$, $P < 0.01$) and phosphate concentration ($r = 0.2$, $P < 0.01$), the highest density of Cyanophyta which was observed in S5 may be related to the pollution of S5 by nitrate and phosphate especially in the rainy seasons of fall and spring. Inversely, the Cyanophyta density was at least in S2 (as control station) due to S2 vicinity to un-utilized regions and lack of pollution by nitrate and phosphate (*Fig. 4*). Williams and Tonnessen stated that increased concentration of nitrate and phosphate caused to increase in phytoplankton density in water due to the essential role of nitrogen and phosphorous for phytoplankton activity (Williams and Tonnessen, 2000). The main consequence for enrichment of surface waters by nitrate and phosphorus is eutrification (Stoddard, 1994; Khan and Ansari, 2005). One of the indications for eutrification is the growth of Cyanophyta or green-blue algae which causes to water appears greenish. They assimilate phosphate at a faster rate than green algae (Lam and Silvester, 1979).

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Bacillariophyta density in S2 was more than S5 (*Fig. 4*) which is likely due to the lower concentration of nitrate in S2. There was a negative correlation between Bacillariophyta density and nitrate concentration (r = -0.5, P < 0.01, (*Table 7*). Due to dominance of Bacillariophyta in the lakes with less pollution (Song et al., 2007), so the lowest abundance of Bacillariophyta in S5 represents highest pollution of the wetland in this station. Also, Bacillariophyta is the most algal taxonomic group represented in mesotrophic systems (Rosén, 1981) such as S2 in our work.

The highest density of Dinophyta was observed in S5 followed by S3 (*Fig. 4*) while phosphate concentration was highest in S3 followed by S5 (*Fig. 2A*). Higher concentration of phosphate in S3 than S5 was related to agricultural runoffs entry to the station due to it vicinity to farming lands. So, considering negative correlation between the Dinophyta density and phosphate (r = -0.7, P < 0.01) (*Table 7*), the vicinity of S3 to farming lands may be a main factor to receive more phosphate and less growth of Dinophyta. Watson and colleague (1997) believe that in eutrophic regions (such as S3 and S5 in our work) Dinophyta generally decline (Watson et al., 1997).

Also, Dinophyta in the summer were most frequent than other seasons and they were not found in the winter in any stations (*Fig. 4*). This is because of highest temperature (r = 0.8, P < 0.01) and pH (r = 0.8, P < 0.01) as well as lowest dissolved oxygen (r = -0.7, P < 0.05) in the summer (*Fig. 2C, D* and *E*) (*Table 7*) which caused to more growth of Dinophyta.

Euglenophyta and Chrysophyta in S5 were more abundant than other stations (*Fig. 4*). In S2, Euglenophyta was at least and Chrysophyta was absent in all seasons. There were positive correlations between the nitrate concentration and density of Euglenophyta as well as Chrysophyta (r = 0.4, P < 0.01) (*Table 7*). Higher content of nitrate in S5 -due to the vicinity to residential regions- may be main factor to increase Euglenophyta and Chrysophyta in S5. Due to negative correlation between Euglenophyta density and pH (-0.2, P < 0.05) (*Table 7*) it seems that higher density of Euglenophytain in the winter (*Fig. 4*) is related to the lower pH in this season (*Fig. 2C*).

Phytoplankton diversity

The diversity indices (H′) in S4, S3 and S5 (3.55, 3.51 and 3.50, respectively) were not significantly different (P < 0.05) (*Fig. 3*) and were more than S1 and S2 (3.46 and 3.35, respectively). *Table 7* showed a positive correlation between total diversity of phytoplankton and phosphate concentration (r = 0.4, P < 0.01). Therefore, higher phosphate concentration in polluted stations (S3, S4 and S5) may be the cause of greater diversity of phytoplankton than S1 and S2. Also, Diversity was highest in the summer (3.52) followed by winter (3.51), fall (3.45) and spring (3.414) which is due to the change in diversity of different divisions during the seasons. For example, Dinophyta in the summer were most frequent than other seasons and they were not found in the winter in any stations. Chrysophyta in S1 and S2 was absent.

The normal range of H′ is from 0 to` 4 and indicated the diversity value and pollution status of a water body. The values > 3, 1-3 and < 1 indicate clean water, moderate pollution and heavily polluted, respectively (Williams and Tonnessen, 2000). In this study, Shannon diversity index (H′) ranged from 3.28 up to 3.57 and it was clear that the wetland water was still clean. Higher concentration of phosphate causes more diversity of individual taxonomic groups (Watson et al., 1997) which is similarly observed in S5.

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DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

Phytoplankton composition

In this study, 59 genera and 43 families belonging to 6 divisions were identified which Bacillariophyta phylum and *Nitzschia* genus were the most common ones by the abundances of 46.6% and 14.8%, respectively (*Table 2* and *3-6*). Similar result has been obtained by Arimoro et al. (2008). In each division, the highest density was recorded in the genus *Nitzschia* (Bacillariophyta, 6054 ± 1915 cell/L) as a bio-indicator for eutrophic status of the water, followed by *Cosmarium* (Chlorophyta, 2869 ± 1855 cell/L) as an oligotrophic status bio-indicator, *Microcystis* (Cyanophyta, 1969 ± 714 cell/L) as an eutrophic status bio-indicator, *Peridinium* (Dinophyta, 1293 ± 1310 cell/L), *Euglenaria* (Euglenophyta, 717 ± 605 cell/L) and *Dinobryon* (Chrysophyta, 314 ± 337 cell/L) (*Tables 3, 4* and *6*) (Wu, 1984; Sakset and Chankaew, 2013; Bellinger and Sigee, 2015). Also, the genus *Pinnularia* (Bacillariophta, 743 ± 237) is indicated an oligotrophic status while *Cyclotella* (Bacillariophyta, 2544 ± 967 cell/L), *Anacystis* (Cyanophyta, 925 ± 405 cell/L) and *Surirella* (Bacillariophyta, 557 ± 178 cell/L) indicating mesotrophic status of the wetland (Song et al., 2007; Fonge et al., 2012). Considering the highest density of the genus *Microcystis* indicating eutrophication in S5, S4 and S3 (*Fig. 5*) and due to the variations of nitrate and phosphate concentration in these stations (*Fig. 2A* and *B*), it seems that the high nutrient content in the stations especially nitrate and phosphate caused to initiate eutrophic status in the wetland. In fact, the vicinity of these stations to the residential and agricultural land uses caused to the high nutrient concentrations specially in the raining seasons of the fall and spring.

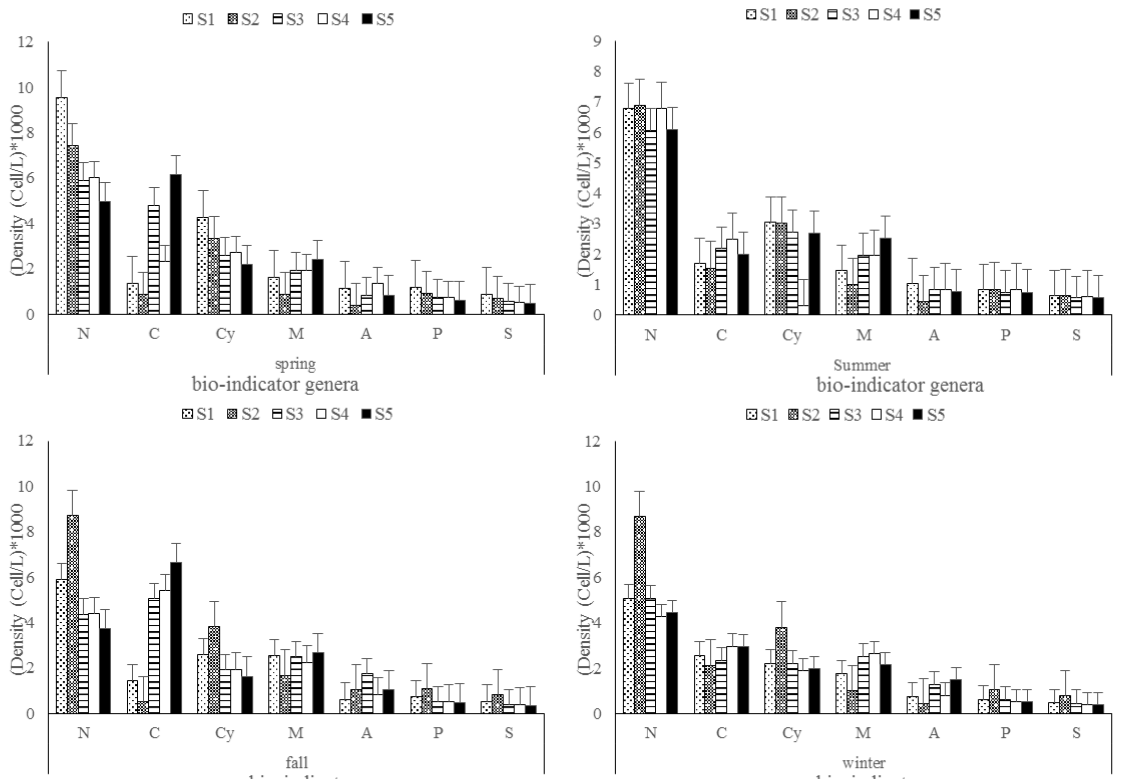

Figure 5. *Composition of the bio-indicator genera (N: Nitzschia, C: Cosmarium, Cy: Cyclotella, M: Microcystis, A: Anacystis, P: Pinnularia, S: Surirella) for water trophic status in different stations and seasons in Choghakhor wetland*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Considering a positive correlation between the Cyanophyta density and nitrate (0.6, $P < 0.01$) as well as phosphate concentration (0.2, $P < 0.05$) (*Table 7*) the *Microcystis* as the most abundant genus of *Cyanophyta* must be seriously controlled due to its ability to develop bloom in the wetland and to produce toxic compounds which threat the life of the organism living in the wetland (Lam and Silvester, 1979).

The total density of bio-indicator genera for eutrophic, oligotrophic and mesotrophic status were 8023 ± 2888, 3612 ± 1503 and 4026 ± 1057 cell/L, respectively. Although, H′ index was greater than 3 and so indicating the wetland water is clean, but according to the greater density of bio-indicator genera for eutrophic status, it seems that the wetland moves from mesotrophic and oligotrophic to the eutrophic status. It may be caused by the human activity mainly residential wastewaters and farming runoffs which makes entry of nitrate and phosphate to the adjacent parts of the wetland through the rainfall.

Conclusion

The phytoplankton status in relation to physico-chemical changes were evaluated in different seasons of 2011 as well as different stations in the margin of Choghakhor wetland. The stations were: S1 in north west affected by residential wastewaters; S2 in the north was adjacent to un-utilized regions (control); S3 in southwest was receiving agricultural runoffs; S4 and S5 in south east get residential wastewaters.

Phytoplankton community was included 59 genera, 43 families and 6 divisions which Bacillariophyta was the most common (46.6%) with 25 genera followed by Chlorophyta (32.0%), Cyanophyta (13.3%), Dinophyta (4.4%), Euglenophyta (3.0%) and Chrysophyta (0.8%). The most abundant genus was *Nitzschia* (14.8%) from Bacillariaceae.

Phytoplankton density (S5 > S4 > S3 > S1 > S2), as well as nitrate (S5 > S4 > S3 > S1 > S2) and phosphate (S3 > S5 > S4 > S1 ≈ S2) were different in different stations and seasons ($P < 0.05$). Also, there were correlations between phytoplankton density and nitrate ($r = 0.6$, $P < 0.01$), Chlorophyta density and phosphate ($r = 0.3$, $P < 0.01$), Cyanophyta density and phosphate ($r = 0.2$, $P < 0.05$) which supports the nitrate and phosphate role to increase the density of the phytoplankton in the stations with high nutrient contents (S5, S4 and S3). Chlorophyta and Cyanophyta abundance were maximum in S5 and minimum in S2. Inversely, Bacillariophyta density was highest in S2 and lowest in S5. The highest and lowest density of Dinophyta was observed in S5 and S3, respectively. In S5, It was greater than S2. Dinophyta in the summer were most frequent than other seasons. Euglenophyta was most abundant in S5 and least abundant in S2. Chrysophyta in S5 was most abundant and in S2 was absent.

The composition of the genera within the divisions showed that the highest density was recorded in the genus *Nitzschia* (Bacillariophyta, eutrophic status bio-indicator), followed by *Cosmarium* (Chlorophyta, oligotrophic status bio-indicator), *Microcystis* (Cyanophyta, eutrophic status bio-indicator), *Peridinium*, *Euglenaria* and *Dinobryo*. Also, *Microcystis* was maximum in S5 followed by S4 and S3. Generally, the total density of bio-indicator genera for eutrophic, oligotrophic and mesotrophic status were 8023 ± 2888, 3612 ± 1503 and 4026 ± 1057 cell/L, respectively.

Mean diversity index was varied as: S4 ≈ S3 ≈ S5 > S1 ≈ S2 ($P < 0.05$) and represented that the wetland water is still clean (H′ > 3). Although, H′ index was greater than 3 and so indicating the wetland water is clean, but according to the greater density

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of bio-indicator genera for eutrophic status, it seems that the wetland moves from mesotrophic and oligotrophic to the eutrophic status. It may be caused by the human activity mainly residential wastewaters and farming runoffs which makes entry of nitrate and phosphate to the adjacent parts of the wetland through the rainfall.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51735192

ADVANCING SMALLHOLDERS' SUSTAINABLE LIVELIHOOD THROUGH LINKAGES AMONG STAKEHOLDERS IN THE CASSAVA (*MANIHOT ESCULENTA* CRANTZ) VALUE CHAIN: THE CASE OF DAK LAK PROVINCE, VIETNAM

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(Received 21st Jan 2019; accepted 6th Mar 2019)

Abstract. Cassava (*Manihot esculenta* Crantz) is a versatile crop that plays a vital role in sustaining smallholders' livelihoods, and in increasing farmers' income. This research assessed the cassava value chain in Dak Lak province, Vietnam. It demonstrated the need for enhanced efficiency in the production of cassava thus enhancing the cassava value chain. Value chain analysis was conducted through a questionnaire-based survey of 300 household farmers, in-depth interviews with key informants, and focus group discussions in seven communes in three districts. The aim was to explore how to improve and develop the value chain, increase stakeholders' incomes and particularly, to ensure sustainable household livelihoods. The findings relating to the sharing of value added among the stakeholders showed that farmers create the highest value added but that intermediaries derive the most profit. In addition, relationships exist amongst different stakeholder ranging from input providers to the final users which are overwhelmingly starch and ethanol factories. There is a local linkage between input suppliers and farmers, both spot-market and persistent relationships which exist between farmers and intermediaries. Furthermore, the distribution of both gross and net profits overwhelmingly favors the traders and processors. However, intermediaries play an important role and the farmers would not secure full benefits without their support.
Keywords: *gross profit, household, intermediaries, stakeholders relationship, value added*

Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial drought-resistant crop cultivated mainly in dry areas, which contributes significantly to the nutrition and livelihood of many farmers (Sewando, 2012). This crop is a versatile plant that is used in the production of a large number of products ranging from traditional food products to livestock feeds as well as having uses in the industrial sector as a raw material for ethanol and starch and its numerous derivatives (Pingmuanglek et al., 2017). The Vietnamese cassava value chain is characterized by numerous intermediaries due to inadequate commercial infrastructure as well as a fragmented pattern of land use for cassava production (Thao et al., 2013). As a result, cassava has a lower value compared to other crops in the same region. Dak Lak province is in the middle of the Central Highlands where four other provinces, Gia Lai, Kon Tum, Lam Dong, and Dak Nong are also located (Dak Lak, 2018). In Dak Lak, cassava is grown as a cash crop by rural

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

households and the income earned from cassava plays a vital and increasing role in farmers' livelihoods (Son et al., 2016). The area cassava plantations in Dak Lak province has grown from 30,732 ha in 2014 to 36,300 ha in 2017 (GSO, 2018a), and the yield of cassava during that period fluctuated only slightly from 18.3 to 18.8 tonnes per hectare (GSO, 2018b). Cassava farmers also face various challenges stemming from a lack of access to modern inputs and improved varieties, as well as the problems relating to the local infrastructure and a lack of access to credit (Njukwe et al., 2014). Hence, improvements in the productivity of cassava, the processes used, their products, in the means of distribution in the value chain would lead to an improvement in output (Masamha et al., 2017).

This would in turn contribute to an increase in household income which would lead to increased spending in areas such as education and health services as well as other aspects of daily life (Rutherford et al., 2016). Nevertheless, unpredictable cassava prices in developing countries have tended to increase the vulnerability of farming household incomes (Ouma and Jagwe, 2010). In addition, the farmers have limited to connection with the market as well as a lack of comprehensive information. Consequently, building relationships among stakeholders in the cassava value chain and increasing cassava productivity is necessary in this region. The aim of the study is to improve and develop the cassava value chain, increase stakeholders' income and particularly, to find out the appropriate strategies to ensure sustainable household livelihoods.

Review of literature

Cassava is a crucial source of food in Africa because it can be planted in unfertile soil and is also able to resist severe weather (McNulty and Oparinde, 2015; Meridian Institute, 2012), and it is, for instance, a vital staple crop in Liberia (Coulibaly et al., 2014a). Africa is one of the leading cassava producing regions contributing over 56% of the total global supply (FAO, 2018). It has been proposed that labour, transportation systems and novel technologies, as well as the coordination of agents, are all factors by which the efficiency of the cassava value chain could be improved (Trienekens, 2011). The cassava market is primarily based on the cassava tuber and products made from it. However, cassava leaves also offer an additional source of nutrition although there have been few studies to date of their benefits or of how they are consumed and whether there is a wider market for them (Andersson et al., 2016). The utilization of locally available resources such as cassava, not only creates value-added products but also brings benefits to the local society, with benefits accruing to all stakeholders within the cassava value chain in the region (Mcnulty and Adewale, 2015). Olukunle (2013) found that income and employment for farmers in Nigeria could be created from the cassava value chain as well as other actors in the value chain with over one million jobs being created in rural areas of Nigeria and an increase of approximately US$450 per year in the income of 1.8 million participating farm families. However, although a strong long-standing market has been established in the cassava sector it was found that the farmers gained a smaller percentage of the total profits, compared to traders who received the largest part of the profit. Naziri et al. (2014) studied the cassava value chain and the diversity of post-harvest losses arising in four countries on two continents, Thailand and Vietnam in Southeast Asia, Nigeria and Ghana in sub-Saharan African. They found that post-harvest losses at different stages of the cassava value chain were due to cassava cultivation, processing and consumption methods and the relationships and linkages

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among the value-chain stakeholders. However, they suggested that there was no "one-size-fits-all" solution for dealing with post-harvest losses but those solutions depended on the specific characteristics of different value chains. They estimated that in Ghana, economic losses due to partial spoilage of cassava root were be between 16 and 28% caused by the long distance between the production site and the place of final consumption. Similarly, in Nigeria, economic losses suffered by cassava root due to breakage and deterioration during harvesting and distribution were estimated to be between 10 and 30%. Daniels et al. (2011) were able to provide a complete picture of the value chain in Nigeria including farmers, processors, traders, input suppliers and other stakeholders

In Cambodia, the yield of cassava is from 8 to 10 tonnes per ha with the average area under cassava being 0.2 to 1 ha/household. A study of the cassava value chain was conducted by the IBC (Inclusive Business for promoting sustainable Cassava smallholders) project in Tboung Khmum province and the study particularly examined government policy (SNV Cambodia, 2015) as well as examining gender-related aspects of the value chain. The linkages among stakeholders were analyzed with the aim of improving knowledge as well as proposing appropriate measures for further strengthening the cassava value chain. In recent years, the productivity and yield of cassava in Vietnam has increased and it now ranks third in terms of agricultural production after rice and corn. The area planted to cassava has reached 560,000 ha, with an average yield of 17.63 tonnes/ha and a production of 9.87 million tonnes annually (Hieu, 2016). This is because the area in which cassava is grown has been widened and hybrid varieties have been applied with farmers now cultivating 70% hybrid varieties including KM94 and 30% local varieties (Phuc, 2015). The average yield of cassava in 2016 was approximately 19 tonnes/ha (FAO, 2018), which was more than double that in 2000 (8.4 tonnes/ha). Nguyen et al. (2005) presented a complete picture of the cassava value chain and also the relationship among the stakeholders in the chain. The study also examined the role of the government as well as their operations and the sharing of benefits between stakeholders in the value chain, finding that as (Olukunle, 2013) noted in Nigeria, the farmer received unfairly small share of the benefits while intermediaries obtained most of the profit in the cassava value chain. According to (Naziri et al., 2014) study of postharvest losses there were differences between the northern and southern parts of Vietnam. In the north, production was mostly by small-scale farmers while in the south, it was characterized by both larger scale production and processing units normally consisting of from 20 to 30 hectares. That study found that post-harvest losses mainly occurred on large sized and small-sized plots because the local people lack of knowledge as well as in harvested. The kind of losses experienced were breakage and deterioration of roots as were found in other areas studied. Vietnam is a tropical country and it is unsurprising that cassava has become one of its most vital crops (GSO, 2018c). In Dak Lak the area planted with cassava increased from 32,000 ha to over 36,300 ha between 2011 and 2017, with the average cassava yield fluctuating around 19 tonnes/ha and the total production reaching a peak of 703,300 (*Fig. 1*) tonnes in 2017. The province is, therefore, a region where cassava is one of the most prominent crops. The study reported discusses the major aspects of the cassava value chain in Dak Lak province. Firstly, it systematically identifies and maps the stakeholders participating in the distribution channel and marketing of cassava and details the profit and cost structures. Secondly, it identifies the sharing of benefits between the various actors and analyzes the gross and net profits within the chain. It also examines how and by whom

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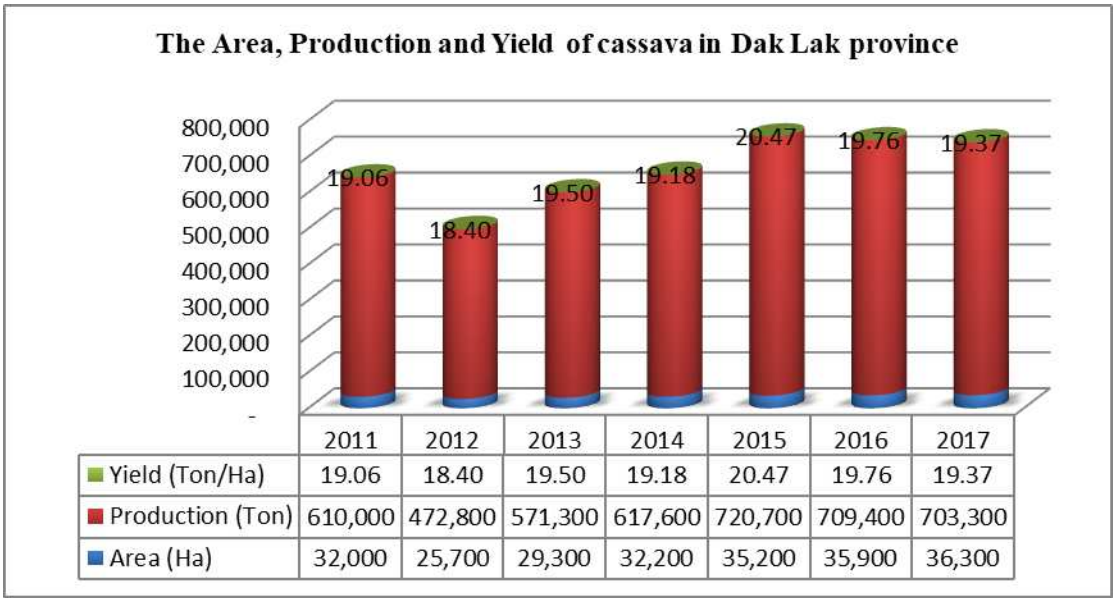
DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

benefits could be gained from enhancing the relationships among the actors and organizations involved. Thirdly, it examines ways in which the cassava value chain could be upgraded by improvements in cassava productivity (Naziri et al., 2014). Finally, it highlights the role of relationships and coordination mechanisms in improving farming-related policies in order to enhance the cassava value chain and to increase the earnings of farmers.



	2011	2012	2013	2014	2015	2016	2017
Yield (Ton/Ha)	19.06	18.40	19.50	19.18	20.47	19.76	19.37
Production (Ton)	610,000	472,800	571,300	617,600	720,700	709,400	703,300
Area (Ha)	32,000	25,700	29,300	32,200	35,200	35,900	36,300

Figure 1. *The area, production and yield of cassava in Dak Lak 2011-2017*

Materials and methods

Study area

Dak Lak province is located in the Central Highlands between longitude 107°28'57" and 108°59'37" east, and between latitude 12°9'45" and 13°25'06" north (*Fig. 2*). It occupies an area of 13,125.37 km^2 (Dak Lak, 2018). Currently, the population is over 1.8 million people with a provincial population density of over 137 people per square kilometre. There are 47 ethnic groups living in the province, the largest; of which, the Kinh account for about 70% of the people, with other ethnic minority communities including Ede, Thai, Tay, M'nong, and Nung people representing the remaining approximately 30%. The climate of the province is separated into two sub-regions with the North West being quite hot and dry in the dry season, while the climate in the south east is cool and pleasant.

The Krong Bong district is located in the south east and the centre of the district is 55 km from Buon Ma Thuot city in the north west of the province (Krong Bong, 2018). Krong Bong has an area of 1,257.49 km^2 and a population of 90,126 people. It is affected by the tropical monsoon, and it's climate, which has two distinct seasons, rainy and hot, is not only impacted by the generally high altitude but is also influenced by the Cu Yang Sin mountain, which rises to over 2,400 m.

Ea Kar district was established on 13th September 1986 under decision No. 108/1986/QĐ-HĐBT of the Vietnamese government. It is 52 km from Buon Ma Thuot city, along the No. 26 National Highway. It is located to the north of Dak Lak province

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and has a land area of 1,037.47 km^2 and the population density is over 138 inhabitants per square kilometre. Cassava is one of the staple crops produced to meet the material needs of local industry and it is the largest producer among agricultural crops in Dak Lak province (Ea Kar, 2017).

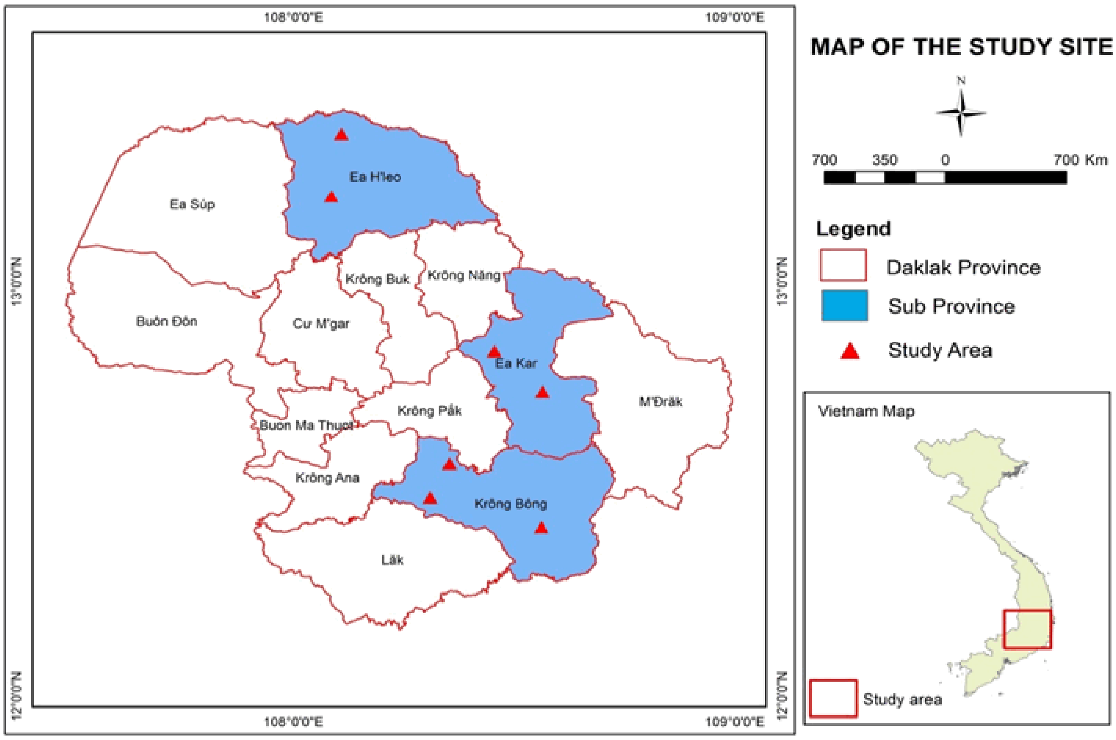

Figure 2. *The location of the study area*

Ea H'Leo district is the northern gateway of Dak Lak province. The district is approximately 80 km from Buon Ma Thuot city and was separated from Krong Buk district on 8th April 1980 under government decision No.110/QĐ-HĐBT. It has an area of 1,335.12 km^2 and a population of 125,123 people. Cassava is a prominent crop in the region (Ea H'leo, 2018). The study reported was conducted by surveys in three districts, Ea Kar, Krong Bong and Ea H'leo which are prominent areas of cassava production in Dak Lak. The major source of household income is from cassava, other cash crops and cattle husbandry. The average area planted to cassava by households is from 0.5 ha to 3.5 ha and the average yield is approximately 20 tonnes/ha. Most of the households growing cassava consist of northern ethnic minorities such as the Ede, Dao, Tay and Nung.

Data collection and analyses

Data collection

The study was conducted using a case study approach, which is one of several methods of conducting social science research (Yin et al., 2009). Data collection was conducted with the aid of structured questionnaires (Fonji et al., 2017). Data relating to household characteristics (Mukete et al., 2018) came from a household survey, interviews with key informants and focus-group discussions with heads of household

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and actors in the cassava value chain. The cross-sectional design enabled the researchers to address the study objectives and this method was adopted in order to save time during the data collection process (Kothari, 2004; Masamha et al., 2017). This method has been employed in a number of previous studies of the cassava value chain and related issues (Komen et al., 2010; Masamha et al., 2017; Son et al., 2016). The researchers selected seven communes from the three districts, Krong Bong, Ea Kar and Ea H'leo, using the multi-stage sampling method. In each commune, a questionnaire-based survey was conducted to collect relevant data from stakeholders in the cassava value chain based on visits to households. In this study, the interviews covered at least 70% of the households in each commune, where the farmers grow cassava. The questionnaire employed consisted of a mixture of open and closed ended questions. It was written in English then translated into Vietnamese before being used to interview the indigenous people (Echato and Echato, 2018). To complement the survey data, both primary and secondary data were collected from stakeholders who directly participate in the value chain, including from input providers, traders and processors (Masamha et al., 2018). Both in-depth interviews using a semi-structured questionnaire and direct observation in the field were also applied in this study (Coulibaly et al., 2014b). The in-depth interviews were conducted with key informants, including directors of local cassava starch factories (Son et al., 2016), input suppliers, cassava growers, collectors, traders, in Cu Kty, Hoa Son and Cu Pui (Krong Bong district), Ea Sar and Ea Pal (Ea Kar district), and Ea Tir and Ea H'leo (Ea H'leo district). These key informants were surveyed using a different questionnaire, which covered core processes (Naziri et al., 2014) traded quantities and prices of inputs as well as the selling price of cassava in the local market compared to other areas. Focus group discussions with cassava farmers were conducted during 2018 in the seven different communes included in the study. Field trips were used to gather primary as well as secondary data. The basic unit for this research was the *household farmer* which was defined as a group of people living together in the same house and taking part in the same daily activities (Mukete et al., 2018). The populations of the study were the cassava growing household farmers in Dak Lak province. Goal-directed sampling, a commonly used sampling approach was adopted, with the sample of participants being selected based on preselected criteria appropriate to a specific research question (Mack et al., 2005). The total sample size was 300 households across the seven communes, and 20 both collectors and traders, and key informants were interviewed as well as the owner of a cassava starch factory in the area.

Statistical analyses

Both qualitative and quantitative methods of analysis were applied in this study to understand the roles and actions of the major's actors (Apata, 2013). The analysis of the data from the questionnaire-based survey involved coding, data entry and analysis using the SPSS Version 20 statistical program (Masamha et al., 2017; Mukete et al., 2018) and Microsoft Excel. Frequencies and means for socio-economic and demographic data were described based on descriptive statistics (Masamha et al., 2017).

The qualitative data from the in-depth interviews with stakeholders and focus group discussions were analyzed by specific content analysis in order to identify and examine the most important topics (Masamha et al., 2018). A value chain details the many activities that are required to take a product or service through the different phases of production and delivery to the final consumers, and its disposal after use (Kaplinsky and

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Morris, 2000). The analysis of the cassava value chain was based on the value-chain analysis method (VCA) (Naziri et al., 2014) and value-chain upgrade solutions were computed in this study using a quantitative method. The VCAs were designed using techniques to determine specific relationships and linkages at different stages among actors who participate in the cassava value chain. Analyzing supply stages, marketing and trading relationships between actors in chain analysis has become a key tool since it can enable an understanding of the whole chain (Meaton et al., 2015).

This study identified major aspects of the cassava value chain in Dak Lak province. The production cost, intermediate input (II) value added (VA) and other economic parameters, including gross profit (GPr), net profit (NPr). These were evaluated based on specific actors' perspectives. Revenues were calculated according to the following equation:

$$\text{Revenues} = (Q \times P) + \text{income from by-products}$$

where Q = quantity sold and P = price paid by buyer (Purcell et al., 2008).

Components of total value generated by the value chain such as output value (Y) and product value were also calculated using the Q × P formula, based on analytical frameworks for value chain analysis proposed by international organizations such as Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ, 2007).

Value added (VA) was calculated to measure the new wealth created by a productive activity and thus the creation of wealth and the contribution of the production process to the growth of the economy. VA was calculated according to the following equation:

$$VA = Y - II$$

where Y = total sales (output) value and II = intermediate input such as fertilizer, pesticide and seedlings.

Profit elements were calculated as follows:

$$GPr = VA - (\text{wages and salaries} + \text{interest charges} + \text{taxes})$$

$$NPr = GPr - \text{depreciation}$$

GPr expresses the economic gain, or loss, to an actor once all current production costs have been met. NPr indicates the economic gain or loss taking into account the predictable costs of actual investment.

Results

Description of stakeholders in the cassava value chain

Input provider

The study found that there were both backward and forward linkages in the cassava value chain. Input providers were backward-linked from the farmer. Thus, input providers were important actors who supply agricultural products to meet farmer demands for items such as seedlings, fertilizer and pesticide as well as being a source of informal credit for agricultural activities. A good relationship between providers and

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farmers has the potential to improve the value chain by giving farmers access to informal credit without them needing to resort to the banking system or other sources of credit, and can help small-scale household farmers to pool their resources to deal with activities which cannot be done by individual farmers.

Farmer/producer

This is the first actor in the chain and they are mostly located in rural areas where an inequitable infrastructure has developed, with the farmer being at a disadvantage by needing to supply fresh cassava to the buyer (Njukwe et al., 2014). *Table 1* shows the socio-economic characteristics of household farmers across the study sites. The average age of the respondents is one of the major factors that affect the chain with 82.33% being over 35 years of age and 71.67% having received at least secondary formal education. Furthermore, it was notable that males represented almost 90% of the respondents suggesting that cassava production is overwhelmingly dominated by men. In addition, most of the farmers (92.32%) had more than 5 years' experience of cassava cultivation. The fresh cassava produced by farmers is mostly sold locally to large traders or factories as well as to other collectors at both the village market and the farm gate. However, this spot linkage only exists during the harvest period. The seedlings for the cassava varieties cultivated in the region are derived from multiple sources such as producers' own farms or those of neighbours, local seeding centres or from donations by international organisations conducting research about cassava cultivation. The land is generally prepared with a machete, hoe or dibble and the cassava plant cutting is inserted vertically, horizontally or at an angle (El Bassam et al., 2010). Some farmers who own larger areas prepare their land using tractors.

Table 1. *Socio-economic characteristics of respondent at the study sites*

Profile		COMMUNE							Total	%
		CUKTY	CUPUI	EAHLEO	EAPAL	EASAR	EATIR	HOASON		
Gender	Female	3	7	5	5	4	5	3	32	10.67
	Male	41	35	40	38	38	37	39	268	89.33
Total		**44**	**42**	**45**	**43**	**42**	**42**	**42**	**300**	**100**
Age of the respondent	< 25	0	0	0	0	1	0	1	2	0.67
	> 55	8	5	8	7	5	11	13	57	19
	25-35	3	14	7	9	12	1	5	51	17
	35-45	17	19	16	14	13	20	11	110	36.67
	45-55	16	4	14	13	11	10	12	80	26.66
Total		**44**	**42**	**45**	**43**	**42**	**42**	**42**	**300**	**100**
Level of formal education	0	1	6	5	2	3	3	2	22	7.33
	1	10	15	6	9	9	9	5	63	21
	2	26	18	27	28	23	24	29	175	58.33
	3	7	3	7	4	7	6	6	40	13.34
Total		**44**	**42**	**45**	**43**	**42**	**42**	**42**	**300**	**100**
Number of years of cultivation by farmer	< 5	1	10	9	5	5	6	2	38	12.67
	> 15	12	4	15	7	6	8	11	63	21.00
	10-15	18	14	14	21	18	19	18	122	40.67
	5-10	13	14	7	10	13	9	11	77	25.66
Total		**44**	**42**	**45**	**43**	**42**	**42**	**42**	**300**	**100**

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Transportation of the harvested cassava from the field may be by truck, motorbike or bicycle, but is mostly accomplished by a tractor-pulled trailer known locally as a *xe cay* (*Fig. 3*). Cassava is widely grown as a mono-crop by small-scale farmers on fragmented land (0.1 to 4.5 hectares) for food purposes and for use in the industrial sector. Nevertheless, larger scale inter-cropping models are also practiced by a small number of household farmers who sell their produce to processing factories. Cassava production is labour intensive (Masamha et al., 2017) so it is one of the best options to improve a farmer's livelihood in a rural area where there is an excess of labour available.

Collectors and traders

Collectors can be grouped based on the quantity of cassava which they buy, namely local, small and large and they include local people as well as those who come from a different region. They play a vital role in the linkage between the cultivation and consumption of fresh and dried-chip cassava. Depending on the buying capacity of the collector, the cassava is collected by different means. Local collectors usually gather directly from both small-scale cassava farmers and from indigents who collect the cassava residue from the harvested fields in or near the village. Larger collectors are able to buy fresh cassava from previous actors in the value chain as well as providing information to those other actors. Most of the product is then sold to starch or ethanol factories with a small volume being delivered to the Tay Ninh province cassava factory.

Figure 3. *Transportation in the cassava value chain. (Photo by author)*

Processors

Most of the cassava produced in Dak Lak is bought by starch or ethanol factories in each district. There are seven starch factories in Dak Lak province with capacities ranging from 150 to 250 tonnes of cassava starch per day, all of which are owned by the Dak Lak Starch Cassava Company. In addition, cassava is used as a raw material by Dai Viet, which was established in 2010, and is one of the biggest ethanol factories in Vietnam with an installed capacity of 54,000 tonnes of ethanol per year. The factories buy cassava in the form of dried chip or fresh root from small collectors as well as from traders and farmers who have large cultivation areas. Thus, most of the cassava tubers are transported to factories by collectors or traders (Son et al., 2016). In addition to its industrial use, fresh cassava is also processed into cassava chips by small-scale chip producers for use both in household animal husbandry and by animal feed companies. There is also a small amount of cassava root used for human consumption including in the form of local traditional cakes known as *Banh Trang* or *Bot Loc*.

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Cassava value chain distribution channel in Dak Lak province

Currently, there are a number of distribution channels for cassava derived from cultivation by farmers to the final customer. However, there are only two major channels and these have an effect on the income of the households surveyed in this study. Firstly, the cassava is supplied to starch companies in order to produce starch (Son et al., 2016) for the export market (85%) with the rest (15%) for the domestic market. The second channel is to the ethanol factory which uses cassava as its feedstock to produce ethanol for a diverse range of domestic consumers (*Fig. 4*).

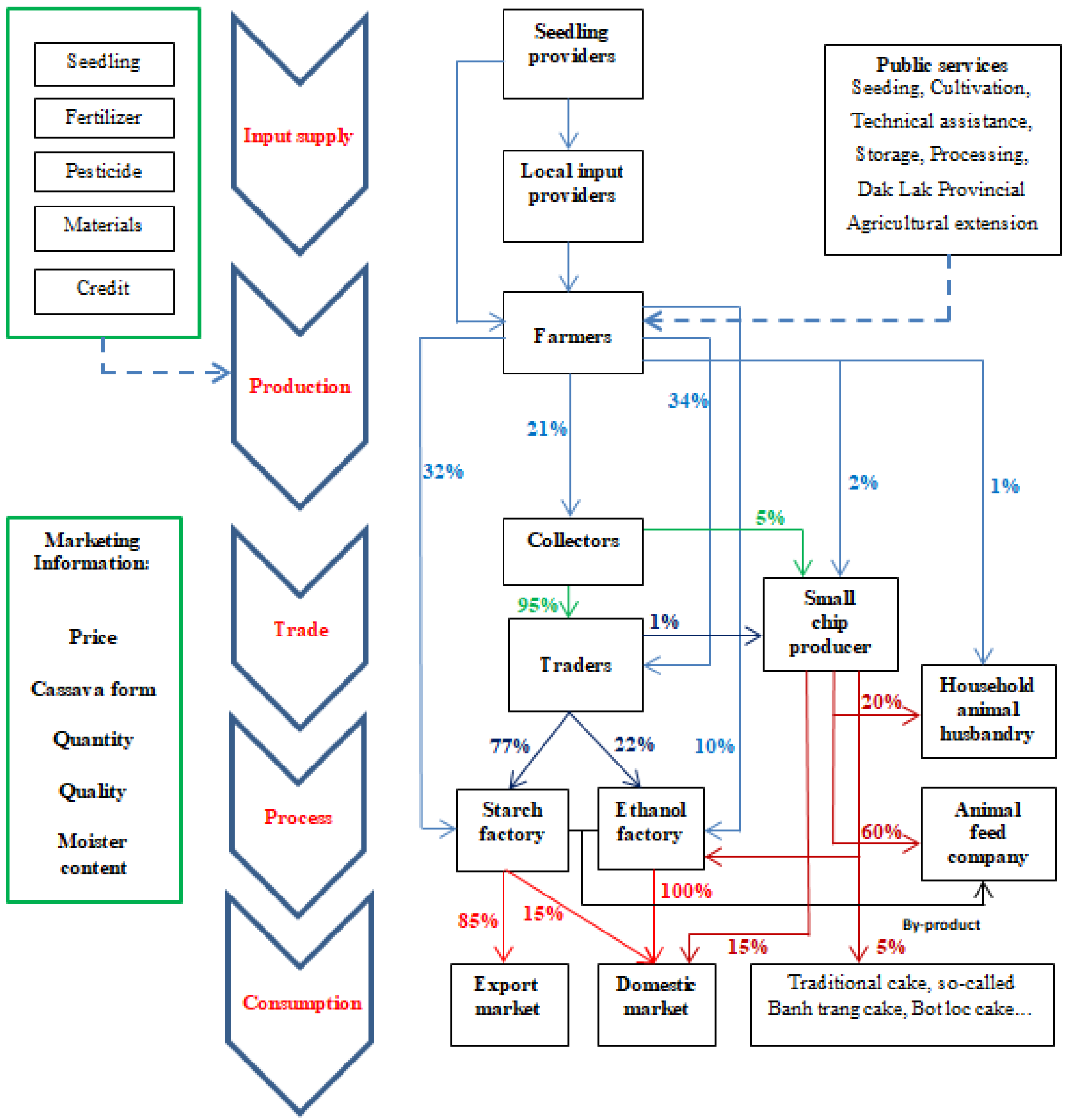

Figure 4. *Dak Lak cassava value chain map*

Based on our findings, it was estimated that 97% of the total cassava production is sold in fresh-root or dry chip form for industrial use with the rest being used to meet household requirements. In the first distribution channel, the fresh cassava tubers are

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either sold by the farmer to intermediaries (collectors, 21% and traders, 34%) and then sold to a starch factory, or are supplied directly by the farmer to the factory (32% - all percentages based on total production) to produce starch. In the second distribution channel, the farmers sell either directly to the ethanol factory (10% of total production) or through traders (22%) (*Fig. 4*). In addition, small chip producers and household animal husbandry account for 2% and 1% of total production respectively.

Financial analysis of stakeholders in the cassava value chain

Cassava growers

The yield of cassava production in the study site fluctuated in a range of 20 to 25 fresh root tonnes/ha. However, in exceptional cases, the yield was as low as 5.5 tons per hectare. This is because some households make an inadequate investment in growing cassava. Moreover, the low fertility of the land and the use of poor quality varieties also contribute to low productivity. The average yield was approximately 20 tons of cassava tubers per hectare. The conversion rate was 50%, and the farmer can thus obtain around 10 tons of dried chip cassava from the fresh quantity harvested. The producers had various options to sell their products to buyers. Depending on individual farmer's targets and market price information, the cassava growers sold 21%, 34%, 32% and 10% of their produce respectively to collectors, traders, starch factories and the ethanol factory. In terms of the price obtained for cassava, there is great variability depending on the price of exported starch (Son et al., 2016). In this study, the total farmer's income was VND47.895 million or US$2,048 per hectare (US$1 = VND23,390). The price of cassava was approximately VND2 million per tonne and the average yield was around 23.64 ton ha^{-1}. In the value chain analysis of cassava, the cost of intermediate inputs represented 8.52% of the total income of the producers, of which the highest percentage (over 50%) related to seedlings (VND4.081 million) (*Table 2*).

Table 2. *Major indicator analysis of fresh cassava value chain per hectare*

Item	Value (VND1,000)	Value USD	Proportion %
Output	**47,895**	**2,048**	**100.00**
Intermediate input	**4,081**	**174**	**8.52**
Seedlings	2,257	96	4.71
Fertilizer	1,000	43	2.09
Pesticide	239	10	0.50
Transporting	556	24	1.16
Fuel	29	1	0.06
Value added	**43,814**	**1,873**	**91.48**
Land preparation	3,338	143	6.97
Planting labour	3,570	153	7.45
Fertilizer labour	214	9	0.45
Weeding labour	1,702	73	3.55
Pesticide labour	133	6	0.28
Harvesting labour	6,019	257	12.57
Transport labour	97	4	0.20
Interest	122	5	0.25
Gross profit (GPr = NPr)	**28,618**	**1,224**	**59.75**

Exchange rate: 1 US$ = 23,390 Vietnamese dong (VND) (HSBC, 2018)

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The value added was calculated to be VND43.814 million (US$1,873) which accounted for 91.48% of the total production and this confirms that cassava is a favourable crop, which contributes to household farmer income with high economic efficiency, producing a GPr of VND28.618 million per hectare with low intermediate input costs.

In the case of chip cassava, the value added and the NPr accounted for 92.33% of the total production and 55.64% per hectare, respectively (*Fig. 5*). Although the total cost incurred also involved incurring an additional VND4.320 million (US$67) per hectare for peeling and drying the fresh cassava. However, both the income and profit received by the producers was higher for chip cassava than for fresh cassava since chips attract a higher price than fresh cassava, selling for VND4,500 per kg compared to VND2,026 per kg respectively. Furthermore, processing cassava into dried chips at the farm level provides employment for the indigenous people and thus helps to deal with rural employment, which is a growing problem in the region, and also helps to diversify the farmers' income. Finally, the farmer is able to negotiate the selling price with other actors in the value chain because they can store their product in dried chip form for a longer period in order to wait for the optimal market price (Viet et al., 2013).

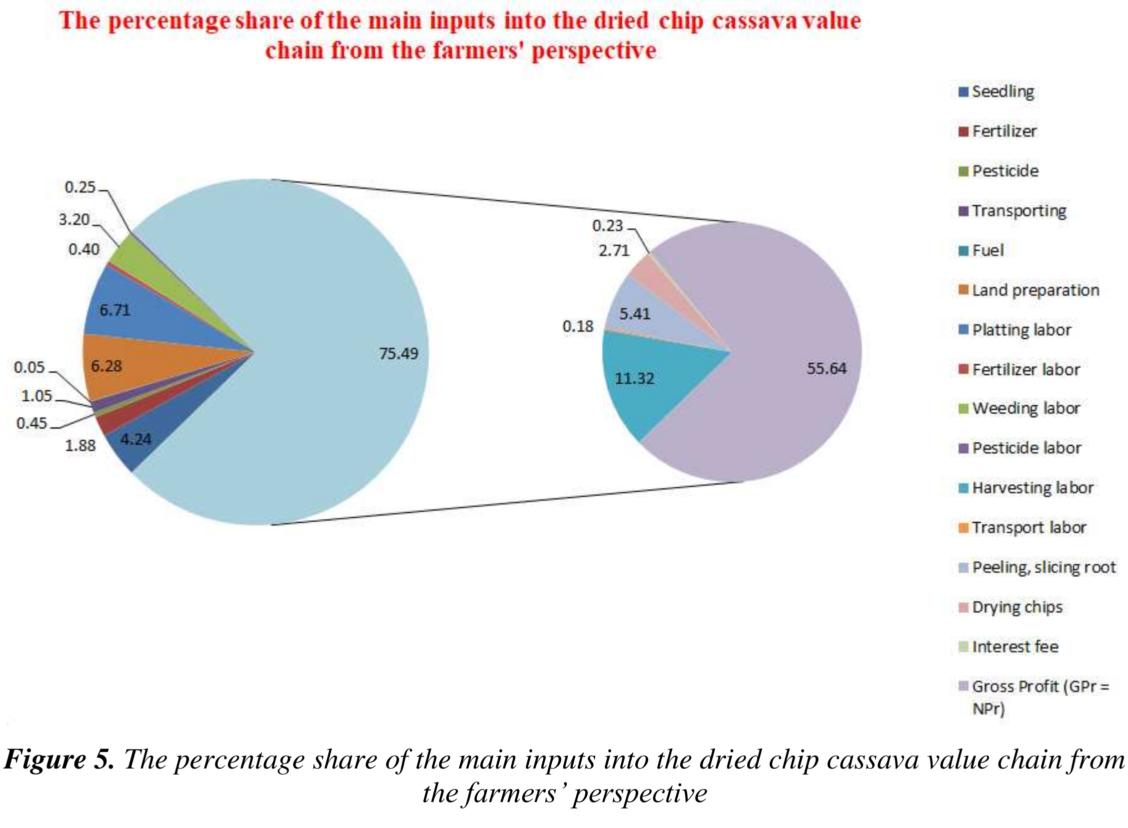

Figure 5. *The percentage share of the main inputs into the dried chip cassava value chain from the farmers' perspective*

The study found that based on different market price and productivity scenarios, the producer can always gain profit from their production. This is an advantageous situation and one that is attractive to household farmers who participate in the cassava value chain. In the worst-case scenario with both a low selling price and productivity, the farmers GPr was VND215 per kg, while the producer's GPr was VND1,729 per kg (*Fig. 6*) for the best scenario with a lucrative market and high productivity coinciding.

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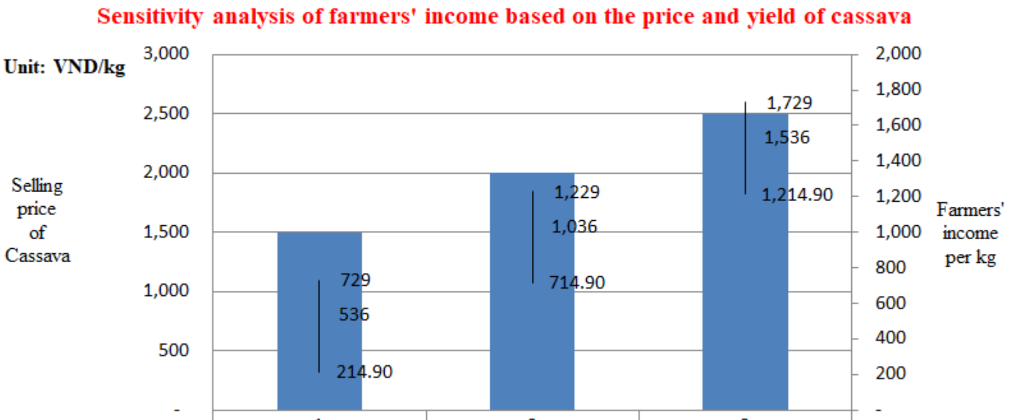

	1	2	3
.	1,500	2,000	2,500
Low productivity	215	715	1,215
Average productivity	536	1,036	1,536
High productivity	729	1,229	1,729

Figure 6. *The sensitivity analysis of farmers' income based on the price and yield of cassava*

The collector perspective

The analysis of the financial situation from the collectors perspective shown in, *Table 3* indicates that, the amount expended by collectors for fresh cassava was approximately VND2,338/kg of which, the cost of fresh cassava accounted for the highest proportion of approximately 85%, followed by transport costs accounting for 9.42%. At this stage, the value added was created less than farmer with 6.15% compared to mostly over 90% in dried chip cassavas. However, a large quantity of produce was purchased by the collectors and the GPr and NPr of VND62,650 and VND61,530 per ton respectively were both higher than those earned by the farmer. According to Viet et al. (2013), it is normal for an amount to be deducted from the price paid by collectors to cover impurities in the cassava supplied by the farmer. This ranges from 7 to 10% of the purchase price depending on the time after harvesting as well as the moisture content and this finding was supported by our study. The quality of cassava tubers deteriorates depending on the harvesting season, the length of cultivation and the time to storage. Moreover, the collector is usually faced with having to provide finance due to delays in payment by the starch factories and this will also affect the purchase price paid to the farmer. The business pattern is also similar for the sale of dried chips by farmers to traders, and starch and ethanol factories. The GPr and NPr are actually little different from the fresh root model at VND62,550 (US$2.67) and VND57,550 (US$2.46) per tonne, respectively. However, in the case of dried chips, the collectors have more opportunity to locate the best market for their produce. The quality of cassava tubers depends on the harvesting time and length of storage. Generally, the percentage of starch reduces proportionally based on the length of storage by as much as 10% after a matter of days. In addition, the processing factories often postpone payments to collectors for several weeks because the factories face short-term financial constraints. The collectors do not have any other options although their business activities are affected by this problem. One of the reasons is that the purchasing system in this area has not yet developed and the local market for cassava is excessively dependent on demand from the processing factories (*Fig. 7*).

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Table 3. *Major indicator analysis of fresh cassava value chain per ton from the collectors' perspective*

Item	Value (VND1,000)	Value USD	Proportion %
Output	**2,400.00**	**102.61**	**100.00**
Intermediate input	**2,252.00**	**96.28**	**93.83**
Cassava root	2,025.00	86.58	84.38
Transportation	226.00	9.66	9.42
Communication	1.00	0.04	0.04
Value added	**148.00**	**6.33**	**6.17**
Labour wages	47.50	2.03	1.98
Interest	12.85	0.55	0.54
Handling	25.00	1.07	1.04
Gross profit (GPr)	**62.65**	**2.68**	**2.61**
Depreciation	1.12	0.05	0.05
Net profit (NPr)	**61.53**	**2.63**	**2.56**

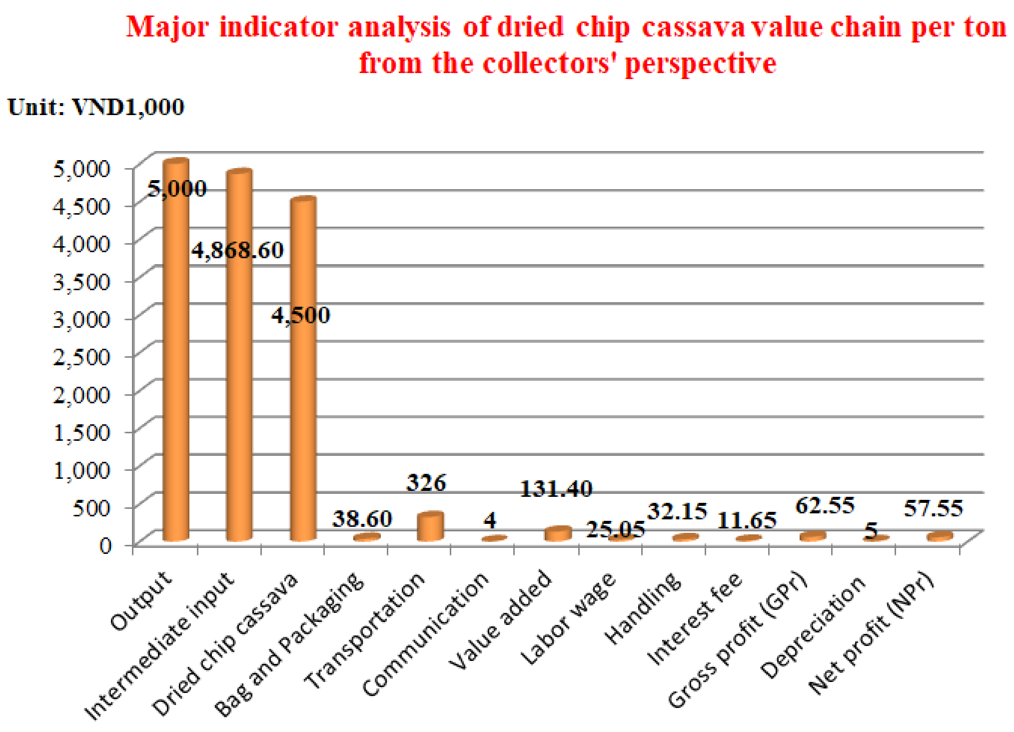

Figure 7. *The major indicators analysis of dried chip cassava per ton from the collectors' perspective*

The trader perspective

The traders' business pattern is similar to that of collectors, with traders buying some fresh cassava as well as dried chip from producers in February and March but also obtaining most (95%) of their cassava from collectors in both root and chip forms (*Fig. 4*). The quantity purchased ranges between 20 and 25 tons per day with this figure reaching a peak of 40 to 50 tons per day during the harvesting season. It is notable that

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the purchase price paid by traders is very similar to that paid by collectors who buy cassava directly from farmers.

Furthermore, traders usually negotiate with farmers in order to fix the price of cassava before it is harvested. However, changes in the market can affect this practice and, for instance, if the market price is greater than the price fixed before harvesting, then the purchase of the cassava will be concluded based on the market price. In contrast, if the market price is lower, the sale will be concluded based on the price fixed which represents a fair trading relationship between these actors in the cassava value chain. The average selling price of fresh and dried chip cassava were VND2.85 million (US$121.85) and VND5.5 million (US$235.14) per ton, respectively, and the gross profit gained was VND75,930 (US$3.25) (*Table 4*) per ton for cassava root and VND75,040 (US$3.21) per ton for dried chip cassava (*Fig. 8*). Nevertheless, traders play a vital role through their relationship with the farmer because they usually facilitate the supply of intermediate inputs, such as fertilizer, pesticide and herbicide, as well as financing the living costs of household farmers. The farmers can borrow money from the traders in order to deal with day-to-day demands which they have to face, including the cost of food, education and health care.

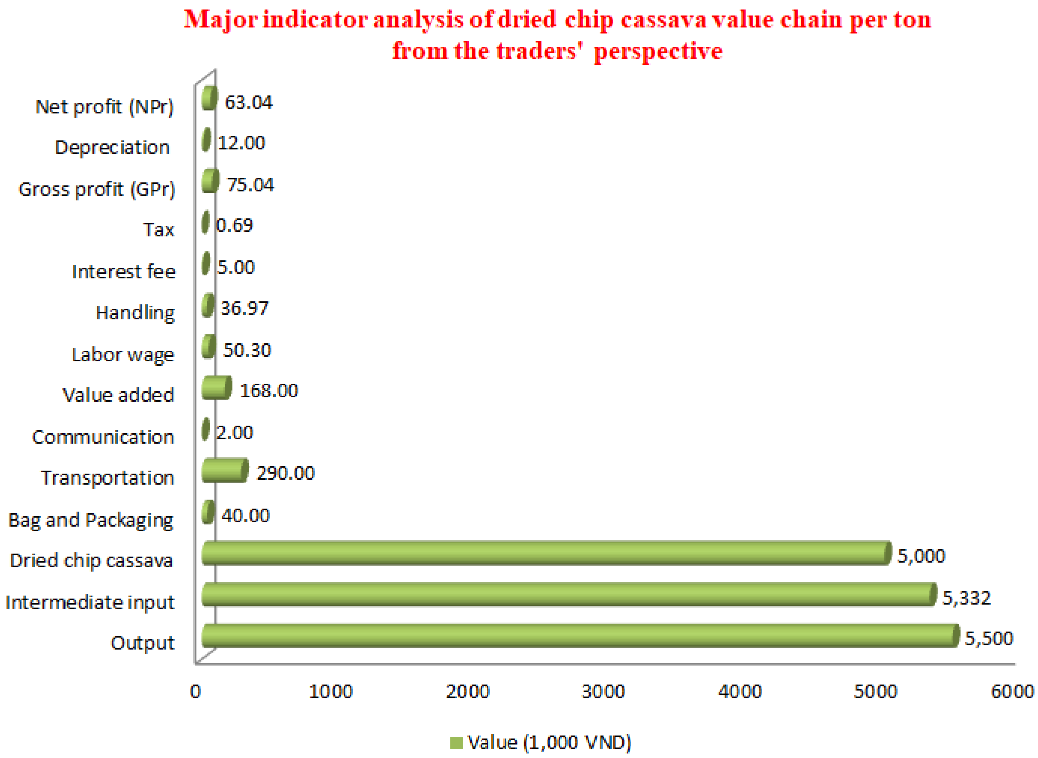

Figure 8. *The major indicators analysis of dried chip cassava per ton from the trader' perspective*

The starch factory perspective

The feedstock used by starch factories is mostly derived from traders (77%) as well as directly from producers (32% their production). The intermediate cost to produce 1 ton of cassava starch is approximately VND11.2 million (US$479.5) (*Fig. 9*). The conversion rate for fresh cassava is approximately 3.5 tons of cassava to produce 1 ton

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of cassava starch (Viet et al., 2013) and the factory is also left with a pulp residue for which there is a market after processing. Hence, the gross profit derived by the factory was found to be approximately VND1.14 million per ton (US$48.57) including the revenue from both cassava starch and pulp residue. However, there is great variability in the price of cassava starch depending on the export price of starch (Son et al., 2016). Thus, the total cost and profit have been unstable over the years. This is a difficulty that most cassava starch factories are currently facing.

Table 4. *Major indicator analysis of fresh cassava value chain per ton from the traders' perspective*

Item	Value (VND1,000)	Value USD	Proportion %
Output	**2,850.00**	**121.85**	**100.00**
Intermediate input	**2,676.50**	**114.43**	**93.91**
Cassava root	2,400.00	102.61	84.21
Transportation	275.00	11.76	9.65
Communication	1.50	0.06	0.05
Value added	**173.50**	**7.42**	**6.09**
Labour wages	69.00	2.95	2.42
Interest	0.65	0.03	0.02
Tax	0.42	0.02	0.01
Handling	27.50	1.18	0.96
Gross profit (GPr)	**75.93**	**3.25**	**2.66**
Depreciation	10.00	0.43	0.35
Net profit (NPr)	**65.93**	**2.82**	**2.31**

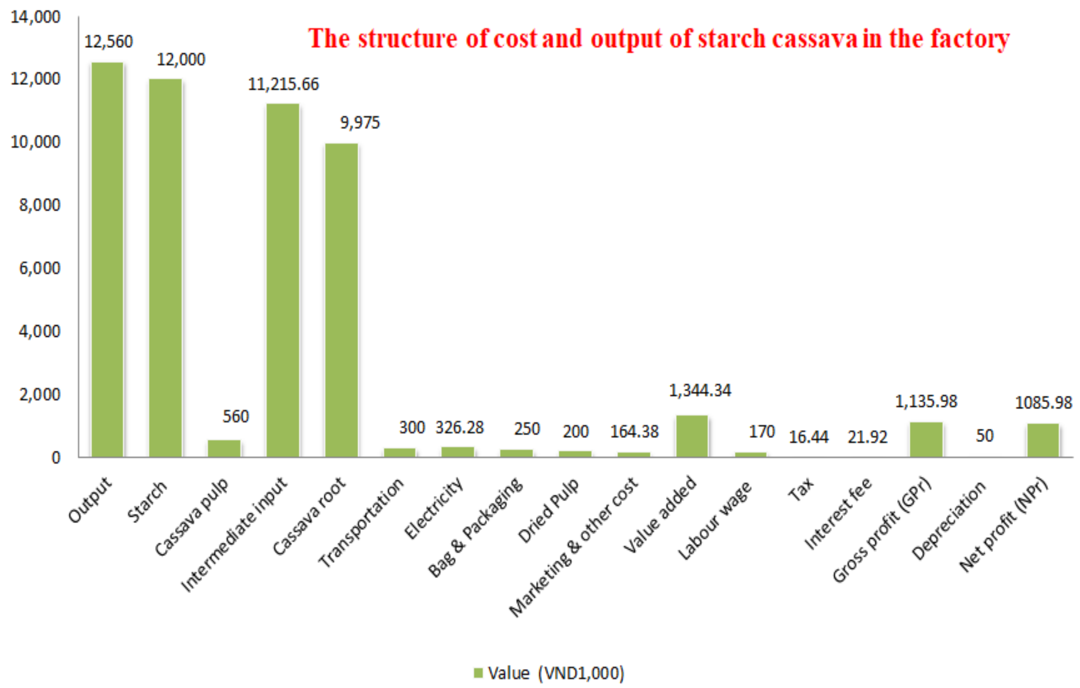

Figure 9. *The structure of cost and output of starch cassava per ton from starch factory perspective*

The ethanol factory perspective

The production capacity of the ethanol factory in this area is approximately 54,000 tons per year or around 4,500 tons per month based on market demand. The ethanol produced is used for numerous purposes in industries including food, cosmetics, and pharmaceuticals as well as others. There is a large quantity of cassava available to the factory and there is the potential to export its product to the Chinese market as well as to other overseas markets. However, currently, the total production of the ethanol factory is used domestically since its current processing costs are not competitive with that of similar products from other countries, particularly with those in Brazil which dominates world ethanol processing. *Table 5* shows that the production cost in the Dak Lak ethanol factory is around VND15.74 million (approximately US$673) per ton while the price of ethanol processed in Brazil is currently US$600 per ton at Ho Chi Minh City port in Vietnam. Therefore while the factory currently earns a GPr of nearly US$29 per ton based on current production costs, according to Mr. Dao Trong Tuan (personal communication), who is the Chairman of the board of directors of the Dak Lak ethanol factory, it will be very difficult to be competitive with prices in the global market in the near future without government support.

***Table 5**. Financial analysis of the operation of the Dak Lak ethanol factory per ton*

Item	Value (VND1,000)	Value (USD)	Proportion %
Output	**16,565.26**	**708.22**	**100.00**
Ethanol	15,900.00	679.78	95.98
Cassava pulp	560.00	23.94	3.38
CO2	105.26	4.50	0.64
Intermediate input	**15,200.00**	**649.85**	**91.76**
Cassava root	12,540.00	536.13	75.70
Transportation	500.00	21.38	3.02
Electricity	480.00	20.52	2.90
Energy	1,200.00	51.30	7.24
Dried pulp	180.00	7.70	1.09
Marketing and other cost	300.00	12.83	1.81
Value added	**1,365.26**	**58.37**	**8.24**
Labour wages	420.00	17.96	2.54
Interest	277.78	11.88	1.68
Tax	22.22	0.95	0.13
Gross profit (GPr)	**667.48**	**28.54**	**4.03**
Depreciation	324.69	13.88	1.96
Net profit (NPr)	**342.79**	**14.66**	**2.07**

The ethanol products produced in the ethanol factory are therefore at a disadvantage compared to ethanol at the global market scale, largely because of the small scale farming by which the raw material is produced, a lack of advance cultivation technology, and the provision of short-term subsidies from the Vietnamese government. *Table 5* indicates how the value added is shared among the stakeholders comprising the farmers, the ethanol and starch factories, and the collectors and traders who obtain approximately 38%, 28%, 27%, 4% and 3% respectively. From these figures, it can be

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seen that the greatest value-added is created by the farmers which is fair based on their contribution to the value chain among the stakeholders. Shifting to cassava cultivation from other crops is considered as an appropriate strategy in poverty alleviation for household farmers in rural areas. In the cultivation phase, it is the farmers who as the producers commit almost all the resources required to produce fresh and dried chip cassava. In addition, the farmers are also the stakeholders who gain the highest percentage of the NPr (48%). However, the absolute value of both their GPr and NPr was the lowest among the stakeholders in the chain due to the volume of cassava that is provided by each farmer. In contrast, while the value added by collectors and traders accounted for less than 5% of the cassava value chain their absolute profit was higher than that of the farmers since they are undertaking cassava transactions representing from 30 to 40 tons per day while most household farmers harvest less than 30 tons per hectare per year (*Figs. 10* and *11*).

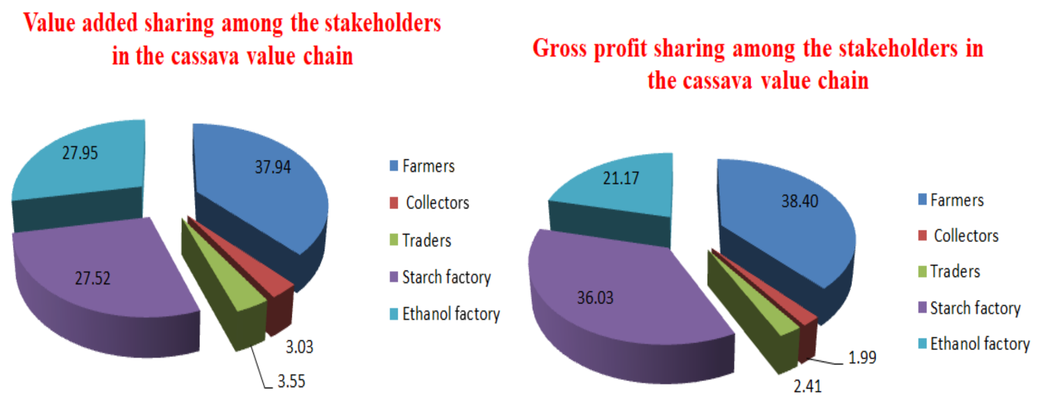

Figure 10. *Value added and gross profit sharing among the stakeholder in cassava value chain*

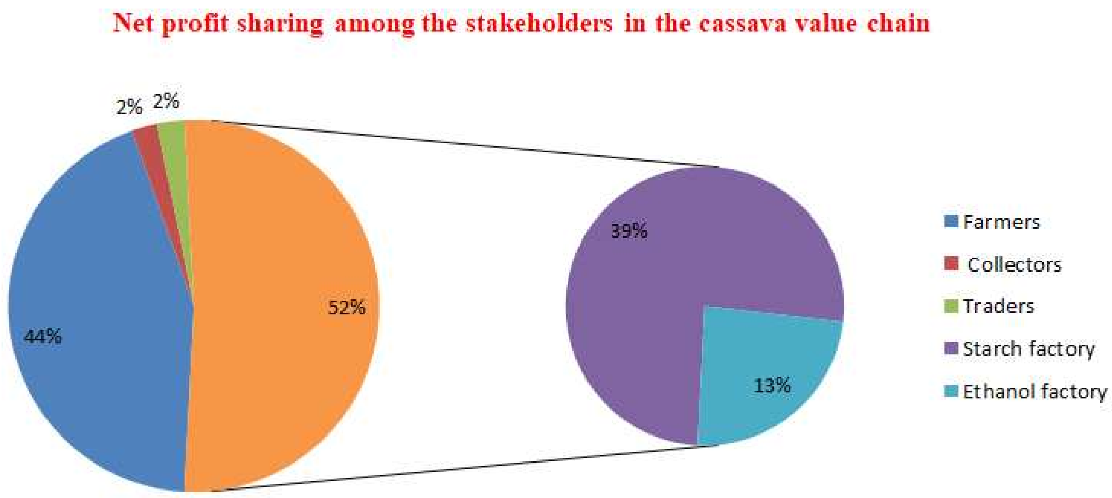

Figure 11. *The net profit sharing among stakeholder in cassava value chain*

With regard to the starch and ethanol processing factories, it is undeniable that they obtain the greatest absolute benefit from the cassava value chain since they are responsible for processing the largest volumes of cassava into products which they can

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sell. Moreover, they must meet the market demands of consumers and the value of the cassava will be increased through processing it as a raw material into starch, ethanol and other products. Hence, in order to increase the value of cassava, appropriate strategies involving linkages and collaboration among these actors is vital.

The relationships and linkages among cassava value chain stakeholders

Relationships exist amongst actors at different process stages in the cassava value chain by which input providers are connected with producers (farmers), farmers are connected with intermediaries and finally by which farmers and processors in the guise of both starch and ethanol factories are linked. Further, each of the actors has indirect relationships at each stage of the chain. The findings, of this study, identify a local relationship between input suppliers and farmers, which manifests its importance in the cassava planting season. However, this relationship does not solidify linkages since farmers generally prefer to change the location of their purchases every year. In addition, poor farmers cannot afford to buy input materials such as fertilizer and pesticide which might improve cassava productivity. Linkages between farmers and intermediaries are manifested as both spot-market and persistent relationships. On the one hand, the farmers can sell their products to any collector or trader who will pay the highest price. However, there is a potential risk in cassava value chain transactions that the selling price may be unstable over time. Buyers will always try to find any reason to reduce the purchasing price and sellers are under pressure to sell since the quality of cassava deteriorates rapidly and fresh cassava can only be preserved for two days after harvesting (El Bassam et al., 2010). This is a disadvantage for farmers who live far from the market particularly if the local infrastructure in their area is poor. On the other hand, the intermediaries can assist farmers not only by providing necessary input materials but also by extending informal credit to them. This, therefore, creates a persistent linkage; and generally, the buyer and seller meet, come to an agreement which is confirmed in a contract. In this relationship, therefore, there is a higher level of trust and some level of interdependence. Hence, the farmer and collector or trader are responsible for agreeing both the quantity of cassava and the price to be paid which is formalized by a contract agreed for each succeeding season. This linkage, therefore, tends to ensure a sufficient volume of cassava for forward actors in the value chain (i.e. end users). Moreover, the farmers will be assured of a sufficient volume of production from which they can support their occupation and that of their families. Thus, both actors gain benefit from persistent relationships.

Discussion

Our results concurred with the finding of Naziri and Bennett (2014) in both African and Southeast Asia that Cassava is mostly cultivated as a mono-crop by household farmers in small plots but was somewhat at variance with, the findings of Njukwe et al., 2014 that in Cameroon, cassava is cultivated by over 90% of the farming population, mostly as an intercrop in small plots of between 0.4 to 12 hectares. Further, the study reported herein revealed that the education status of the respondents was different from those studied by Njukwe et al. who found that 21% of the farmers had only primary education while the present study found that the majority of the participants (76.67%) had received at least secondary school education which may represent an advantage when cultivating cassava. Further, some previous studies found that not only were

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DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

cassava tubers consumed but the leaves of the cassava planted were also used as a foodstuff constituting a part in the daily food intake of some African people (Andersson et al., 2016). However, in our study, the cassava leaves were left in the field after harvesting. Son et al. (2016) found that the percentage of cassava converted to dried chip in Quang Binh province was 50% of the fresh cassava harvested. However, this rate depended on various factors such as the variety grown as well as the period that the cassava was kept post-harvest. This finding is in accord with the findings of our research. Additionally, our findings suggest that in order to improve the value chain the first priority is to ensure that the products meet the needs of the market, which was also suggested by (Thanh et al., 2017) as a means of enhancing the value chain for exported agricultural products. These researchers also suggested that particular solutions need to be adopted to overcome individual problems to ensure that exported agricultural products meet the needs of the export market in order to create a sustainable value chain. The present study also reveals that low productivity and competitiveness in the cassava value chain may be due to various factors such as poor infrastructure, lack of farmer skills and limited capital resources, as well as a lack of synchronized mechanisms amongst competent authorities (Fonji et al., 2017). Our results agreed with those of Fonji et al. (2017) relating to cassava cultivation in the central region of Cameroon. Moreover, the results of the present study were in agreement with those reported by Leo (2015) with regard to the effect of growing cassava on the income of small-scale farmers in Abia State, Nigeria which suggested that in order to increases the income level of farmers, they should apply advanced technologies and enhance the capacity of buildings (Leo, 2015). Further, as some previous studies have noted, in order to be successful, all partnerships should be formalized by appropriate contracts which clearly state the roles and responsibilities of the actors across the value chain (Njukwe et al., 2014). Our findings also determined that improving the cassava value chain can be achieved by cooperation among the stakeholders. Other scholars have emphasized farmers' participation in profitable stages of the cassava value chain by strengthening coordination, growing new cassava varieties and applying novel processing technologies (Sewando, 2012). Most previous studies have observed that the most common means by which farmers transport cassava involves the farmer carrying the cassava from the field to their home, and Njukwe et al. (2014) found that transportation from the growing area in Cameroon was dominated by head or back-loads, and that 32.5% of the cassava grown was consumed in fresh form due to inadequate infrastructure. This agrees with research by Tshiunza et al. (1997) in six major African cassava producing countries where transportation from field to home was accomplished by various means but most (70%) was by head-load or back-load and that women accounted for over 80% of that form of transport. In our study, it was found that currently, more than 90% of the cassava is sold as cassava root and that most of the farmers bring their products home as well as supply it to their market using the local vehicle known as the xe cay (*Fig. 3*). In addition, our study also found that almost all (97%) of the cassava tubers are consumed as raw material for starch and ethanol production, either based on direct sales or sales facilitated through collectors or traders, with only 3% processed for other purposes. This is important and quite different from some previous studies, for instance that of Nweke (1994) who found that an average of 40% of the cassava in South and Centre, West and North West and East regions in Cameroon as provided for sale in the market while Tshiunza et al. (1997) discovered that 85% of the farm production in six major African cassava producing countries was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

consumed in households while 10% was sold directly in markets with only 5% being used for processing purposes (Njukwe et al., 2014).

Conclusion

This research used structured and semi-structured interviews with numerous cassava value chain actors, focus group discussions with farmers (Andersson et al., 2016), and in-depth interviews with key informants to construct a comprehensive overview of the cassava value chain which was also supplemented by direct participant observations in Krong Bong, Ea Kar and Ea H'leo districts in Dak Lak province. Our findings revealed that activities in the cassava value chain are dominated by males and that it is the farmers who create the highest value added while the role of intermediaries is the most profitable. It was also found that the household farmers from among the ethnic minority groups suffered most physical losses at the stage of selling their products because they tend to keep the fresh cassava for some days after harvesting it and then sell it to a collector or trader. Additionally, most of the farmers lack sufficient capital for investment in technologies etc. to improve cassava productivity and they, therefore, obtain less profit than other groups who trade in or process cassava. In the light of the foregoing result, improving the profitability of household farmers is a major challenge that will require critical and specialized budgetary and political support at national and global levels (Sattar et al., 2017). In particular, the household farmers in the Dak Lak cassava production areas have limited resources although the household farmers' incomes are improving because of the currently increasing cassava price. The study found that intermediaries play an important role in the relationship between the cultivation and consumption step and they not only provide input material but also supply informal credit that can help farmers conduct good cassava cultivation. However, these intermediaries purchase cassava at low prices from household farmers depending on the contracts they sign with them. Depending on their financial capacity, cassava is collected either as fresh cassava or as dried chips. In either case, however, most of the profits from the cassava value chain accrue to the intermediaries.

With regard to the processors, the factories who were the end users of 97% of the cassava currently grown specialize in the production of starch and ethanol. It would be beneficial for them to diversify into other cassava products to meet different market demands in the future. Our findings suggest that these actors currently receive less profit than before because of increasing input-material costs. It is undeniable that the demand from factories makes the cassava market more competitive and it is also leading to the price of the fresh cassava produced by Vietnamese farmers becoming dependent on the Chinese market, which is a very volatile and risky market. Therefore, processing factories play a vital role in the cassava value chain and their demand has led to the enhancement of the value of cassava root, which in turn has mitigated hazards inherent in the traditional market by diversifying the uses to which cassava is put to include products such as starch, flour and ethanol. On the other hand, this study revealed that although there are various distribution channels, there are, in fact, two major channels which dominate the cassava value chain. Therefore, addressing the emerging opportunities and challenges in the cassava market requires cross-sectorial participation from the full range of stakeholders in the value chain notably the government through the Ministry of Agriculture and rural development which can supply credit in the form of soft loans for fertilizer or pesticide. This would probably represent a cheaper form of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

capital financing that is currently available through intermediaries. Once interdisciplinary cooperation is committed from all sides, this will improve the operation of the cassava value chain in this region.

Finally, this finding has just focused on value added and financial cost as gross and net profit of stakeholders in the cassava value chain. It has not yet referred to the role of gender in the household such as females' decision to participate and level of participation cassava production as well as market participants. In addition, this study has not clearly analysed the role of government policy in reducing the risk of cassava market (such as price subsidy, rural credit policy). Thus, it is highly recommended for further researches regarding inequality gender and, or improving females' role in the cassava value chain as well as policy reform in order to give a boost to cassava value chain for both farmer and the rest of stakeholders.

Acknowledgements. The authors would like to thank Thailand's Education Hub for the Southern Region of ASEAN Countries (TEH-AC) and the Faculty of Environmental Management at Prince of Songkla University, Thailand for funding while conducting this research. We are grateful to the directors of the starch factory in Dak Lak as well as the Director of the ethanol factory in Dak Nong province who kindly contributed to this research by sharing their experiences in in-depth interviews. We would like to acknowledge the contribution of all the respondents as well as the local authorities in the three districts. They all give us valuable information during the field research and farmer group discussions and interviews. Finally we would also like to say thank you to both Mr. Le Khac Dong who given us valuable recommendations relating to environmental aspects and to Mrs. Vu Trinh Vuong who made a valuable contribution associated with economics aspects of the research.

Conflict of interests. The authors confirm that there is no conflict of interests related to the content of this article.

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APPENDIX

Figure A1. *Group discussion with farmers. (Photo by author)*

Figure A2. *Cassava field trip. (Photo by author)*

Figure A3. *In-depth interview with the director of the factory. (Photo by author)*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_51935217

ANALYSIS OF PROMISING BARLEY (*Hordeum vulgare* L.) LINES PERFORMANCE BY AMMI AND GGE BIPLOT IN MULTIPLE TRAITS AND ENVIRONMENT

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(Received 21st Jan 2019; accepted 28th Feb 2019)

Abstract. The development of stable and adaptable new cultivars are based only on positive results obtained from the interaction between the genotype and the environment. Therefore, the study aimed to test the stability and general adaptability of promising barley lines in terms of grain yield and traits in multi-environments. For this purpose, twelve barley genotypes were used in the study. The trials were carried out with four replications in a random design at seven environments in years 2012-13 and 2013-14. The superior and stable genotypes were identified with GGE biplot and AMMI (Additive main effects and multiplicative interaction) models. The AMMI analysis showed that the major treatment sum of squares was affected by environments (80.6%), GE (14.0%) and genotypes (5.4%), respectively. On the other hand, the first two principal component axes (PCA 1 and PCA 2) contributed to the complete interaction with 88.1%, whereas, PCA 3 and PCA 4 axes only with 12.0%. The GGE biplot indicated that G4 is adaptable for all environments, while Altikat, G2 and G3 showed specific adaptation to E1, E3 and E5, G6, G7 and G8 to E6, respectively. According to both techniques, G2, G3, G6, G7, G8 and Altikat were the best genotypes with high yield, whereas G4 was the best with high yield, and stable and general adaptation. The results of biplot indicated that G4 (ARUPO /K8755//MORA/3/CERISE/SHYRI//ALEL I/4/CANELA/5/HART-BAR) was recommended for release and it was released as HEVSEL in 2017. On the other hand; G7 and G6 were protected as genetic material to use as parent in breeding programs for yield stability and quality respectively.
Keywords: *breeding, barley, stability, GEI, grain yield*

Abbreviations: AMMI, Additive main effects and multiplicative interaction; AEA, average-environment axis; AEC, average-environment coordination; GE, genotype by environment interaction; GGE, G + GE; MET, multi-environment trials; PC, principal component; PCA, principal component analysis; E, environment; G, genotype; TGW, thousand grain yield; HW, hectoliter weight; PC, protein content; HT, time of heading; PH, Plant height

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most commonly used crop after wheat, rice and corn worldwide and this crop is more durable than other grains for stress conditions (drought, salinity, temperature stress), which is important in arid and semi-arid regions based on rainfall grows (Vaezi et al., 2017). The grains of barley are used as animal feed, food and biodiesel as well as raw material in malt industries to obtain

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52195233

malt. Approximately 65-70% of the produced barley in the world is used as animal feed, 33-35% as malt in beer, whiskey and biodiesel production and 2-3% as human food in food production. The consumption of barley in Turkey 90-92% is used as animal feed and the rest of it as malting in brewing and food industry (Anonymous, 2019).

The potential of a genotype in any environment is determined by the effect of environmental (E), genotype (G) or interaction (GE or GEI) factors. Because the breeders need quite stable cultivars in different environmental conditions and main some traits for agronomic, new varieties must have reliable results in a wide range of environments (Solonechnyi et al., 2015). The reason for the basic differences in the performance of genotypes in wide environments is due to the interaction of the genotype with the environment (Kendal and Aktas, 2016; Neisse et al., 2018). Genotype environmental interaction (GEI) analysis is of primary importance, as is the case for other crops inn barley breeding and many other intermediate studies (Kilic, 2014).

The most effective method to predict G, GE and GEI effects is multiple environmental trials (METs). METs are the most appropriate method to select the best genotypes for any environment and to identify genotypes that keep their genetic potential in many different environments (Farshadfar et al., 2012). Since the data obtained from METs are quite high, it is difficult to interpret these data without graphs. Therefore, different models have been used recently by many investigators to evaluate the data obtained from studies conducted in different environments. At the beginning of these methods, AMMI and GGE biplot methods are used and the shapes obtained from these methods visually provide the breeders with very important facilities in terms of the performance of the genotypes (Mortazavian et al., 2014; Solonechnyi et al., 2018). The AMMI model provides more information to researchers about the stability of genotypes in terms of grain yield, while GGE biplot on the relationships between genotypes and properties (Mohammadi et al., 2016).

The aim of this study was to evaluate the multiple traits data obtained from the studies conducted with advanced barley lines in multiple environmental conditions with AMMI and GGE biplot models and to determine the most stable genotypes as registration candidates and to present the benefits of these models to the breeders.

Materials and methods

Material

Ten spring barley promising lines and two checks were evaluated in seven locations across 2012-2013 and 2013-2014 years. The introductory information of genotypes presented in *Table 1* and the introductory information about locations presented in *Table 2*. **Altıkat** cultivar used in the study as control; because this cultivar was released in 2011in research area. So, it is very stable among cultivars which used in research on grain yield. Because it is regional and majority barley growers prefer the 6 rows cultivar in this area. **Şahin 91** cultivar used in the study as control; because this cultivar is national a cultivar, and it is facultative type and used in common in north part of region, because it is resistant to cold damage which is sometime happening in spring time. Therefore the genotypes compared with this cultivars for grain yield and other properties of traits).

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DOI: http://dx.doi.org/10.15666/aeer/1702_52195233

Method

The trials was carry out in a randomized block design with four replications and planting density was used 450 seeds in per m^{-2}. Plot size was 7.2 m^{-2} in sowing time (6 m long × 1.2 m wide) planted at 20 cm spaced and composed of a total of 6 rows. Sowing of trials were done in October month and Harvest were done in June in both of season and across locations. The sowing dates and harvest dates in all locations are not very effective, because the harvesting time is dry usually and sowing were done after rainfall. The fertilizing percentages were used 60 kg N ha^{-1} and 60 kg P ha^{-1} with planting and 60 kg N ha^{-1} was used to each plots at tillering time for all plots. The chemical struggle was done against weeds in the period of 2-4 leaves in all locations and seasons. Harvesting was done using a Hege 140 harvester in an area of 6 m^2 in each plot.

Table 1. *The information's about genotypes, used in experiment*

Genotypes	Pedigree of genotypes	Spike rows
G1	NK1272/Moroc 9-75/6/ VICTORIA/4/GL/COPAL/TERAN 78/5/ SHYRI/7/ CUNH./3/.. SEA01 04-OS.0S-0SD-0SD-0SD-0SD-0SD-0SD-0SD	2 row
G2	ROBUST//GLORIA-BAR/COBAL/3/KASOTA CBSS00M00027S.0S-0SD-0SD-1SD-0SD--0SD-0SD-0SD	6 row
G3	CABUYA/JUGL CBSS00M00060S.0S-0SD-0SD-01SD-0SD-0SD-0SD-0SD	6 row
G4	ARUPO/K8755//MORA/3/CERISE/SHYRI//ALELI/4/ CANELA/5/HART-BAR CBSS00M00098S.0S-0SD-0SD-1SD-0SD-0SD-0SD-0SD	2 row
Altikat	Arta/4/Arta/3/Hml-02//Esp/1808-4L (ICB96-0601-0AP-10AP-0AP)	6 row
G6	ARUPO/K8755//MORA/3/CERISE/SHYRI//ALELI/4/ CANELA /5/HART-BARCBSS00M00098S.0S-0SD-0SD-2SD-0SD-0SD-0SD-0SD	2 row
G7	ARUPO/K8755//MORA/3/CERISE/SHYRI//ALELI/4/ CANELA/5/HART-BAR CBSS00M00098S.0S-0SD-0SD-4SD-0SD-0SD-0SD-0SD	2 row
G8	RECLA 78/SHYRI 2000 CBSS00M00122S.0S-0SD-0SD-4SD-0SD-0SD-0SD-0SD	2 row
G9	CUCAPAH/PUEBLA/7/ROBUST//GLORIA-BAR/COPAL/3/ TOCTE /6/GLOR/… CBSS00M00206S.0S--0SD-0SD-5SD-0SD-0SD-0SD-0SD	6 row
Şahin 91	YEA 1553-1/Eskişehir.	2 row
G11	TAPIR-BAR/PETUNIA 1 CBWS00WM00056S.0S-0SD-0SD-1SD-0SD-0SD-0SD-0SD	6 row
G12	ROBUR-BAR/142-B//ASTRIX/SUTTER334.3/3/3CABUYA CBSS00M00053S.0S- 0SD-0SD-2SD-0SD-0SD	6 row

Table 2. *Years, sites, codes, coordinate status of environment long term of precipitation*

Years	Sites	Code of sites	Altitude (m)	Latitude	Longitude	Annual rainfall (mm)
2012/13	Diyarbakir	E1	496	36° 97' N	38°42' E	680.6
	Adiyaman	E3	483	37° 46' N	40^0 56' E	592.0
	Hazro	E2	895	38° 15' N	40° 49' E	743.9
2013/14	Diyarbakir	E4	496	36° 97' N	38°42' E	356.7
	Adiyaman	E6	685	37° 46' N	40^0 56' E	592.0
	Hazro	E5	895	38° 15' N	40° 49' E	743.9
	Ceylanpinar	E7	363	36° 51' N	40° 20' E	260.3

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The described growing seasons 2012-2013

After exiting the plants, the barley was developed with the cool weather and cold damage occurred in extreme winter days. Seasonal conditions were favorable for barley cultivation in the development period, but in March and April, partly due to arid history, the expected high yield was not obtained. Adiyaman location during the spring period to partially heat stress, Hazro location was exposed to frost damage in late spring. In Diyarbakir location, conditions were more favorable than other locations.

The described growing seasons 2013-2014

Seasonal conditions were negative in terms of barley cultivation in the development period and snowfall occurred on March 31, 2014, especially when the barley was in the period of stalking. Genotypes were exposed to drought in Ceylanpınar location. In the Adiyaman location, it was partially exposed to temperature stress and cold damage. In the Hazro location, frost damage occurred during the period of erasing. In Diyarbakır, cold stress were effective in March and heat stress after heading period. Therefore the grain yield of 2012-2013 season was suitable than 2013-14 growing season. The conditions of Ceylanpınar location is usually hard than other location, because the the total of rainfall of seasons usually is low (*Table 2*).

The protein content was measured in the NIT (Near-infrared transmittance) instrument.

Statistical analysis

The AMMI analysis was used to ranking of the genotypes based on grain yield and GGE biplot was used to see the relation hip between genotypes and tarits across seven environment and two years The AMMI model used as recommended by Verma et al. (2016), and GGE biplot were used as recommended by Yan and Thinker (2005), to identify the mega- environments and superior genotypes. Statistical analyzes of data were performed using JMP and GenStat 12 analysis programs.

The graph (1) introduced by AMMI analysis based on grain yield of across years, and other graphs (2-6) generated by GGE biplot, (2) the ranks genotypes based on stability in aceoss environments, (3) which-won-where/what of GT biplot based on across years, (4) the ranks genotypes based on ideal genotype, (5) the relationship between genotype by trait, (6) the relationship between environment by trait.

Results

The AMMI analysis showed that the major treatment sum of squares were effected by environments (80.6%), GE (14.0%) and genotypes (5.4%), respectively (*Tables 3* and *4*). The major variation of yield explained by the circles showed that the environments varied and the majority of the variation in yield was due to environmental changes. On the other hand, the first two principal component axes (PCA 1 and PCA 2) distributed to the complete interaction 88.1%, whereas, PCA 3 and PCA 4 axes only 12.0% (*Table 5*). The majority percent of multiplicative variance of the sum of the squares based interaction influenced by the first two PCA scores.

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Table 3. *The average yield performance at each E and over environments (kg ha^{-1})*

Genotype	E1		E2		E3		E4		E5		E6		E7		Mean of E.	
1	5268	bc	4042	bd	4810	b	3675	be	4299	ac	3535	bd	1751	d	3911	CD
2	6310	a	4604	ac	4969	b	3467	cf	4109	ac	3056	de	1715	de	4033	BC
3	6399	a	4494	ad	5163	ab	3150	df	4385	ab	3321	ce	2033	c	4135	BC
4	6360	a	4775	ab	5008	ab	4617	a	4732	a	3967	bc	2695	a	4593	A
Altıkat	5748	ab	4925	a	5925	a	3860	ad	4220	ac	3977	bc	1159	f	4259	B
6	4806	c	3746	d	3721	c	4288	ab	5147	a	4867	a	2354	b	4133	BC
7	5650	ac	3896	cd	5035	ab	4171	ac	4295	ac	3925	bc	2847	a	4260	B
8	5008	bc	4021	bd	4710	b	4066	ac	4571	a	4893	a	1891	cd	4166	BC
9	5494	ac	4525	ad	5071	ab	2705	f	3488	bc	2545	e	1518	e	3621	DE
Şahin 91	4877	bc	4167	ad	4988	ab	2995	ef	3478	bc	4216	ab	1255	f	3711	DE
11	5692	ac	4092	bd	4646	bc	3170	df	3292	c	3474	bd	834	g	3600	E
12	5313	bc	3746	d	5188	ab	3689	be	4100	ac	3264	ce	1760	d	3866	CE
Mean	5577	A	4253	C	4936	C	3654	F	4176	CD	3753	DE	1818	E		
CV(%)	11.4		12.8		13.4		14.6		17.5		14.6		19.6		14.2	
LSD	92.1*		78.6*		94.8*		77.5**		104.8*		78.8**		19.7**			

Table 4. *The variance of AMMI analysis on grain yield of barley*

Source of Variance	DF	Sum of square	Mean of squares	F Ratio	Explained (%)
Treatments	83	500019991	6024337	18.48	
Genotypes	11	26817395	2437945	7.48**	5.4
Environments	6	403068142	67178024	61.74**	80.6
Block	21	22849272	1088061	3.34	
G x E	66	70134454	1062643	3.26**	14.0
Interaction PCA 1	16	43155999	2697250	8.27**	65.0
Interaction PCA 2	14	15320921	1094352	3.36**	23.0
Interaction PCA 3	12	4841983	403499	1.24	7.3
Interaction PCA 4	10	3093078	309308	0.95	4.7
Residuals	14	3722472	265891	0.82	
Error	231	75323191	326074		
Total	253	598192454	1785649		

Table 5. *AMMI selections the first four genotypes for per environment and PCA scores*

Sites	Mean (kg/ha^{-1})	Score	1	2	3	4	PCA [1]	PCA [2]	PCA [3]	PCA [4]
E1	5577	-25.89	G4	G2	G3	Altikat	-25.90	13.36	15.60	1.13
E2	4253	-20.52	Altikat	G3	G4	G2	-20.52	-9.37	11.20	-4.94
E3	4936	-26.34	Altikat	G12	G9	G4	-26.34	-16.11	-21.93	2.20
E4	3654	16.59	G4	G6	G8	G7	16.59	4.24	-3.67	22.48
E5	4176	15.53	G6	G4	G7	G8	15.53	8.48	5.73	3.97
E6	3753	29.19	G6	G8	Şahin 91	Altikat	29.19	-26.21	6.03	-9.28
E7	1818	11.43	G7	G4	G6	G3	11.44	25.59	-12.96	-15.56

The AMMI model showing Genotype x Environment means

The AMMI method is interpreted as bi-directional, the genotypes and environment main effect indicate by *x* axis and the effects of interaction indicate by *y* axis (*Fig. 1*).

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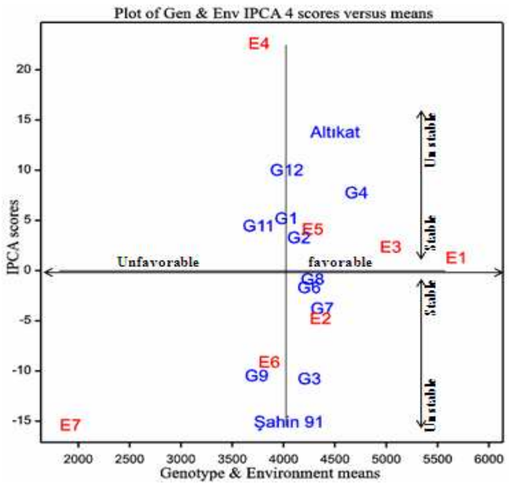

Figure 1. *The AMMI model based on grain yield (kg/ha^{-1}) of genotypes(G) in 7 environments(E)*

The main effects of genotype and environment are seen along the x-axis while the interaction effects are seen along the y-axis. On this figure; genotypes and environments are evaluated according to the mean (y axis) with stability (x axis). If the genotypes are close to the x axis, they are interpreted as stable, ıf they located the far from x axis it means that they are unstable. According to the AMMI analysis model, the genotypes showed a high variation and were found in different regions on the graph and the performance of the circles could be clearly seen. According to AMMI, E1, E2, E3 and E5 looking as high yielding environments, because these environments located upper on y axis, while E4, E6 and E7 looking as low yielding environments, because they located below of y axis. On the other hand, G2, G3, G4, G6, G7,G8 and G5 (Altikat variety) showed good performance, because of they located above on y axis (mean yield axis). It is believed that these genotypes were high yielding. But, G1, G9, G11, G12, G10 (Sahin 91) showed low performance, due to they located under on y axis (mean yield axis). Moreover, G4 is looking as high yielding genotype, but moderate stable because it was locate the far from x axis (stable line), while G8 is looking as stable, but this genotype is looking low yield potential than G4 genotype. The IPCA scores indicated that E1 could be recommended to tested genotypes, because of high yield potential of it (*Table 5*). According to Mirosavlievic et al (2014), the genotypes have small IPCA1 values are more stable, and similar outputs were recorded by Mohammadi et al (2013), in barley.

The recommendation of genotype for environment

The average grain yield of spring barley genotypes ranged from 3600 kg/ha to 4593 kg/ha in seven environments (*Table 6*). The genotype G4 was existence in the top of four environments. On the other hand, some genotypes estimated for special environments. For example; the genotype G6 dawn in the top genotype for E6, Altikat for E1, E3 and E5. Also, G4 can advise in the first to E1, E4 and the second to E5 and E7. The results of *Table 5* and *Table 6*, showed that AMMI is an effective method to evaluate the perfect genotype for all environments or specific genotype for desirable environments. Moreover, the AMMI analysis is describe recommendations of correct genotype for special environment (Bantayehu, 2013).

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The recommendation of environment

The average yield of seven environments was changed from 1818 kg/ha to 5577 kg/ha of spring barley genotypes (*Table 5*). The AMMI analysis indicated that E1 is the best among test environments, followed E3 and E2.On the other hand, E7 is looking very poor among test environments, because the rainfall of this environment is very low. According to results of AMMI analysis showed that we can recommend the E1 to describe the top yield of genotypes, while E7 for describe the genotypes on drought conditions (*Figure 1*). Xu et al. (2014) reported that the GGE biplot is the best way to determine the best environment to tested genotypes.

Table 6. *The traits value of average for twelve genotypes*

Genotypes	Heading time (date)	Plant Height (cm)	Thousand grain weight(g)	Hectoliter weight(g/l)	Protein content (%)
G1	98	83	38.1	74.2	13.2
G2	96	89	33.7	71.4	11.1
G3	97	83	40.1	72.2	12.1
G4	96	84	39.1	74.3	12.0
Altıkat	99	86	34.9	68.5	11.0
G6	97	82	42.9	74.8	13.0
G7	95	85	39.1	72.6	11.6
G8	95	82	40.5	73.4	12.4
G9	98	85	39.0	65.0	12.1
Şahin 91	107	79	42.6	70.9	13.0
G11	97	85	37.2	72.9	12.2
G12	96	87	39.0	71.2	12.3
Mean	**97**	**84**	**38.9**	**71.8**	**12.2**

Ranking genotypes based on stability and environment

The ranking genotypes indicate the ideal and stable genotype in across environments, as showed in *Figure 2*.

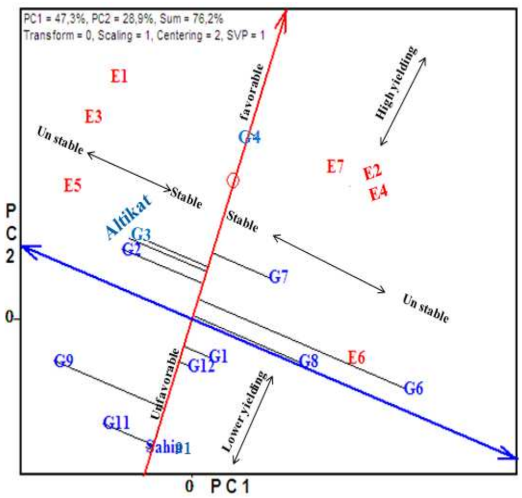

Figure 2. *The rank genotypes based on stability*

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The horizontal line with double arrow pointed the average of environment the upper of arrow points to higher mean yield through the environments, the vertical line with one arrow is ordinate the points the stability of genotypes across environments (Yan and Tinker, 2006). Thus, the genotype G4 with short genotype vector had both the highest mean yield and stability values, while Sahin 91 had low mean yield through across environment, but stable. On the other hand, G6 had above average yield, but unstable because of long genotype vector. The genotypes have large PC1 values mean that this genotype is high yielding and stable. The results of this study showed that G4 had wide adaptability for across environments, while G1, G9, G11, G12 and Sahin 91 for none environments. Some genotypes (G2, G3 and Altikat) showed special adaptability to special environments (E1, E3 and E5), G6, G7 and G8 to E6 (*Fig. 2*). Therefore, the ranking of genotypes are depend on their stability performance in across environments. Taheripourfard et al. (2017) reported that the GGE is good method to tested genotypes for multi-environment.

Which-won-where/what of GE biplot based on across environments

The GE biplot based on across season data is visualize the polygon of which-won-where/what. The figure divided by thick axis from center figure, and each zone separated by two thick lines is referred to as the "sector" and is indicated by numbers 1, 2, 3 etc., starting from the lower right part of the graph, and if the genotypes and traits located in the same sector it means that are closely related each other (Yan and Tinker, 2006; Dogan et al., 2016). Considering the *Fig. 3* with this prediction; thick axis from center figure divide the biplot into five sectors. The environments (E1, E2, E5, E6, E7) with G4 down in the sector 2; G2 and Altikat with E3 in sector3; G1, G6 and G8 with E6 in sector1; while other genotypes did not relation with any environment. Consequently, G4 had high yielding at five environments (E1, E2, E5, E6 and E7), while G6 at E6. Therefore, the best genotype is definete by the best performance in mega-environments.

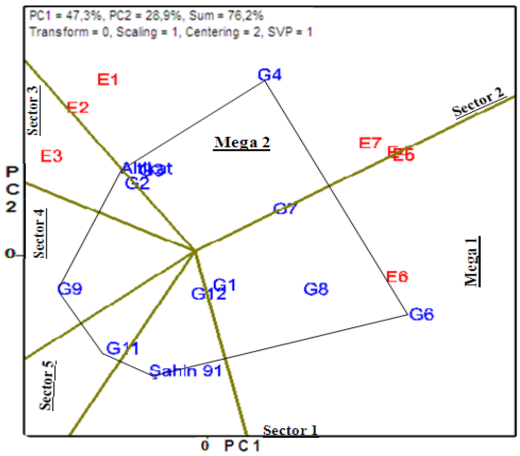

Figure 3. *Which-won-where/what of GE biplot based on across season data*

The ranks genotypes based on ideal genotype

The discriminating and representativeness of genotypes based traits are visualize the "ideal center" over the mean values of the environments and offers the opportunity to evaluate genotypes according to their proximity or distance to this center(Yan and Tinker, 2005; Oral, 2018). If the genotypes are located in the center, they are the most ideal, if they

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are located above the average vertical axis, but far from the center, it means that they are ideal, if they are located below vertical axis, it means that they are undesirable. Considering on this prediction the *Fig. 4* explained that the G4 located center of AEA, and so, it is more desirable than other genotypes, while Sahin 91 is the poorest genotype, because it is located under mean axis. The term "ideal genotype" is meaningful only when associated with mean performance. According to *Fig. 4*, the G4 is highly "ideal", other genotypes (G2, G3, G7 G6, G8 and G5 (Altikat)) are desirable genotypes, and because of G4 took places in center of AEA and other genotypes took places on above averages yield axis, and so it means that they are just yielding for specific environments. On the other hand, G1, G9, G11, G12 and Sahin 91 are very poorest for average yield, so they are undesirable genotypes across environment. From this example, we can say that the ideal genotype (G4) is the best among genotypes for across environments.

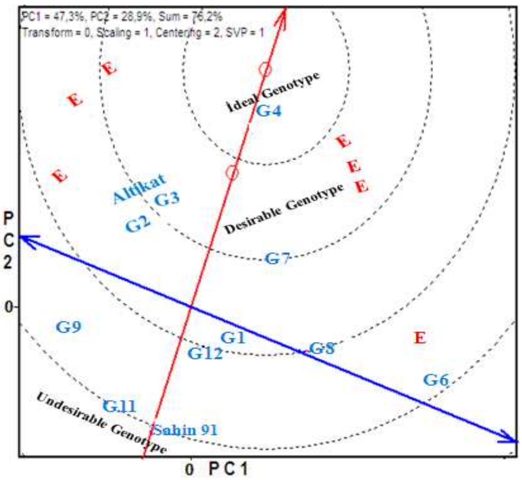

***Figure 4.** The ranks genotypes based on ideal genotype*

The relationship genotype by trait (quality) in seven environments

The relationships between genotype by trait visualize the performance each genotype on traits (*Fig. 5*).

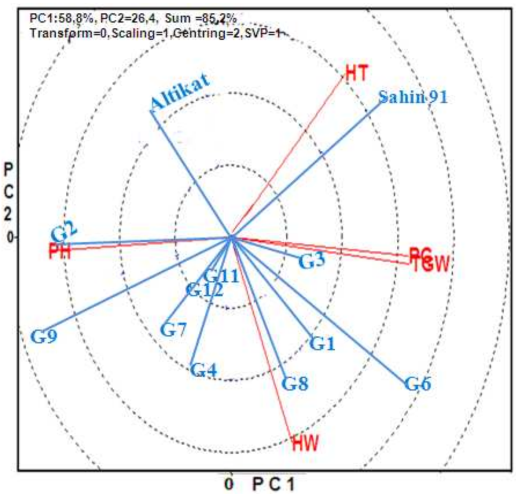

***Figure 5.** The relationship between genotype by trait*

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A biplot such a graph to be interpreted bi-directionally has the following comments (Yan et al., 2000; Yan and Tinker, 2006; Kendal and Dogan, 2015). 1) The cosine of the angle between the vectors of the two properties approaches the Pearson correlation between them. Therefore, an angle of less than 90° shows a positive correlation, an angle greater than 90° shows a negative correlation and an angle of 90° shows zero correlation. 2) If the vector of a trait is longer than other vectors, the variation of this trait on genotypes is higher than the other traits, ıf the vector length of any trait is very short than other traits vector then the variation of this trait is very low. 3) The angle between the vector of any genotype and any trait gives information about the state of the genotypes. If the angle is quite sharp and narrow indicates that the genotype is below of average for that trait if the angle is too large then the genotype is under. 4) The length of the vector of a genotype indicates the strength or weakness of the genotype for all trait profiles. Depend on these principles described in the GT biplot technique, the following observations can be made about *Fig. 3*. Considering the observations on this figure showed that there was positive correlation (the angel of vectors <90°) among protein content (PC), thousand grain weight (TGW) and hectoliter weight (HW), whereas negative correlation (the angel of vectors >90°) with plant height (PH) and these three traits. Also, the relationship between genotypes and traits were observed. Therefore, G6 associated with PC, TGW and HW, while G3 with PC and TGW G2 with PH, Şahin 91 with heading time (HT). Because of the genotypes were positioned on these traits. Consequently, the biplot showed excellent discriminating to select special genotypes with special trait and results confirmed that barley parameters were affected by G, GE, and GEI as reported by (Dogan et al., 2016).

The relationship between environments by trait (quality) of four genotypes

The relationship between environment-trait showed in *Figure 6* and *Table 7*. The information and explanations related to the identification of *Figure 6* are already given in the upper section (3.5). The biplot showed that there was positive correlation between TGW and HW, between PH and HT, whereas negative correlation among PC and other traits except PH.

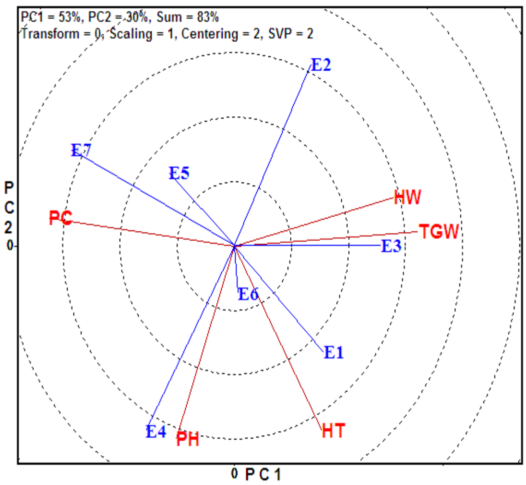

Figure 6. *The relationship between environments by trait*

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Also, the relationship between environments and traits were observed. Therefore, E3 associated with TGW, HW, while E1 with HT and E4 with PH and E5, E7 with PC. Because of these environments were positioned on definite traits. Consequently, the biplot showed excellent discriminating to select special environment with special traits and to work environments for recommendation special traits.

***Table 7.** The traits value of average over environments*

Environments	Heading time (date)	Plant Height (cm)	Thousand Grain weight(g)	Hectoliter weight(g/l)	Protein content(%)
E1	105	98	38.1	71.8	12.0
E2	97	60	40.1	70.5	13.4
E3	105	80	38.4	73.1	11.1
E4	109	98	33.6	67.1	15.5
E5	93	85	31.9	69.8	13.8
E6	101	95	35.8	71.1	14.4
E7	92	85	30.6	69.4	18.3
Mean	100	86	35.5	70.4	14.1

Discussion

The methodology of AMMI biplot approach have been reported to be a comprehensive and effective method since it classifies genotypes according to their levels in combination with target environments and graphically rank the genotypes with their strengths and weaknesses in different environments (Yan and Tinker, 2006). On the other, GGE biplot method allows breeders to establish an relations between genotype by traits or environment by traits (Dehghani et al., 2006; Stanisavlievic et al., 2013; Karami et al., 2018; Oral, 2018). The selection genotype by AMMI and GGE biplot method helps breeders to evaluate genotype on grain yield, and to seen the relations between genotype by traits or environment by traits (Sayar and Han, 2015; Dogan et al., 2016).

The AMMI analysis showed that the main effects of genotype (G), environment (E) and GE interaction are very important (*Fig. 1* and *Table 4*). The results of AMMI showed that a large variation explained by environments (80.6%), while a little variation by genotype effect (5.4%). Therefore, the effect of the environment on the fluctuation and variation of the grain yield was found to be higher than the genotype effect and interaction. Many researchers have reported that they have achieved similar results in their AMMI studies (Bantayehu, 2013; Shukla et al., 2015; Kendal and Tekdal, 2016). According to Stanisavlievic et al (2013), majority percent of treatment variation explained by environment, while genotype and interaction effect is very low. On the other hand, the majority percent of multiplicative variance of the sum of the squares based interaction influnced by the first two PCA scores. Vaezi et al. (2017) reported that in the AMMI model in different parameters, because of stability analysis was performed using multiple IPCA numbers, and so it is better than stability analysis using the first IPCA score. Hense, the study showed that G4 is looking as high yielding genotype, but moderate stable, while G8 is looking as stable, but this genotype is looking low yield potential than G4 genotype. According to these explanations, it is possible to determine the best registration candidate for the Southeastern Anatolia Region of Turkey with AMMI method and this study can be used successfully for other regions, countries in different plants as well as.

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GGE biplot analysis establishes a framework for classifying target test environments that differ between genotypes which are stable and yielding (*Fig. 2*).The study showed that G4 is stable for all environment, while G6 is unstable for majoirty environments. If the effect of genotype in the variation (G) is quite large (47.3%), PC1 scores will be highly correlated with G and PC2 (28.9%) is controlled by GE interaction (Yan and Tinker, 2005; Kendal and Aktas, 2016; Kendal et al., 2016; Vaezi et al., 2017; Oral et al., 2018). GGE biplot gives an idea to evaluate the relationships between all circles (*Fig. 3*). The GGE biplot defines the relationships between all circles based on the general model of MET data, whereas the simple correlation coefficients define only the relations between the two environments (Farshadfar et al., 2013). In the study, there was positive and significant correlation among E1, E2, E3 and also E4, E5, E7 in which-won-where graphical pattern. In fact, the GGE biplot polygon image is the best method used to determine the best genotype in every mega environment (Elakhdar et al., 2017). The study indicated that G4 is the best genotype for two mega-environment (*Fig. 3*), while G6 only for mega 1. The genotypes which are favorable discriminating and representative of across environments (Dehghani et al., 2006; Jalata, 2011; Kendal and Sayar, 2016).The study indicated that G4 is favorable genotype on discriminating and representative across test environments (*Fig. 4*). According to these explanations, it is possible to determine the best registration candidate for the Southeastern Anatolia Region of Turkey with GGE biplot method and this study can be used successfully for other regions, countries in different plants as well as. On the other hand, the results of the study showed that it was possible to make environmental-genotype relations, the stability of genotypes in all circles, the most ideal genotypes and mega environment in all circles with the figures formed by GGE biplot method.

Conclusion

The results of the study evaluated with AMMI and GGE biplot; the genotype (G), environmental (E) main effects and the and GE interaction effect was significant for tested advanced barley genotypes warm and warm-half environments of Turkey and other similar countries. The genotype (G4) showed best performance on grain yield among genotypes across environments, therefore this genotype was desirable in terms of high mean yield and stability. On the other hand; specific genotypes were appropriate for specific environments (G2-E5, G7-E2, G9-E6) and E1 was the best yielding. The result of study indicated that G4 is suitable to recommend for release and G7 desirable origin for yield stability and G6 valuable source for quality to use in barley breeding program. These three lines were obtained from ARUPO /K8755//MORA/3/CERISE/ SHYRI//ALELI/4/CAN ELA/5/HART-BAR hybrid. Depend on the results G4 was released as **HEVSEL** in 2017. On the other hand; G7 and G6 protected as genetic material to use as parent in breeding program for improve grain yield and quality, respectively. The multiple environment data can be evaluated by AMMI and GGE biplot analysis, because these two analysis methods allow a meaningful and useful summary of genotype performance across test environments.

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DOI: http://dx.doi.org/10.15666/aeer/1702_52195233

APPENDIX

Picture 1. *The locations of research was presented in Southeast Anatolia of Turkey*

Picture 2. *A photo of the genotypes in the trials*

Picture 3. *A photo of before and after maturity time of genoteypes in the trials*

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DOI: http://dx.doi.org/10.15666/aeer/1702_52195233

ENDOSULFAN CONCENTRATIONS IN ASSOCIATION WITH SERUM BIOCHEMICAL PARAMETERS AND RISK OF CANCER

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(Received 19th Jan 2019; accepted 27th Feb 2019)

Abstract. The present study was conducted to evaluate the association between residues of α-endosulfan, β-endosulfan, endosulfan sulfate and total endosulfan (Σ endosulfan) with serum cholesterol, alkaline phosphatase (ALP) and total proteins (TP) in diagnosed cancer patients and healthy subjects of Karachi, Pakistan. Donors provided fasting blood samples with informed consent and serum was separated within two hours of blood collection through centrifugation at 2500 rpm. Levels of serum cholesterol, ALP and TP were quantified on Roche/Hitachi spectrophotometers while gas chromatograph (GC-17A) coupled with 63Ni electron capture detector was used for the quantification of endosulfan residues. Detected mean concentrations of α-endosulfan, β-endosulfan and endosulfan sulfate were higher in the cases compared with controls *($p > 0.05$)*. Mean levels of serum cholesterol and TP were found significantly reduced in the cancer cases *($p < 0.05$)* while ALP activity was found significantly elevated in the cancer cases compared with controls *($p < 0.05$)*. An association between endosulfan sulfate and cholesterol levels ($r = 0.333$) and between Σ endosulfan and cholesterol ($r = 0.245$) was detected in the cancer cases but no such association was found in the control group. The presence of higher serum concentrations of α-endosulfan, β-endosulfan, endosulfan sulfate and Σ endosulfan associated with abnormal serum biochemical parameters may be a risk factor of various malignancies in humans.
Keywords: *insecticide, cholesterol, ALP, total proteins, environmental pollutants*

Introduction

Endosulfan is a broad spectrum organochlorine insecticide used around the globe in household and agriculture to control insect pests (Dilna et al., 2018). Endosulfan has been reported in human tissues, milk and serum samples (Latif et al., 2012; Khawaja et al., 2012; Khwaja et al., 2013; Saeed et al., 2017; Attaullah et al., 2018). Endosulfan consists of α and β isomers in the ratio of 7:3 (Wan et al., 2005). The main endosulfan metabolite is endosulfan sulfate which is liphophilic and mainly accumulated in the adipose tissues (Cerrillo et al., 2005). Endosulfan causes a variety of disorders in humans including testicular cancer, prostate cancer and breast cancers (Soto et al., 1994; Romeo and Quijano, 2000; Saiyed, 2003; Ibarluzea et al., 2004), teratogenicity (Grumfeld and Bonefeld-Jorgensen, 2004; Singh et al., 2007; Silva and Beauvais, 2010), DNA damage (Bhalli et al., 2009) and neurotoxicity (ATSDR, 2015). Endosulfan damages human RBCs at concentrations ranging from 1ppb to 1ppm (Daniel et al., 1986).

Serum cholesterol is a useful biomarker of several metabolic disorders. Levels of serum cholesterol are mostly found diminished in the cancer patients compared

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DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

with the healthy subjects (Ding and Hu, 2008). There is a potential association between serum concentrations of chlorinated pesticides and serum cholesterol levels (Aminov et al., 2013; Singh and Chan, 2018). Alkaline Phosphatase (ALP) is found almost in all tissues and is comparatively stable in serum. ALP levels are found elevated in the cancer cases compared with controls as reported in colorectal carcinoma (Saif et al., 2005), breast cancer (Prabasheela et al., 2012) and osteosarcoma (Kim et al., 2017; Sahran et al., 2018).

Serum total protein contents is a good indicator for the assessment of various malignancies and stage of the disease as reported in squamous cell carcinoma (Nayyar et al., 2012). Several studies have shown a correlation between persistent organochlorine pesticide residues and alterations in enzyme levels (Azmi et al., 2006; Arshad et al., 2007; Dyk and Pletschke, 2011), serum proteins and ALP (Khan et al., 2008) and serum cholesterol, TP contents and ALP levels (Paccagnella et al., 1971).

Endosulfan is banned in Pakistan but is sold and used illegally on vegetables, fruits, crops and for other miscellaneous purposes as confirmed in recent studies across the country (Anwar et al., 2011; Randhawa et al., 2016; Ahmad et al., 2018; Hayat et al., 2018). It was therefore necessary to evaluate endosulfan residues with respect to health risk in humans. The present study was aimed to comparatively evaluate the concentrations of α-endosulfan, β-endosulfan and endosulfan sulfate in the cancer cases and healthy subjects and to find out any potential association between endosulfan concentrations with serum cholesterol, ALP and TP contents.

Materials and Methods

Analysis of endosulfan isomers (α and β) and endosulfan sulfate, cholesterol, ALP and Total Proteins was carried out in sera of the diagnosed cancer cases (n= 51) and healthy control subjects (n = 30) at Karachi, Pakistan. Mean ages and male to female ratio was (39.5 years, 47:53) in the cancer cases and (33.3 years, 90:10) in the controls respectively. The study was conducted in general population of Karachi, Pakistan with no past history of occupational exposure of the subjects to endosulfan. The study subjects were indirectly exposed to endosulfan due to intake of contaminated vegetables, food, water, fruit and milk. This shows exposure of the general population to endosulfan indirectly through food chain. Blood samples (8 ml each) were collected randomly from the cancer patients and healthy subjects with informed consent at various hospitals of Karachi. Serum was separated from the blood within two hours of collection through centrifugation and was stored at – 20 ºC at the Toxicology Laboratory, Department of Zoology, University of Karachi. Extraction, purification and quantification of endosulfan in the serum samples was carried out according to the previously described methods (Dale et al., 1970; Atuma and Aune, 1999; Moreno Frias et al., 2001). The detailed protocol for the extraction of endosulfan residues, clean-up with florisil column and quantification through Gas Chromatography coupled with Electron Capture Detector (GC-ECD) has been described elsewhere (Attaullah et al., 2018).

Cholesterol analysis was carried out according to (Allain and Roeschlau, 1974) with Cholesterol Kit catalogue no. (CHOL 12016630 122), COBAS Registered was used for this purpose. About 3.0 µl of the sample was mixed with 250 µl of R1 reagent. A photometric determination of the color intensity produced by hydrogen peroxide was made on Roche/Hitachi 902 analyzer.

ALP in the serum samples was processed according to (Bowers and McComb, 1975) with the help of ALP Kit catalogue no. (AP 7927) and quantification was carried out on HITACHI 717 Spectrophotometer, Randox Laboratories Ltd., UK. About 0.025 ml (25 µl) of serum sample was mixed with 1.0 ml of reagent and heated up to 37 °C for 5 min. A yellow colored p-nitrophenol was produced at 405 nm. The color intensity is a measure of the level of ALP in the sample.

Total Proteins in the serum were determined according to (Weichselbaum, 1946) with Kit catalogue no. (TP-11553836 316), COBAS Registered. Serum sample (5 µl each) was mixed with 90 µl of R1 reagent, then R2 reagent (90 µl) was added which formed a purple colored biuret complex. The color intensity of the biuret complex was quantified with Roche/Hitachi 902 Spectrophotometer at 546 nm.

Statistical evaluation of the data was carried out using MS Excel Office Version 365 by calculating the mean values of endosulfan concentrations, cholesterol, ALP and Total Protein contents in the cancer cases and controls. The calculated mean values of the studied parameters were compared in the cases and controls through independent t Test (two samples assuming unequal variances) with *p* values less than 0.05 showing statistically significant difference between the cancer cases and controls. Values of Pearson correlation coefficient (r) between endosulfan and serum biochemical parameters were calculated in MS Excel and were confirmed statistically through regression analysis.

Results

Elevated mean concentrations of endosulfan isomers and endosulfan sulfate (mg/kg wet weight serum) were detected in the cancer cases compared with controls. Mean levels of endosulfan in the cases versus control subjects were: α-endosulfan (0.043 *vs.* 0.03, $p = 0.56$), β-endosulfan (0.041 *vs.* 0.021, $p = 0.42$), endosulfan sulfate (0.104 *vs.* 0.1, $p = 0.62$) and Σ endosulfan (0.189 *vs.* 0.153, $p = 0.37$) (*Table 1*).

Table 1. *Mean levels ± SD of the studied parameters in the cancer cases and controls*

Studied Parameters	Cases			Controls			*P values*
	Mean	SD	Range	Mean	SD	Range	
α-endosulfan (mg/kg)	0.043	0.124	0 – 0.712	0.03	0.06	0-0.231	*0.56*
β-endosulfan (mg/kg)	0.041	0.161	0 – 1.118	0.021	0.044	0-0.183	*0.42*
Endosulfan Sulfate (mg/kg)	0.104	0.236	0 – 1.422	0.1	0.183	0-0.771	*0.62*
Σ Endosulfan (mg/kg)	0.189	0.364	0 – 2.134	0.153	0.178	0-0.771	*0.37*
Chol. (mg/dl)	116	22.7	78 – 165	157	34.9	82 – 225	*<0.05*
ALP (U/L)	128	49.5	60 – 419	109	28.6	71 – 223	*0.03*
TP (g/dl)	6.2	0.57	4.5 – 7.3	7.2	1.06	4.6 – 9.1	*<0.05*

Legend: Σ endosulfan = Sum of the endosulfan isomers (α, β) and endosulfan sulfate, SD = Standard Deviation. Normal ranges in an average adult human for cholesterol: < 200 mg/dl; ALP: 30-120 U/L; Total Proteins: 6.0 – 8.0 g/dl

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DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

Cholesterol levels were found significantly diminished in the cancer cases compared with controls. The detected mean levels of cholesterol in the cancer cases versus controls were (116 ± 22.7 mg/dl *vs.* 157 ± 34.9 mg/dl; $p < 0.05$) (*Table 1*). ALP activity was found elevated in the cancer cases compared with controls. Mean ALP levels in cases versus controls were detected as 128 ± 49.5 U/L *vs.* 109 ± 28.6 U/L; $p < 0.05$) (*Table 1*). Total Proteins in cases were found significantly reduced (6.2 g/dl) compared with controls (7.2 g/dl; $p < 0.05$) (*Table 1*).

In the cancer cases, Pearson correlation coefficient between endosulfan sulphate and cholesterol (r = 0.333; $p = 0.02$) and between Σ endosulfan and cholesterol (r = 0.245; $p = 0.08$) indicate a positive association between endosulfan and cholesterol levels as shown in *Fig. 1*. Correlation between endosulfan and ALP was found very weak in the studied cohort (*Fig. 1*). Statistical significance was not achieved between endosulfan residues and the studied parameters because significance level obtained was higher than 0.05 in all of the cases except between endosulfan sulphate and cholesterol (r = 0.333; $p = 0.02$) and between α-endosulfan and TP (r = - 0.303; $p = 0.03$) in the cancer cases (*Fig. 1*).

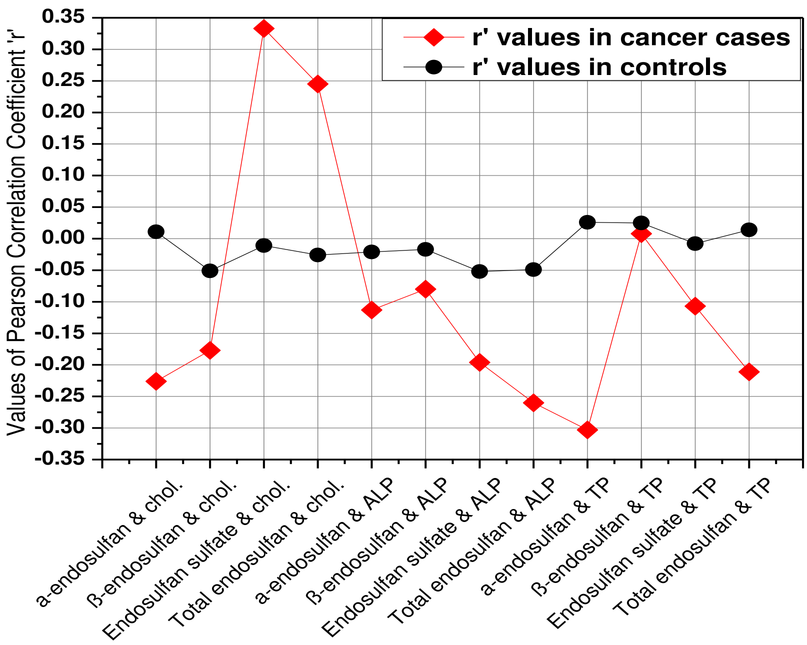

Figure 1. *Correlation between endosulfan residues with serum Cholesterol (chol.), alkaline phosphatase (ALP) and total protein (TP) contents in the cancer cases and controls*

In the major cancer sites, the Lymphatic system showed the lowest mean cholesterol level (99.6 mg/dl) with a higher concentration of endosulfan sulfate (0.107 ± 0.152 mg/kg), reduced mean ALP level (99 ± 4.2 U/L) and reduced mean TP level (5.6 ± 0.1 g/dl) (*Table 2*). Highest ALP activity was detected in the blood cancer cases (148 ± 30.7 U/L) with reduced mean cholesterol level (102 ± 19.1 mg/dl) and a mean Σ endosulfan level of 0.131 ± 0.244 mg/dl (*Table 2*).

In the sub sites of cancer cases, lowest mean level of cholesterol was detected in Hodgkin's Lymphoma (HL) as 83 mg/dl with mean level of endosulfan sulfate as 0.215 mg/kg and lowest mean level of TP (5.5 g/dl) (*Table 2*). Highest mean cholesterol level

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was detected in the ovarian carcinoma (151 mg/dl) with significantly reduced level of α-endosulfan (0.007 mg/kg) (*Table 3*).

***Table 2.** Mean levels ± SD of the tested parameters in the studied major cancer sites*

Major Cancer Sites	α-endosulfan (mg/kg) Mean±SD	β-endosulfan (mg/kg) Mean±SD	Endosulfan Sulfate (mg/kg) Mean±SD	Σ endosulfan (mg/kg) Mean±SD	Chol. (mg/dl) Mean±SD	ALP (U/L) Mean±SD	TP (g/dl) Mean±SD
Oral Cavity, Pharynx	0.007±0.013	0.089±0.082	0.07±0.121	0.167±0.054	104±23.4	112±6.65	6.5±0.5
Digestive System	0.022±0.071	0.106±0.309	0.075±0.11	0.204±0.336	127±20.7	133±87.3	6.2±0.6
Respiratory System	0.004±0.008	0	0.085±0.17	0.089±0.179	111±23.3	141.5±15.9	5.9±0.1
Breast	0.144±0.252	0.003±0.006	0.344±0.515	0.492±0.708	125±20	108±21.8	6.6±0.4
Skin	0.007±0.016	0.006±0.014	0.06±0.107	0.074±0.101	107±14.6	125±16.4	6±0.3
Female Genital System	0.002±0.003	0.007±0.014	0	0.009±0.012	136±24.1	105±24.1	6.1±0.8
Blood	0.056±0.117	0.032±0.067	0.042±0.049	0.131±0.224	102±19.1	148±30.7	6.1±0.5
Lymphatic System	0	0	0.107±0.152	0.107±0.152	99.5±23.3	99±4.2	5.6±0.1

***Table 3.** Mean levels ± SD of the tested parameters in the sub sites of cancer cases*

Sub sites of cancer cases	α-endosulfan (mg/kg) Mean±SD	β-endosulfan (mg/kg) Mean±SD	Endosulfan Sulfate (mg/kg) Mean±SD	Σ endosulfan (mg/kg) Mean±SD	Chol. (mg/dl) Mean±SD	ALP (U/L) Mean±SD	TP (g/dl) Mean±SD
Tongue	0.011±0.016	0.081±0.115	0.105±0.149	0.198±0.017	111.5±27.5	110±6.3	6.8±0.4
Pharynx	0	0.106±0	0	0.106±0	89±0	118±0	6±0
Esophagus	0.005±0.008	0	0.04±0.053	0.046±0.05	125±17.3	116±24.2	6.4±0.8
Stomach	0	0.559±0.789	0.042±0.059	0.601±0.849	125±24	119±5.65	6.2±0.1
Colon	0.065±0.13	0.059±0.101	0.005±0.011	0.131±0.228	131±18.4	106±16.7	6.3±0.3
Rectum	0	0.007±0.011	0.146±0.135	0.153±0.147	137±24.3	205±186	6±1.3
Appendix	0.01±0	0	0.318±0	0.328±0	91±0	113±0	5.9±0
Larynx	0	0	0	0	102±0	161±0	6.2±0
Lung	0.005±0.009	0	0.113±0.196	0.119±0.206	114±27.6	135±11.2	5.9±0.1
Breast	0.144±0.252	0.003±0.006	0.344±0.515	0.492±0.708	125±20	108±21.8	6.6±0.4
Neck	0	0	0.13±0.171	0.13±0.171	97±2.8	112±19.7	5.8±0.5
Cheek	0.012±0.02	0.011±0.01	0.013±0.01	0.037±0.02	113±16.4	134±8	6.2±0.1

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DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

	1	9	1	8			
Cervix	0.002±0.002	0.014±0.019	0	0.016±0.016	145±20.5	108±41	5.8±1.2
Ovary	0.007±0	0	0	0.007±0	151±0	104±0	6.1±0
Vagina	0	0	0	0	105±0	98±0	6.8±0
ALL	0.007±0.012	0.004±0.006	0.024±0.036	0.036±0.029	113±5.13	136±8.5	6.2±0.6
AML	0.092±0.146	0.053±0.083	0.049±0.055	0.195±0.281	92.8±11.5	143±34.9	6.2±0.6
CML	0	0	0.048±0.067	0.048±0.067	120±39.5	182±1.4	5.7±0.2
HL	0	0	0.215±0	0.215±0	83±0	102±0	5.5±0
NHL	0	0	0	0	116±0	96±0	5.7±0

Legend: ALL= Acute Lymphoblastic Leukemia; AML= Acute Myeloid Leukemia; CML= Chronic Myeloid Leukemia; HL= Hodgkin's Lymphoma; NHL= Non-Hodgkin's Lymphoma.

Discussion

Endosulfan is a widely used insecticide worldwide and has been associated with various malignancies in human and animal studies. The present study was conducted to evaluate the potential association of endosulfan residues with serum biochemical parameters and to ascertain the possible role of endosulfan residues in causing various malignancies in humans. Findings of the present study are consistent with the previous cases-control studies on the association of endosulfan with various cancers in humans (Kumar et al., 2010; Arrebola et al., 2015; Shah et al., 2018). Serum mean cholesterol levels and Total Proteins were found significantly reduced in the cancer cases compared with controls ($P < 0.05$) while serum ALP levels were found elevated in the cancer cases than controls ($P < 0.05$) (*Table 1*). Similar findings in cancer cases versus controls have been detected in previous studies for cholesterol levels (Mufti and Baseer, 1996; Memon et al., 2007; Chawda et al., 2011; Ahmad et al., 2012), for Total Protein levels (Nayyar et al., 2012) and for ALP levels (Saif et al., 2005; Prabasheela et al., 2012).

Variations in levels of serum cholesterol, ALP and TP from the normal levels indicate the severity of disease in various cancers and can be utilized as diagnostic and prognostic biomarkers.

An association between endosulfan sulfate and cholesterol ($r = 0.333$) and between Σ endosulfan and cholesterol ($r = 0.245$) has been detected in the cancer cases. A very weak association between α-endosulfan and cholesterol ($r = 0.011$) has been detected in the control subjects (*Fig. 1*). This indicates that endosulfan concentrations are associated with cholesterol levels and support the previous literature on the subject (Aminov et al., 2013; Singh and Chan, 2018; Fadaeipuor et al., 2016). Endosulfan and organochlorine pesticides residues increases with increasing levels of serum cholesterol and declines with decreasing levels of cholesterol. In the present study, although levels of cholesterol were significantly lower in the cancer cases but the residues of endosulfan were higher in the cancer cases in comparison with controls (*Table 1*). Apparently, reduction in cholesterol level may be an immune mechanism for decreasing the lipid pool in serum and thus reducing the storage reservoirs for lipophilic toxic chemicals in the body. Another reason for the reduced cholesterol levels in the cancer cases may be due to the adverse effects of higher endosulfan

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DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

residues in the cancer cases. A weaker association was found between endosulfan and ALP in the cases compared with controls (*Fig. 1*).

Serum endosulfan concentrations detected in the present study may not be capable to affect ALP activity as indicated by the weak association from the Pearson correlation coefficient (*Fig. 1*). Highest ALP activity (148 U/L) was found in the blood cancer cases while lowest was found in cases of the lymphatic system (99 U/L) (*Table 2*). In the blood cancer cases, detected mean levels of endosulfan were: α-endosulfan (0.056 mg/kg), β-endosulfan (0.032 mg/kg), endosulfan sulfate (0.042 mg/kg), Σ endosulfan (0.131 mg/kg) with reduced mean level of cholesterol (102 mg/dl) and elevated level of ALP (148 U/L) (*Table 2*). This indicates that the altered levels of cholesterol and ALP might be due to the presence of high endosulfan concentrations. Lowest cholesterol level (99.5 mg/dl), lowest ALP level (99 U/L) and lowest TP level (5.6 g/dl) were detected in cases of the lymphatic system with detected mean conecentration of endosulfan sulfate as 0.107 mg/kg (*Table 2*). The altered parameters in lymphatic system is indicative of the role of endosulfan sulfate in cases of the lymphatic system. A similar pattern was also detected in cases of AML, Neck and Appendicular Carcinoma (*Table 3*).

In cases of Hodgkin's Lymphoma, reduced mean TP level (5.5 g/dl), lowest mean cholesterol level amongst sub sites (83 mg/dl) and significantly elevated mean level of endosulfan sulfate (0.215 mg/kg) were detected (*Table 3*). This indicates a possible role of endosulfan sulfate in the risk of HL and an association with cholesterol and TP levels.

In the sub sites of cancer cases, highest ALP activity was found in cases of the rectal carcinoma and CML while lowest was detected in cases of NHL (*Table 3*).

The overall results indicate that cancer cases are having higher residues of endosulfan compared with controls. Serum cholesterol and total proteins are significantly lower in the cancer cases compared with controls while ALP activity was higher in the cancer cases compared with controls. There was a positive association between endosulfan residues and serum cholesterol levels in the cancer cases, but no such association was detected in the controls. Presence of lower cholesterol levels and higher endosulfan residues simultaneously may act as risk factors of various malignancies in humans. The overall results indicate that the cancer cases are having higher serum concentrations of endosulfan associated with abnormal levels of serum biochemical parameters compared with the control group.

Conclusion

The presence of higher serum concentrations of α-endosulfan, β-endosulfan, endosulfan sulfate and Σ endosulfan associated with abnormal serum biochemical parameters may be a risk factor of various malignancies in humans. Further work is recommended to ascertain the actual causes of endosulfan toxicity focusing on the mechanisms underlying alterations of serum biochemical parameters and associated health risks in humans and experimental animals.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52355244

SOIL PHYSICAL PROPERTIES AFFECTED BY SOIL PLANKING AND ROOT GROWTH OF COTTON (*GOSSYPIUM HIRSUTUM* L.)

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(Received 23rd Jan 2019; accepted 8th Mar 2019)

Abstract. In the South East Anatolia region of Turkey as well as in many parts of world, soil planker is used to level and firm the soil prior to planting of cotton (*Gossypium hirsutum* L.) at least four times. However, excessive soil compaction due to using of planker can increase soil strength and hamper root growth. This study aimed to investigate the effects of soil planking on soil physical properties, the root growth of cotton and the density of weed species. In this purpose, a field experiment was carried out at different pass numbers of a planker by using a randomized block design with three replications in 2018. The results showed that penetration resistance was increased by the pass number of the planker at 0-15 cm soil depths. While soil planking increased the soil moisture content at 15 cm soil depth, it reduced the soil temperature. Multiple passes of planker decreased the root length and dry root weight of cotton. Soil planking levels differently influenced the density of weed species. Consequently, it can be said that the planking of soil before cotton seeding may influence the root growth and the density of weed species due to changing the physical properties of soil.
Keywords: *soil packing, penetration resistance, moisture content, root length, root weight, weed density*

Introduction

The main condition for a successful cotton production is early stand of uniform seedlings that are rapidly emerging and developing. One of the key factors to accomplish this goal is a well-prepared, smoothened and firmed seedbed. The smoothening of seedbed is required for proper operation of sowing machines. Also, a firm seedbed is considered desirable for increasing emergence and seedling growth of cotton because the close contact between the soil particles and between soil and seed results in faster germination and allow more effective use of existing soil moisture (Berti et al., 2008; Tong et al., 2015). Therefore, planker are commonly used before seeding in cotton production in the South East Anatolia region of Turkey as well as in many parts of world because the using of planker can create appropriate soil condition for planted seeds by compacting soil particles to a suitable density, providing better soil-seed contact and encouraging capillary rise of water from subsoil (Tong et al., 2015; Zuo et al., 2017). However, excessive soil compaction due to using of planker can hamper root growth and decrease soil aeration, and consequently affect yield of crops. Also, it decreases infiltration rate and hydraulic conductivity by increasing bulk density and penetration resistance of soil (Nawaz et al., 2013; Singh et al., 2015).

There have been contradictory findings reported in the literature about the effects of the change of soil physical properties due to compaction on plant root growth and yield.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52455258

The effect of the same compaction degree on root growth and plant yield depends on the crop grown, soil type, and weather conditions (Dias et al., 2015). Baker (2014) reported that the effect of soil compaction was different under dry and wet conditions. He stated that although slight compaction is beneficial but too much is detrimental to yield under dry conditions, any amount of compaction can decrease yields as a result of inhibiting root respiration due to reduced soil aeration under wet conditions.

Using of a planker before seeding results in important changes in the movement and content of heat, air, water and nutrients in the seedbed. These changes within soil significantly affect plant root growth, which has an important role in the nutrient uptake and plant growth. Although several researchers (e.g. Lipiec and Hatano, 2003; Botta et al., 2010; Sivarajan et al., 2018) determined the axle load on some soil properties and yield, most of them did not investigate the correlation between the soil physical properties caused by compaction and plant growth. Also, there is little research on using of the planker before seeding in the literature. It is important to know how much the amount of packing is necessary for smoothening of seed bed and allowing more effective use of soil moisture without hampering root growth. The work by Johnston et al. (2003) showed that a packing force of 333 N per press wheel provided adequate emergence and grain yield across varied environmental conditions. They found that the higher packing force influenced emergence in canola, and not yield. Planking level of a particular soil is affected by many factors such as moisture content, type of soil, contact pressure, number of planker passes, and working speed of planker. In South-East Anatolia region of Turkey, farmers usually pass the planker at least four times before cotton seeding. An understanding of how the planking pressure and the passes number of a planker before seeding influence the soil properties and root growth would help producers apply the optimum number of passes for given type of planker in their farm and soil conditions.

The objective of this study is to investigate the effects of different pass numbers of a planker on soil physical properties (moisture content, temperature, penetration resistance), the root growth of cotton (root length, root thickness, root dry weight) and the density of weed species. Also, the relationship between the soil physical properties caused by compaction, and soil physical properties and root growth parameters will be evaluated by correlation analysis.

Materials and methods

Experiment site description and experimental design

An experiment was conducted in a farmer's field in Diyarbakır, Turkey during cotton growing season in 2018. Experimental field (latitude: 37°55’36”N, longitude: 40°13’49’E, altitude: 630 m above sea level) is located at South-East Anatolia region of Turkey. The region is characterized by a semi-arid climate (humid winters and dry summers). The annual rainfall, based on the long-term average (1929-2017) is 483.5 mm, about 80% of which occurs from November to May. Monthly rainfalls and temperature records during the experimental year and over the long term are shown in *Figure 1*. The rainfall at experimental growing season was significantly higher than long-term average at May after cotton planting. There was no much difference between mean monthly air temperatures of long-term years and 2018. Post-planting rainfall and air temperature may be expected to influence seedling emergence and plant growth by affecting the oxygen content and temperature of soil (Berti et al., 2008).

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The soil (0-20 cm) of the experimental field was clay loam with pH of 8.03, organic matter content of 15.8 g kg^{-1}, total salt of 0.08% and $CaCO_3$ of 60.8 g kg^{-1}. Average values of soil moisture content, temperature, penetration resistance at different soil depths before the application of treatments are listed in *Table 1*. The moisture content (dry basis) of soil at 10 cm depth was 10.87%. This moisture content is suitable for planking operations on a clay loam soil. The values of soil temperature and penetration resistance were within the appropriate limits for cotton emergence and seedling root growth.

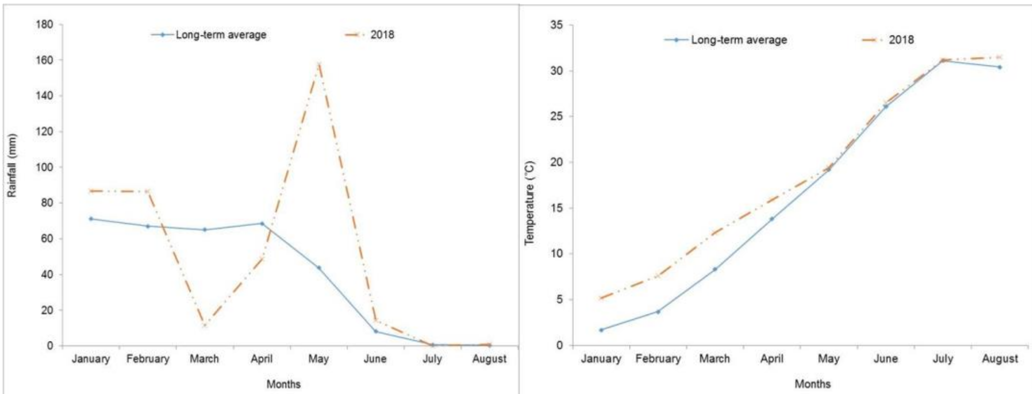

Figure 1. *Total monthly rainfall and mean temperature during experiment year*

Table 1. *Initial soil physical properties at 0-30 cm soil depths before the application of the treatments*

Soil parameters	10 cm		20 cm		30 cm	
	Mean	SD	Mean	SD	Mean	SD
Moisture content (%)	10.87	2.96	44.96	2.42	74.53	10.18
Temperature (°C)	21.30	0.8	20.85	0.45	20.40	0.20
Penetration resistance (kPa)	76.33	44.64	510.60	204.60	1007.20	146.84

The previous crop at the experimental site was cotton. The seed bed preparation included chopping the post-harvest cotton stalk, an autumn moldboard plowing (20 cm) followed by cultivator to 10 cm in April after broadcasting the fertilizer (20-20-0%, N-P_2O_5-K_2O for supplying 60 kg N plus 60 kg P_2O_5 ha^{-1}). The different pass numbers of a planker (200 kg m^{-1}) were tested by using a randomized block design with three replications. Treatments included the pass numbers of planker as untreated control (planking0), soil planking by planker in one (planking1), two (planking2), three (planking3) and four (planking4) passes. Plot size was 84 m^2 (30 m x 2.8 m). The first passing of planker was applied on 15 April 2018, and other passing was applied two days after the first passing of planker. DP-499, standard cotton cultivar for the region, was planted at seed rate of 20 seeds m^{-2} by using a pneumatic precision seed drill on 18 April 2018. The sowing depth of drill was adjusted as approximately 5 cm. First hoeing was done at about 15-20 days after sowing (DAS), second and third hoeings were done at about 30 and 45 DAS to loose, aerate the soil and control the weeds two times. Also, a lister was used to apply the top-dress fertilizer (100 kg ha^{-1} ammonium nitrate) and make the furrow before the first irrigation. The first irrigation was applied by furrow

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irrigation methods 60 days DAS. The latter furrow irrigations were applied by 7 times at approximately 11-13 days intervals during growing season, with nearly 150 mm at each irrigation. For a given irrigation, all plots received the same amount of water.

Description of planker

The planker tested was a metal rectangular with length of 600 cm and width of 20 cm, attached to a carriage frame (*Fig. 2*). Total weight of the planker was 1200 kg, calculating the weight per meter as 200 kg m^{-1}. The calculated contact pressure of the planker was 9.81 kPa. The tractor, New Holland T6600 with 4-wheel-drive developing 119 kW, was operated at 5.4 km h^{-1} for pulling the planker.

Figure 2. *The packer tested in the experiment*

Measurements

Soil moisture content and temperature was measured by Aquaterr - Model T300 - Moisture Measurement Instrument (*Fig. 3a*). The measuring range of this Instrument was 0% d.b.–100% d.b., and the precision was ±1.5 F.S. Measurements was taken at 15 cm soil depth before the application of treatments and 5 days DAS. Soil penetration resistance were measured using a digital cone penetrometer FieldScout SC 900 (Spectrum Technologies, Aurora, IL) recording the pressure applied in Pascals every 2.5 cm, to a depth of 45 cm at the same time when soil moisture content and temperatures were measured (*Fig. 3b*). All the measurements were conducted at three random locations in each plot and the average value was taken as the result.

Figure 3. *Soil moisture and temperature measurement instrument (a) and soil penetrometer (b)*

Root samples were obtained by digging out plant roots to a depth of 30 cm at the early flowering period. A cylindrical soil corer with a 130 mm inner diameter was used to dig out the roots (*Fig. 4a*). Five plants were randomly selected in each plot to determine the root length, root thickness and root dry weight. Root thickness was recorded with a digital vernier caliper (Mitutoyo UK Ltd., Hampshire, UK) on the collar

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and root length was measured with a meter ruler (*Fig. 4b*). To determine dry root weight, roots was cut on collar from plant and thoroughly washed with water for devoid of soil particles. Then, roots samples were oven-dried to a constant weight at 65 °C for 72 h and weighed to determine the root weight (g).

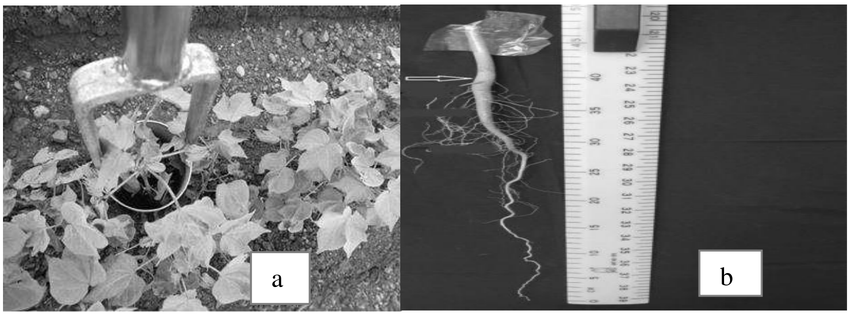

Figure 4. *The soil corer for taking root sample (a) and measurement of root length and the measurement point of root thickness (b)*

Weed species and their densities were determined by counting their numbers in 1 m^2 quadrats which were located randomly in each plot before cotton harvest. The density of weed species was calculated by taking the arithmetical mean of plots for each treatment and classified according to A-E scale used by Uremis (2005). In this scale,
a represents weed density ≥ 3 weeds m^{-2}
b represents 2 weeds m^{-2}≤ weed density < 3 weeds m^{-2}
c represents 1 weeds m^{-2} ≤ weed density < 2 weeds m^{-2}
d represents 0.1 weeds m^{-2} ≤ weed density < 1 weeds m^{-2}
e represents weed density < 0.1 weeds m^{-2}.

Statistical analysis

Analysis of variance (ANOVA) was performed on all the measured soil properties and root growth variables using the JMP statistical software package (SAS, 2002, Cary, NC, USA). The means between treatments were compared using LSD’s multiple range tests at the significance level of 0.05. Simple correlation coefficients between the soil properties and root growth in the study were calculated using correlation analysis.

Results and discussion

Soil physical properties

Soil moisture content and temperature

Figure 5 shows that the soil moisture content was affected by the passing numbers of planker five DAS. Using the planker significantly increased the soil moisture content at 15 cm soil depth. However, there was no significant difference among the pass numbers of planker. Unlike the soil moisture content, the soil temperature was the highest in the untreated plots that planker was not used. As their effects on soil moisture content, the difference among the pass numbers of planker was not statistically significant (*Fig. 5*). Soil moisture and temperature are two important soil parameters which have significantly influenced crop growth by affecting many physical and biological

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DOI: http://dx.doi.org/10.15666/aeer/1702_52455258

processes such as soil N-mineralization within the soil (Wang et al., 2006). Therefore, it is very important to know how planking practices affected the soil moisture content and temperature. Soil planking decreases air pockets in which evaporation occurs. Therefore, soil drying and heating slow down as the result of soil compaction. Also, soil compaction encourage capillary rise of water from subsoil to topsoil (Tong et al., 2015).

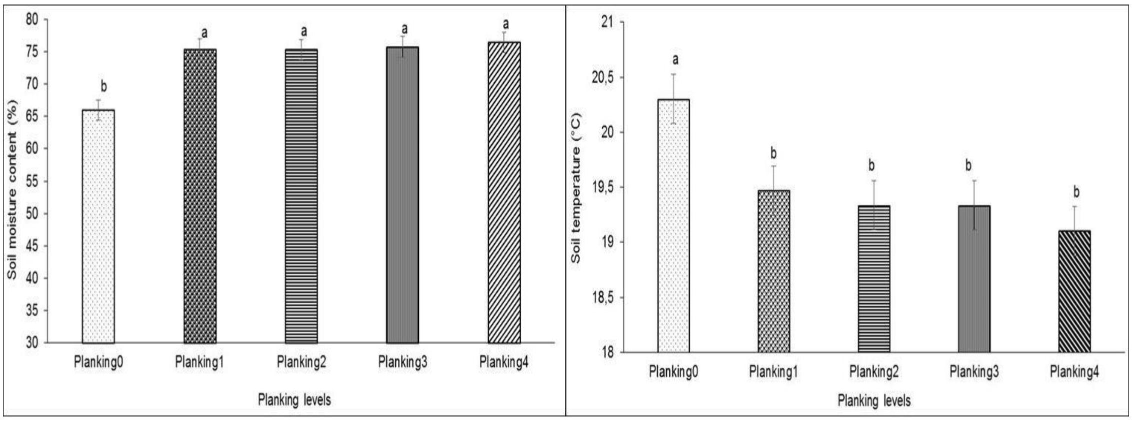

Figure 5. *The effect of different pass numbers of planker on soil moisture content and temperature at 15 cm depth. Means followed by different letters are significantly different according to LSD's multiple range test at the significance level of 0.05. Planking0: untreated control; Planking1: soil planking by planker in one pass; Planking2: soil planking by planker in two passes; Planking3: soil planking by planker in three passes; Planking4: soil planking by planker in four passes*

Soil penetration resistance

Mean values of penetration resistance at different planking levels and different soil depths are presented in *Figure 6*. While the pass number of planker significantly influenced the penetration resistance at the soil surface (~0 to 15 cm depth), there was no significant difference between treatments at subsurface layers (20-30 cm soil depths). Using the planker significantly increased the penetration resistance at the soil layers of 0-15 cm depth. The soil planking by planker in four passes (Planking4) resulted in a significantly higher soil penetration resistance which was the highest (1356.67 kPa) at 5 cm depth. There was no significant different between planking3 and planking4 at 0-10 cm soil depth. In general, it can be said that penetration resistance was linearly increased by the pass number of the planker at all soil depths of surface layer (~0 to 15 cm). However, the peak values of penetration resistance for all the pass numbers of planker were commonly obtained at 5 cm soil depth. In all treatments, the values of the penetration resistance at all soil depths were below critical compaction (2000 kPa) reported by Hamza and Anderson (2005).

Root growth parameters

Root growth is a critical component in overall plant performance during production. Main root growth parameters include: root length, root thickness, root weight, root volume, root:shoot ratio, specific root length, branching pattern, horizontal distribution, root hair density, root uptake ability, root hydraulic conductance, and root viability

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(Judd et al., 2015). In this study, we could determine only root length, root thickness and root dry weight at the early flowering period.

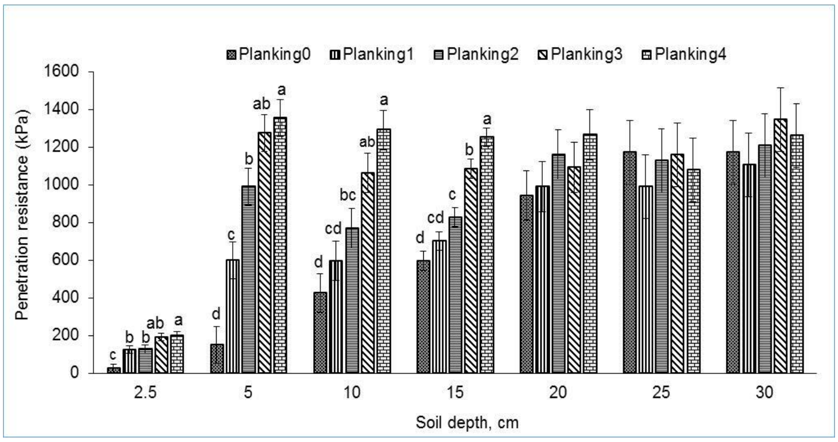

Figure 6. *The effect of different pass numbers of planker on soil penetrometer resistance at different depths. Means followed by different letters are significantly different according to LSD's multiple range test at the significance level of 0.05. Planking0: untreated control; Planking1: soil planking by planker in one pass; Planking2: soil planking by planker in two passes; Planking3: soil planking by planker in three passes; Planking4: soil planking by planker in four passes*

Figure 7 shows a picture of the characteristic growth patterns of individual roots under different soil planking levels. As seen in picture, the direction of the root growth change from vertical to horizontal immediately above the restricting layer with increasing the pass number of the planking because roots are physically restricted by a dense layer containing few pores suitable for root penetration. Several researchers (e.g. Goss and Russell, 1980; Tsegaye and Mullins, 1994) reported that soil compaction cause the root tip exert a force to deform the soil due to pores much smaller than the root diameter. They stated that this process may considerably decrease root elongation rates, increase the root diameter and change the pattern of lateral root initiation.

The root length was the highest in the Planking0 (untreated control) treatment and the difference between the Planking0 and Planking1 was not statistically significant. Increasing the pass number of planker from one to two significantly decreased cotton root length and there was no significant difference among Planking2, Planking3, Planking4 treatments (*Fig. 8*). Our results are consistent with general results from the previous researches. For example, in a recent review, Bengough et al. (2011) reported that root elongation significantly slowed in soils with penetrometer resistances in the range 800-2000 kPa. Similarly, Logsdon et al. (1987) determined that the root length of corn (Zea mays L.) seedlings decreased with increased mechanical impedance in a fine-textured soil. In our study, multiple passes of the planker significantly increased the penetration resistance at 0-15 cm soil depths as seen in *Figure 6*. This increase in penetration might decrease the root elongation. Geisler-Lee et al. (2010) reported that the higher ethylene production in roots due to a lack of oxygen in compacted soils is associated with the inhibition of elongation. Also, the decreased root elongation in compacted soil might be resulted from a decreased rate of cell division in the meristem together with a decrease in cell elongation (Bengough and Mullins, 1990).

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Figure 7. *The characteristic growth patterns of individual roots under different soil planking levels at the early flowering period. Planking0: untreated control; Planking1: soil planking by planker in one pass; Planking2: soil planking by planker in two passes; Planking3: soil planking by planker in three passes; Planking4: soil planking by planker in four passes*

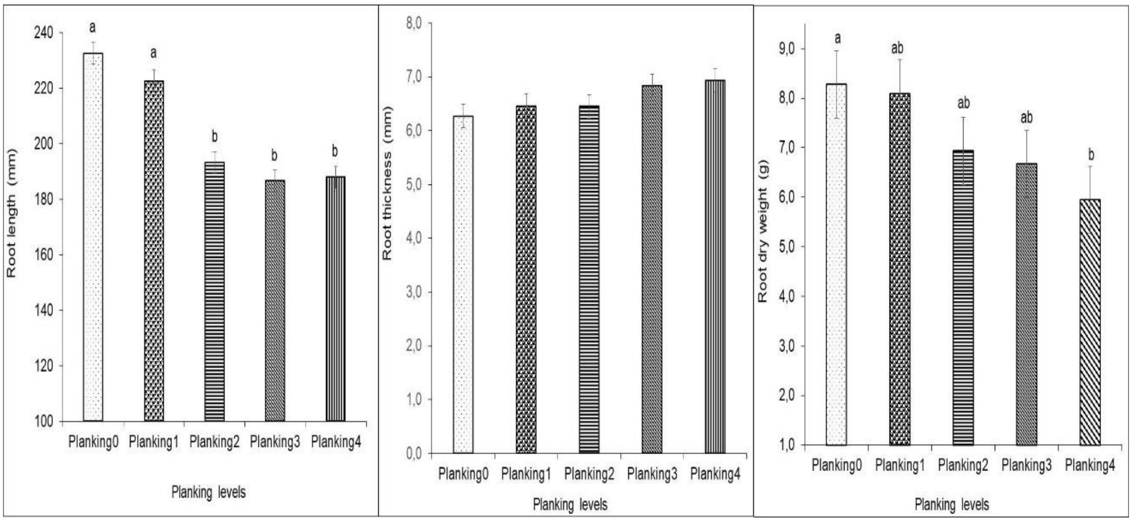

Figure 8. *The effect of different pass numbers of planker on some root growth parameters (root length, root thickness, root dry weight) at the early flowering period. Means followed by different letters are significantly different according to LSD's multiple range test at the significance level of 0.05. Planking0: untreated control; Planking1: soil planking by planker in one pass; Planking2: soil planking by planker in two passes; Planking3: soil planking by planker in three passes; Planking4: soil planking by planker in four passes*

Soil compaction can increase root thickness in order to reduce the risk of root buckling and the mechanical stress acting on the root during penetration (Kirby and Bengough, 2002; Colombi et al., 2017). Hettiaratchi et al. (1990) reported that a

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variable apex geometry's cyclic growth routine enabled plant roots to penetrate compact soil and this growth mode resulted in thickened roots. Also, Bengough and Mullins (1990) reported that soil mechanical impedance caused the increased root thickness due to increased thickness of the cortex and increases in the diameters of the outer cells as well as increases in the number of cells per unit length of root. *Figure 8* shows that the pass number of the planker did not statistically influence the root thickness. In this study, root thickness was determined on the root base near the soil surface not on root cap. No difference among treatments in terms of their effect on root thickness might be resulted from the measurement point of root thickness. Additionally, in our experiment, the highest soil penetration resistance was 1345 kPa. This resistance value might be apparently not high enough to modify root diameter. Rosolem and Foloni (2002) reported that the effect of soil compaction on root diameter depends largely on the level of penetration resistance.

The dry root weight was significantly reduced with the increased pass number of planker (*Fig. 8*). While untreated control treatment had the highest dry root biomass, the four passes of the planker resulted in the lowest dry root weight. There was significantly no difference among the other treatments. As seen in *Figure 6*, four passes of the planker significantly increased soil penetration resistance. This increase in soil penetration decreased the root length, consequently root dry weight (*Fig. 8*). Similarly to our results, several researchers found that the root dry weight was significantly reduced by soil compaction during growing of other crops including wheat (Rahman et al., 1999; Wu et al., 2018), alfalfa (Hakl et al., 2007), sugar beet and cotton (Gemtos and Lellis, 1997), soybean and common bean (Buttery et al., 1998). However, Sarto et al. (2018) determined that the root dry matter of safflower genotypes decreased as penetration resistance levels were increased from 200 to 1770 kPa. They stated that the effect of increased penetration resistance on root growth can change according to each plant species or genotype.

Correlations between soil properties and root growth parameters evaluated

The relation between the soil properties, and soil properties, seed cotton yield per plant and root growth parameters measured in the study was evaluated by Pairwise Correlation analysis. The results are shown in *Table 2*. The soil moisture content was positively correlated with the penetration resistance at 0-15 cm soil depths. This indicate that the increase in the penetration resistance due to planking significantly increase moisture content at 0-15 cm soil depth. However, these findings are contrary to the previous studies reported by several researches (e.g. Veronese et al., 2006; Van Quang et al., 2012; Lomeling and Möri Lasu, 2015) that show that penetration resistance is negatively correlated to soil moisture content. In this study, positive correlation between soil penetration resistance and moisture content can be attributed to the change in bulk density and macro pores caused by soil planking, prevention of the loss of soil moisture content and the capillary rise of water from subsoil to topsoil. The soil temperature was negatively correlated with both penetration resistance and moisture content. This indicates that the increase in soil moisture content and penetration resistance decreased the soil temperature. Lakshmi et al. (2003) reported that the changes in soil temperature affect soil moisture and vice versa. While the root thickness was positively correlated with penetration resistance at 10 cm soil depth, no significant correlations were found between the root thickness and penetration resistance at other soil depths. This indicates that increase in penetration resistance at 10 cm soil depth increased the root thickness.

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Root length was positively correlated with soil temperature whereas it was negatively correlated with penetration resistances at 2.5, 5.0, 10, 15 cm soil depths and moisture content. This means that the increase in penetration resistance decreased the root length. Bengough and Mullins (1990) reported that mechanical impedance decreased the rate of root elongation because of both the decrease in the rate of cell division in the meristem, and the decrease in cell length. Negative correlation between soil moisture content and root length indicates that root length was increased with decrease in soil moisture content at 15 cm soil depth. This can be resulted from physiologically increasing root elongation to uptake available water in deeper soil zone (Serraj and Sinclair, 2002). Similarly, Malik et al. (1979) reported that the initial rate of root elongation after emergence was faster in the soil at low water content compared with that at high water content. There was no significant correlation between root length and thickness. While the root dry weight was positively correlated with soil temperature, it was not correlated with soil penetration resistance and moisture content. Besides, the root dry weight was positively correlated with the root length whereas no significant correlation was found between root dry weight and root thickness. This shows that while the root dry weight was affected by root length, effect of root thickness on root weight was not significant.

Table 2. *Correlation coefficients among parameters considered in the study*

	PR2.5[1]	PR5[1]	PR10[1]	PR15[1]	PR20[1]	PR25[1]	PR30[1]	MC[2]	T[3]	RT[4]	RL[5]
PR5	0.9011***										
PR10	0.7565**	0.8147***									
PR15	0.8392***	0.9028***	0.8720***								
PR20	0.3559ns	0.4186ns	0.6609**	0.4414ns							
PR25	0.0306ns	0.025ns	0.2417ns	0.0503ns	0.5975*						
PR30	0.1420ns	0.2941ns	0.2991ns	0.1775ns	0.4140ns	0.4645ns					
MC	0.8245**	0.7268**	0.6674**	0.6602**	0.4846ns	0.0988ns	0.2157ns				
T	-0.5754*	-0.5370*	-0.4973*	-0.4996*	0.0197ns	0.2623ns	-0.1710ns	-0.6754**			
RT	0.4524ns	0.4969ns	0.6318**	0.3880ns	0.4230ns	0.3894ns	0.4090ns	0.3510ns	-0.2690ns		
RL	-0.6249*	-0.7114**	-0.5403*	-0.6027*	-0.0268ns	0.0521ns	-0.2324ns	-0.5170*	0.7098**	-0.4391ns	
RW[6]	-0.3389ns	-0.3406ns	-0.4288ns	-0.3697ns	0.0743ns	0.1448ns	-0.0489ns	-0.2819ns	0.7138**	-0.1327ns	0.5045*

[1]PR2.5…PR30: penetration resistance at 2.5…30 cm soil depths; [2]MC: moisture content at 15 cm soil depth; [3]T: temperature at 15 cm soil depth; [4]RT: root thickness; [5]RL: root length; [6]RW: root weight; *: significant effect at 0.05 level of probability; **: significant effect at 0.01 level of probability; ***: significant effect at 0.001 level of probability; n.s: non significant effect

The density of main weed species affected by planking levels

Soil firming by planker significantly affects the species richness, composition, and diversity of weed phytocenoses because it may change soil properties and weed seed emergence. The weed community in this experiment area was composed of 13 species including Cultivated licorice (*Glycyrrhiza glabra* L.), Common mallow (*Malva neglecta* Wallr.), Field bindweed (*Convolvulus arvensis* L.), Field Mallow (*Malvella sherardiana* (L.) Jaub. & Spach), Mexican Ground-cherry (*Physalis philadelphica* Lam.), Black nightshade (*Solanum nigrum* L.), Cocklebur (*Xanthium strumarium* L.), Redroot amaranth (*Amaranthus retroflexus* L.), Johnsongrass (*Sorghum halepense* (L.) Pers.), Giradol (*Chrozophora tinctoria* (L.) Raf.), Purple nutsedge (*Cyperus rotundus* L.), Common reed (*Phragmites australis* (Cav.) Trin. ex Steud.), Bermudagrass (*Cynodon dactylon* (L.) Pers.). The weed species observed in quadrats was Black nighshade (*Solanum nigrum* L.), Bermudagrass (*Cynodon dactylon* (L) Pers.), Mexican Groundcherry (*Physalis philadelphica* Lam.), Common reed

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(*Phragmites australis* (Cav.) Trin ex. Steud), Purple nutsedge (*Cyperus rotundus* L.) and Field bindweed (*Convolvulus arvensis* L.).

Figure 9 shows the effect of soil planking levels on the density of those weed species. Considering the total density of weed species, while the highest weed density was observed in untreated control (17.21 weeds m^{-2}) and the four passes of the planker (16.99 weeds m^{-2}), planking1 treatment had the lowest (4.54 weeds m^{-2}). Total weed density was 10.32 weeds m^{-2} in Planking2 and Planking3 treatments. As seen in *Figure 9*, while the increase in the pass number of planker decreased the density of the Bermudagrass and Field bindweed species, it increased density of Common reed and Purple nutsedge. Planking1 treatment had the highest density of Black Nighshade among all treatments. While the density of the Bermudagrass was > 3 plant m^{-2} at untreated control plots, no plant was observed in in quadrats at other treatments (*Fig. 9*). The two passes of planker resulted in the highest density of Mexican Ground-cherry. Density of Common reed and Purple nutsedge was observed to be more than 3 plants m^{-2} when planker was passed four times. However, the density of Purple nutsedge was the highest at the Planking2 treatment as seen in *Figure 9*. The untreated control plots had the highest density of Field bindweed.

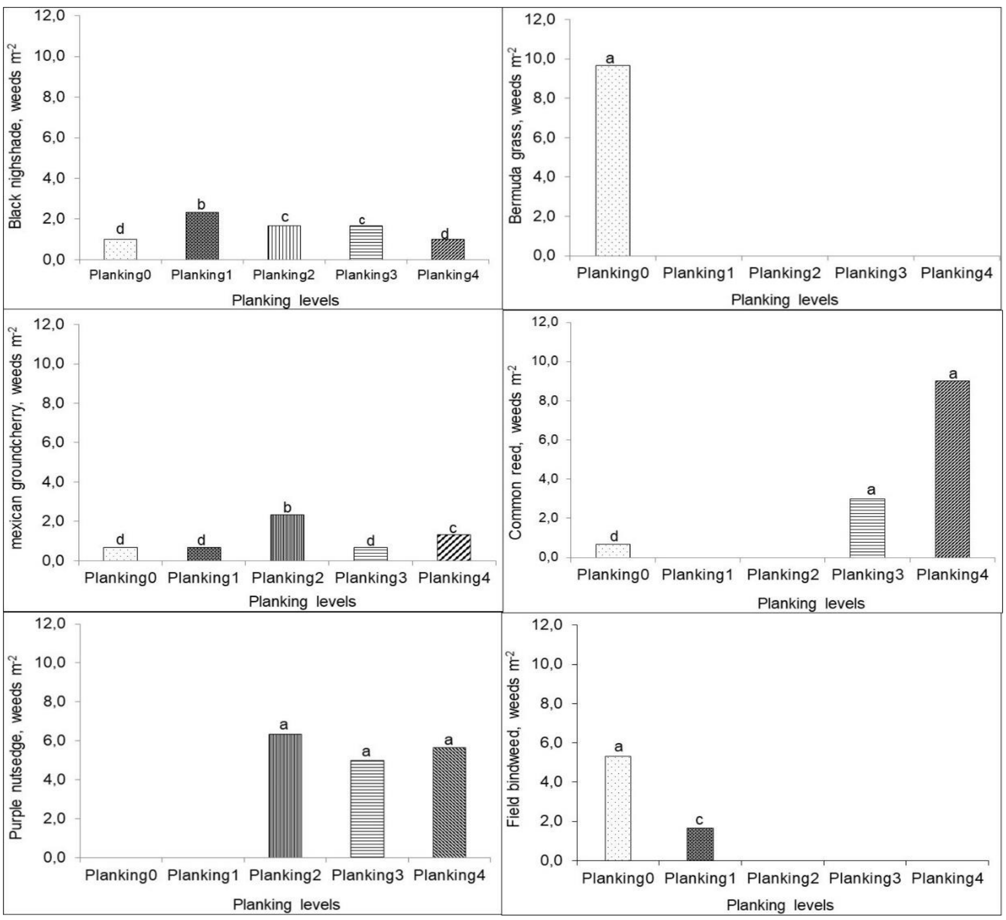

Figure 9. *The effect of different pass numbers of planker on the density of some weeds species. a:> 3 weeds m^{-2}; b: 2-3 weeds m^{-2}; c: 1-2 weeds m^{-2}; d: 0.1-1 weeds m^{-2}; e: <0.1 weeds m^{-2}. Planking0: untreated control; Planking1: soil planking by planker in one pass; Planking2: soil planking by planker in two passes; Planking3: soil planking by planker in three passes; Planking4: soil planking by planker in four passes*

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The seedling emergence and growth of weed species can change in different soil conditions because of their genetic characteristics (Whitely and Dexter, 1984; Materachera et al., 1991). Therefore, the changes in soil physical and chemical properties due to soil planking may differently influence the seedling emergence and growth of weed species. Similarly, Lenssen (2009) determined that land rolling differently affected weed species after crop emergence and at harvest. Reintam and Kuht (2012) reported that the soil physical and chemical properties, and the position of weed seeds within the soil matrix play an important role in seedling emergence and seed survival.

Conclusions

In this study, the effects of soil planking levels on soil physical properties, the root growth of cotton and density of weed species was determined. The results of study revealed that the soil planking had an important effect on the root length and dry root weight of cotton for significantly changing the penetration resistance, moisture content and temperature of soil. Soil planking significantly increased the soil moisture content at 15 cm soil depth although there was no significant difference among the pass numbers of planker. Unlike moisture content, the soil temperature was the highest in the untreated plots and there was no significant difference among the pass numbers of planker. The penetration resistance was increased by the increased pass number of the planker at all soil depths of surface layer (0 to 15 cm) although soil planking had no significant effect on the penetration resistance at 20-30 cm soil depths. Multiple passes of planker significantly decreased cotton root length. Also, the dry root weight was significantly reduced with the increased pass number of planker. Correlation results indicated that while the increase in the penetration resistance due to planking significantly increased moisture content at 15 cm soil depth, it reduced soil temperature and root length. There was positive correlation between root thickness and penetration resistance at 10 cm soil depth but not other depths. Also, the density of weed species was affected by the pass number of planker. While the increase in the pass number of planker decreased the density of the Bermudagrass and Field bindweed species, it increased the density of Common reed and Purple nutsedge.

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EVALUATION AND MODELLING OF METHANE YIELD EFFICIENCY FROM CO-DIGESTION OF WASTE ACTIVATED SLUDGE AND OLIVE MILL WASTEWATER

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(Received 24th Jan 2019; accepted 6th Mar 2019)

Abstract. This research aims to enhance the biodegradation efficiency of waste activated sludge co-digested with olive mill wastewater in a batch system at a laboratory scale in (600 ml) digester. The potential biomethane production was investigated. Different concentration ratios were tested out at a thermophilic temperature (55°C) for a retention period of 32 days. The results showed an increase in methane amount in the case of co-digestion. A height methane yield was obtained (71% of CH_4) at a mixing ratio: 87.5/12.5 of waste activated sludge/olive mill wastewater. The kinetic modelling was done to analyze the digestion performance with two models: the modified Gompertz equation and the modified logistic equation. The kinetic data and the concentration ratio give a peak correlation, whose the Gauss amplitude equation is convenient to predict the optimum mixing ratio and the limited concentration to avoid the inhibition of process. The synergistic effect is limited if olive mill wastewater mixing ratio exceeds the limited ration (22%).
Keywords: *anaerobic co-digestion, waste activated sludge, olive mill wastewater, kinetic study, synergistic effect*

Introduction

Anaerobic digestion is a waste management method aimed at the reduction of harmful effects on the environment (Manyi-Loh et al., 2013; Ali Shah et al., 2014). Anaerobic digestion (AD) has been recognized as an efficient bioprocess for the management of waste activated sludge (WAS) (Kardos et al., 2011), by offering many environmental and economic benefits (Mulat and Horn, 2018). In this method, microorganisms play a crucial role in treating organic matter and returning the chemical elements into the active cycle. Thus, they are effective in mineralization of the complex organic matter through a sequential breakdown and release of chemically stabilized compounds, mainly methane (CH_4) and carbon dioxide (CO_2) (Gopinath et al., 2014; Ali Shah et al., 2014; Zeng et al., 2017). However, WAS is known for its low biodegradability mainly due to its low carbon to nitrogen (C/N) ratio. This limits its digestion under traditional mesophilic conditions (Coelho et al., 2011; Mahanty et al., 2014). Therefore, the co-digestion of sludge with other organic wastes can offer numerous potential benefits for the AD process, such as: dilution of the potentially toxic compounds eventually existing in any treated materials, augmenting the organic matter biodegradability, better biogas yield due to synergistic effects, tuning of the moisture content and pH, strengthening the essential buffer capacity to the mixture and the enlarging of bacterial range strains taking part in the process (Anjum et al., 2017; Kashi et al., 2017; Xu et al., 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

Therefore, notable issues have been performed by digesting simultaneously the WAS with different biological wastes (Heo et al., 2004; Bolzonella et al., 2006; Carrere et al., 2008; De Vrieze et al., 2013; Sun et al., 2013; Qiao et al., 2015; Mulat and Horn, 2018).

Olive Mill Wastewater (OMW) is a very attractive co-substrate option for the anaerobic co-digestion of municipal sludge because, carbon source addition like OMW as a substrate, enhanced the total VS and therefore the biogas yield (Ma et al., 2008). OMW is becoming a serious environmental problem, especially for its high chemical oxygen demand (COD). It is generally acknowledged that the high toxicity of OMW is entirely ascribable to phenols (Perez et al., 1992).

This article focuses on the anaerobic co-treatment of two typical wastes in Algeria, which are totally unexploited and in some cases harmful to the environment. WAS (production period whole year) and OMW (production period October–March) and as two representative types of biomass wastes produced in Boumerdes (Algeria) and other mediterranean countries. The precise aim of the present research was to investigate biochemical methane potential assays for raw WAS alone and mixed with varying amounts of OMW.

Material and methods

Waste sampling

The sampling of WAS was carried out in a municipal wastewater treatment plants in Boumerdes (Geographical coordinates are 36°45'0"N and 3°40'0"E in DMS), Algeria. When the sludge age, is 12 days in the extended aeration. The OMW used in this study was taken from a three-phase olive mill processing plant located at the Issers city (Geographical coordinates are 36°43'0"N and 3°40'0"E in DMS) in Boumerdes during the harvesting season. The biochemical compositions of wastes are revealed in *Table1*.

Table 1. *Characteristics of substrate*

Parameters	Waste activated sludge	Olive Mill Wastewater
pH	7.8 ± 0.15	4.8 ± 0.1
CODt (g l^{-1})	90.7 ± 1.8	128.1 ± 5.4
CODs (g l^{-1})	35.0 ± 0.6	64.7 ± 1.4
TS (g l^{-1})	150 ± 0.8	69.5 ± 3.1
VS (g l^{-1})	71.7 ± 0.5	57.4 ± 4.5
TN (g l^{-1})	3.8 ± 1.8	1.26 ± 2.2
TP (g l^{-1})	0.903 ± 0.07	0.48 ± 0.09
TPc (eq gallic acid. g l^{-1})	/	4.11 ± 0.3
Oil and grease (g l^{-1})		17.4 ± 1.7
Cd (mg l^{-1})	201	$<1\times10^{-3}$
Cr(mg l^{-1})	508.9	0.655
Pb (mg l^{-1})	335.5	0.186
Mn(mg l^{-1})	922.5	$<1\times10^{-3}$
Ni (mg l^{-1})	$<1\times10^{-3}$	3.96×10^{-2}
Fe(mg l^{-1})	4520	1.504
Zn(mg l^{-1})	30.63	0.24
Cu(mg l^{-1})	1116	0.33

CODt total chemical oxygen demand , CODs: Soluble chemical oxygen demand
TS :total solids, VS: Volatile solids , TN:total nitrogen / TP total phosphorus
TPc: total phenolic compounds

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Digester and operation

A 600 ml digester used for producing biogas from biomass through AD (*Figure 1*). The functioning volume of each digester was maintained at 450 ml and run under uncontrolled pH. For these experiments, the inoculum was an anaerobic sludge treating WAS which was diluted to 0.64 g/l of volatile solids (15 ml /for each digester). When the mixtures were prepared at different weight ratios (WAS % / OMW %): (87.5 / 12.5, 75 / 25, 50 / 50, 25 / 75, 12.5 / 87.5) and the mono- digestion of both wastes (100/0, 0/100), the bioreactor was purged with helium gas to eliminate air from the reactor. Experiments were carried out at a thermophilic temperature of 55°C by incubation in Marie-bath. Biogas volume generated was measured by liquid displacement (NaOH 2%) (Esposito et al., 2012). The chemical compositions of each mixture ratio are revealed in *Table 2*.

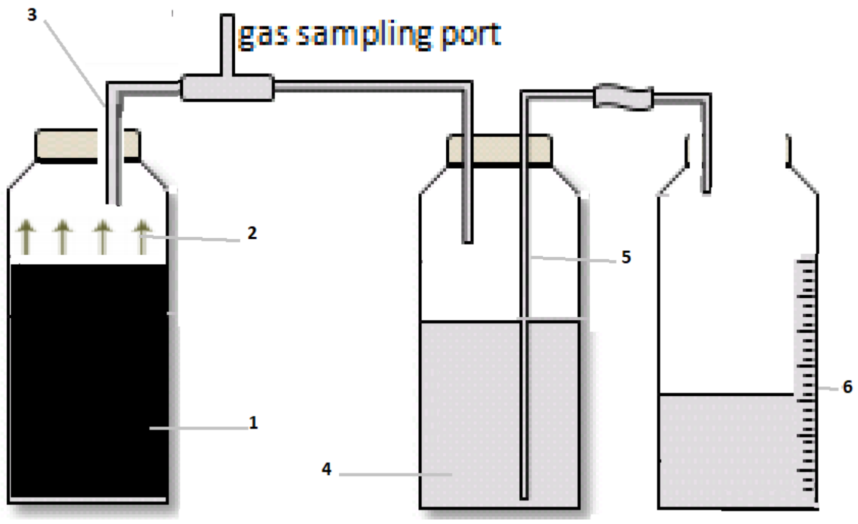

Figure 1. *Anaerobic digestion system: From each digester placed in a water bath (1) a silicone tube (2) led the generated gas (3) out. This tube was led to the top of another glass bottle which contained NaOH 2% solution (4). There was another tube (5) from the bottom of that bottle, through which the gas pumped the solution to the graduated bottle meter (6). This way, it was possible to measure the volume of the generated gas accurately, on a daily basis*

Analytical method

Soluble and total chemical oxygen demand (CODt and CODs) total nitrogen (TN) and total phosphorus (TP), Total solids (TS) and Volatile solids (VS) were quantified according to Standard Methods (Apha, 1998). The pH of the wastes measured according to NF ISO 10390 by a portatif pH-Metre (HANNA HI8424, France) (Rodier et al., 2009). Total phenolic compounds (TPc) were extracted and purified in ethyl acetate using the method of Macheix et al. (1990). The concentration of TP compounds was determined spectrophotometrically (according to the Folin–Ciocalteu method (Singleton and Rossi, 1965)) Heavy metals were determined by the atomic absorption spectrophotometer (Perkin Elmer, Optima8000) according to the Method cited by Liu et al. (2001). The biogas composition (CH_4 + CO_2) was measured using a gas chromatograph (GC-HP 5890) coupled with a thermal conductivity detector (*TCD*) and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
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stainless steel column that was 2 m long with a 5 mm OD and 2 mm ID and contained Porapak Q 100 that had a mesh range from 80 to100. The carrier gas was N_2, and the analysis was carried out at a carrier gas flow rate of 30 ml min^{-1} with the injector, column, and detector temperatures at 120, 90, and 120ºC, respectively.

GC-MS analysis of ethyl acetate extract of OMW was performed on a BRUKER SCION 365 GC System (NS-GC 1409S312), Gas chromatography (GC) coupled to a triple quadruple mass spectrometer fitted with an Rtx-5MS capillary column (30 m X 0.25 mm inner diameter, X 0.25 µm film thickness; maximum temperature, 350°C). Ultra-high purity helium (99.99%) was used as a carrier gas at a constant flow rate of 1.0 ml min^{-1} using the condition cited by Al Owaisi et al. (2014). The percentage composition of the ethyl acetate extract components was expressed as a peak area percentage. The identification and characterization of chemical compounds was based on GC retention time. The mass spectra were computer matched with those of standards available in mass spectrum libraries.

Table 2. *Characteristics of the mixture prepared with different waste activated sludge/Olive Mill Wastewater mixing ratios*

WAS /OMW ratios	TS (g l^{-1})	VS (g l^{-1})	CODt/TN	pH
100 /0	150± 0.8	71.7± 0.5	18.95	7.8± 0.15
87.5 /12,5	138.78± 0.9	68.61± 0.8	21.66	6.79± 0.1
75/25	129.75± 0.8	67.5± 1.1	25.12	6.39± 0.14
50/50	109.5± 0.9	64± 1	34.43	5.55± 0.11
25/75	89.25± 1	60.5± 0.9	50.10	5.4± 0.13
12.5/87,5	78.03± 1.7	58.11± 2.2	63.25	5.04± 0.09
0/100	69± 3.1	57.4± 4.5	82.05	4.8± 0.1

Ratio WAS /OMW: mixing ratio of waste activated sludge /Olive Mill Wastewater
TS: Total solids ,VS: Volatile solids
CODt/TN: the ratio of total chemical oxygen demand /total nitrogen

Methanogenic activity test

To control the biomass composition of anaerobic co-digested waste the methane production potential of the test biomass is measured under an unlimited substrate. The acetoclastic methanogenic activity of each biomass was evaluated in shaken batch assays on the end of each kinetic. All assays were carried out in glass serum bottles (250 ml), and each biomass sample was washed with 25 mM phosphate buffer to remove any extra substrate and was dispersed by a homogenizer. The bottles containing 230 ml of 25 mM phosphate buffer were inoculated with the washed anaerobic biomass directly to a final concentration of 2 g VSS l^{-1}. The test substrates used were acetate, COD strength was set at 2000 mg COD•l^{-1}. Nutrients were not added in order to limit the biomass growth during the test period. The medium and the headspace were filled with N_2 gas at 1 atm (101 k Pa). The bottles were incubated in the dark at 55ºC. All measures other than specifically described here are given elsewhere by Pat-Espadas et al. (2015). Methane gas production was determined through the liquid (11.2% w/v KOH Solution + Thymol Blue Indicator) displacement method according to Jawed and Tare (1999) and Esposito et al. (2012). The maximum specific methanogenic acetoclastic activity (SMAA) (ml CH_4/VSS /h) was calculated from the slope of the cumulative CH_4 versus time graph.

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Kinetic models

Tow models to estimate performance parameters were used. The cumulative methane production data from the experiments were fitted to the modified Gompertz equation (MGE) given by (*Eq. 1*), so this equation plot the cumulative methane production according to the time (Maamri and Amrani, 2014).

$$M = \mathrm{P.exp} \quad \text{- exp } [\frac{R_m.e}{P}(\lambda \text{ - t}) + 1] \qquad \text{(Eq.1)}$$

where M is the cumulative methane production (l), P the methane production potential (l), R_m the maximum methane production rate (l d^{-1}), λ the duration of lag phase (d) and t is the duration of the assay (time) at which cumulative methane production M is calculated (d).

The Logistic equation (LGE) a model which has been used for anaerobic fermentation, as well as, for estimate the methane generated from sewage sludge (Donoso-Bravo et al., 2010). In this case, a modified version of the logistic function was used (*Eq. 2*).

$$M = \frac{P}{1 + \exp(4\mathrm{R}_m(\lambda \text{ - t})/\mathrm{P} + 2)} \qquad \text{(Eq.2)}$$

The parameters P, R_m, and λ were estimated for each of the digesters using the OriginPro8 software.

Statistical analysis

All assays were conducted in triplicate. The data on characteristics of the substrate with different mixing ratios were expressed as mean ± standard deviation. The data on performances of each digester were expressed as mean ± standard deviation during the operation period. A one-sample t-test was used to test the significance of the results and $p < 0.05$ was considered statistically significant. The statistical analysis of regression was qualified by an analysis of variance (ANOVA) and Akaike’s test by OriginPro8.

Results

Identification of phenolic compounds extracted from OMW

The identification of phenolic compounds was performed by relevant molecular mass data from GC-MS. GC provided the separation of the major biophenols in the OMW extracts as illustrated in *Figure 2*. The phenolic composition of the OMW ethyl acetate extract is summarized in *Table 3*.

Anaerobic digestion

The cumulative methane production (ml) during the codigestion of WAS/OMW is shown in *Figure 3*. As was the case for different ratios of a mixture for a retention time of 32 days. Methane production started immediately from the first day of digestion in all the digesters.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
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***Table 3.** Phenolic compounds (or related analytes) found in ethyl acetate extract of olive mill wastewater identified by GC-MS*

Fraction	Compounds	Retention time (min)	Fragments	Molecular weight	Formula	(%)
1	SuccinicAcidDimetyhlEster	2.89	55. 115	146	$C_6H_{10}O_4$	5.723
2	MethylCatechol	3.522	53.81.109	124	$C_7H_8O_2$	1.327
3	4-Ethylphenol	4.575	77. 107. 122	122	$C_8H_{10}O$	1.173
4	Vanillic acid	4.846	70 . 78. 126	168	$C_8H_8O_4$	0.455
5	Pyrocatechol	5.011	64. 110	110	$C_6H_6O_2$	1.987
6	A-Terpinolene	6.76	41 .91	136	$C_{10}H_{16}$	1.545
7	Tyrosol	7.89	41 .81.123.138	138	$C_8H_{10}O_2$	0.668
8	Vanillin	7.97	109.122.151	152	$C_8H_8O_3$	0.668
9	3,4,5 TrimethoxybenzoicAcid	9.062	39.53.93	212	$C_8H_{10}O_3$	0.040
10	Dihydroeugenol	9.29	31. 137	166	$C_{10}H_{14}O_2$	0.352
11	p-Coumaric Acid	9.916	147. 164	164	$C_9H_{12}O_4$	0.294
12	DecarboxymethylElenolicAcid	10.38	139.08	184	$C_9H_{12}O_4$	1.037
13	Hydroxytyrosol	10.694	109.137	154	$C_8H_{10}O_3$	7.227
14	Protocatechuic acid	10.87	76.107.126	154	$C_7H_6O_4$	5.955
15	3,4,5 TrimethoxybenzoicAcid	11.02	39.53.93	212	$C_8H_{10}O_3$	0.015
16	Syringic acid	12.59	155.180.182.197	198	$C_9H_6O_5$	0.038
17	4-Hydroxycinnamic acid	13.597	46.104.146.163	164	$C_9H_8O_3$	0.577
18	Gallic acid	14.371	135.150	170	$C_7H_6O_5$	0.438
19	Pinorisinol	14.90		358	$C_{20}H_{22}O_6$	0.010
20	MethylLinoledaite	15.13	41. 55. 65. 81.95	294	$C_{19}H_{34}O_2$	0.612
21	Luteolin	15.842	77. 135	285	$C_{15}H_{10}O_6$	0.042
23	DecarboxymethylLigstrosideAglycon	16.14	41.97	304	$C_{17}H_{20}O_5$	0.017
23	palmitic acid	16.446	29. 69	256	$C_{16}H_{32}O_2$	0.021
25	Dehydrodieugenol	16.66	164	326	$C_{20}H_{22}O_4$	0.002
26						
27	9-Octadecanoic Acid(Z)methyl ester	17.35	41. 55. 69. 81.97 .264	282	$C_{18}H_{34}O_2$	1.198
28	Ferrulic acid	17.569	88.118.149	194	$C_{10}H_{10}O_4$	0.008
29	Cafeic acid	19.31	89.134.151			0.018
30	Octadecanoic acid	17.79	43.69. 73.284	372	$C_{22}H_{44}O_2$	0.018
31	10-Hydroxy DecarboxymethylAglycon	17.861	336.01	336	$C_{17}H_{20}O_7$	0.017
32	Bis(2-Ethylhexyl)Phthalate	18.696	57. 149.167. 279	390	$C_{24}H_{38}O_4$	0.05371
33	Linoleic acid	19.54	139	280	$C_{18}H_{32}O_2$	0.012
34	Oleic acid	19.68	69.85	282	$C_{18}H_{34}O_2$	0.008

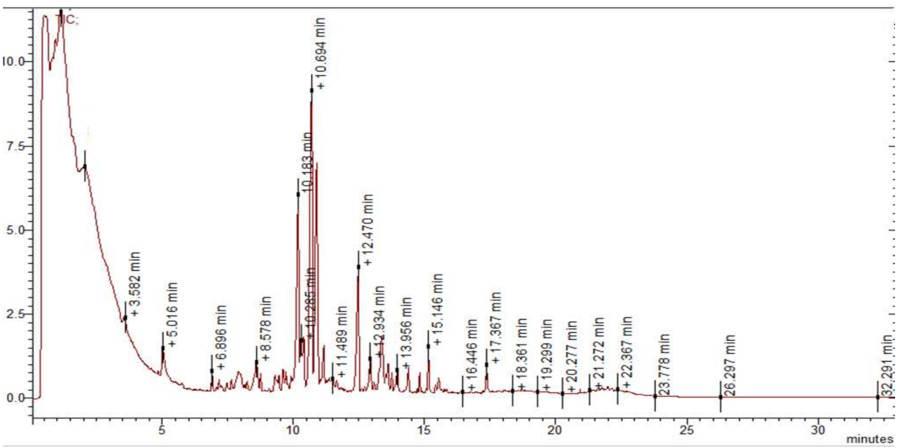

***Figure 2.** GC-MS chromatograms of Phenolic monomer (or related analytes) found in ethyl acetate extract of olive mill wastewater*

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The cumulative methane is better in case of codigestion than monodigestion (WAS /OMW =100/0 and 0/100), especially at the ratio 87.5 / 12.5 of WAS/OMW with the highest specific methane yield value (*Figure 3*) comparatively. The kinetic parameters of the AD process are constantly used to analyze the performance of digesters and design appropriate digesters, which are also useful to considerate inhibitory mechanisms of degradation (Kabouris et al., 2009). With an assumption that methane produced is a function of bacterial growth in batch digesters, to quantify analytically parameters of the batch growth curve, the MGF and LGF were selected to fit the cumulative methane production data. Values of parameters obtained are summarized in *Tables 4 and 5*.

It has been observed that the cumulative methane produced is well fitted with the two models as is evident from the correlation coefficient R^2 (0.9) between the experimental and predicted values along with the parameter estimated.

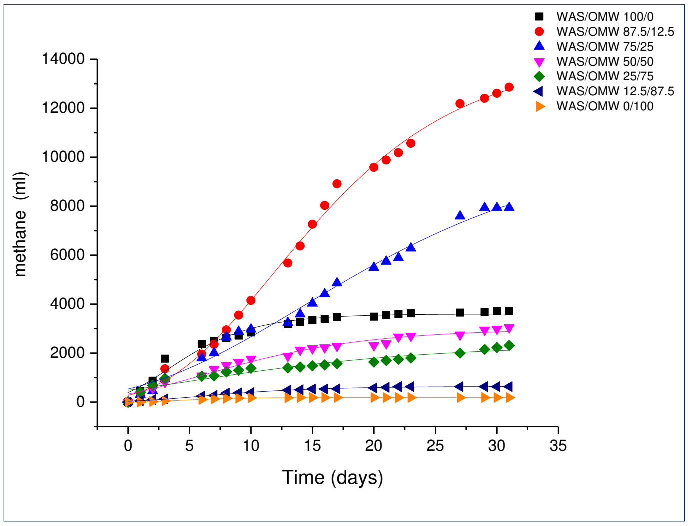

Figure 3. *Cumulative methane production at a different mixing ratio of waste activated sludge /Olive Mill Wastewater (WAS /OMW)*

Table 4. *Values of modified Gompertz equation and statistical measures for the kinetic model for Cumulative methane productions at a different waste activated sludge/Olive Mill Wastewater mixing ratio*

Ratio WAS /OMW (%)	R^2	P (ml)		R_m (ml/day)		λ (day)		F Value	Prob>F
		Value	Stand Error	Value	Stand Error	Value	Stand Error		
100 /0	0.97	3597.53	0.51	332.28	62.09	2.18	0.73	2048.39	0.0000
87.5 /12.5	0.99	14197.76	0.63	608.85	320.61	10.77	0.72	6651.40	
75/25	0.98	10582.33	2.47	301.16	816.69	13.14	0.20	1898.26	
50/50	0.97	2997.91	0.48	143.70	101.17	5.07	0.55	1605.77	
25/75	0.90	2348.29	1.70	76.10	227.52	4.18	0.32	553.41	
12.5/87.5	0.99	641.43	0.02	42.73	8.69	4.83	0.81	5271.44	
0/100	0.99	184.06	0.008	25.57	1.35	3.05	0.88	8718.94	

Ratio WAS /OMW: mixing ratio of waste activated sludge /Olive Mill Wastewater
P: the methane production potential
R_m: the maximum methane production rate
λ: the duration of the lag phase

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Table 5. *Values of modified logistic equation and statistical measures for the kinetic model for Cumulative methane productions at a different waste activated sludge/Olive Mill Wastewater mixing ratio*

Ratio WAS /OMW (%)	R^2	P (ml)		R_m (ml/day)		λ (day)		F Value	Prob>F
		Value	Stand Error	Value	Stand Error	Value	Stand Error		
100 /0	0.95	3555.08	1.05	456.63	71.79	3.88	0.63	1258.96	
87.5 /12.5	0.99	12856.97	1.05	968.24	266.89	13.05	0.66	3155.21	
75/25	0.97	8920.91	2.38	477.63	489.07	15.25	0.02	1243.84	
50/50	0.95	2877.57	0.78	203.73	98.22	7.81	0.36	983.20	0.0000
25/75	0.89	2272.75	1.87	101.71	199.61	8.20	0.89	465.32	
12.5/87.5	0.98	623.21	0.07	62.35	10.60	6.99	0.70	2381.88	
0/100	0.99	182.71	0.01	38.70	1.44	4.40	0.87	6752.96	

Ratio WAS /OMW: mixing ratio of waste activated sludge /Olive Mill Wastewater
P: the methane production potential
R_m: the maximum methane production rate
λ: the duration of the lag phase

The Akaike test (*Table 6*) confirms that the model of the MGF has lower AIC value and so is more likely to be correct. This model is 6139.83 times more likely to be correct.

Performance data (*Table 7)* shows that the WAS/OMW ratio of 87.5 / 12.5 favored the degradation of the organic matter, considering the best VS reduction (69.91 ±1.72). The methane yield increases slightly with the addition of OMW and remains stable until the WAS/OMW ratio of 75/25.

Table 6. *Akaike test result*

	Residual sum of squares	N° parameters	AIC	Akaike Weight
modified Gompertz equation	6031.5483	3	133.85471	0.99984
modified logistic equation	13329.2795	3	151.29981	1.63E-04

AIC: Akaike information criterion

Table 7. *Performances of mono- and co-digestion*

Ratio WAS /OMW (%)	pH_f	SMAA (ml CH_4/Gvss/h)	Specific Production (l/g VS)	CH_4 (%)	Methane yield (l/g VS_r)	VS_r (%)
100 /0	8.35 ±0.17	1.09± 0.07	0.125	70	0.629	19.94 ±2.78
87.5 /12.5	8.16±0.2	3.13 ± 0.03	0.517	71	0.74	69.91 ±1.72
75/25	7.89±0.3	1.51 ± 0.4	0.391	45	0.67	58.49 ±1.03
50/50	6.24±0.2	0.63 ± 0.06	0.117	27	0.25	46.84 ±0.83
25/75	5.89±0.16	0	0.097	18	0.18	53.90 ±1.64
12.5 /87.5	5.29±0.1	0	0.027	13	0.15	18.39 ±1.1
0/100	5±0.09	0	0.008	10	0.1	8.01 ±0.87

pH_f:final pH
SMAA: specific methanogenic acetoclastic activity
VS_r: Volatile solids reduction

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DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

We can easily observe that the increase in the amount of OMW in the mixture gives a peak correlation (*Figure 4*) in the methane production parameters. Then the SMAA also gives a peak profile.

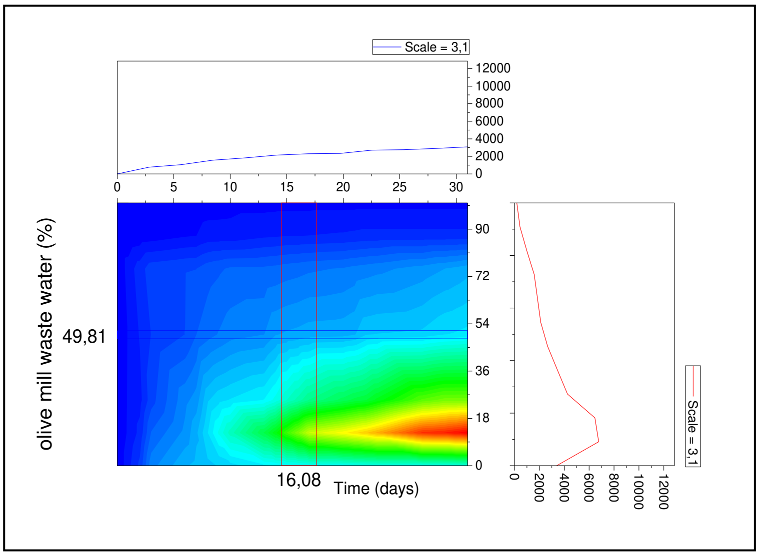

***Figure 4.** Correlation between cumulative methane production and Olive Mill Wastewater amount and retention time*

In light of these results, the investigate the WAS/ OMW optimum mixture ratio by the modelling of these correlations (*Figure 5*) via the Gauss amplitude equation (*Eq. 3*) has given a good statistical significance (*Tables 8 and 9*).

$$Y = Y_0 + Ae^{-\frac{(X-Xc)^2}{2W^2}} \quad \text{(Eq.3)}$$

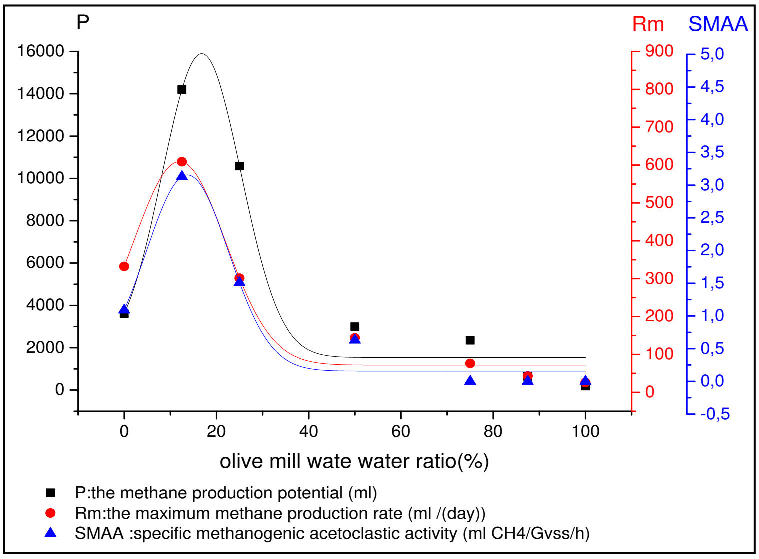

***Figure 5.** Gauss amplitude correlation between Olive Mill Wastewater ratio and the methane production potential, the maximum methane production rate, and specific methanogenic acetoclastic activity*

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DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

Table 8. *Values of Gauss amplitude function for the correlation between Olive Mill Wastewater amount and the methane production potential, the maximum methane production rate and Specific methanogenic acetoclastic activity*

	R^2	Y_0		xc		w		A		FWHM	Area
		Value	SE	Value	SE	Value	SE	Value	SE	Value	Value
P	0.93	1537.03	672.9	16.79	1.04	8.54	1.5	14360.36	1814.2	20.11	307413.4
R_m	0.93	71.77	26.06	12	1.19	9.98	1.2	537.33	58.15	23.50	13443.7
SMAA	0.92	0.15	0.15	13.69	1.25	8.96	1.1	2.99	0.35	21.11	67.41

Y_0; xc; w ; A; FWHM : Gauss amplitude function parametres
S E: Standard Error

Table 9. *ANOVA analysis of regression*

	DF	Sum of Squares	MeanSquare	F Value	Prob>F
P	4	3.36048E8	8.4012E7	46.64	0.005
R_m	4	592609.95	148152.48	54.76	0.004
SMAA	4	13.36	3.341	33.76	0.008

P: the methane production potential
R_m: the maximum methane production rate
SMAA: specific methanogenic acetoclastic activity

Based on the adjusted correlation coefficient (R^2 > 0.9), we can approve a good agreement and advocates greater significance of the model.(Niladevi et al., 2009). ANOVA of the fitted model for the P, R_m, and SMAA (*Table 9*) demonstrates that the model is significant due to the F-value of 46.64, 54.76, 33.76 respectively and the low probability P-value ($p \leq 00$). Generally, an F-value with a low probability P-value suggests a significant regression model (Rene et al., 2007). The maximum value of P, R_m and SMAA are obtained from the (x,y) coordinates of amplitude, and that the Limit of synergistic effect is calculated from the addition of Xc to the W value, *Table 10* summarizes these results.

Table 10. *Optimization parameters*

Parametres	Maximum value	Olive mill wastewater ratio %	
		Optimum ratio	Limit of the synergistic effect
P (ml)	15897.39	16.79	25.33
R_m (ml /d)	609.1	12	21.98
SMAA (ml CH_4/VSS/h)	3.14	13.69	22.65

P: the methane production potential
R_m: the maximum methane production rate
SMAA: specific methanogenic acetoclastic activity

To verify the limited mixing ratio, by considering the waste activate sludge (WAS) as the main compound and the olive mill wastewater (OMW) as the additional mixing compound in this study, the calculation of the relative fraction "*f*" from the specific production of the co-digestion dived to the WAS mono-digestion alone was done. This

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DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

factor give an exponential correlation (*Figure 6*) with good R^2 (0.962) and statistical significance (p = 0.016). Logically to obtain synergistic effect the "*f* "must be upper than1 (*f* > *1*).This condition is verified when the Olive mill wastewater ratio is lower than 22 (%).This result validate the result presumed by the Gauss amplitude model 21.98 and 22.65 (%) predicted by R_m and *SMAA* data, respectively.

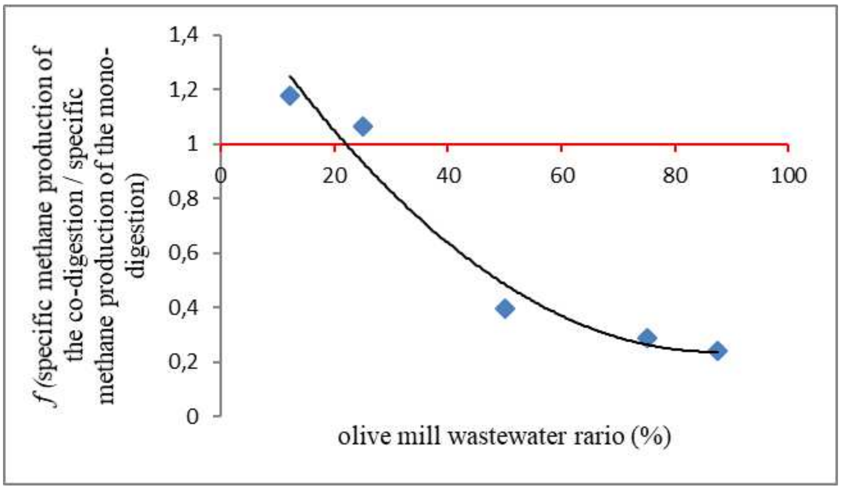

Figure 6. *Exponential correlation between Olive mill wastewater ratio and factor "f" of increase in the specific methane production*

Discussion

The GC-MS identification of phenolic compounds present in olive mill wastewater showed qualitative differences amongst the different research paper according to cultivars and their geographical origin (La Cara et al., 2012; Leouifoudi et al., 2014).

The best cumulative methane production was done at WAS/OMW ratio of 87.5/12.5 (12000 ml) without adjusted pH (neutral), this result is comparable to the result of codigestion of olive mill wastewater and swine manure established by Azaizeh and Jadoun (2010) with a 14000 ml of biogas under adjusted pH (neutral), at 38 ± 2°C for 11 days using Gadot sludge (25 g) or Prigat sludge (25 g) added to 250 ml of olive will waste water (Azaizeh and Jadoun, 2010). In our study the addition of OMW (WAS/OMW: 87.5/12.5) up to 72.34% in the cumulative production, this result is better comparativly to the codigestion of pig manure and OMW at pig/OMW ration equal to 60/40 which up to 40% the production of both substrates (Kougias et al., 2010).

The synergistic effect for co-digestion of waste activated sludge and olive mill wastewater at 87.5/12.5 mixture ratio was mostly attributed to a greater extent of volatile solids reduction and higher specific methane yield. This is a result of a supplementary requirement of nutrients and micro/trace elements from co-substrates, as the catalytic centers of the involved enzymes in methanogenic pathways (Pagés-Díaz et al., 2014; Xie et al., 2017). The olive mill wastewater is an additional source of Ni metal which is implicated in three recognized pathways of methanogenesis. This last one is regularly metal-rich enzymatic pathways when Fe is the most abundant metal, followed by Ni and Co, and smaller amounts of Mo (and/or W) and Zn. Fe is primarily present as Fe–S clusters used for electron transport and/or catalysis. Ni is either bound to Fe–S

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DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

clusters or in the center of a porphyrin unique to methanogens, cofactor F430. Zn occurs as a single structural atom in several enzymes (Glass and Orphan, 2012). However, other origins of synergisms must additionally be considered, such as the optimization of the C/N ratio (Xie et al., 2017).

However, OMW alone and other mixtures have acidic pH and low biogas yield because the methanogenic bacteria are most efficient at pH 6.5–8 (Mao et al., 2017). Based on the pH value at the end of digestion (*Table.7*) the WAS/OMW ratios: 75/25 and 50/50 reinforce the system buffer capacity.

The obtained results show clearly that SMAA and the lag phase λ values are moderately varied with initial condition COD/ N, pH, TS, VS, and waste type. This can be done in the dynamic of biomass composition and to the selective synergistic effect of bacterial communities. Li et al. (2015) in their study of AD system, of cattle and/or swine manure by metagenomics assays, noted that the substrate type, the ratio of co-substrate, play major roles in the COD/N ratio of substrate and free ammonia which play a central factors in the development and structuring of the bacterial communities in AD systems.

This peak profile of correlation can be explained by the limitation of the AD at high amounts of OMW % in the medium of fermentation. Though the WAS microorganisms have a limited capacity to degrade the high molecular-mass polyphenols in OMW biotreatment (Sayadi et al., 2000) and the inhibition of AD of OMW imply polyphenolic compound and the long chain fatty acids, which are considered as a toxic compound in the system of the AD (Hamdi, 1996; Hernandez and Edyvean, 2008; Saha et al., 2011; Oz and Uzun, 2015; Al-Mallahi et al., 2016). According to Borja et al. (1997) the cinnamic, benzoic, caffeic and protocatechuic acids are an inhibitor of acetoclastic methanogenesis at a limit concentration.

The long-chain fatty acids present in OMW are also responsible for its toxicity to methanogenic bacteria (Hamdi, 1992). The oleic acid is present in high concentration in OMW (Sayadi et al., 2000; Visioli and Galli, 2002; Elkacmi et al., 2017) which gives a high concentration of oleates. Comparatively to Sousa et al. (2009) an oleate added have given a stoichiometric value considering complete oleate oxidation. This indicates that acetoclastic activity was affected by oleate, so methane production in these cultures could be justified just by hydrogenotrophic activity or a limited acetoclastic activity (Sousa et al., 2013). Referring to Wu et al. (2017) the improvement in methane production rate was limited to the oleic acid concentration.

Conclusion

These results are consistent with the batch conducted tests, where the best performance was observed through a clear peak correlation which describe that the optimum settings for the maximum value of methane yield and acetoclastic activity are delimited by OMW components. The lower performance degrees achieved for a high OMW amount in codigestion. Gauss amplitude function is a good model to predict the area limits of the microbial communities synergistic effect which are not able to avoid inhibitory effects associated with the inhibitors present in OMW. Further research studies are needed to determine the microbial and biochemical properties of substrates. In addition, a follow-up study on the effects of individually isolated inhibitors on process performance.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ●ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52595274

FORMATION CHARACTERISTICS OF THE MUDFLOW PROCESS IN AZERBAIJAN AND THE DIVISION INTO DISTRICTS OF TERRITORY BASED ON RISK LEVEL (ON THE EXAMPLE OF THE GREATER CAUCASUS)

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(Received 25th Jan 2019; accepted 6th Mar 2019)

Abstract. In Azerbaijani part of the Greater Caucasus, which has been intensively developed in recent years in order to exploit recreational resources. Based on the interpretation of the ASP within the Azerbaijani part of the Greater Caucasus based on the derived from the effect of mudflow processes (the amount of material taken out, the erosive effect of the flow on the valley, the accounting of the mudflows and the basin as a whole, and the prevailing types and classes of mudflows, the geomorphological conditions of formation and passage mudflows, and statistical data on past mudflows) on the actual and possible damage affecting the population from mudflows a map-scheme was drawn up according to five-point scale. On the scale there are zones with a high (once in two-three years, one strong mudflow is possible) - V, with an average (possibility for one strong mudflow every three-five years) - IV, with a weak (every five-ten years is possible 1 strong mudflow) - III, with potential mudflow hazard - II and where no mudflow processes are observed - I. The synoptic-climatic conditions of mudflow formation have been studied, the mudflow links with macro forms of circulation over Europe and Siberia have been revealed.
Keywords: *modern dangerous geomorphological processes, recreation, precipitation, meteorological conditions, types of circulation, anthropogenic impact*

Introduction

Occurrence of catastrophic mudflows is one of the most serious problems, faced when developing mountain areas. Mudflow streams destroy various buildings, disrupt the functioning of infrastructure facilities, and may even lead to human casualties. Some recent mudflows that occurred in the world can be noted. On the night of April 1, 2017, the rivers of Mokoa, Sangoyako and Mulatos caused mudflow in Colombia, passing through 17 districts of the Mokoa city with 45 thousand dwellers. Eventually 254 people were killed, 400 people were injured, and 220 were missing [https://ria.ru/20170401/1491281111.html]. On August 8, 2017 a mudflow was responsible for killing of 23 people in the Sichuan province of China, destroying more than 70 buildings as well [https://ria.ru/20170808/1499983623.html]. On December 23, 2017, the mudflow and floods caused the death of about 90 people in the Philippines. Several dozens went missing [https://ria.ru/20171223/1511564592.html]. On the same day, 15 people were killed in the Los Lagos region of Chile as a result of the mudflow. About 200 houses were destroyed as well. The disaster disrupted supply of water and electricity [https://ria.ru/20171223/1511563855.html]. Many similar examples more can be noted. The above mentioned facts serve as an evidence for the topicality of studying mudflow hazard in the regions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52755291

Mudflows are observable in almost all mountain and foothill regions of Azerbaijan. The mountain areas occupy about 60% of the territory of Azerbaijan, where over 17% of the population lives (Chernomorets, 2005). However, in some cases, the mudflow-resistant installations are ineffective and may aggravate the consequences of mudflow processes, since they were built without sufficient scientific justification. Development of scientifically grounded methods of protection of settlements, industrial enterprises, agricultural regions, etc. from mudflows is the most urgent problems of further development in the mountainous regions of Azerbaijan.

In May-June of 2018 heavy rains in the mountainous areas of the Greater Caucasus caused mudflows to occur that left serious problems in Zagatala, Balakan, Agsu, Gabala, Guba, Shamakhi, Ismayilli and other districts. The mudflows on Agsu River flooded the sown areas of the villages of Basgal, Kohne Dahar, Sulut and Zarnava of the Ismayilli region, and destroyed the Basgal-Sulut Bridge. The mudflow, observed on the Goychay River in Goychay district damaged two two-storey residential buildings and also several private houses. 45 families were evacuated. In the Mashadganly village of Agsu district, the Agsu River washed off the road that connects the village with a population of 1500 people with the district center. The poles installed for power transmission and telecommunications were destroyed, water- and gas supply of the village was disrupted, and irrigation channels became useless completely. In the village of Madrasa, the Shamakhi district, a road connecting the village with the district center was damaged. Relatedly, the bridge was broken down, and communal facilities were heavily affected. In the villages of Buynuz and Diyalli of the Ismayilli district, the mudflows on Pirsaat River resulted in destruction of the telephone- and electricity lines. Mudflow on Dalichay River nearby the Katekh village of Balakan district affected more than 50 houses, leaving livestock and poultry killed and 8 small bridges destroyed as well. Electricity facilities and sources of drinking water were also damaged. The territory area of flooded areas made up 150 ha in total, including 10 ha of tobacco, and 140 ha of wheat, barley and corn plantations. The village roads, as well as part of the road of Yevlakh–Zagatala–Lagodekhi were damaged. Mudflow on Bum River (tributary of the Kishchay river) destructed the bridge connecting the Tikanly village with the city of Gabala. Residents of the village received assistance (*Fig. 1*). Similar processes on a smaller scale were recorded in the adjacent territories as well.

Figure 1. *Bridge demolished by the mudflow in the valley of the Bum River (tributary of the Kishchay River)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_52755291

Mudflows do not occur accidentally. Typically, they are preceded by a long period (stage) of pre-catastrophic changes (Alizade, et al., 2015). At present, there is no unambiguous answer to the question of the mechanisms of the occurrence of mudflows. In most cases even in the presence of all the necessary conditions, origination of this natural disaster is hardly possible without some additional impulse, whether it is the impact of one process (abundant snowmelt, rainstorm and others) or the total effect of many factors. Some territories in this regards, despite all the prerequisites, may remain stable for years. Others, on the contrast, quite unexpectedly become the place of the formation of catastrophic mudflows. However, “unexpectedly” does not mean “illogically” (Barinov, 2010).

In Azerbaijan, the most hazardous region in terms of debris mudflows is the territory of the Greater Caucasus (41°55’ n.l.-39°56’ n.l.; 46°46’ e.l.-50°33’ e.l.), the region that has become increasingly popular among tourists in recent years (*Table 1*).

Here, practically the whole spectrum of contemporary hazardous geomorphological processes (CHGP) such as high seismic activity, avalanches, rockfalls, landslides, screes, mudflows, etc. are observable.

Recreational and tourism nature of the development in the Greater Caucasus includes also the use of the territories in remote and inaccessible places where construction of various facilities is already underway. Relatedly, it is necessary to develop principles for forecasting, protecting and managing the CHGP.

In the Greater Caucasus, the duration of mudflows is different by various regions. On the southern slope, 45.5% of the mudslides typically last up to 6 hours. Short-time mudslides are observed in the basins of the rivers of Goychay, Girdiman and Agsu. To the west of the Goychay basin, the duration increases. The most continuous mudflows are observed in the western part of Greater Caucasus. The duration of mudflows in the northeastern Greater Caucasus and also Gobustan is 1 to 4 h as usual (80% of the total mudflows). On the southern slope of the Greater Caucasus, the maximum of mudflow is observed between 20.00 and 22.00, whereas on the northeast slope and Gobustan in 16.00-18.00 hours. Falling rainfall and passing mudflows in the evening hours are conditioned by convective processes, since invading cold air masses are heated above the plains. This heating leads to the development of convective ascending currents and the formation of cumulonimbus clouds and mudflow-forming rainfall.

The purpose of this study is to research the conditions of the formation of mudflows, to identify the main geomorphological factors that play a large role in the formation of mudflows, as well as to establish the connection of the passage of mudflows with circulation types with the identification of primary features.

Materials and methods

The issues of exploring distribution and differentiation of mudflow foci by different altitudinal belts, as well as the study of synoptic and climatic conditions of mudflow formation and the identification of primary mudflow hazard features are the important problems, solution of which can allow develop specific measures on reducing the formation of mudflow foci and the passage of mudflows.

The materials of satellite imagery (SRTM) were used as the initial data for creating the map-scheme of mudflow hazard of the studied region.

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DOI: http://dx.doi.org/10.15666/aeer/1702_52755291

Table 1. *Dates of passage of the dangerous mudflow processes in the Azerbaycan part of the Greater Caucasus for 2016-2018. (Compiled by Tarikhazer, 2018)*

№	River	Date of passage of mudflow	Duration of mudflow (hours)	The causes of beginnings of mudflow	Consequences of mudflow
1.	Gudialchay	17.04.2016	2	Shower	The bridge in Guba district is destroyed
2.	Pirsaatchay	18.04.2016	2	Shower	The material damage was caused to the villages of Ranjbar and Navai of the Shamakhi district
3.	Pirsaatchay	09.05.2016	2	Shower	The crop areas were destroyed and perished livestock in the villages of Archiman and Chagan of Shamakhi district
4.	Gusarchay	19.05.2016	2	Shower	The road in the village of Guzun of the Gusar district is destroyed
5.	Gudialchay	20.05.2016	2	Shower	The bridge in the village of Tuler of the Guba district was destroyed
6.	Kurmukhchay	12.06.2016	2	Shower	The material damage was caused to the houses of the villages of Gakhbash and Ibakhly of the Gakh district
7.	Pirsaatchay	12.06.2016	5	Shower	The damage was caused to Kurdmashy, Gushendzhe, Garakollug, Balik, Enishdibi, Talysh, Yenikend, Shukurchyu and Ashygbayramli villages of the Ismayilli district. The mudflows destroyed, orchards, grains and grape fields, and livestock perished. Part of the road of Garamyaryam-Ismayilli was destroyed. In the village of Gushendzhe mudflows destroyed one house, whose resident was seriously wounded
8.	Gusarchay	25.06.2016	2	Shower	Damage was caused to more than 20 houses, crop areas of the village of Guzun of the Gusar district. The livestock perished. Ruined bridge
9.	Kishchay	03.07.2016	2	Shower	The bridge connecting the village of Kish of Sheki district with the district center is damaged. 35 villagers were evacuated
10.	Lazachay	01.09.2016	3	Shower	53 people of village of Laza of Gusar district evacuated. Partially destroyed the Guba-Khinalig road and the bridge for 29 km this route. The traffic was interrupted. The mudflows also caused damage to residential buildings in the village of Utug in the same district, electricity was not delivered to this settlement
11.	Gudialchay	01.02.09.2016	5	Shower	About 10 villages of the Guba region were damaged. The mudflow destroyed the supports of power lines in the village of Krasnaya Sloboda. The section of the road Guba-Khinalig was destroyed. The mudflow destroyed almost 30 pillar of telecommunications, as a result, communication with 20 settlements of the district was interrupted. The greatest damage was caused to the automatic telephone exchange, functioning in the villages in the directions of Gonagkent-Sokhub, Kupchal-Gechresh, Zargova-Chichi and Khinalig
12.	Damiraparanchay	02.09.2016	2	Shower	The mudflows made traffic difficult on the central roads of the Gabala district. The mudflow washed away the car (VAZ-21015 model)
13.	Geychay	02.09.2016	4	Shower	In the villages of Istisu, Chaygovushan, Galynjag and Sumagallli of Ismayilli district, damage was caused to crop areas and gardens. The poultry has perished

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14.	Agsuchay	20.10.2016	3	Shower	Mudflows destroyed the coastal dams near the villages of Mashadganly and Agalarbeyli of Agsu district, as well as in the city of Agsu. The mudflows flooded the mountain village of Kyalva, interrupting the communication with the rest of the district
15.	Turyanchay	17.05.2017	2	Shower	Damage to crop areas and gardens of Gabala district is damaged
16.	Agsuchay	25.05.2017	2	Shower	The material damage was caused to the villages of Kechmaddin, Archiman and Talishnuru of the Shamakhi district. Power poles are destroyed
17.	Shinchay	29.05.2017	3	Shower	Roads were destroyed in the village of Kichik Dehne in the Sheki district
18.	Jeyrankechmez	02.07.2017	2	Shower	76 km of the Baku-Shamakhi road is damaged
19.	Delichay (tributary of the river Talachay)	14.04.2018	5	Shower	In the village of Mazikh of Zagatala district homestead land of 200 houses flooded, 15 houses are damaged, poultry has perished. The mudflows were washed away the a private car of the “VAZ 2106” model. Damage to power lines and material damage was causedto houses and household lands of the villages of Mamrukh, Hoytala, Yolayryj, Mago
20.	Agsuchay	11.05.2018	2	Shower	The crop areas in the villages of Basgal, Kekhni Dahar, Sulut and Zarnava of the Ismayilli district, were flooded, and the Basgal-Sulut bridge was destroyed
21.	Talachay	14.05.2018	4	Shower	In the village of Mazikh of Zagatala district the crop areas are flooded. 4 people injured
22.	Damarchin (the tributary of the Kishchay river)	02.06.2018	4	Rains	A pedestrian bridge has been destroyed in the city of Sheki on the road to the fortress Naryngala and Shambagy
23.	Geychay	03.06.2018	3	Rains	Damaged two two-story residential buildings and several private houses. 45 families were evacuated
24.	Agsuchay	03.06.2018	2	Rains	In the village of Mashadganly in the Agsu district, the mudflow was washed away the road that connects the village (with a population of 1500 people) with the district center. The supports of power lines and telephone communications were destroyed, water and gas supply to the village was disrupted, irrigation channels were completely destroyed
25.	Pirsaatchay	04.06.2018	2	Shower	In the villages of Buynuz and Diyalli of the Ismailli district, telephone and power transmission lines were destroyed
26.	Dalichay	12.06.2018	4	Shower	In the village of Katekh of the Balakan district, more than 50 houses were flooded, livestock and poultry were perished, 8 small bridges were destroyed. Power lines, sources of drinking watere was damaged corp areas of a total area of 150 hectares, including 10 hectares of tobacco, 140 hectares of wheat, barley and corn was flooded. The village roads and part of the road Yevlakh-Zagatala-Georgia are damaged
27.	Agsuchay	12.06.2018	2	Shower	In the village of Madrasa of the Shamakhi district a road has been damaged that connects the village with the district center. The bridge was destroyed, livestock was perished
28.	Bumchay	13.06.2018	3	Shower	The mudflow destroyed the bridge connecting the Tikanly village of the Gabala district with the district center. Residents of the village received assistance
29.	Agsuchay	17.06.2018	2	Shower	The road and power lines in the Machachy village of Ismayilli district have been destroyed. Residents were left without light, water supply was disrupted. The cultivated areas and orchards are blurred

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Data SRTM represent the processed results of a radar topographic survey of the surface of the globe, made by the method of radar interferometry, produced from the American ship “Shuttle” in February 2000.

The result of the survey was a digital model of relief of 85% of the Earth’s surface. For data processing SRTM we used a software ArcGIS package.

The main operations were performed in ArcGIS Spatial Analyst, 3D Analyst and Geostatistical Analyst applications.

In this work, the materials about overpast mudflows (1891–2016), rainfall ≥20 mm/day for the mudflow period (1964–2014), and about types of circulation according to B. D. Dzerdzeevsky (1899-2015) and G. Ya. Wangenheim (1891-2002) were used.

Results

In the territory of Azerbaijan, mudflow processes are observed more often and on larger scale in the Greater Caucasus. Formation, development and geographical spread of these processes are considerably driven by geological and tectonic conditions (which, combined with other contemporary exogeomorphological processes, contribute to the formation of scress, rockfalls, landslides, etc.), lithological conditions (sedimentary deposits of the Jurassic, Cretaceous and partly Paleogene-Neogene systems such as clay shales, sandstones, limestones, marls, which, in connection with the intensive weathering, create huge loose and detrital materials), tectonic structure of the mountains (the latest tectonic movements and discontinuous infractions with a large amplitude), seismicity of the territory (7-8 and higher seismicity points), the amount of precipitation (on the southern slope, within the heights of 1500-2700 m it falls at 900-1600 mm), etc. (Alizade, et al., 2015). The CHGP data in the conditions of high anthropogenic load reflects the base for hazard posed to infrastructure of Sheki-Zagatala, Shamakhi-Ismayilli, Guba-Khachmaz and other territories. In these regions, cities, significant number of settlements, tourism complexes, highways, and electricity and communication lines that contribute to the demographic and industrial potential, are typically located on the cones of the rivers of mountain areas, as well as terraces (*Fig. 2*) and seismogenic-gravitational blocks that are linked to risk zones.

***Figure 2**. The right bank of the river Dashagylchay. The threat of destruction of the village of Bash-Dashagyl*

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Contemporary and latest tectonic movements. Endogenous processes

The Greater Caucasus was formed as an alpine orogenic structure between the Scythian and Transcaucasian epihercine plates (Alizade, 2004). This is a megantiklinorium that covers a narrow strip of the Caucasian geosyncline and the adjacent parts of the Scythian and Transcaucasian epihercine plates.

Longitudinal faults of the north-western stretching play an important role in the development of the Greater Caucasus, since they contributes to high seismicity of the region. Transverse (Transcaucasian) deep faults play an important role in the structure of the Greater Caucasus as well (*Fig. 3*).

The Greater Caucasus is characterized by the most intense manifestation of the latest tectonic movements. In the late Sarmatians, most of the territory (with the exception of the Watershed Range) was below sea level, and the mountain peaks had a height of 600-800 m. The total sweep of the newest uplifts of the Greater Caucasus at the tops of mounts of Bazarduzu, Shahdag and Tufandag during the Pliocene-Quaternary period exceeded 3600 m (Budagov, 1993).

The existence of a system of longitudinal faults separating the zones of uplifts from the descending zones in the region indicates the folded-block character of the development of the latest tectonic movements and the block structure of the morphostructures of the Greater Caucasus (Alizade, et al., 2016).

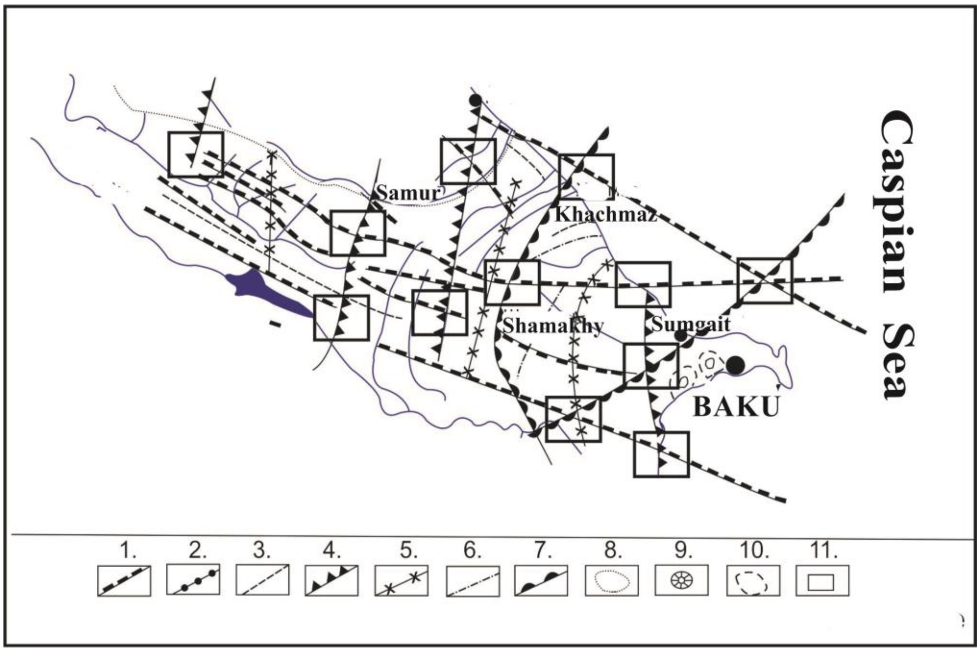

Figure 3. *Map of transverse (Transcaucasian) deep faults of the Greater Caucasus (Compiled by E. K. Alizade)*

Lineaments corresponding to faults (thrusts and reverse faults) of the longitudinal (general Caucasian) direction:

1. Regional deep faults, which limit large longitudinal fold-block stages
2. Local faults corresponding to the boundaries of longitudinal fold-block morphostructures
3. Discontinuous violations that determine the details of morphostructures

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Lineaments corresponding to faults (fault-shifts) of the transverse (anti-Caucasian) direction:

1. Regional deep faults, limiting transverse megablocks
2. Local faults corresponding to the boundaries of transverse block segments
3. Breaking violations
4. Large interregional diagonal disjunctive zones
5. The contour of the Samur ring object
6. Large volcanic centers
7. Ring structures (tectono-volcanogenic)
8. "Geodynamically tensional fields"

The morphostructures of the Greater Caucasus are characterized by the alternation of longitudinal (general Caucasian) and transverse steps. Their development is due to differentiated neotectonic uplifts along longitudinal and transverse deep faults with an amplitude of uplift at +3–3.5 km. Features of the neotectonic regime have determined the formation of high-altitude morphographic steps in the Greater Caucasus and the corresponding spectra of high-altitude landscape belts, as well as the specifics of the manifestation of CHGP within their boundaries. Seismic phenomena are confined to areas of contrasting relief and active modern tectonic movements as well as to zones of active deep faults and places of their intersection, i.e. morphostructural nodes.

The seismotectonic factor defines the development of avalanches, mudflows, landslides, screes and rockfalls. Intensive gravitational displacements of detrital masses are observed particularly in the earthquake foci. On the tectonically tense slopes with high relief energy, the regime and intensity of the gravitational processes are sensitive in regard to earthquakes of 3-4 points. The talus and scree processes become activated on the slopes of 40° or more steepness, where conditions for the formation of mudflow foci are shaped. Landslides are activated on slopes of 15° of steepness. Among all factors of mudflow development, the seismotectonic factor serves as the basis for the formation of foci of the solid phase of mudflows and the unpredictability of the hazard level (Laperdin et al., 2011).

Exogenous processes

The development and intensity of exogenous processes are significantly influenced by two factors of fracturing:

1) The conditions of rocks and the grade of fissuring of rocks. In the high mountains of the Greater Caucasus, sandstones, sandy, clayey and calcareous shales, limestones and other rocks are widely spread. The rocks are easily destroyed under the influence of physical weathering. In the mid-mountain and low-mountain belts clay rocks are most common, and are subject to intense erosion;

2) Climatic conditions (regime, amount, annual distribution and formation conditions of mudflow-forming precipitation; air temperature, etc.). The highest precipitation falls in the middle belt of Greater Caucasus region (up to 1600 mm), whereas the lowest amount is recorded on the southern coast of the Absheron Peninsula (up to 200 mm). The average annual air temperature is also unevenly distributed: in the highland belt it is from below 0 to +2 °C; in the mid-mountain belt it is from 6 to 10 °C; and in the lowland belt and lowland zones from 11 to 14.5 °C. The diurnal amplitudes of air temperature in the zone of formation of mudflow foci, as an indicator of physical weathering, fluctuate within 8-12° (Ayyubov, 1962).

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It should be noted that climatic factors are involved in the pre-formation of loose mud material, while other factors, including heavy rainfalls are directly involved in the process of mudflow formation. As analysis of data on mud-forming precipitation shows, mudflows in the territory of Azerbaijan are formed if diurnal precipitation is 20 mm and higher per day (Nabiyev, 1985).

Extreme values of mudflow-forming rains in different mudflow-prone areas vary within different limits. For example, on the southern slope of the Greater Caucasus the amount makes up 20-188 mm, while in the northeastern Greater Caucasus and Gobustan it is 20-132 mm. The maximum amount of mudflows in the main mudflow areas is observed during the period of the greatest frequency of abundant precipitation. The number of days with a daily rainfall of ≥20 mm on the southern slope of the Greater Caucasus increases towards an altitude of 2200-2400 m, whereas on the northeastern slope precipitation increases towards 3000-3200 m. It is defined that the main amount of precipitation with a daily amount of ≥20 mm falls out at relatively short intervals, during which the average intensity was very high.

According to the analysis, the share of the storm (duration and amount of precipitation) in the overall abundant precipitation of ≥20 mm is various by mudflow-prone areas, since on the southern slope of the Greater Caucasus, they constitute 20.1 and 58.2% respectively, whereas on the northeastern slope of the Greater Caucasus and Gobustan 15.2% and 50%. It was revealed that mudflows occur during continuous rains that last for several hours, accompanied by short showers, with high intensity.

Quantitative and qualitative data of macrocirculation were determined to analyze large-scale processes leading to downpour precipitations and at their high intensity also to mudflows in the study area. Meantime, to quantify the types of circulation, the criteria of the general index, proposed by Katz (1973) were used. The study showed that in days of mudflows, mainly meridional processes predominated, but in some cases the main role was played by zonal processes. It should be noted that, as a rule, zonal processes in the territory of the South Caucasus, including Azerbaijan, do not result in heavy rainfall and mudflows. Typically, the fall of intense downpours and the formation of mudflows can be observed only in the conditions of invasion of cold, moist and unstable-stratified air masses, which occurs only in meridionally-developing processes. A study of mudflows affected by zonal processes showed that a few days before the beginning of mudflows over Western Europe, a meridional conversion of circulation occurred, as a result of which cold air masses in the rear part of the high-rise hollow trough invade the southern regions of Western Europe across the British Isles, where the upper-level frontal zone (ULFZ) and cyclogenesis occur.

Further, the high-rise hollow and the cyclone gradually shift to the east – towards the territory of Asia Minor and the South Caucasus. In other words, the formation of mudflows occurred in condition of interaction of cold air masses, entering the ULFZ system during the meridional transformation period. While meridional transformations happen, anticyclones are formed under the high-level crest in the lower troposphere over the north of Europe. In the days of mudflow formation they shift to the central regions of the European part of Russia. The stationing of these anticyclones takes place when the cyclone is blocked over Asia Minor – the east of the Mediterranean Sea. It should be noted that the division of atmospheric processes in a quantitative way into zonal and meridional processes gives a general idea of the directions of air currents at certain period of time, but this is not always sufficient for a detailed description of the processes entailing abundant downpours and mudflows. Therefore, we preferred the use

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of qualitative methods for determining the nature of the circulation as the more expedient way. To identify the relationship between atmospheric circulation and mudflows in Azerbaijan, circulation types were used in accordance with the classification of Wangenheim (1935) and Dzerdzeevskij (1975). The simpler form of typing of atmospheric processes was considered by Wangenheim (1935), who proposed three types or forms of basic atmospheric processes: western (W), meridional (C) and eastern (E). In the case of type W, the western conveyance of air masses prevails; in the case of C, transportation occurs from the north-west and the north, whereas for E the direction is from northeast. The study found that there is a direct connection between the mudflows occurring in Azerbaijan and the E processes, namely, the intensification of these processes makes mudflows more active in the research area. Conversely, during the W processes, mudflow formation is enfeebled. The obtained results are justified not only in the Caucasus, but also in the regions of Central Asia (Dzerdzeevskij, 1975), since on the rivers of these territories the activation of mudflow phenomena is observed when E processes prevail, whereas it weakens during W processes. When macroforms E prevail, then precipitation falls out above the norm in Central Asia, Kazakhstan and also the Caucasus, which leads to an increase in the likelihood of mudflow formation. When W processes dominate, precipitation is low in those regions, and mudflow activity is weakened. In the case of western transportation, the high-pressure axis is not indifferent at what latitude it is.

Therefore, the processes W can be divided into two subtypes (W_1 and W_2). This division shows that the weather conditions in the territory of the South Caucasus, as well as along its latitudes are different. In the first case (W1), the high-pressure region covers the southern regions of Europe and consequently, while this subtype is present, as in Southern Europe, dry weather prevails.

In the case of subtype W_2, on the contrary, the anticyclone area is located at latitudes north of 50° northern latitude, while cyclonic weather with sufficient moisture prevails over the southern regions of Europe, including the South Caucasus (Sheкo, 1980).

To estimate the synoptic situations responsible for the formation of mudflows, the classification of atmospheric circulation types, proposed by Dzerdzeevskij (1975), was used as well.

It was established that the mudflows in Azerbaijan are mainly formed under the subtype 12a (the subtype of the northern meridional circulation) and 13l (the subtype of the southern meridional circulation that prevails in the summer). These subtypes were observed during the all months with high probability of occurrence of mudflows in the period of 1960-2014. They led mudflow-forming precipitation. In certain years, some types were more intense than others, and differed in terms of the increase in frequency. For example, during the decrease in the frequency of subtype 12a (1980-1992 years), an increase in the subtype 13l was observed, whereas in 2008-2014, the contrary was observed. In the period of 1899-2014, the participation of macroprocesses of the subtype 12a was always noticeable in the mudflow formation. However, the subtype 13l processes became intensified since the beginning of the 1960s, and their frequency increased from year to year (*Figs. 4* and *5*).

Under the subtype 13l, the exit of southern cyclones to the territory of the Northern and Southern Caucasus was observed. These cyclones are characterized by high speed of movement and significant temperature contrasts. Relatedly, within the short time, they entail rainfall leading to activation of mudflows. In the case of subtype 12a a blocked anticyclone is formed over the Siberia and stretched to the southern regions of

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Western Siberia. At this time, the cyclones formed over the Mediterranean Sea, move to the territory of the South Caucasus, and farther to the north-east. When warm and moist masses formed over the Mediterranean Sea meet cold arctic air masses, the atmospheric fronts become activated, as a result of which precipitation falls out above the norm in the study area that causes the mudflow activity of the rivers to increase.

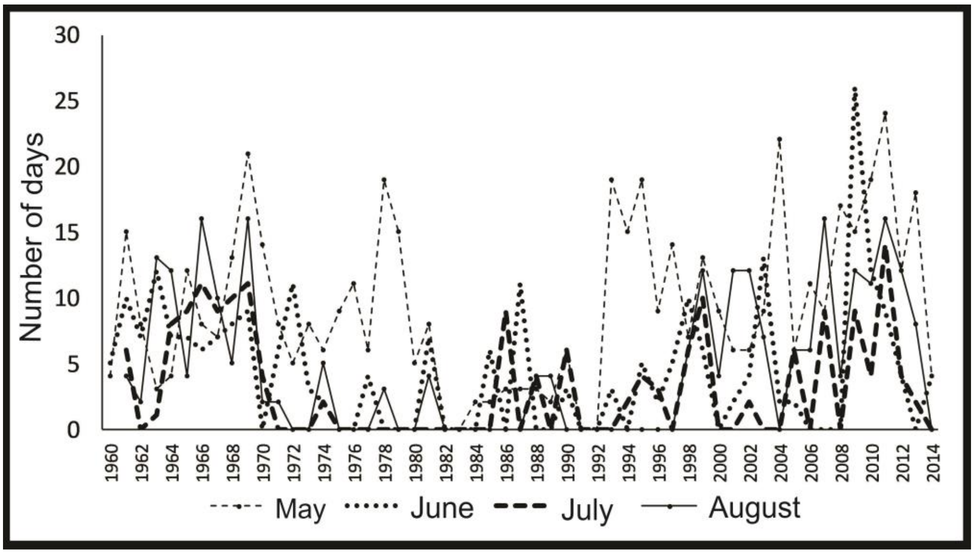

Figure 4. *Repeatability of the circulation type 12a in the mudflow dangerous months*

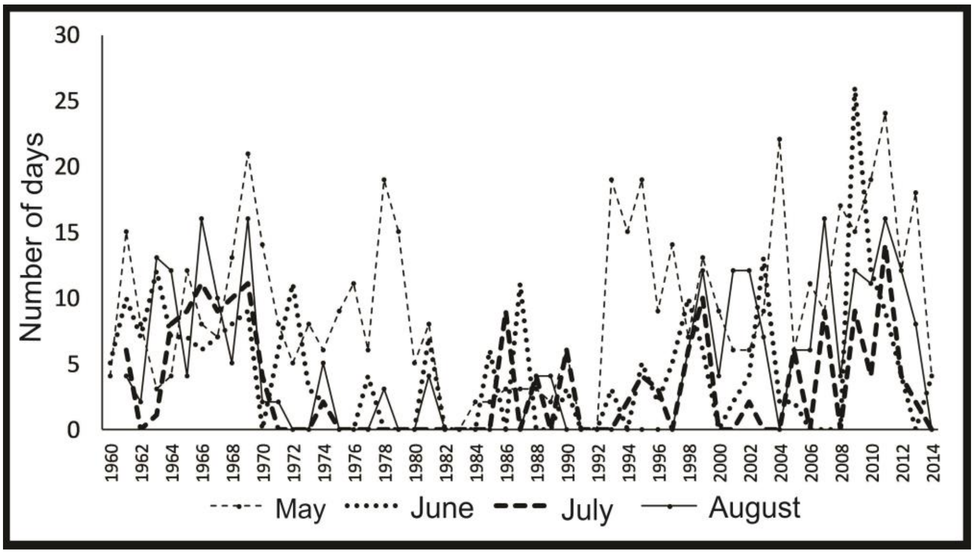

Figure 5. *Repeatability of the circulation type of 13l in the mudflow dangerous months*

3) Relief (depth of dismemberment, change of absolute heights, exposure of slopes, direction of watersheds, etc.). A significant part of the territory of Greater Caucasus consists of slopes, the steepness of which exceeds 10-15°. This feature left an imprint on the nature of the relief-forming processes. Steep slopes cover significant (upper and middle) parts of river basins. Within the high mountains, intensively dissected by erosion, denudation and nival processes, they are characterized by the development of

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glacial and gravitational exogenous processes. The energy of the relief and the slopes are the determining conditions for the speed, nature and massive motion of the soils on the slopes. The slopes of the mountains and ridges of the Greater Caucasus, composed of clayey shales, sandstones, marls and limestones, are distinguished by the development of planar and linear flushing, different rates of drift and accumulation intensity. For example, in the headwaters of the Gusarchay, Velvelichay and Gudialchay rivers, the steepness of slopes of river valleys is more than 45° (Alizade, et al., 2015; Tarikhazer, et al., 2017).

Here, the movement of detrital masses occurs at a high speed, and the removal of the weathered material predominates over the speed of accumulation of debris. Consequently, the slope factor here is the main condition for the mass movement of detrital material, regardless of the physical and mechanical properties of the soils. However, such types of mass movements as landslides in most cases do not depend on the slope of the slopes, rather being a result of the breaking of stability in relationship with the lithological differences. In the highlands, where the energy of the relief is more than 1000 m, and the steepness of slopes is more than 30-40°, the flushing intensity exceeds 1 mm/year, while in the low mountains it is 0.10–0.5 mm/year.

The largest washout from the surface is observed in the mid-mountain zone, which is apparently due to the denudation features of the bedrocks and the destructive influence of the climatic factor. In general, the speed of denudation is 0.5 mm/year in the Greater Caucasus, while in the highland and in part the middle part, it is 1 mm/year.

4) Density of the river network has a great influence on the depth of the relief fragmentation. In the high-mountain and mid-mountain zones of the Greater Caucasus, the density of the river network is 0.4-0.7 km/km^2, and in the low-mountain and plain zones, it makes up 0.1-0.2 km/km^2. Eventually, the mudflow-forming factors, depending on their combination, serve as the basis for the development of mudflow streams which poses the level of risk in this territory.

The flows are typical for all the high-altitude belts of the Greater Caucasus, and the most typical are the mud flows, the mud- and stone flows, and also the debris (stone) flows which are typical for the southern slope of the Major Caucasus Ridge. The rivers of the southern slope of the Greater Caucasus are typical mudflow rivers, which are different in terms of frequency, strength and type of mudflow. The most powerful mudflows are typical for Kurmukhchay, Shinchay, Kishchay, Balakanchay, Demiraparanchay, Tikanlychay and other rivers. Flows of mud composition are typical for rivers, the basins of which are composed of clays and shales (tributaries of Talachay, Mukhakhchay, Jeyrankechmaz, Sumgaitchay and other rivers). Mudstone and stone flows (debris flows) are developed in river basins, the upper parts of which are composed of shales, sandstones, limestones and other rocks (Gamidova, 2011).

Within boundaries of the basin of the Kish River, the most powerful mudflow tributary river is Damarjig, the territory area of basin of which is 66 km^2. It lies from north to south for a distance of 12 km (*Fig. 6a* and *b*). Forests are developed only in the lower parts of the valley up to 2000 m of elevation. Above this altitude, steep rocky slopes, devoid of vegetation are found. The thickness and spatial distribution of slope deposits are in relationship with the nature of the relief and the lithology of the rocks, as well as the impact of mudflow-forming torrential rains.

In the basin of Kurmuk River, the main mudflow-sensitive tributary is Bulanigsu (with total area at 18 km^2). This river originates at an altitude of 3200 m. In its lower

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part, there is a forest with an area of 4 km^2, and the rest of the territory is devoid of vegetation because of the steep slopes. The natural conditions of the basin determine the intensive co-development of subnival and gravitational, as well as gravitational and denudation processes. The main tributary of Shin River with high prone to mudflow process is Shikhgaflan (with total area at 12 km^2). Its waters are formed on the slope of the Garagaya Mount (3460 m). In the lower part of the river there is a forest with an area of 2 km^2, while the rest of the basin consists of rocky slopes with a small area of mountain meadow landscapes. Babachay is another tributary of Shin River characterized by occurrence of mudflows. Mudflows are observable also in the basins of the rivers Katekhchay, Talachay, Mukhakhchay, Dashagylchay (*Fig. 7a* and *b*), Kungutchay, Filfilichay, Tikanylychay, Dyamiraparanchay, Gumbashchay, Lyakitchay, Zayzitchay, Gashgachay, Gamzalichay and others flowing on the southern slope of the Greater Caucasus (Tarikhazer, et al., 2013, 2014).

In this research, the hearts feeding the mudflows are grouped morphologically as follows: screes (the feeding areas of Shinchay, Kishchay, Damiraparanchay, Mazymchay and other rivers); rockfalls (high-mountain areas in the basins of Kurmukhchay, Tikanylychay and other rivers); moraines (Kishchay, Kurmukhchay, Vandamchay and other rivers); landslides (the upper parts of Kishchay, Balakanchay, Filfilichay, Khalkhalchay, Mukhakhchay, Gabalachay, Dashagylchay and other rivers) (Tarikhazer, et al., 2013, 2014, 2015).

On the northeastern slope of the Greater Caucasus, mudflows are observed mostly in the basins of Gilgilchay, Atachay, Shabranchay, Devechichay, etc. rivers. Traces of mud-stone mudflows in the form of mud-shafts and mud masses are well seen in the wide floodplains of the rivers Gilgilchay and Atachay. Origination of flows of mud and stone composition in these river basins is obviously associated with the widespread development of landslides as well as the co-development of landslides and mudflows in the catchment areas of these rivers (Tarikhazer, 2018).

In the arid-lowland zone, typically the formation and stream of mud as well as the related flat and diffuse forms of hardened debris are observed. Often solidification of muddy mass can be observed at the bottom of ravines and girders due to their "enrichment" with silt and clay inclusions in conditions of the short-term downpours, the waters of which are one of the main driving factors (Khain, 1984).

a b

***Figure 6. a** The section of the highway Sheki-Oguz, blurred as a result of mudflow on the river Kishchay. **b** Bridge damaged by the mudflow in the valley of the Chukhadurmaz river (tributary of the Kishchay river)*

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a b

Figure 7. ***a*** *Upper detrital cone of the Dashaghlachay River.* ***b*** *The detrital cone of the Dashaglychay River on 1.5-2 km below the village of Bash-Dashagyl*

Hearts of mudflow formation are confined mainly to extensive erosion catchments and massifs of landslide- and mudflow origin. Foci of mudflow of such types as erosion funnels are typical for high-mountainous parts of the basins of the rivers Gudialchay, Jimichay, Babachay and Gusarchay. The mudflow foci of basins of such rivers as Gilgilchay, Atachay, Tugchay, Shabranchay and Takhtakorpuchay are associated with flows of landslide, as well as flows of landslide and mudflow origin. In this regard, Atachay River can be shown as the most typical case. Here, the landslide massif of Bakhyshly serves as the foci of the mudflow formation in general related to watercourse stretched from Bakhishly village to Halanj village at a distance of 6-8 km. Such similar hearts are available in the valley of the Velvelechay River between the settlements of Gonagkend and Afurja. In the southeastern Greater Caucasus, Jeyrankechmaz, Sumgaitchay, Pirsaatchay, etc. can be shown as examples for mudflow rivers (flows of mud composition) as well (Alizade, et al., 2015 Tarikhazer, 2018).

Proceeding from the above, it follows that in the process of mudflow formation the considerable role is played by the high-mountainous (rocky) belt (2200-3000 m of altitude and more), since here, contemporary exogenous processes (especially physical weathering) are active. In the mid-mountain belt at 1200-2200 m of altitude, the slopes are densely covered with forest vegetation, which protects the surface from intensive erosion and weathering. In the low-mountain belt (from 400-800 m to 1200 m), the vegetation cover, the decrease in the energy of relief, the moderate contrast of the daily temperature variation and other factors prevent erosion and weathering (Alizade, et al., 2016)

Based on the interpretation of the aerospace images within the Azerbaijani part of the Greater Caucasus, a map-scheme reflecting hazard rate of mudflow processes (in terms of the volume of transported materials; the erosive effect of the flow on the valley; occurrence of mudflows in the tributaries and the basin as a whole; types of the prevailing types and classes of mudflows; the geomorphological conditions of origination, formation and passage of mudflows; statistical data on the happening mudflows), as well as actual and possible damage posed on the population, was drawn up (*Fig. 8*).

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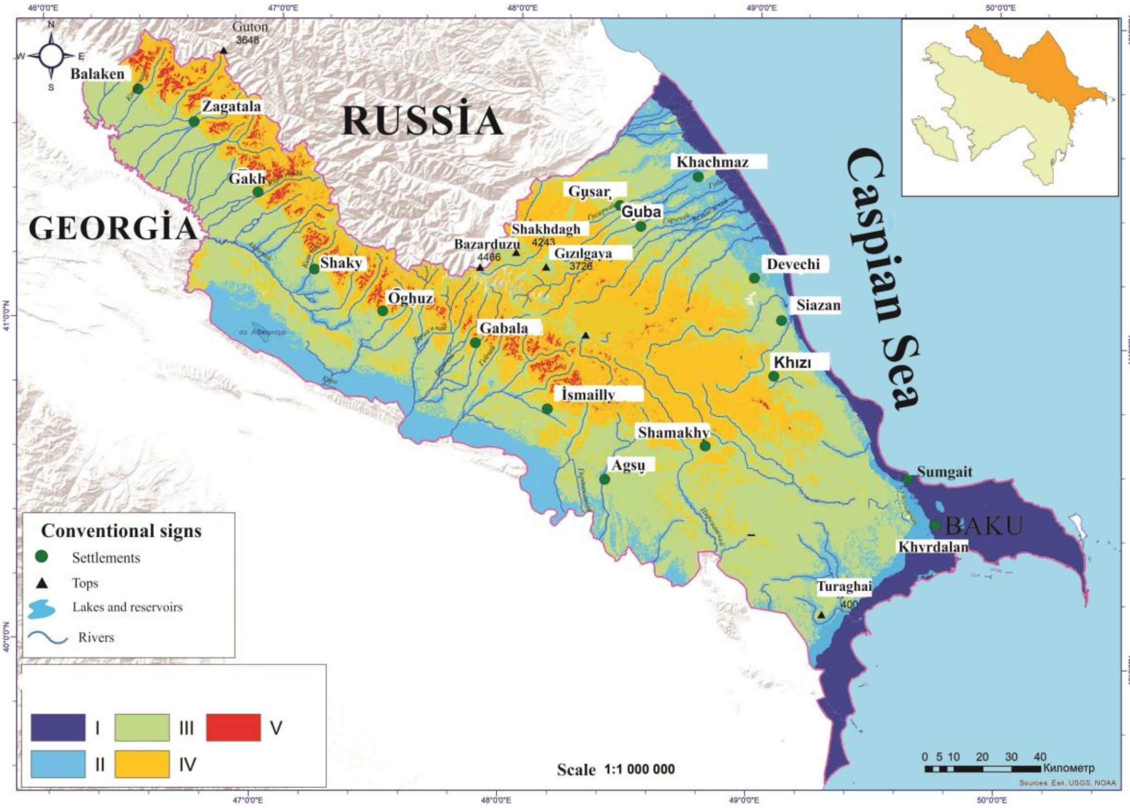

Figure 8. Map-scheme of mudflow hazard in the Azerbaijani part of the Greater Caucasus (compiled by Tarikhazer, 2018). Territories, where there are no mudflow processes - I point. Territories with potential mudflow hazard - II points. Territories with a weak mudflow hazard (once in 5-10 years is possible 1 strong mudflow) - III points. Territories with an average mudflow hazard (once in 3-5 years is possible 1 strong mudflow) - IV points. Territories with high mudflow hazard (once in 2-3 years is possible 1 strong mudflow) - V points

As follows from the map-scheme, the areas with high mudflow hazard include the basins of the rivers Mazymchay, Balakanchay, Katekhchay, Talachay, Mukhakhchay, Gashgachay, Kungutchay, Dashagylchay, Filfilichay, Vandamchay, Goychay (mudflows of water- and stone composition); the basins of the rivers Kurmukhchay, Shinchay, Tikanlychay, Bumchay, Demiraparanchay, the upper parts of the Girdimanchay, Pirsaatchay, Sumgaitchay, Tugchay, Gilgilchay, Velvelichay and Atachay (flows of mud- and stone composition); and the basins of the rivers Devechichay and Shabranchay (mud flows).

The areas with medium mudflow hazard include the basins of Garachay, Gudialchay, Gusarchay, Agchay, Jagajugchay and other rivers.

The areas with low mudflow hazards are the arid valleys and gulches of the Ajinohur foothills, the Gobustan, the southeastern extremity of northeastern Azerbaijan, and the eastern extremity of the Gusar inclined plain. Here mudflows occur in ravines and gulches, being a muddy and stony in their nature.

Intra-mountain hollows, valleys and plains are included by us in the areas of attenuation of mudflows and deposition of their outflows. Absheron Peninsula, the coastal zones of the Caspian Sea are the areas where mudflow processes are not seen.

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Conclusion

1. It was defined that in the territory of the studied region the mudflows are formed in conditions of abundant precipitation with high intensity, and cannot be formed without the invasion of cold and transformed air masses from the northern regions of the Europe.

In the conditions of all circulation processes, such air masses penetrate the territory of the Greater Caucasus beyond the cold fronts under the impact of meridional transformations of the tropospheric altitudinal deformation fields. In this case, the altitude frontal zone becomes more powerful, while the value of the specific and relative humidity of air, as well as the instability of stratification, which leads to mudflow-forming torrential rains, grows. It was established that when the E process is available, the mudflow activity increases in this mudflow-prone region, whereas in the case of W, it decreases. In the process of mudflow formation, active role is played by the subtypes of circulation 12a and 13l in accordance with the classification of Dzerdzeevskij.

2. From the above mentioned it follows that mudflow processes are one of the most significant risk factors fixed within the Azerbaijani part of the Greater Caucasus. They intensify ecological tension and cause huge damage to the economy of the country and human settlements. The most important issues are:

- The detailed study of the dynamics of the development of mudflow foci, as well as the features of the accumulation of mudflow materials and their readiness for demolition
- The study of the nature of changes in river beds, the state of coasts and protective structures (currently, one of the main measures is the construction of mudflow dams)
- The study of potential mudflow routes and, on this basis, carrying out ecological and geomorphological measures to stabilize the ecological situation

3. Timely informing about the threat of development of such hazardous phenomena as mudflows would significantly reduce the risk and amount of damage, posed from these processes.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52755291

REGRESSION TREE ANALYSIS OF FACTORS AFFECTING FIRST LACTATION MILK YIELD OF DAIRY CATTLE

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(Received 28th Jan 2019; accepted 6th Mar 2019)

Abstract. The objective of this study was to examine the effects of management and environmental effects on first lactation milk yield by means of regression tree method. Regression tree method is useful to determine the effects of several factors on specified depended variables. The independent variables used in the model were factors such as farm, breed, year of calving, season of calving, age at calving and days in milk. Data used in this study were obtained from three state farms. The resulting data set consisted of 754 records from 1st farm, 1120 records from 2nd farm and 324 records from 3rd farm. The average total milk yield and days in milk were 5413.11 ± 2033.18 kg and 324 ± 64 days, respectively. Year of calving and days in milk were important variables affecting first lactation milk yield of dairy cattle, followed by farm and breed ($p < 0.01$). The determination coefficient of the prediction was found as 78.9%. As a result of the study 14 distinct paths from the root node to the leaves were presented.
Keywords: *multiple linear regression, farm, breed, year of calving, days in milk, cow*

Introduction

First lactation milk yield is a very important characteristic in all dairy cattle. Factors affecting this trait can be divided into genetical (breed) and environmental, such as farm, year of calving, season of calving, age at calving and days in milk. Understanding the factors that change the environment of the dairy cattle can be used to take advantage of some improvement in milk yield that occur in normal lactation. During first lactation at an average age of 2.5 years cow produces approximately 76% of the milk produced by a mature cow (Nirish, 2010). Milk production is usually less during the summer because of the higher environmental temperatures and the prevalence of green-forage scarcity. Thus the season of calving has got a marked effect on the total production. Cows freshening shortly before winter months produce more total yield than those calving at other times of the year. The increase is probably due to more favorable temperature and more digestible feeds available during the winter. The farmer has no effect on the physiological factors of the cow, but has some effects on environmental factors affecting the cow. Factors that alter the environment of dairy cattle can be used to take advantage of some changes in milk composition and the yield during the lactation (Irshad, 2015).

An alternative approach to nonlinear regression is to sub-divide the space into smaller regions, where the interactions are more manageable. The general model consists of two parts: one is only a recursive section, and the other is a simple model for each cell of the section. The regression trees use the tree to represent the recursive part.

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DOI: http://dx.doi.org/10.15666/aeer/1702_52935303

Each terminal node or leaf of the tree represents a cell of the section and has just added a simple pattern applied to it in this cell. This is due to CART's desirable properties as, automatic handling of the variable selection, variable interaction modeling, local effect modeling, nonlinear relationship modeling. CART also is more robust in the presence of outliers and not affected by monotonic transformations of variables. Not all variables have to be in the same type; some of them can be continuous, some can be discrete, etc. Regression trees have several advantages: there are no complicated calculations and hence the estimation is fast: it is easy to understand what variables are important when estimating. In practice, it is possible to build CART models with dirty data (i.e. missing values, lots of variables, nonlinear relationships, outliers, and numerous local effects).

Many studies have been carried out in the application of regression trees in animal husbandry. Lots of them concerning determination of factors effecting milk yield (Mirtagioglu et al., 2008; Bakır et al., 2010; Topal et al., 2010; Cak et al., 2013; Eyduran et al., 2013) and body weight (Topal et al., 2010) of animals. But also there are some researches concerning the application of regression trees to the different data from animal science (Eyduran et al., 2008, 2016; Koç et al., 2017; Takma et al., 2017)

In the present study we are interested in examining the management and environmental effects affecting cows at first lactation by means of regression tree analysis.

Materials and methods

Materials

Data on total lactation milk production were obtained from Tahirova, Konuklar and Malya farms. These farms are the state enterprises belonged to General Directorate of Agricultural Enterprises (*Fig. 1*). Konuklar Farm is located in the 57 km from Konya. The climate is a typical continental climate of Central Anatolia; summers are hot and dry, winters are cold and rainy (GDA, 2016). The average annual rainfall is 322.4 mm, and the altitude is 1050 m. The average annual temperature is 11.6 °C (GDM, 2017). Malya Farm is located 27 km north east of Kırşehir. This farm is located in the Central Anatolia Region, at an altitude of 985 m. Again, the climate is the typical continental climate of Central Anatolia, summers are hot and dry and winters are cold and rainy (GDA, 2016). The average annual rainfall is 378.4 mm. The average annual temperature is 11.4 °C (GDM, 2017). Tahirova Farm is located in Balıkesir province; the farm is situated at an altitude of 166 m. Summers are hot, winters are rainy. The average annual rainfall is 583.7 mm. The average annual temperature is 14.6 °C (GDM, 2017).

At all three farms the cows were grazed and winter supplement was also provided. In the current paper, 2198 first lactation records were assessed for 1874 Brown-Swiss dairy cattle reared at Konuklar and Malya State Farms, and 324 Holstein dairy cattle reared at Tahirova State Farm. Records covered the period of calving from 1987 to 2007.

Methods

Regression tree method

Multiple linear regression (*Eq. 1*) is a way of making quantitative predictions with multiple independent variables $X_i \equiv \{X_1, X_2, . . . Xn\}$

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$$Y = \beta_0 + \beta^T X + \varepsilon, \qquad \text{(Eq.1)}$$

Independent variables have an additive effect on Y, also an interaction (*Eq. 2*),

$$Y = \beta_0 + \beta^T X + \gamma X X^T + \varepsilon \qquad \text{(Eq.2)}$$

Prediction trees use the tree to represent the recursive partition. Each of the terminal nodes, or leaves, of the tree represents a cell of the partition, and has attached to it a simple model which applies in that cell only (*Fig. 2*).

Figure 1. *Locations of investigated farms (Google Earth, 2019)*

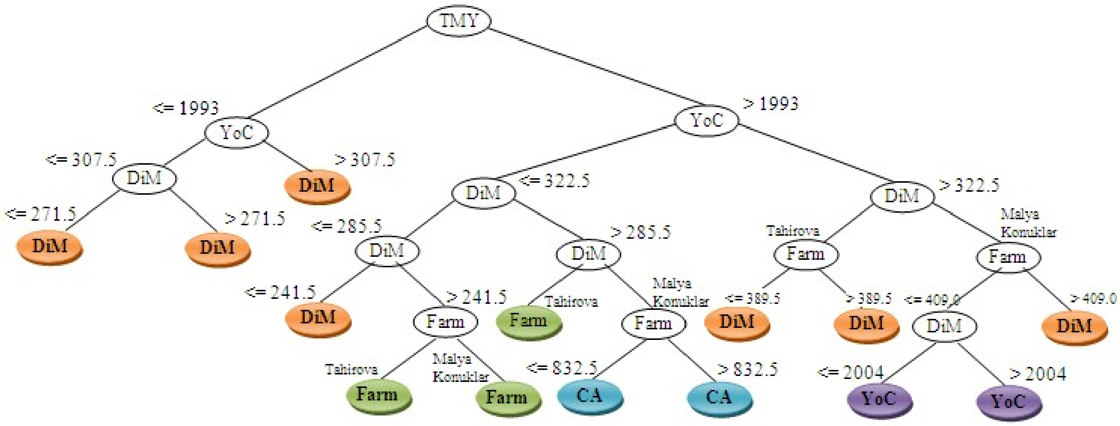

***Figure** 2. Scheme of CART of present work. TMY - total milk yield, YoC - year of calving, DiM - days in milk, CA - calving age*

Classification and Regression Tree splits the data into segments that are as homogeneous as possible with respect to the dependent variable (*Fig. 3*) (Lahmann and Kottner, 2011).

A regression tree can be seen as a kind of additive model (Hastie and Tibshirani, 1990) of the form:

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$$m(x) = \sum_{i=1}^{n} k_i \times I(x \in D_i) \quad \text{(Eq.3)}$$

where k_i are constants; I(.) is an indicator function returning 1 if its argument is true and 0 otherwise; D_i are disjoint partitions of the training data D such that $\bigcup_{i=1}^{n} D_i = D$ and $\bigcap_{i=1}^{n} D_i = \emptyset$.

A terminal node in which all cases have the same value for the dependent variable is a homogeneous, "pure" node. Regression trees (RT) is a method, where the target variable is continuous and tree is used to predict its value.

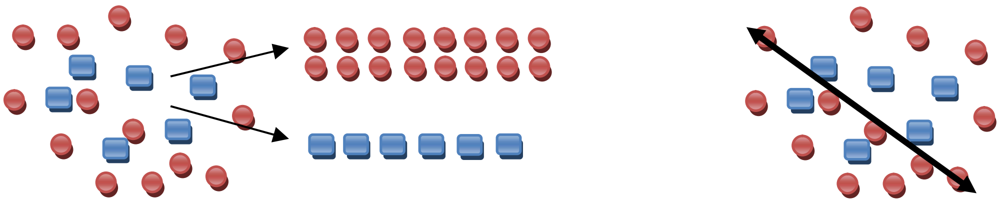

Figure 3. *Classification (left) and regression tree structure (right) for a model with 2 classes*

Each path from the root of the tree to a leaf corresponds to a region. Each inner node of the tree is a logical test on a predictor variable. In the particular case of binary trees, there are two possible outcomes of the test, true or false. This means that associated to each partition D_i we have a path p_i consisting of a conjunction of a logical tests on the predictor variables. This symbolic representation of the regression function is an important issue when one wants to have a better understanding of the regression surface (Soman et al., 2006).

For all analysis handled in this study IBM SPSS Statistics version 20 was used.

Goodness of fit

The coefficient of determination (*Eq. 4*) was used for determination of goodness of fit of the regression tree model.

$$R^2 = 1 - \sum_{i=1}^{n} (y_i - \tilde{y}_i)^2 / \sum_{i=1}^{n} (y_i - \bar{y})^2 \quad \text{(Eq.4)}$$

Results and discussion

There are 2 continuous (days in milk (DIM), calving age (CA) and 4 categorical (farm, breed, season of calving and year of calving) variables used to predict total milk yield (TMY). *Table 1* shows descriptive statistics of continuous variables. *Table 2* represents the frequency and percentage of categorical variables.

Table 1. *Descriptive statistics for the continuous variables*

Traits	N	Mean	SD	Min.	Max.	CV (%)
Total milk yield (kg)	2198	5413.11	2033.178	1098	12032	37.56%
Days in milk (days)	2198	324.19	63.899	137	675	19.7%
Calving age (days)	2198	858.46	90.160	702	1194	10.5%

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Table 1 shows that the most variation is in the total milk yield. At the same time, the lowest number of days in milk is 137 days and the lowest calving age is 702 days.

Table 2. *Frequency and percentage values for several categorical variables*

Farm	Frequency	Rate	Season	Frequency	Rate	Breed	Frequency	Rate
Konuklar	754	34.3%	Winter	522	23.7%	BS	1874	85.3%
Malya	1120	51.0%	Spring	656	29.7%	HO	324	14.7%
Tahirova	324	14.7%	Summer	559	25.4%			
			Autumn	461	21.0%			
Total	**2198**	**100**	**Total**	**2198**	**100.0**	**Total**	**2198**	**100.0**

YoC	Frequency	Rate	YoC	Frequency	Rate	YoC	Frequency	Rate
1987	27	1.2%	1995	101	4.6%	2003	162	7.4%
1988	63	2.9%	1996	104	4.7%	2004	98	4.5%
1989	52	2.4%	1997	131	6.0%	2005	231	10.5%
1990	55	2.5%	1998	130	5.9%	2006	134	6.1%
1991	87	4.0%	1999	141	6.4%	2007	49	2.2%
1992	84	3.8%	2000	127	5.8%			
1993	72	3.3%	2001	134	6.1%			
1994	75	3.4%	2002	141	6.4%			
Total = 2198								

Figure 4 shows the average TMY of different farms according to the calving year.

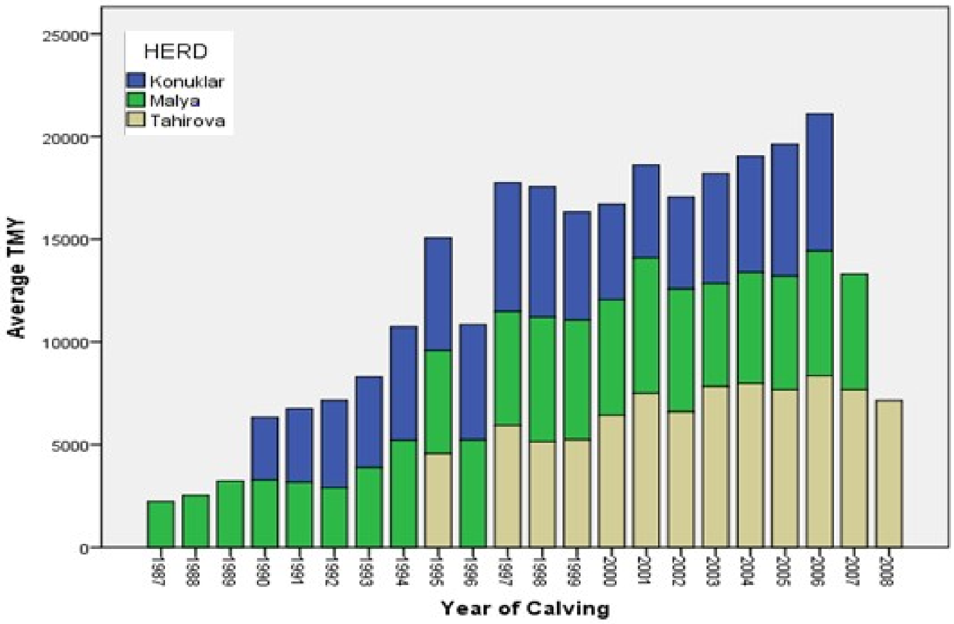

Figure 4. *The average TMY of different farms according to the calving year*

The CART algorithm is a process structured as a series of questions that determine what the next problem will be. The result of these questions is a tree-like structure with

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endpoint nodes. On each intermediate node, a state goes to the lower left node if the condition is met (Loh, 2011).

The regression tree diagram is depicted in *Figure 5*.

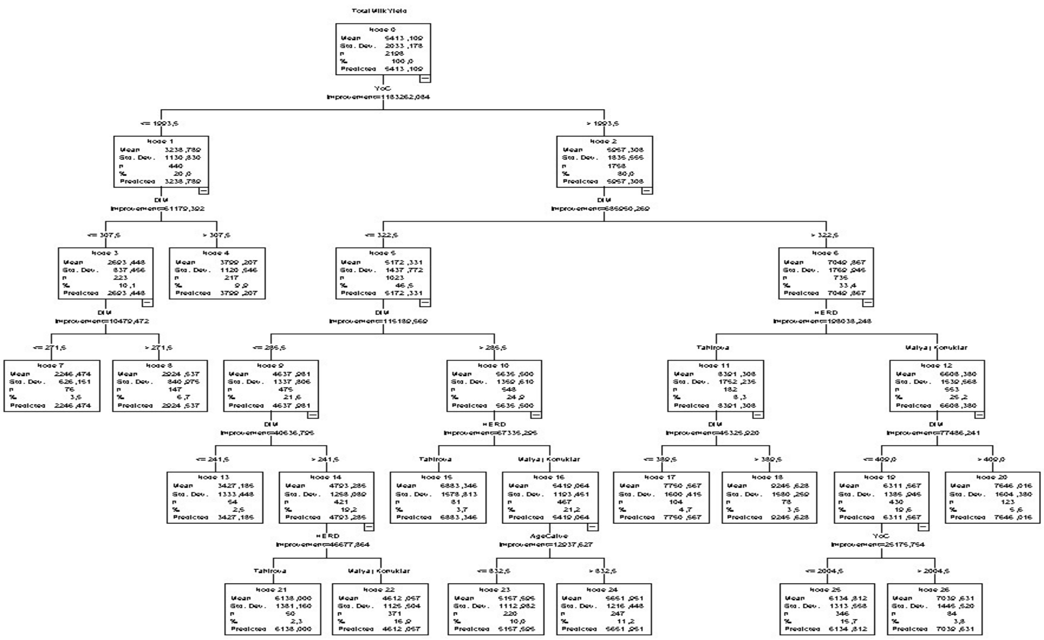

Figure 5. *Regression tree diagram for total milk yield*

The node definitions and splitting values for TMY were given in *Table 3*.

Table 3. *Node definitions and splitting values for TMY predictions*

Node	Predicted mean	SD	N	Percent	Parent node	Independent variable	F value	df1	df2	Split value
0	5413.11	2033.178	2198	100.0						
1	3238.79	1130.830	440	20.0	0	YoC	55.548**	21	2176	<= 1994
2	5957.31	1835.555	1758	80.0	0	YoC	55.548**	21	2176	> 1994
3	2693.45	837.456	223	10.1	1	DiM	3.897**	323	1874	<= 307.5
4	3799.21	1120.546	217	9.9	1	DiM	3.897**	323	1874	> 307.5
5	5172.33	1437.772	1023	46.5	2	DiM	3.897**	323	1874	<= 322.5
6	7049.87	1769.945	735	33.4	2	DiM	3.897**	323	1874	> 322.5
7	2246.47	626.151	76	3.5	3	DiM	3.897**	323	1874	<= 271.5
8	2924.54	840.975	147	6.7	3	DiM	3.897**	323	1874	> 271.5
9	4637.98	1337.806	475	21.6	5	DiM	3.897**	323	1874	<= 285.5
10	5635.50	1359.610	548	24.9	5	DiM	3.897**	323	1874	> 285.5
11	8391.31	1752.235	182	8.3	6	Farm	269.741**	2	2195	Tahirova
12	6608.38	1539.568	553	25.2	6	Farm	269.741**	2	2195	Malya; Konuklar
13	3427.19	1333.448	54	2.5	9	DiM	3.897**	323	1874	<= 241.5
14	4793.29	1258.089	421	19.2	9	DiM	3.897**	323	1874	> 241.5
15	6883.35	1578.813	81	3.7	10	Farm	269.741**	2	2195	Tahirova
16	5419.06	1193.451	467	21.2	10	Farm	269.741**	2	2195	Malya; Konuklar

17	7750.57	1600.415	104	4.7	11	DiM	3.897**	323	1874	<= 389.5
18	9245.63	1580.259	78	3.5	11	DiM	3.897**	323	1874	> 389.5
19	6311.57	1385.945	430	19.6	12	DiM	3.897**	323	1874	<= 409.0
20	7646.02	1604.380	123	5.6	12	DiM	3.897**	323	1874	> 409.0
21	6138.00	1381.160	50	2.3	14	Farm	269.741**	2	2195	Tahirova
22	4612.06	1125.504	371	16.9	14	Farm	269.741**	2	2195	Malya; Konuklar
23	5157.60	1112.982	220	10.0	16	CA	0.907	392	1805	<= 832.5
24	5651.95	1216.448	247	11.2	16	CA	0.907	392	1805	> 832.5
25	6134.81	1313.558	346	15.7	19	YoC	55.548**	21	2176	<= 2005
26	7039.63	1445.520	84	3.8	19	YoC	55.548**	21	2176	> 2005

**$p < 0.01$, $R^2 = 78.9\%$

At the top of regression tree diagram, Node 0, which gave general descriptive statistics of TMY, was divided into new two child nodes, with respect to YoC factor. The TMY averages of the cows were born in 1994 and early on (Node 1) was estimated as 3238.79 kg and the TMY average of the other cows (Node 2) was estimated as 5957.31 kg. Right side nodes always show high level mean. With an average of 5957.31 kg, Node 2 gave the highest TMY. Numbers (proportions) of lactation records were 440 (20%) for Node 1 and 1758 (80%) for Node 2. The YoC factor yielded a significant influence on TMY of cows whose lactation records were included in Node 1 ($F = 55.548$, df1 = 21, df2 = 2176, $P < 0.01$). This shows the increase in total milk yield over the years. Afterwards Node 1 was divided into two child nodes (Nodes 3 and 4), depending on DiM factor. Corresponding average values for these two new child nodes were 2693.45 kg and 3799.21 kg, respectively. Numbers of lactation records were established as 223 (10.1%) and 217 (9.9%). While TMY values of cattle with DiM of 307.5 days and less were 2693.45 kg and TMY values of cattle with DiM more than 307.5 days, were found as 3799.21 kg. On the other hand, Node 3 is divided into 2 branches according to DiM factor. While TMY value of 76 cows with DiM 271.5 days and less was found to be 2246.47 kg, TMY value of 147 cows with DiM more than 271.5 days was found as 2924.54 kg. DiM is the first criterion in the TMY classification of records from 1993 and after (Node 2). DiM factor remarkably influenced the TMY of cows whose lactation records were consisted of DiM less than 322.5 days in the Node 2. The TMY averages for these two nodes (Node 5 and Node 6) were found as: 5172.333 kg and 7049.87 kg, respectively. Numbers (proportions) of lactation records were established as 1023 (46.5%) and 735 (33.4%), respectively. According to DiM factor these records are also divided into animal classes which are less than 285.5 days (Node 9) and more than 285.5 days (Node 10). Node 9 is divided into classes with DiM less than 241.5 days (Node 13) and more than 241.5 days (Node 14). The Node 13 consists of 54 records and the average TMY value is 3427.19 kg. The Node 14 was divided into two child nodes: the Tahirova farm with an average TMY of 6138.00 kg and the Malya and Konuklar farms with an average TMY of 4612.06 kg. The records with DiM over 285.5 (Node 10) was divided into two groups. 81 animals with an average TMY value of 6883.35 kg were belonged to Tahirova (Node 15) farm and 467 animals with an average TMY value of 5419.06 kg belonged to the Konuklar and Malya farms (Node 16). The Node 16 was then further classified (Node 23 and Node 24) according to the CA factor that was not statistically significant. Animals with DiM of 322.5 days and more were divided into two child nodes on the basis of farm (Node 11

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and Node 12). 182 animals with an average TMY value of 8391.31 kg were belonged to the Tahirova (Node 11) and 553 animals with an average TMY of 6608.38 kg were belonged to the Malya and Konuklar farms (Node 12). 182 animals (Node 11) in the Tahirova farm were divided into 2 homogeneous classes according to the DiM criteria: 104 animals with an average of 7750.57 kg milk and less than 389.5 DiM (Node 17) and 78 animals with an average of 9245.63 kg of milk and 389.5 and higher DiM (Node 18). The animals in the Malya and Konuklar farms (Node 12) were classified according to the DiM factor. Accordingly, the average TMY value of the 430 animals (Node 19) with a DiM of less than 409.5 days was 6311.57 kg, and the 123 animals (Node 20) with a DiM of more than 409.5 days were 7646.02 kg. Then, Node 19 was divided into two child nodes according to the YoC factor as before 2005 (Node 25) and after 2005 (Node 26).

On account of the fact that Nodes 4, 7, 8, 13, 15, 17, 18, 20, 21, 22, 23, 24, 25 and 26 are terminal nodes, there is not any separation for providing homogeneity in those nodes.

The following algorithms were used for the estimation of the TMY in the regression tree method we applied:

$P_1 = (\boldsymbol{YoC \leq 1994}) \cap (\boldsymbol{DiM > 307.5})$	$k_1 = 3799.2$ kg
$P_2 = (YoC \leq 1994) \cap (DiM \leq 307.5) \cap (DiM \leq 271.5) = (\boldsymbol{YoC \leq 1994}) \cap (\boldsymbol{DiM \leq 271.5})$	$k_2 = 2246.5$ kg
$P_3 = (YoC \leq 1994) \cap (DiM \leq 307.5) \cap (DiM > 271.5) = (\boldsymbol{YoC \leq 1994}) \cap (\boldsymbol{271.5} < DiM \leq 307.5)$	$k_3 = 2924.5$ kg
$P_4 = (YoC > 1994) \cap (DiM \leq 322.5) \cap (DiM \leq 285.5) \cap (DiM \leq 241.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{DiM \leq 241.5})$	$k_4 = 3427.2$ kg
$P_5 = (YoC > 1994) \cap (DiM \leq 322.5) \cap (DiM \leq 285.5) \cap (DiM > 241.5) \cap (Farm = Tahirova) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{241.5} < DiM \leq 285.5) \cap (\boldsymbol{Farm = Tahirova})$	$k_5 = 6138.0$ kg
$P_6 = (YoC > 1994) \cap (DiM < 322.5) \cap (DiM < 285.5) \cap (DiM > 241.5) \cap (Farm = Malya \cup Konuklar) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{241.5} < DiM \leq 285.5) \cap (\boldsymbol{Farm = Malya \cup Konuklar})$	$k_6 = 4612.1$ kg
$P_7 = (YoC > 1994) \cap (DiM \leq 322.5) \cap (DiM > 285.5) \cap (Farm = Tahirova) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{285.5} < DiM \leq 322.5) \cap (\boldsymbol{Farm = Tahirova})$	$k_7 = 6883.4$ kg
$P_8 = (YoC > 1994) \cap (DiM \leq 322.5) \cap (DiM > 285.5) \cap (Farm = Malya \cup Konuklar) \cap (CA \leq 832.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{285.5} < DiM \leq 322.5) \cap (\boldsymbol{Farm = Malya \cup Konuklar}) \cap (\boldsymbol{CA \leq 832.5})$	$k_8 = 5157.6$ kg
$P_9 = (YoC > 1994) \cap (DiM \leq 322.5) \cap (DiM > 285.5) \cap (Farm = Malya \cup Konuklar) \cap (CA > 832.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{285.5} < DiM \leq \boldsymbol{322.5}) \cap (\boldsymbol{Farm = Malya \cup Konuklar}) \cap (\boldsymbol{CA} > 832.5)$	$k_9 = 5652.0$ kg
$P_{10} = (YoC > 1994) \cap (DiM > 322.5) \cap (Farm = Tahirova) \cap (DiM \leq 389.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{322.5} < DiM \leq 389.5) \cap (\boldsymbol{Farm = Tahirova})$	$k_{10} = 7750.6$ kg
$P_{11} = (YoC > 1994) \cap (DiM > 322.5) \cap (Farm = Tahirova) \cap (DiM > 389.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{DiM} > 389.5) \cap (\boldsymbol{Farm = Tahirova})$	$k_{11} = 9245.6$ kg
$P_{12} = (YoC > 1994) \cap (DiM > 322.5) \cap (Farm = Malya \cup Konuklar) \cap (DiM > 409.5) = (\boldsymbol{YoC} > 1994) \cap (\boldsymbol{DiM} > 409.5) \cap (\boldsymbol{Farm = Malya \cup Konuklar})$	$k_{12} = 7646.0$ kg
$P_{13} = (YoC > 1994) \cap (DiM > 322.5) \cap (Farm = Malya \cup Konuklar) \cap (DiM \leq 409.5) \cap (YoC \leq 2005) = (\boldsymbol{1994} < YoC \leq 2005) \cap (\boldsymbol{322.5} < DiM \leq 409.5) \cap (\boldsymbol{Farm = Malya \cup Konuklar})$	$k_{13} = 6134.8$ kg
$P_{14} = (YoC > 1994) \cap (DiM > 322.5) \cap (Farm = Malya \cup Konuklar) \cap (DiM \leq 409.5) \cap (YoC > 2005) = (\boldsymbol{YoC} > 2005) \cap (\boldsymbol{322.5} < DiM \leq 409.5) \cap (\boldsymbol{Farm = Malya \cup Konuklar})$	$k_{14} = 7039.6$ kg

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DOI: http://dx.doi.org/10.15666/aeer/1702_52935303

where, P_i shows the algorithm to the terminal Node. TMY obtained from each algorithm is shown with the variable k_i.

There are 14 distinct paths from the root node to the leaves. This three divides the input data in 14 different regions. Using the (*Eq. 3*), we obtain:

$$m(x) = \sum_{i=1}^{14} k_i \times I(P_i)$$

Figure 6 shows importance level of the independent variables. According to *Figure 6,* YoC was significantly identified as the most important factor influencing TMY.

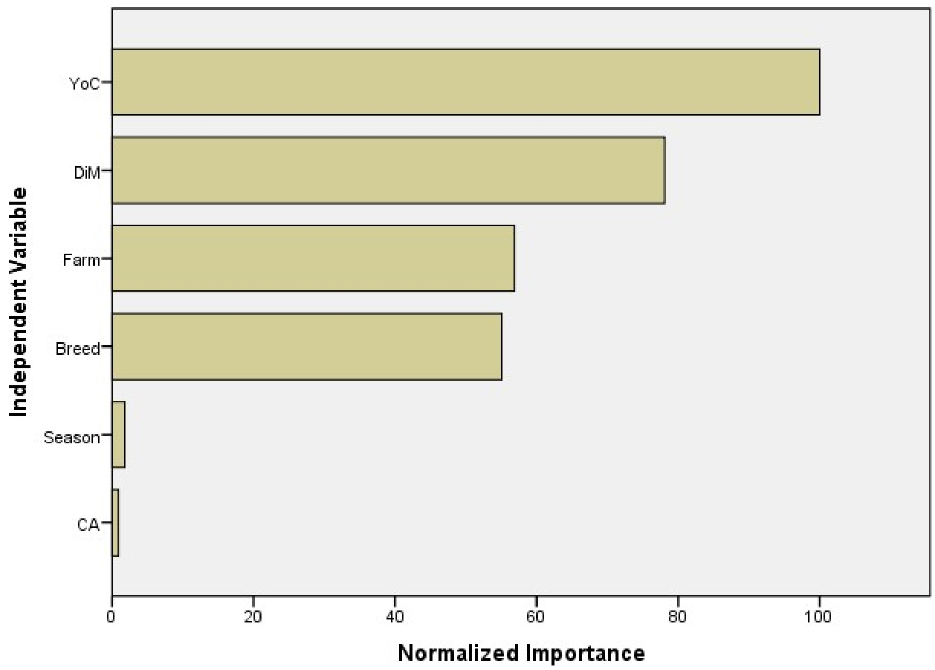

Figure 6. *Importance level of independent variables affecting TMY*

Their importance rate was given in *Table 4*.

Table 4. *Importance level of independent variables affecting TMY*

Independent Variable	Importance	Normalized importance (%)
Year of Calving	1299236.915	100.0
Days in Milk	1052872.027	81.0
Farm	765055.898	58.9
Breed	741247.200	57.1
Season	29218.096	2.2
Calving Age	24202.305	1.9

The R^2 of the tree is 78.9%, which is significantly higher than that of a multiple linear regression fit to the same data (R^2 = 59.1%). In current YoC was found most

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52935303

important factor affecting total milk yield followed by days in milk (81%). Teke and Akdağ (2010); Bayril and Yılmaz (2010) in their study indicated that the effect of calving year on lactation milk yield were significant in Holstein cows. The categorical variable farm, as we expected, is grouped separately Malya and Konuklar (close to each other geographically) and Tahirova. The other common characteristics of Konuklar and Malya farms are that they have the same breed animal (Holstein). As a result of the analysis, it is expected that the importance rate of both farm and breed characteristics are close (58.9% and 57.1%, respectively). Gürses and Bayraktar (2012) reported that effects of enterprise, calving year were found significant on 305 days milk yield. Season and age at calving was not found so important in the total milk yield prediction (2.2% and 1.9%, respectively). In the study of Topal et al. (2010) season of calving of Swedish Red cattle also was found the least important factor (5.9%) affecting actual milk yield. But there are some other works were claiming the opposite (Bakır and Çetin, 2003; Özçakır and Bakır, 2003; Sehar and Özbeyaz, 2005; Erdem et al., 2007; Akçay et al., 2007; Çilek, 2009).

Conclusion

Due to the fact that CART is not a parametric method, the data used here is not required be belonged to a particular type of distribution. Also, this method can easy determine effects of both continuous and categorical variables on dependent continuous variable.

In regression tree diagram, the year of calving trait was determined to be the most affective factor for total milk yield prediction, followed by days in milk, farm and breed ($P < 0.01$). Thus, while the average total milk yield in the first lactation was 3238.79 ± 1130.83 until 1993, this yield increased in years and reached 6134.81 ± 1313.56 kg in 2005. This yield has increased to 7039.63 ± 1445.52 kg in Malya and Konuklar farms after 2005. In the Tahirova farm, we can say from the P_{11} path that the total milk yield increased after 1994 and reached the average of 9245.63 ± 1580.26 kg. Insignificant input variables such as season and age at calving were excluded from the regression tree.

As a result, the routing of the algorithms leading to the terminal node will help to determine the criteria for the classification in the regression tree and the application of the test data to this algorithm.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_52935303

EFFECTS OF DIFFERENT TYPES OF BIOCHAR ON BASIC PROPERTIES AND BACTERIAL COMMUNITIES OF BLACK SOIL

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(Received 30th Jan 2019; accepted 8th Mar 2019)

Abstract. In order to explore the effects of biomass carbon on the basic properties of black soil and bacterial diversity, in this study, the combination of indoor culture and high-throughput sequencing was used to analyze the nutrient changes and bacterial communities of black soil after adding biochar. The results showed that compared with CK, soil pH, organic matter and available potassium increased significantly after adding biochar ($P < 0.05$), and catalase, invertase, urease, and phosphatase increased substantially, while polyphenol oxidase decreased. A total of 17 phyla, 51 known genera were detected by high-throughput sequencing, among which *Proteobacteria* was the dominant phyla. The dominant genera including *Sphingomonas, Mizugakiibacter, Bacillus, Marmoricola, Rhodanobacter*, and *Streptomyces*. From the analysis of bacterial diversity, it can be concluded that the bacteria ace, chao1, Shannon and Simpson index of black soil increased after adding biochar, compared with CK in redundancy analysis (RDA), AN, NO_3^--N, NH_4^+-N had a greater impact on microbial communities. The microbial functional bacteria AOA and cbbl were significantly correlated with pH, SOM, AN, NH_4^+-N, and NO_3^--N ($p < 0.05$). Therefore, the addition of biochar can not only improve soil nutrients but also plays an important role in maintaining the diversity of soil bacterial communities.
Keywords: *biochar, enzyme activity, high-throughput sequencing, bacterial diversity, microbial functional genes*

Introduction

As a good soil improver, biochar could not only improve soil pH and base saturation but also reduce soil aluminum saturation (Cantrell et al., 2012; Zwieten et al., 2010; Liang et al., 2006). Zhou et al. (2017) found that biochar can also increase soil respiration in temperate forests. In recent years, more and more scholars have found that biochar could reduce greenhouse gas emissions (Weber et al., 2018). Biomass carbon can promote nutrient availability and increase carbon sequestration and soil fertility (Tian et al., 2016). The restoration of heavy metal pollution in soils and sediments by biochar is also considered by more and more scholars (Oleszczuk et al., 2012). So many characteristics of biochar are applied to the improvement of soil. As a big agricultural country, China's black soil is one of the three black soils in the world. Because of the unscientific fertilization and other factors in recent years, black soil is also facing environmental pollution. Therefore, the material charcoal is of great significance to the improvement of soil. However, the research on the microbial community of black soil by biochar is relatively scarce. The diversity of the microbial community is different when different biochars are added to different soils. Castaldi et al. (2011) found that the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

addition of wood biomass charcoal had little effect on soil microbial communities, while the addition of cotton biomass charcoal increased microbial activity. Some scholars have shown that biochar can provide suitable habitat for microorganisms due to its high porosity and cation exchange capacity (Cui et al., 2013). Some scholars have also shown that adding biomass charcoal to fine textured soil reduces microbial activity (Wang et al., 2017). The effect of biochar on soil enzyme activity is also the focus of current scholars. According to recent research, the addition of biomass carbon increases the enzyme activity associated with the N and P cycles, while at the same time reducing the enzyme activity associated with the C cycle (Bailey et al., 2011). Anyway, some studies have the opposite result.

The relationship between physical and chemical properties of black soil, enzymatic activity, microbial community diversity, and functional bacteria and environmental factors can help to further understand the improvement mechanism of biochar on black soil, and explore the influence of environmental factors on microbial community. Good use of biomass carbon in black soil, and the relationship between environmental factors and functional bacteria can more clearly understand the mechanism of action of biomass carbon, enzyme activity plays an indispensable role in soil activity, through A series of detailed studies on black soil by biochar can clearly identify the types and amounts of biomass carbon that are beneficial to soil improvement. This experiment mainly used three kinds of biomass charcoal to explore the improvement effect of black soil. In order to provide theoretical basis to the application of charcoal in Agriculture.

Materials and methods

Test soil and biochar

Test soil

The test soil was a typical black soil and was collected from the long-term location test points (43°47'42" N, 123°20'45"E) of the Jilin Agricultural University. Corn is the major crop mostly cultivated on this soil. The fertilizer mostly applied during corn production are N 180 kg hm^{-2}, P 39.3 kg hm^{-2}, K 75 kg hm^{-2}, soil bulk density of 1.15 g cm^{-3}. The soil sample was collected from 0-20 cm plow layer soil, and after air drying, it was sieved by 2 mm. The basic physicochemical traits are shown in *Table 1*.

Table 1. *Physicochemical characteristics of the test soil*

pH	**SOM (g kg^{-1})**	**AN (mg kg^{-1})**	**AP (mg kg^{-1})**	**AK (mg kg^{-1})**	**clay (%)**	**silt (%)**	**sand (%)**
5.55	21.52	84.25	15.68	192.05	34.75	25.47	39.77

SOM: Soil organic matter; AN: alkali-hydrolyzable nitrogen; AP: available phosphorus; AK: available potassium

Test biochar

Three kinds of biomass carbon were provided by Dalian Xinglongxi Development Co., Ltd. These included corn stover, rice husk, and pine wood pyrolyzed at 350-550 °C. The basic physical and chemical properties of biomass carbon are shown in *Table 2*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

Table 2. *Physicochemical characteristics of the test biochar*

Measurement items	CB	RB	PB
Ash (%)	41.61±1.2	38.62±2.1	11.60±1.0
pH	9.82±0.6	8.48±0.5	8.08±0.8
Organic carbon (g.kg^{-1})	463.54±1.6	708.50±2.5	210.20±1.4
Total phosphorus (g.kg$^{-1)}$	16.20±1.1	13.16±1.4	11.07±1.7
AP (mg·kg^{-1})	219.97±2.2	385.28±2.1	5.01±1.8
N (%)	1.37	1.33	0.39

CB: corn stover biomass carbon; RB: rice husk biomass charcoal; PB: pine biomass carbon

Training test design

Bio-char-soil preparation: black soil over 2 mm sieve and biomass charcoal over 20 mesh sieve were initially obtained. The air-dried black soil sample was mixed with certain quantities of each biomass charcoal to obtain 1000 g for each treatment. A total of 7 treatments were included in the trial (*Table 3*). Treatments were put in plastic bowls and randomly placed in an incubator at constant temperature and humidity (*Fig. 1*). Samples were incubated at 25 °C for 6 months and moisture was always adjusted to 70% of field capacity. We had three repeats per treatment (Note: 1.2% is 1.2% of total weight; 6.0% is 6.0% of total weight).

Figure 1. *Constant temperature and humidity incubator*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

Table 3. *A total of 7 experimental treatments*

Treatments	Composition
CK	Black soil
1.2%CB	Black soil + 1.2% corn stover biomass carbon
6.0%CB	Black soil + 6.0% corn stover biomass carbon
1.2%RB	Black soil + 1.2% rice husk biomass charcoal
6.0%RB	Black soil + 6.0% rice husk biomass charcoal
1.2%PB	Black soil + 1.2% pine biomass carbon
6.0%PB	Black soil + 6.0% pine biomass carbon

Research methods

The sampling of mixed culture soil

After the biomass carbon-black soil mixed treatments were cultured for six months, two soil samples were taken from each treatment. One for DNA extraction and the other for basic data determination. The soil was passed over 2 mm sieve for the removal of impurities. The same treatment was mixed three times in parallel. Samples for DNA extraction was stored at -80 °C and samples for basic data determination was air dried.

Determination of basic data

Soil pH was measured on a 1:2.5 (w/v) ratio in distilled water using a pH meter, Soil organic matter (SOM) was determined after dichromate oxidation and titration with ferrous ammonium sulphate, alkali-hydrolyzable nitrogen (AN) was determined alkaline solution diffusion method, available phosphorus (AP) was determined colorimetrically using the molybdate method, and available potassium (AK) was extracted by incubation with 1.0 mol L^{-1} ammonium acetate for 0.5 h, followed by filtration, available potassium content was determined using a flame photometer (Brookes et al., 1985). Ammonium nitrogen and Nitrate nitrogen (NH_4^+-N and NO_3^--N) was extracted with 2 M KCl and determined by flow injection analysis (TRAACA-2000, Germany). Polyphenol oxidase and Catalase were measured calorimetrically in a clear 96-well microplate according to Ai et al. (2015). Urease activity was determined according to Kandeler and Gerber (1988) and was expressed as mmole NH_4^+ g^{-1} dry soil h^{-1}. Invertase activity was determined by using sucrose solution as the substrate as described by Chen et al. (2013). The activity of Phosphatase was measured by the transformation of disodium phenyl phosphate to phenol as described by Jin et al. (2016).

Extraction and detection of total soil DNA

Soil microbial DNA extraction was performed using the Power Soil DNA Isolation Kit from MOBIO, USA. 0.5 g of soil samples were weighed and stored in a refrigerator at -80 °C to extract total DNA and purify soil genomic DNA (TIAN quick Midi Purification Kit, TIANGEN) according to the kit manual procedure; Detected by ultra-micro spectrophotometer (Nanodrop 2000) DNA concentration ≥ 20 ng.μL^{-1}, $OD_{260/280}$ between 1.8 and 2.0, and $OD_{260/230}$ between 1.8 and 2.0. The extracted DNA was stored at -20 °C for subsequent measurement.

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High-throughput sequencing of the Illumina MiSeq platform

The construction of a high-throughput sequencing library was completed by GENEWIZ Corporation (Shanghai, China). The V3–V4 hypervariable regions of the Bacteria 16S rRNA was amplified by PCR using 5 to 50 mg of DNA as a template. The end of the PCR product of the 16S rRNA genes were amplified using the primer set U341F (ACTCCTACGGGAGGCAGCAG) and U806R (GGACTA CHVGGGTWTCTAAT). The library quality was detected using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the library concentration was detected by Qubit and real-time quantitative PCR (Applied Biosystems, Carlsbad, CA, USA). After the DNA library was mixed, double-ended sequencing (PE) was performed using the Illumina MiSeq (Illumina, San Diego, CA, USA) instrument instruction manual. Image analysis and base calling were performed using the MiSeq Control Software (MCS) in the MiSeq tool. Finally, an initial classification analysis was performed on the Illumina base space cloud computing platform.

All sequences were deposited in the NCBI sequence read Archive (SRA) database (Accession numbers: SRR7275367-SRR7275373).

Real-time PCR amplification

The abundances of genes (nifH, archaeal amoA (AOA), bacterial amoA (AOB), nosZ, *cbbl* and *gdh3*) encoding the key enzymes for biological N, P cycling were quantified by real-time PCR (IBA7500) for all soil samples. These methods were modified from their original descriptions (references following primers) (*Table A1* in the *Appendix*). Gel-purified PCR producgts from a common DNA mixture (equal amounts of DNA from all samples) were used to prepare sample-derived quantification standards (YU et al., 2005). The copy number of genes in each standard was calculated by DNA concentration (ng•μL^{-1}, measured by Qubit) divided by average molecular weight (PCR product length and GC content was obtained from barcoded-pyrosequencing results) of the amplified gene fragment (Mao et al., 2012).

Data processing

OTU analysis uses V-SEARCH (1.9.6) for sequence clustering (sequence similarity is set to 97%), and the aligned 16S rRNA reference database is UNITE ITS database (https://unite.ut.ee/), then RDP is used. Classifier (Ribosomal Database Program) Bayesian algorithm performs species taxonomy analysis on representative sequences of OTUs and gives statistics on the community composition of each sample at different species classification levels. The analytic diversity software Qiime (1.9.1) was used to calculate the alpha diversity indices such as Shannon, Chao1, Ace, Simpson, goods-coverage. The (Un) Weighted Unifrac analysis was used to compare whether there was a significant difference in microbial communities between samples. Based on the (Un) Weighted Unifrac sample distance matrix information, non-metric multi-scale (NMDS) analysis was used to demonstrate β diversity. Correlation analysis between species and environment (RDA) was performed using Canoco 5.0 software. We use Excel 2010, SPSS19.0 for data statistics and analysis.

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Results

Effect of biomass carbon addition on physical and chemical properties and enzyme activity of black soil

The basic physical and chemical properties of the soil after adding biochar are shown in *Table 4*. After adding biochar, the pH, organic matter and available potassium of black soil increased compared with CK treatment, and the effect of 6.0% CB treatment was the most significant. After adding biochar, the available phosphorus content of soil increased significantly with 1.2% RB, 6.0% RB, and 6.0% CB, while 1.2% CB, 1.2% PB, and 6.0% PB treatment decreased compared with CK treatment. After adding biochar, the alkaline nitrogen, ammonium nitrogen and nitrate nitrogen in the soil showed a decreasing trend, and their values were the highest compared with CK treatment.

Table 4. *Basic physical and chemical properties of bio-carbon soil*

Treatment	pH	SOM ($g\ kg^{-1}$)	AN ($mg\ kg^{-1}$)	AP ($mg\ kg^{-1}$)	AK ($mg\ kg^{-1}$)	NH_4^+-N ($mg\ kg^{-1}$)	NO_3^--N ($mg\ kg^{-1}$)
CK	5.31±0.02d	39.10±1.13e	139.86±3.63a	37.00±2.36b	238.79±4.07e	29.00±2.25a	167.58±16.06a
1.2%CB	5.64±0.02b	50.01±1.25d	119.76±2.28b	31.27±1.01b	375.36±7.69d	25.71±4.60a	131.77±8.21bc
6.0%CB	6.23±0.06a	114.52±7.42a	65.65±0.89f	61.60±2.46a	861.77±12.67a	4.27±0.28c	42.58±3.55e
1.2%RB	5.55±0.19bc	60.13±4.45c	94.60±2.79c	69.33±12.20a	549.87±45.57c	13.12±1.71b	139.94±5.66b
6.0%RB	5.68±0.01b	95.88±7.18b	76.15±2.85e	65.20±2.84a	710.77±2.91b	7.19±1.60c	104.51±10.22d
1.2%PB	5.47±0.06c	51.70±3.62d	94.90±0.22c	31.10±1.50b	243.49±0.57e	12.05±0.40b	104.97±6.31d
6.0%PB	5.57±0.03bc	100.29±3.02b	88.65±4.69d	35.07±0.92b	257.92±0.58e	12.81±2.06b	118.33±10.63cd

The changes in soil respiration intensity and enzyme activity after adding biochar are shown in *Table 5*. After the addition of biomass charcoal, the respiration intensity of the soil was significantly enhanced by the addition of corn stover charcoal, while the addition of rice husk biomass charcoal was significantly enhanced by 1.2% RB, and 6.0% RB treatment was lower than CK treatment. The treatment of adding pine biomass charcoal significantly reduced its respiratory intensity compared to CK. After adding biochar, the catalase was increased compared with CK treatment, and the effect of 6.0% CB was the most significant. The addition of rice husk with 6.0% RB increased the effect significantly, and the addition of pine charcoal increased the effect when 6.0% PB was added. The invertase activity of the soil had the best effect when corn straw charcoal was added and decreased with rice husk and pine charcoal as compared with CK. The urease activity of soil also had the best effect when corn straw charcoal was added, and the effect of treatment with 6.0% CB was the most significant. Adding biomass carbon has little effect on phosphatase, and treatment with 1.2% CB was the most significant. Polyphenol oxidase is inversely proportional to the degree of humification of the soil, with 6.0% CB being the best.

Effect of biomass carbon addition on the abundance of bacteria in black soil

As seen from *Figure 2,* a total of 17 known bacterial phylum were detected in the black soil supplemented with biochar, of which *Proteobacteria* was the dominant flora, and the *Proteobacteria* gate was occupied by 1.2% RB treatment compared with CK treatment. The proportion was up to 41.46%, and the proportion in the 6.0% CB treatment was relatively small at 40.56%. The *Bacteroidetes* and *Actinobacteria* gates

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accounted for a large proportion in the CK treatment, which was 30.02% and 17.68%, respectively. The *Acidobacteria* gate was at 1.2% CB. The proportion of 6.0% CB treatment was 12.73% and 11.64%, respectively. *Firmicutes* accounted for a large proportion of 1.2% RB and 1.2% PB at 7.24% and 7.55% respectively.

***Table 5.** Changes in enzyme activity of soil added with biomass carbon*

Treatment	Respiration intensity (ml kg^{-1})	Catalase (ml g^{-1})	Invertase (ml g^{-1})	Urease (mg g^{-1})	Phosphatase (mg g^{-1})	Polyphenoloxidase (ml g^{-1})
CK	98.99±7.84b	0.94±0.01f	0.68±0.07d	2.01±0.11c	9.85±0.71bc	0.049±0.007a
1.2%CB	114.67±5.75a	1.02±0.02cd	1.05±0.03a	3.15±0.12a	10.84±0.08a	0.028±0.007bc
6.0%CB	119.27±3.77a	1.12±0.01a	1.03±0.05a	3.43±0.25a	9.98±0.28abc	0.009±0.002d
1.2%RB	113.73±7.70a	1.00±0.02de	0.74±0.03cd	2.83±0.70ab	10.38±0.91abc	0.035±0.003b
6.0%RB	85.72±2.37c	1.05±0.01b	0.59±0.01e	2.06±0.07c	8.91±0.40d	0.025±0.001c
1.2%PB	62.20±3.83d	0.98±0.02e	0.79±0.01c	2.32±0.16bc	10.64±0.13ab	0.046±0.008a
6.0%PB	65.96±3.45d	1.03±0.01bc	0.86±0.02b	2.16±0.73bc	9.47±0.19cd	0.022±0.005c

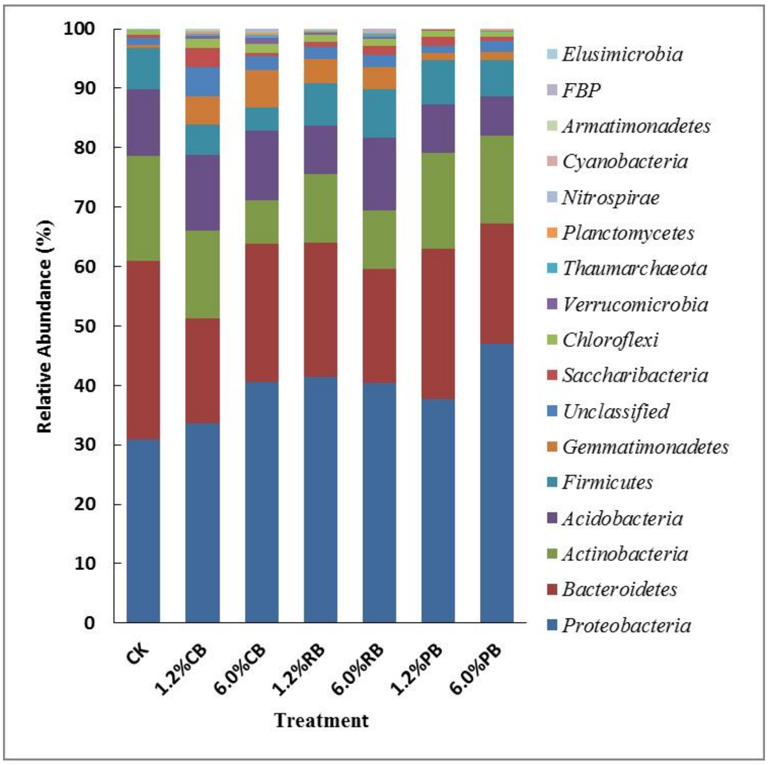

***Figure 2.** Relative abundance of bacterial community at phyla level. (CK: only black soil; CB: black soil +corn stover biomass carbon; RB: black soil + rice husk biomass charcoal; PB: black soil + pine biomass carbon)*

According to the abundance data of OTU, the heat map can cluster different OTUs at the genus level, and the heat map can reflect the relationship between the similarity, difference, and species clustering relationship of different treatments according to the color gradient. A total of 51 bacterial genera were detected from 7 samples in this sequencing. In this study, the top 30 bacterial genera with higher abundance were selected and the heat map was drawn using R language (*Fig. 3*). The dominant species

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included *Sphingomonas, Mizugakiibacter, Bacillus, Marmoricola, Rhodanobacter*, and *Streptomyces*, which accounted for 19.77%, 6.86%, 4.74%, 2.49%, 1.69%, and 1.37, respectively. It can be seen from the figure that, *Sphingomonas* is the lightest color in CK treatment, the darkest in 6.0% PB treatment and the darkest in *Mizugakiibacter* in CK treatment. The *bacillus* is the darkest in 6.0% treatment, at 6.0%. The color is the lightest in CB treatment, the darkest color in the treatment of 1.2% PB of *Marmoricola*, the lightest in 6.0% CB treatment; the darkest color of *Rhodanobacter* in CK treatment, the lightest in 6.0% PB, *Streptomyces* The color is the deepest in the process, and the lightest in the 1.2% CB process. In addition to the above-mentioned several abundance genus species that are significantly concentrated in different treatments, the other genus species also have branching aggregation similarities in different treatments.

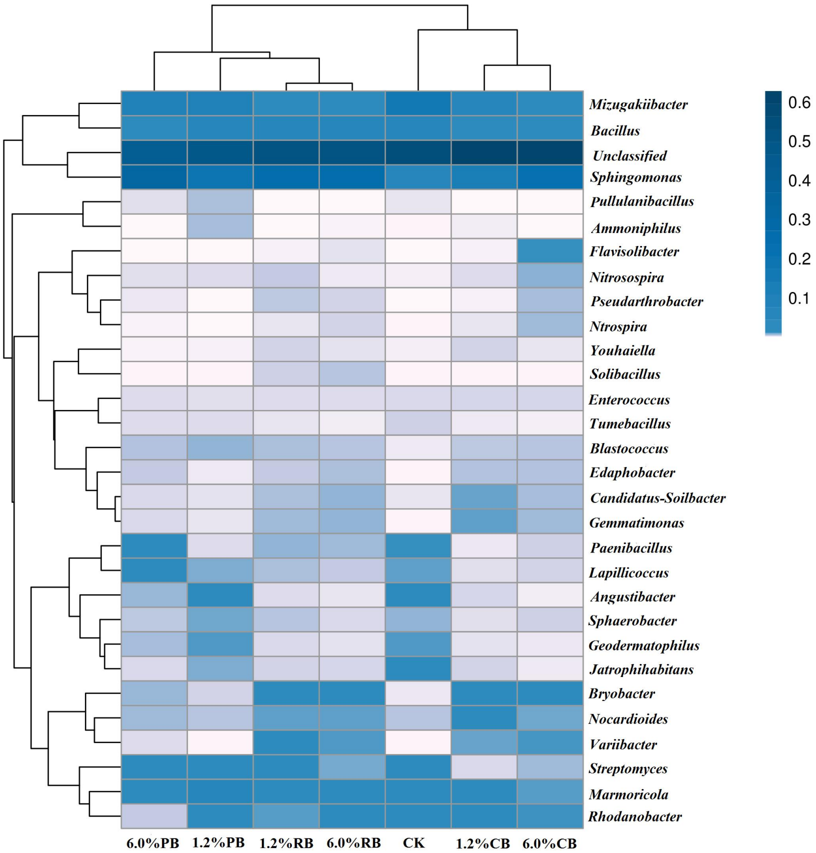

Figure 3. *Heat map of black soil bacterial community level. (CK: only black soil; CB: black soil +corn stover biomass carbon; RB: black soil + rice husk biomass charcoal; PB: black soil + pine biomass carbon)*

The diversity of the bacterial community after adding biochar

It can be seen from *Table 6* that compared with CK treatment, after adding different kinds of biochar, the ace, chao1, Shannon and Simpson indices of black soil increased significantly, which shows that after adding biochar to black soil, the uniformity and richness of the soil have increased. Between the additions of different biochars, the ace and chao1 indices of 1.2% CB and 1.2% RB increased significantly, while the Shannon and Simpson indices of 1.2% CB and 6.0% CB increased most significantly. After

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

adding different amounts of biochar, thus, after adding corn stover biomass charcoal, ace, chao1, Shannon, Simpson index of 1.2% CB treatment were greater than 6.0% CB treatment; after adding rice husk biomass charcoal, the ace and chao1 indices of 1.2% RB treatment were greater than 6.0% RB treatment, while the Shannon and Simpson indices were less than 6.0% RB treatment; after adding pine biomass carbon, the ace, chao1, and Shannon indices of 1.2% PB treatment were less than 6.0% PB treatment, only the Simpson index was greater than 6.0% PB processing.

Table 6. *Statistical table of α diversity index of black soil bacteria*

Sample	Ace	Chao1	Shannon	Simpson	Goods_coverage
CK	239.61	239.05	5.09	0.94	1.00
1.2%CB	275.83	277.14	6.42	0.98	1.00
6.0%CB	271.58	271.14	6.34	0.97	1.00
1.2%RB	277.48	277.11	6.07	0.96	1.00
6.0%RB	273.40	273.50	6.24	0.97	1.00
1.2%PB	260.28	257.13	5.27	0.95	1.00
6.0%PB	266.11	270.50	5.39	0.94	1.00

The dilution curve uses a random sampling method for sequencing sequences to determine whether the sample size is sufficient and predicts the species richness. It can be seen from *Figure 4* that when the sample Reads < 10000, the number of OTUs increases rapidly with the increase of the sequencing depth. When 10000 < Reads < 20000, the number of OTUs increases slowly as the depth increases, when Reads > 20000, as the depth of sequencing increases, the number of OTUs tends to be flat, indicating that the sequencing data at this time is more reasonable. The number of bacteria was higher when treated with 1.2% RB and lower with CK.

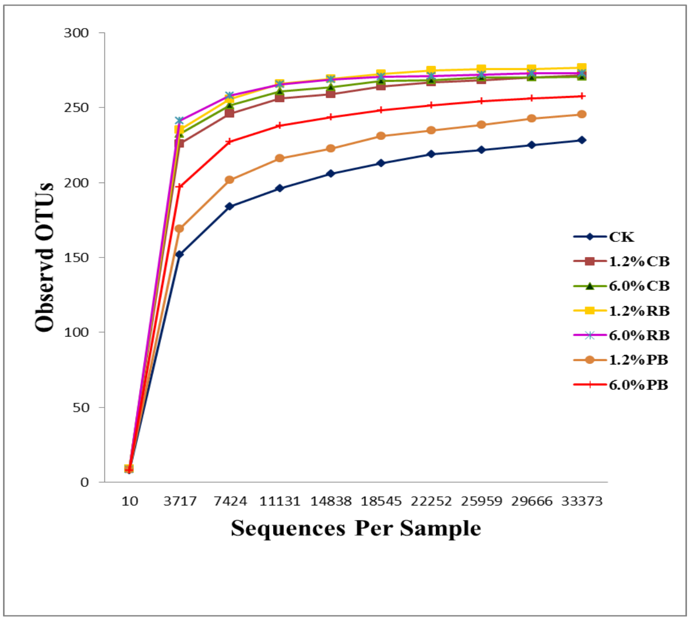

Figure 4. *Dilution curve of soil bacterial in black soil. (CK: only black soil; CB: black soil +corn stover biomass carbon; RB: black soil + rice husk biomass charcoal; PB: black soil + pine biomass carbon)*

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

Effects of environmental factors on bacterial community structure of black soil treated by different biomass carbon

In order to analyze the effects of different biomass charcoal on black soil bacterial community, typical correspondence analysis was carried out in this study. The results are shown in *Figure 5*. As can be seen from the figure, the angle between the pH, SOM, AP, and AK vector arrows is an acute angle, indicating that there is a synergistic effect between the four physical and chemical factors. The 6.0% CB of the sample had the highest correlation with pH and SOM, and the correlation between 6.0% RB and AP was the highest. CK and 1.2% CB had the highest correlation with AN, NO_3^--N and NH_4^+-N. The bacterial *Variibacter* belongs to the AP vector arrow with the smallest acute angle, indicating the highest correlation with AP. The correlation between *Bryobacter* and AK was the highest, the correlation between *Nitrosospira* and pH was the highest, the correlation between *Sphingomonas* and SOM was the highest, and the correlation between *Rhodanobacter* and *Streptomyces* was the highest with AN, NO_3^--N and NH_4^+-N, while AN, NO_3^--N and NH_4^+-N have an acute angle with most species, so AN, NO_3^--N and NH_4^+-N have a greater impact on microbial communities.

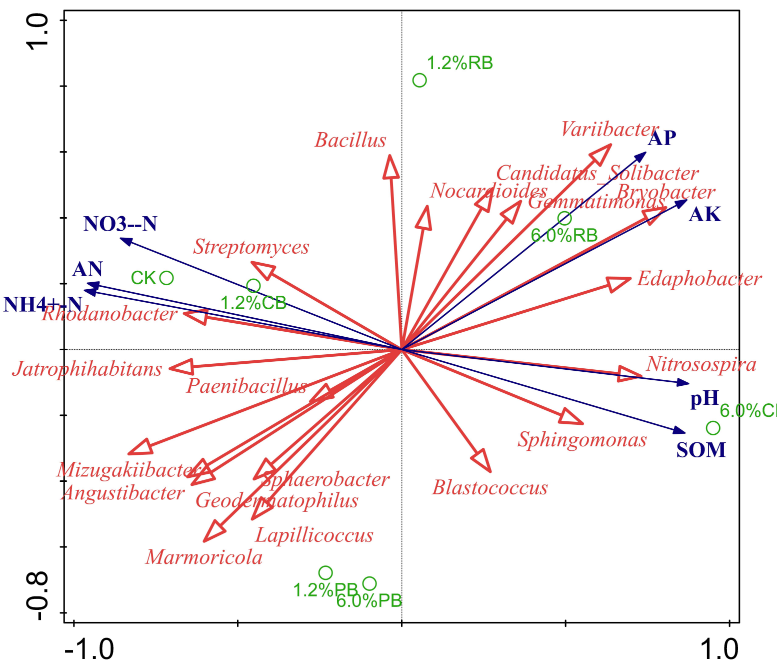

***Figure 5.** RDA analysis between environmental factors and bacterial genera levels (CK: only black soil; CB: black soil +corn stover biomass carbon; RB: black soil + rice husk biomass charcoal; PB: black soil + pine biomass carbon; SOM: Soil organic matter; AN: Alkaline nitrogen; AP: Available phosphorus; AK: Available potassium; NH_4^+-N: Ammonium nitrogen; NO_3^--N: Nitrate nitrogen)*

Correlation analysis between soil functional microorganisms and environmental parameters

Pearson correlation analysis was used to add a correlation between soil nutrient cycling functional genes and soil environmental parameters. It can be seen from *Table 7* that the relative abundance of AOA is significantly positively correlated with pH

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

($r = 0.811$, $p < 0.05$) and SOM ($r = 0.852$, $p < 0.05$), and the relative abundance of AOA is related to AN ($r = -0.823$, $p < 0.005$), NO_3^--N ($R = -0.857$, $P < 0.05$) showed a significant negative correlation, cbbl relative abundance with AN ($r = -0.860$, $p < 0.05$), NH_4^+-N ($r = -0.844$, $p < 0.05$) showed a significant negative correlation.

Table 7. *Correlation analysis between soil functional microorganisms and environmental parameters*

	Ph	SOM	AN	AP	AK	NH4+-N	NO3--N
nifh	0.530	0.410	0.116	-0.153	0.268	-0.210	-0.611
gdh3	0.550	0.239	-0.095	0.620	0.699	-0.016	-0.165
nosZ	0.334	0.681	-0.593	-0.187	0.041	-0.519	-0.514
cbbl	0.375	0.664	-0.860*	0.485	0.449	-0.844*	-0.519
AOB	0.319	0.374	-0.710	0.481	0.436	-0.691	-0.414
AOA	0.811*	0.852*	-0.823*	0.336	0.699	-0.740	-0.857*

SOM: Soil organic matter; AN: Alkaline nitrogen; AP: Available phosphorus; AK: Available potassium; NH_4^+-N: Ammonium nitrogen; NO_3^--N: Nitrate nitrogen; nifh: Nitrogen-fixing bacteria; gdh3: Phosphateolytic bacteria; nosZ: Denitrifying bacteria; cbbl: Carbonaceous bacteria; AOB: Ammonia oxidizing bacteria; AOA: Ammonia-oxidizing archaea

Discussion

Khadem et al. (2017) added scorch and unburned biomass charcoal to the soil. The results showed that the addition of both biochars significantly increased the enzyme activity of the soil. Huang et al. (2017) found that urease activity remained stable when the amount of biomass carbon added was 1% by adding exogenous carbon. When the amount of biomass carbon increased to 5%, urease activity increased significantly, while phosphoric acid. The activity of the enzyme temporarily decreased at the beginning, but eventually increased when the precipitate was treated with 1% biochar. When the concentration of biochar was as high as 5%, the activity of alkaline phosphatase decreased to 0.75 times that of the control. Luo et al. (2016) found that peroxidase activity decreased with the increase of biomass carbon by adding different amounts of biomass charcoal, and the biomass carbon content was significantly negatively correlated with peroxidase activity. Wang et al. (2015) found that by adding biochar, biochar can increase soil pH and CEC. With the addition of biochar, NH_4^+-N in soil gradually decreases peroxidase and fractional oxidation. The activity of the enzyme decreases with the addition of biomass carbon, and the activity of urease increases with the increase of biomass carbon. This study is similar to the results of Wang et al. (2015). After adding biochar, the pH of the soil increased, and the NH_4^+-N, NO_3^--N, and AN in the soil decreased compared with CK, and the polyphenol oxidase in the soil gradually decreased. Because the activity of polyphenol oxidase is negatively correlated with the degree of soil humification, as shown in *Table 5*, the polyphenol oxidase values of 6.0% CB, 6.0% PB, and 6.0% RB are significantly less than CK, indicating that the degree of humification is the best. Catalase is related to soil respiration intensity and microbial activity. In this study, the highest catalase activity was treated with 6.0% CB, the value was 1.12 mL g^{-1}, and the soil respiration intensity of 6.0% CB treatment was 119.27 ml kg^{-1}. Both are higher than other treatments, which also indicates that the catalase activity is proportional to the soil respiration intensity. After adding three different biomass charcoal, the catalase activity was treated with 6.0% activity higher than 1.2%,

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and 1.2% treatment was higher than CK treatment. Urease activity was also highest in 6.0% CB. Most of the urease activity was proportional to the organic matter content, and the organic matter content of 6.0% CB treatment was 114.52 g kg^{-1}, which was higher than other treatments. The urease activity of the other rice husks and pine biomass carbon was treated with 1.2% treatment above 6.0%.

Zhang et al. (2018) found that after adding biochar, the bacterial abundance of bacteria at the bacterial level increased correspondingly, and the effect of *Chloroflexi* gate abundance was significant. In this study, after adding biochar, *Proteobacteria* is the dominant population at the gate level, accounting for 47% of the 6.0% PB treatment, and 41.46% of the 1.2% RB, and the proportion in the CK treatment. Only 30.85%, so adding biochar can increase the abundance of bacterial phylum levels. Taking *Sphingomonas*, a dominant genus of bacteria, as an example, the proportion in the 6.0% PB treatment was 31.22%, the treatment in the 1.2% RB was 24.58%, and the proportion in the CK treatment was 4.74%. The dominant genus *Mizugakiibacter* had the opposite result, accounting for 14.83% of the CK treatment, and the proportion of other treatments is smaller than the proportion of CK treatment. Therefore, the addition of biochar does not necessarily increase the bacterial abundance. Nguyen et al. (2018) found that soils supplemented with biochar had higher bacterial alpha diversity and OTU richness compared to CK. Li et al. (2018) found that by applying biochar, the application of biochar increased the Shannon index of the bacteria compared to the control. This study is the same as the above two studies. After adding biochar, ace, chao1, Shannon, Simpson, CK treatment increased, and the ace index increased significantly with 1.2% RB treatment, both chao1, Shannon, Simpson were significantly increased by 1.2% CB treatment. Du et al. (2018) found that the addition of different doses of biomass charcoal, will change the bacterial community during the composting process, but the composition of the bacterial community did not change much, and they found that the addition of biochar can also change the function such as sexual bacterial community. The smallest acute angle between the genus *Variibacte*r and the AP vector arrow indicates the highest correlation with AP. The correlation between *Bryobacter* and AK was the highest, the correlation between *Nitrosospira* and pH was the highest, the correlation between *Sphingomonas* and SOM was the highest, and the correlation between *Rhodanobacter* and *Streptomyces* was the highest with AN, NO_3^--N and NH_4^+-N, while AN, NO_3^--N and NH_4^+-N had a great influence on the genus. The angle between the pH, SOM, AP, and AK vector arrows is an acute angle, indicating a synergistic effect between the four physical and chemical factors. Fluorescence quantitative PCR was used to detect the abundance of functional genes in soil. Compared with CK, the application of biochar could significantly increase the abundance of functional genes in the soil, and the correlation between environmental factors and functional genes was analyzed by Pearson correlation. As seen from *Table 7*, the relative abundance of AOA is significantly positively correlated with pH ($r = 0.811$, $p < 0.05$) and SOM ($r = 0.852$, $p < 0.05$), and the relative abundance of AOA is related to AN ($r = -0.823$, $p < 0.005$), NO_3^--N ($R = -0.857$, $P < 0.05$) showed a significant negative correlation, cbbl relative abundance with AN ($r = -0.860$, $p < 0.05$), NH_4^+-N ($r = -0.844$, $p < 0.05$) showed a significant negative correlation, which also indicated that the relative abundance of AOA in soil had significant correlation with pH, SOM, AN, NO_3^--N, and cbbl had significant correlation with AN and NH_4^+-N. Therefore, the application of biochar has a relatively large effect on the functional bacteria AOA and cbbl in the soil.

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

Conclusion

Adding biochar can increase the pH, SOM and AK of black soil, and the increase of AP value is not significant, but the content of AN, NH_4^+-N and NO_3^--N in the soil after adding biochar is less than that of CK. The addition of biochar can increase the enzyme activity in soil. The activity of polyphenol oxidase is negatively correlated with the degree of humification. The lower the value, the higher the degree of humification and was best at 6.0% CB. Adding biomass charcoal can also affect the microbial community diversity of black soil. After adding biochar, the α diversity index of black soil was increased compared with CK, and environmental factors also have an effect on the microbial community. Microbial functional bacteria AOA, cbbl and pH, SOM, AN, NH_4^+-N, NO_3^--N had a significant correlation. Based on the above basic properties and changes in microbial communities, in the various treatments of this experiment, the best effect was to add corn stover charcoal, and the best amount to add was 6.0% CB.

Acknowledgements. This work was supported by the article was supported by funding from the National key R&D project sub-project (2017YFD0300405--4), Natural Science Foundation of Jilin Province, China (20170101077JC) and Natural Science Foundation of Jilin Province, China (subject to guide project 20170101004JC).

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

APPENDIX

Table A1. *Primers and real-time PCR conditions used in this experiment*

Function genes	Primers		Real-time PCR protocol
nifH	PolF	5′-TG CGAY CCS AAR GCB GAC TC-3′	94 °C for 5 min; 40 cycles of 94 °C for 30 s, 57 °C for 45 s (54 °C for *nifH* gene, 60 °C for AOB), 72 °C for 1 min
	PolR	5′-ATS GCC ATC ATY TCR CCG GA-3′	
AOA	*Arch-amoA*F	5′-STA ATG GTC TGG CTT AGA CG-3′	
	*Arch-amoA*R	5′-GCG GCC ATC CAT CTG TAT GT-3′	
AOB	*amoA*-1F	5′-GGG GTT TCT ACT GGT GGT-3′	
	amoA-2R	5′-CCC CTC KGS AAA GCC TTC TTC-3′	
nosZ	*nosZ*-F	5′-CGY TGT TCM TCG ACA GCC AG-3′	
	nosZ-1622R	5′-CGS ACC TTS TTG CCS TYG CG-3′	
cbbl	K2f	5′-ACCAYCAAGCCSAAGCTSGG-3′	
	V2r	5′-GCCTTCSAGCTTGCCSACCRC-3′	
Gdh3	Gdh-3F	5′-GTCTGGCGTCGTCTGATTGTG-3′	
	Gdh-3R	5′-CAGGCGTGGATTCTGCGTT-3′	

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DOI: http://dx.doi.org/10.15666/aeer/1702_53055319

MOLECULAR CHARACTERIZATION OF RESISTANCE-BREAKING *TOMATO SPOTTED WILT VIRUS* (TSWV) ISOLATE MEDIUM SEGMENT IN TOMATO

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(Received 31st Dec 2018; accepted 20th Feb 2019)

Abstract. *Tomato spotted wilt virus* (TSWV) is an economically important disease causing significant yield and quality loss in tomato. The most efficient method of disease control relied on the use of the *Sw-5* resistant gene. The new resistance-breaking Tswv isolates have severely infected greenhouses grown tomatoes Eastern Mediterranean area, including Antalya, Turkey. The objective of the study was to identify the cause of the resistance-breaking genetic mutations on the virus genome. The plant materials included resistant *Solanum peruvianum* PI126944 and four commercial hybrids, and a susceptible control. The six resistance-breaking strains (RBS) of the virus were collected from greenhouse-grown tomato plants throughout Antalya, Turkey. Beginning at the seedling stage, five consecutive inoculations were carried out at five-day intervals, using both the resistant and susceptible genotypes. The Medium segment of the virus includes the cell-to-cell movement protein (NSm) and a precursor of the surface glycoproteins (GN/GC) that were sequenced with different primer combination. The medium segment of the RBS genomes was amplified and 4825 bp sequence aligned and blasted using NCBI database. On-structural movement (NSM) domain of the M segment consistently carried C118Y mutation in all RBS analyzed. The multiple mutations on Gc-Gn domain were not associated with resistance break down. Phylogenetic analysis indicated that RB strain identified in Turkey was closely related to Spanish RB strains. The C118Ymutation overcame the resistance conferred by the Sw-5 gene. Hence, a new resistance source is needed to protect the tomato from new RB strains.
Keywords: *orthotospoviruses, NSm protein, avrirulence determinant, hypersensitive response, Sw-5b gene*

Introduction

Tomato production is under threat from viral diseases causing significant yield and quality loss all over the world (Pappu et al., 2009). One of the most common viral diseases occurs with *Tomato spotted wilt virus* (TSWV) on tomato. The TSWV is a member of genus *Orthotospovirus,* family Bunyaviridae (Adams, 2017), limits tomato production especially in greenhouses. The TSWV virus is transmitted by thrips (Thysanoptera, *Thripidae),* the most common vector being *Frankliniella occidentalis*in a persistent and circulative manner (Todd et al., 1995; Mandal et al., 2001).

Orthotospoviruses are enveloped viruses including additional protein package on their RNA genomes. The virus genome contains three RNA segments known as a tripartite RNA genome structure (Cortez et al., 2001). The TSWV has similarly three RNA parts described as small (S), medium (M), and large (L) RNAs (de Haan et al., 1991). The whole genome of TSWV codes six proteins via five different open reading frames (ORFs). The M and S RNAs have special structures where genomes are ambisense The ambisense viruses include both parts of positive and part of negative polarities in their genome (Kormelink et al., 1994; Lewandowskia and Adkins, 2005).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53215339

The genome size of TSWV is 16.6 kbp where small, medium and large segments are 2.9 kb, 4.8 kb, and 8.9 kb, respectively (de Haanet al., 1990). The S segment has two ORFs encoding non-structural RNA silencing suppressor (NSS) and nucleocapsid (NC) proteins (Takeda et al., 2002). The M segment produces both non-structural movement protein (NSm) and envelope glycoproteins Gn-Gc (Kormelink et al., 1994). The last L segment encodes putative RNA-dependent RNA polymerases (RdRp), such as replicase, transcriptase, nuclease, helicase, cap-binding and NTPase proteins responsible for several enzymatic functions of TSWV (de Haan et al., 1991).

TSWV is the second most destructive viral pathogen in the list of economic damage causing plant viruses (Scholthof et al., 2011). The prevention and control of the spread of TSWV is very difficult due to its vectors. For TSWV management, resistant tomato varieties carrying *Sw-5* resistance gene derived from *Solanum peruvianum* have extensively been used (Pappu et al., 2009). In recent years, the resistant tomato plants have been infected with TSWV in Spain (Debreczeni, 2011). Similarly, resistant-breaking TSWV isolates have been reported on genetically resistant pepper plants since 2014 in Samsun, Turkey (Deligöz, 2014). Likely, severe TSWV infections are obtained on resistant gene containing tomato varieties indicating TSWV changed its genetic structure in 2016 in Turkey (Fidan et al., 2016; Batuman et al. 2017). In order to understand the genetic cause of virulence, the study has been conducted. Hence, whole genome structure of avirulent non-resistant breaking isolates and virulent resistance-breaking TSWV isolates are compared in nucleotide sequences and possible functional mutation site(s) have been investigated.

Materials and methods

Determination of the Sw-5 gene containing TSWV resistant plants

Total genomic DNAs were extracted by DNA extraction kit (Thermo Scientific, Germany,) from the resistant *Solanum peruvianum* PI126944, a susceptible commercial tomato hybrid variety (Hazera 5656 (Hazera Seed), and four resistant commercial hybrids, namely Torry F1 (Syngenta), Matatu F1 (RijkZwan), TayfunF1 (De Ruiter), Swanson F1 (Seminis). The DNA samples were PCR amplified using Sw5-2 primers F-AATTAGGTTCTTGAAGCCCATCT, and R-TTCCGCATCAGCCAATAGTGT as reported by Dianese et al. (2010) where the 574 bp and 500 bp amplicons representing resistant and susceptible Sw-5 alleles, respectively.

Inoculations of susceptible and resistant tomato plants with TSWVAntRB isolate

The six resistance-breaking isolates (RBS) were collected throughout Antalya, Turkey, from tomato plants grown in greenhouses. Because sequence analysis indicated the presence of single haplotype, all six RBS were mixed to inoculate the resistant and susceptible tomato samples. The resistant and susceptible samples, five plants in each replicate, were inoculated with RBS and a mock inoculation with phosphate buffer was used as a control. The inoculations were repeated five times at 5 intervals. At 15 days' post inoculations (dpi) when susceptible plant showed virus symptoms, leaf tissue was collected from mock and RBS inoculated plants. Systemic viral infection was subsequently determined by RT-PCR analysis as described previously (Dianese et al., 2010).

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DOI: http://dx.doi.org/10.15666/aeer/1702_53215339

Genome analysis of TSWV isolates

The TSWV isolates were collected from greenhouse-grown tomato plants in Antalya province, Turkey. The TSWV isolates originated from tomato hybrids reported to carry resistance against TSWV in 2016. The TSWV isolates were kept in -20 °C as infected leaf material at Virology Laboratory, Plant Protection Department, Akdeniz University, Antalya, Turkey. Total RNAs were extracted from the samples using an RNA extraction Kit (K0731**,** Thermo-Scientific, Germany). The cDNAs of TSWV were obtained with high capacity cDNA Reverse Transcription Kit (Thermo Scientific, Germany) according to the manufacturer's instructions. The M segment specific primers (Hallwass et al., 2014 and Zhong et al., 2011) presented in *Table 1* were paired (*Table 2*) to amplify TSWV isolates by RT-PCR analyses.

Table 1. *Primer sequences representing M segment of tomato spotted wilt virus (TSWV) used in RT-PCR studies (Hallwass et al., 2014)*

Segment	Primers' name	The sequence of primers from 5' to 3'	Position in M segment
M	M1 (F)	AGAGCAATCAGTGCATCAGAAATATACCTATTA	1-36
	M2 (F)	GTAGATACAAACCATCATATCTCAAACTGG	365-394
	M3 (R)	TCTTTATCAGCTCTGGGTGAATCAC	771-795
	M4 (F)	CAAGGTGAGACAAATCCATAGGTGGCC	1335-1361
	M5 (R)	TGATGAGTATGCTCATGAAGAACAAC	1638-1663
	M6 (F)	CAGGATCATTCAAGTTTGCAATATTTCCAG	2268-2297
	M7 (R)	CTTATTGGGGATGTGAAGAAGCTTGG	2566-2591
	M8 (F)	GATGTTAACCCTAAAGAGCTTCCTG	3029-3053
	M9 (R)	GTCTCAAATGCCCATGTCTATGGCTC	3348-3373
	M10 (F)	GTTATAGGATAATTATCTTGTGTC	4130- 153
	M11 (R)	CCAGAGGTTTATGATGATTCTGCTGAG	4579 4065
	M12 (R)	AGAGCAATCAGTGCAAACAAAAACCTTAATCC	4790-4821

Table 2. *Primer names and sequences used in RT-PCR studies*

Primers' name	The sequence of primer from 5' to 3'	Expected amplicon size
TSWV-M-1F	AGAGCAATCAGTGCATCAGA	955 bps
TSWV-M-1R	CTTCTTCTTCAACTGATCTCTCAAG	
TSWV-M-2F	GCAAGCTGATAATTCCTAAAGG	1351 bps
TSWV-M-2R	AAGGAGATGACATGTCTTGGG	
TSWV-M-3F	CCGCATAGAAGACAGCC	1276 bps
TSWV-M-3R	GTTATAGAAGGTCCTAATGATTGCA	
TSWV-M-4F	GTTAACCCTAAAGAGCTTCCTG	979 bps
TSWV-M-4R	GAGAAGATCATGGGTTATTTGAT	
TSWV-M-5F	CTTATCCAAGAAAAATTGATGC	1051 bps
TSWV-M-5R	AGAGCAATCAGTGCAAACAAAA	

The amplified RT-PCR products were both run on a 1.5% agarose gel, and stained with ethidium bromide and then visualized under UV (Integrated Biometra Gel Imager, Goettingen/(Germany)), and sequenced from both ends at HibriGen

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DOI: http://dx.doi.org/10.15666/aeer/1702_53215339

(Biotechnology Research, Development Industry and Trade Co. Ltd, Turkey). The M Segment were divided into smaller sequences (700 bp to 1200 bp) using primers (*Tables 1* and *2*) to yield overlapping sequences for alignments. The sequence analysis and multiple sequence alignment were carried out using Chromas (Technelsium DNA Sequencing Software Australia) and CodonCode Corporation (Florida) software. The complete M segment sequences were analyzed in National Center for Biotechnology Information (NCBI) using nucleotide BLAST. The agarose gel electrophoresis of M segment amplifications is shown in *Figure 3*.

Results

The plant materials used in this study were tested using Sw-5 specific molecular marker (Dianese et al., 2010). The codominant SCAR *Sw5-2* marker confirmed the resistant vs susceptible status of the plant materials. The resistant *Solanum peruvianum* was homozygous resistant (574 bp), the four commercial hybrids were heterozygous (574 and 500 bp), and the susceptible one was homozygous (500 bp) as expected (*Fig. 1*).

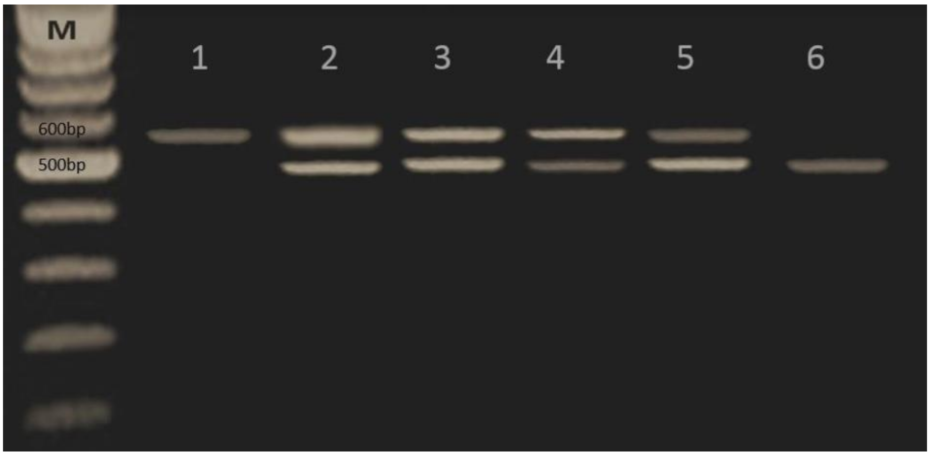

Figure 1. *Agarose gel electrophoresis of Sw-5 primers. M: 100 bpDNA ladder; Lane 1 resistant Solanum peruvianum, is a source of resistance with homozygous alleles (574 bp); Lanes 2 Torry F1 (Syngenta), 3 Matatu F1 (RijkZwan), 4 Tayfun F1 (De Ruiter), and 5 Swanson F1(Seminis). heterozygous resistant alleles known as resistant tomato varieties (574-500 bp); Lane 6 sensitive negative control. Tomato variety (Hazera 5656 (Hazera Seed)) without Sw-5 allele (500 bp)*

Antalya resistance-breaking (AntRB) TSWV isolates are confirmed in mechanical inoculations

After the TSWV mechanical inoculation (Hull, 2014), first typical virus symptoms were observed at 7 dpi on susceptible tomato variety. In resistant *Solanum peruvianum* and 4 commercial tomato varieties, the symptoms were barely evident at 15 dpi, similar in both homozygous and heterozygous plants.

RT-PCRs were performed at 21 dpi for 12 different tomato viruses (*Alfalfa Mosaic Virus* (AMV) (Saleh and Amer, 2013), *Cucumber mosaic virus* (CMV) (Paradies et al., 2000), *Potato virus X* (PVX) (Fidan et al., 2011), *Potato virus Y* (PVY) (Fidan et al., 2011), *Tabaco Etch Virus* (TEV) (Lee et al., 2011), *Tobacco Mosaic Virus* (TMV)

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(Kumar et al., 2011), *Tomato mosaic virus* (ToMV) (Kumar et al., 2011), *Tomato yellow leaf curl virus* TYLCV (Anfoka et al., 2008), *Tomato Chlorosis Virus* (ToCV) (Tiberini et al., 2010), *Tomato ringspot virus* (ToRSV) (Fuchs et al., 2009), *Pepino Mosaic Virus* (PePMV) (Ge et al., 2013) and TSWV (Adkins et al., 2005) (*Fig. 2*) it was confirmed that the samples were infected with only TSWV.

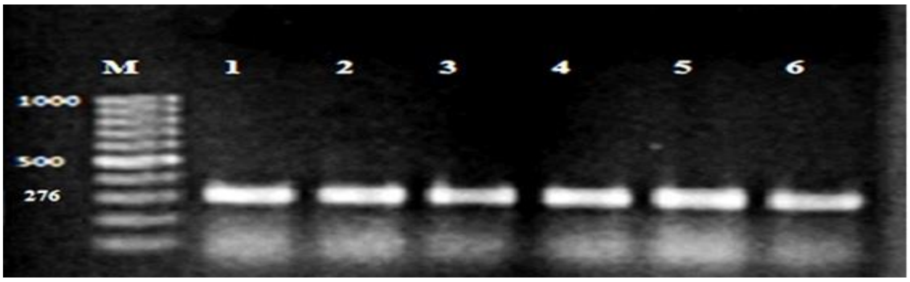

Figure 2. *Agarose gel electrophoresis of PCR products obtained using L1TSWVR and L1TSWVF primers specific to TSWV. The TSWV coat protein specific primers have produced 276 bp amplicon in PCR analyses. Lanes: M: 100 bp Standard Marker; Lane 1 resistant source 'Solanum peruvianum', Lanes 2 'Torry F1' (Syngenta), 3'Matatu F1' (RijkZwan), 4 'TayfunF1' (De Ruiter), and 5 'Swanson F1' (Seminis). Resistant varieties, Lane 6 ('Hazera 5656' (Hazera Seed)) sensitive varieties*

After confirming that the samples got infected with TSWV (*Fig. 2*), the M domain (Medium segment) were sequenced using different primer combinations. The 4825 bp M segment was amplified at five overlapping pieces (*Fig. 3*). The sequence of M Segment parts was aligned and the consensus sequence was subjected to haplotype analysis, using DnaSP 5 program (DNA Sequence Polymorphism. Universidad de Barcelona). Both sequence and haplotype analysis showed that RBS of TSWV collected in Antalya had a single haplotype (Additional file A4), which was named and deposited into NCBI as TSWVAntRB.

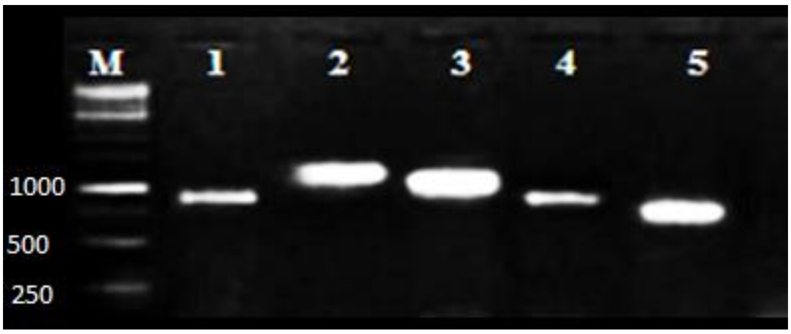

Figure 3. *TSWVAntRB Medium site is belonging to agarose gel electrophoresis. M: marker, Lane 1: M1-M3 primers (759 bp), Lane 2: M2-M5 (1269 bp), Lane 3: M4-M7 primers (1230 bp), Lane 4 primers: M6-M9 primers (1082 bp), Lane 5: M-5F/M-5R- primers (1051 bp)*

Phylogenetic analysis of complete nucleotide sequences of the M segment

The complete nucleotide M segment sequence obtained after assembly was recorded at NCBI database (MH367503). For phylogenetic analyses, the M segment sequences of

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DOI: http://dx.doi.org/10.15666/aeer/1702_53215339

both resistance breaking and non-breaking isolates originating from different parts of the world were retrieved from NCBI to trace the possible origin of resistance-breaking TSWVAntRB isolate. The nucleotide comparison was made in the NCBI BLAST system and the isolates with the highest similarity were selected (*Table 3*). Five of these isolates were RB (Resistance-breaking) and 13 of them were NRB (Non-Resistance-breaking).

Table 3. *The table is nucleotide comparison made in the NCBI BLAST system and the TSWVAntRB (MH367503) with the highest similarity was selected. The first five rows are RB (Resistance-breaking) and 13 of them are NRB (Non-Resistance-breaking) isolates*

	Name of isolate	**Region**	**Host**	**% identity**	**Note**	**Accession No.**
1	TSWVAntRB	Turkey	Tomato		RB	MH367503
2	Pujol1TL3	Spain	*Solanum lycopersicum*	99	RB	HM015520
3	Sala1TL3	Spain	*Solanum lycopersicum*	99	RB	HM015521
4	D-191	Australia	Tomato	97	RB	HM015516
5	Borgo1	Italy	*Solanum lycopersicum*	97	RB	MG457158
6	CA SW21	USA	Tomato	94	RB	KX898453
7	D	USA	Tomato and Tobacco	98	NRB	AF208497
8	p202/3RB	Italy	Pepper	98	NRB	HQ830185
9	LK-1	South Africa	Tomato and *Amaranthus*	96	NRB	KY250489
10	GA-1L	Spain	*Solanum lycopersicum*	92	NRB	FM163371
11	ALPA	Spain	*Datura stramonium*	92	NRB	HQ537114
12	LS3	South	*Leonurus sibiricus*	92	NRB	KM076652
13	TSWV-YN	China	Tomato	93	NRB	JF960236
14	YNta	China	Tobacco	93	NRB	KM657118
15	YN5576	China	*Solanum indicum*	93	NRB	KY495608
16	SPAIN-2	Spain	Tomato	93	NRB	AY744493
17	BasC	USA	*Ocimum basilicum*	93	NRB	KU179514
18	PA01	USA:	Pepper	93	NRB	KT160281

NRB: non-resistance-breaking

The phylogenetic analysis of the isolates selected from NCBI according to the Neighboring method was carried out using the Mega 7 program. The phylogenetic trees showed that the isolate TSWVAntRB is originated from Europe (*Fig. 4*). The genome analysis placed the virus with HM15520.1, Spanish Resistance-breaking isolate.

Of the 17 TSWV isolates with the highest similarity to the RB isolate (TSWVAntRB), five belonged to RB isolates originating from tomato reported in Spain, Italy, USA, and Australia (*Table 3*). The RB isolate TSWVAntRB located on the same branch with one of the Spanish RB isolates. The result indicates that the RB isolate TSWVAntRB identified in Antalya, Turkey most probably originates from Spain. TSWV isolates worldwide have been divided into two roots, Asian and European isolates (Lian et al., 2013). It was determined that the isolates divided into two separate groups and TSWVAntRB isolate was found in group II (*Fig. 4*). The Group II consisted of European resistance-breaking isolates. The most of Group I isolates of Asian origin.

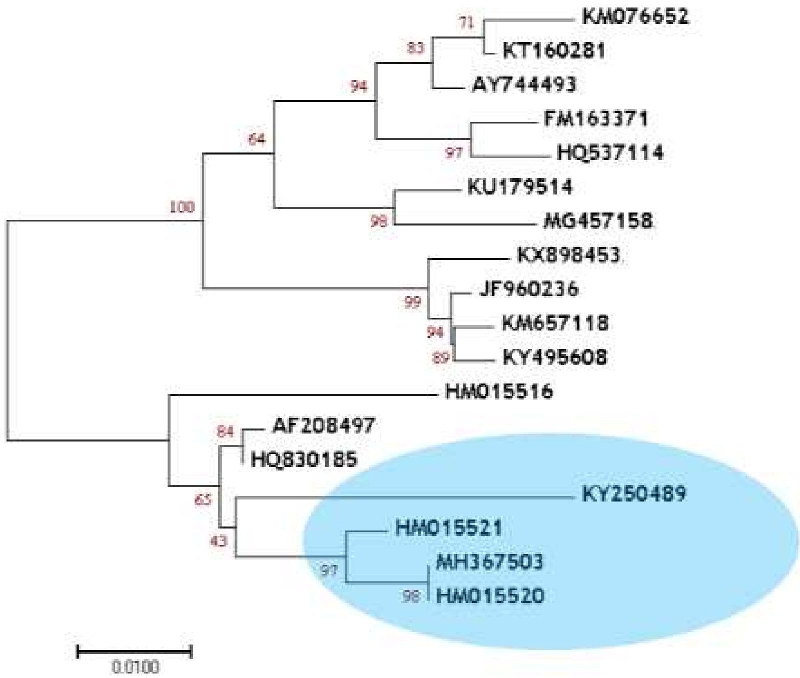

Figure 4. *Phylogenetic analysis showing the relationship between TSWVAntRB (MH367503) isolates with world isolates. The phylogenetic tree MEGA7 program was created using the Neighbor-Joining method. It was determined that the 18 isolates that were taken into consideration were formed by two groups and were calculated using the maximum composite likelihood method, which is the same root as the Spanish isolate (HM015520.1) that breaks down on the same branch with European isolates*

Protein-based comparisons on medium segmentation

Worldwide studies of TSWV indicate that resistance-breaking isolates arise due to a mutation in the NSm protein responsible from cell to cell movement on the Medium segment (Lopez et al., 2011).

The Bioedit and CodonCode Aligner programs were used to process sequence data and ExPASY Tool to obtain the predicted protein sequences of the M segment (*Figs. 5* and *6*). The predicted protein sequences of the TSWVAntRB isolate and the 17 isolates were aligned on which C118Y mutation was highlighted (*Fig. 5*) (Additional files 1, 2). The predicted protein alignment of the M segment further confirmed that a single point mutation on the movement protein located on M segment causes the resistance to break (KY973680.1; KY973679.1, KY973677.1, KT192625.1, KT192624.1, KT192623.1, KM379142.1, KM379141.1, MH367502.1, KX618636.1, KX618635.1, KM407603).

The remaining M segment sequence was also analyzed. The 40 different mutations detected throughout glycoproteins Gn and Gc (1135 aa) were not meaningful for resistance (Additional files 3).

It was previously reported that C118Y point mutation prevents the formation of HR response, causing systemic disease establishment on infected plants (Lopez et al., 2011).

The NSm protein domain on the TSWV's Medium region is the region that allows the virus to move from cell to cell. The resistance provided by the Sw-5 gene on the old isolate both causes rapid deaths in the contaminated tissues through HR reactions and there is no possibility of virus infection and contamination of new tissues in these tissues. However, it is shown that the TSWV resistance-breaking isolates related to

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NSm protein, a point mutation on the cell to cell movement gene, caused resistance breaking on TSWV resistant tomato.

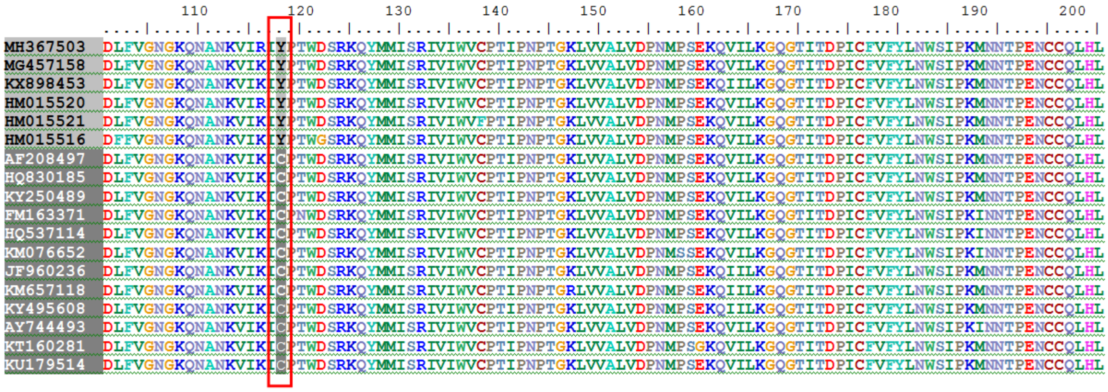

Figure 5. *Sequence alignment of the NSm proteins derived from the six resistance-breaking (RB) and 12 non-resistant-breaking (NRB) strains. The C118Y mutation on predicted amino acids derived from RB and NRB is indicated. The MH367503 (TSWVAntRB Antalya-Turkey RT tomato) line 2 to 6 are resistance-breaking (R.B). Lines 7 to 19 are non-resistance-breaking (N.R.B)*

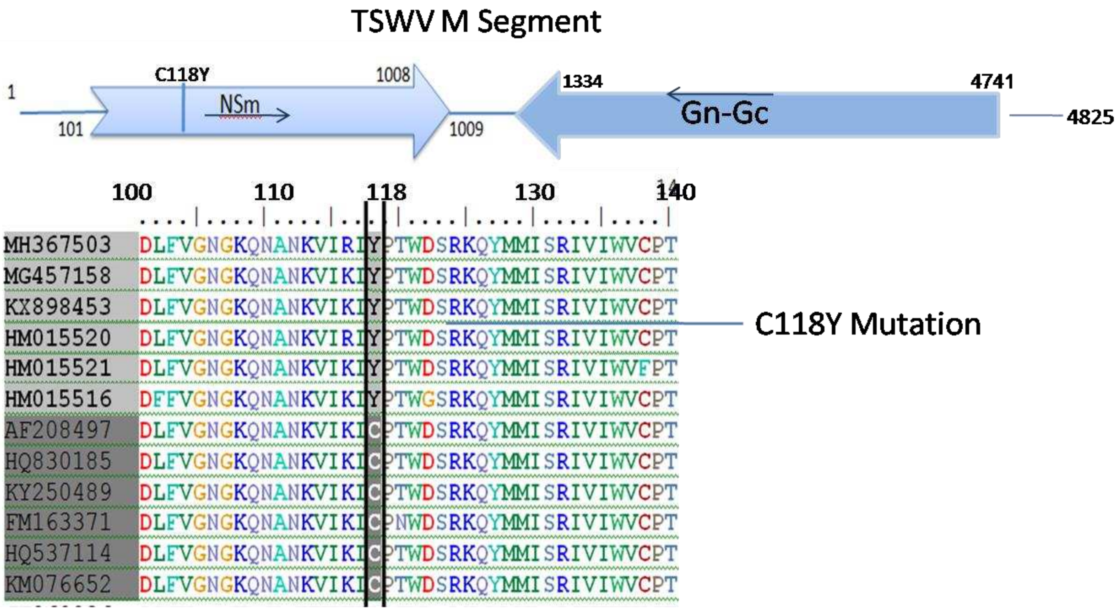

Figure 6. *The mutation site in the NSm protein domain (ORF-open reading frame) of the medium segment of the TSWVAntRB isolate*

Recombination events for single-stranded RNA viruses are thought to be involved in a major evolutionary process. Genetic alterations that occur due to recombination events can be very important for RNA viruses with ambisense character, which has a multi-segmented structure such as TSWV. Earlier studies emphasized the emergence of new isolates due to changes in the form of point mutations on viruses with multi-segmented structures such as TSWV.

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Discussion

Comparison of NCBI isolates and evaluation of the phylogenetic analysis

The TSWV isolates were divided into two origins, namely Asia and Europe (Lian et al., 2013). The sequence comparison of M segment of TSWVAntRB isolates with that of isolates in NCBI returned a similarity ratio of 93-98%, confirming previous reports for two distinct origins (Lopez et al., 2011; Tsompana et al., 2005). However, origins of RB isolates are independent of sequence diversity where the same single point mutation results with resistance break. The extensive use of Sw-5 allele may have caused positive selection, leading to the occurrence of RB isolate (de Ronde et al., 2014). The Sw-5 gene is known to provide resistance to *Orthotospovirus*es through HR response. When infection occurs in plants, cell death occurs rapidly via the Sw-5 gene and the virus is trapped within the dead cells. Recent studies have informed that C118Y mutation on the NSm proteins of *Orthotospovirus*es fails Sw-5 protein to initiate the HR, leading to systemic virus infections in plants (Leastro et al., 2015). The NSm protein is a non-structural protein (Kormelink et al., 1994; Storms et al., 1995) that promotes the generation of *Orthotospovirus*es through plasmodesmata into envelope-free nucleocapsids. Hence, the NSm protein is able to spread to neighboring healthy cells through plasmodesmata at the absence of a functional Sw-5 gene or C118Y mutation that make Sw-5 protein fail to trigger HR.

Lopez et al. (2011) reported that C118Y mutation on the NSm protein in the medium segment is a very important for adaptation on new hosts. This mutation, which is likely to occur on the cysteine, is considered to be a fundamental point in the adaptation of TSWV to the new hosts during the evolutionary process.

The TSWVAntRB may have been transported from Spain via thrips vectors. Persistent propagative transmission of the virus with thrips may explain the long-distance transmission. However, it has been noted that there is no association in the breakdown of the resistance of the TSWV isolate accumulated in thrips body (Debreczeni et al., 2014). It has been argued that the phylogenetic relationship of isolates obtained from different continent may be effective in transporting vector thrips with long-distance migration capability (Lopez et al., 2011). Knowing the factors involved in the evolution of viruses is essential to understanding the processes involved in molecular biology and epidemiology (including the emergence of new viruses), to develop more effective and strong control strategies (Garcia et al., 2001; Moya et al., 2004).

The entire genomic sequences of the Gn-Gc domain in the medium segment were also obtained and compared against the database. The result showed that although this domain carries multiple mutations, none of which plays a role in the breakdown of resistance.

Acknowledgements. The authors are thankful to the Scientific Research Projects (FLY-2017-2619) Coordination Unit of Akdeniz University.

Conflict of interests. The authors declare that they have no conflict of interests.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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APPENDIX

Appendix 1. *Comparison of MH367503 (TSWVAntRB) other TSWV isolates and difference ratios*

MH367503		**0,00**	**0,01**	**0,04**	**0,02**	**0,02**	**0,05**	**0,08**	**0,09**	**0,08**	**0,08**	**0,08**	**0,08**	**0,08**	**0,09**	**0,09**	**0,09**	**0,08**
HM015520	0,00		0,01	0,04	0,02	0,02	0,05	0,08	0,09	0,08	0,08	0,08	0,08	0,08	0,09	0,09	0,09	0,08
HM015521	0,01	0,01		0,04	0,02	0,01	0,04	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,09	0,08
HM015516	0,04	0,04	0,04		0,03	0,03	0,06	0,08	0,09	0,08	0,08	0,08	0,08	0,08	0,09	0,09	0,09	0,08
AF208497	0,02	0,02	0,02	0,03		0,00	0,04	0,06	0,07	0,07	0,06	0,07	0,07	0,06	0,07	0,07	0,08	0,07
HQ830185	0,02	0,02	0,01	0,03	0,00		0,04	0,06	0,06	0,06	0,06	0,07	0,07	0,06	0,06	0,07	0,08	0,07
KY250489	0,05	0,05	0,04	0,06	0,04	0,04		0,09	0,10	0,10	0,09	0,10	0,10	0,09	0,10	0,09	0,10	0,10
FM163371	0,08	0,08	0,08	0,08	0,06	0,06	0,09		0,01	0,03	0,06	0,06	0,06	0,02	0,02	0,04	0,05	0,06
HQ537114	0,09	0,09	0,08	0,09	0,07	0,06	0,10	0,01		0,04	0,05	0,05	0,06	0,02	0,03	0,04	0,05	0,06
KM076652	0,08	0,08	0,08	0,08	0,07	0,06	0,10	0,03	0,04		0,05	0,05	0,05	0,02	0,01	0,04	0,05	0,05
JF960236	0,08	0,08	0,08	0,08	0,06	0,06	0,09	0,06	0,05	0,05		0,01	0,01	0,05	0,05	0,04	0,05	0,01
KM657118	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,05	0,05	0,01		0,01	0,05	0,05	0,04	0,05	0,01
KY495608	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,06	0,05	0,01	0,01		0,05	0,05	0,04	0,05	0,01
AY744493	0,08	0,08	0,08	0,08	0,06	0,06	0,09	0,02	0,02	0,02	0,05	0,05	0,05		0,01	0,03	0,04	0,05
KT160281	0,09	0,09	0,08	0,09	0,07	0,06	0,10	0,02	0,03	0,01	0,05	0,05	0,05	0,01		0,04	0,05	0,06
KU179514	0,09	0,09	0,08	0,09	0,07	0,07	0,09	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04		0,02	0,04
MG457158.1	0,09	0,09	0,09	0,09	0,08	0,08	0,10	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,02		0,05
KX898453.1	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,06	0,05	0,01	0,01	0,01	0,05	0,06	0,04	0,05	

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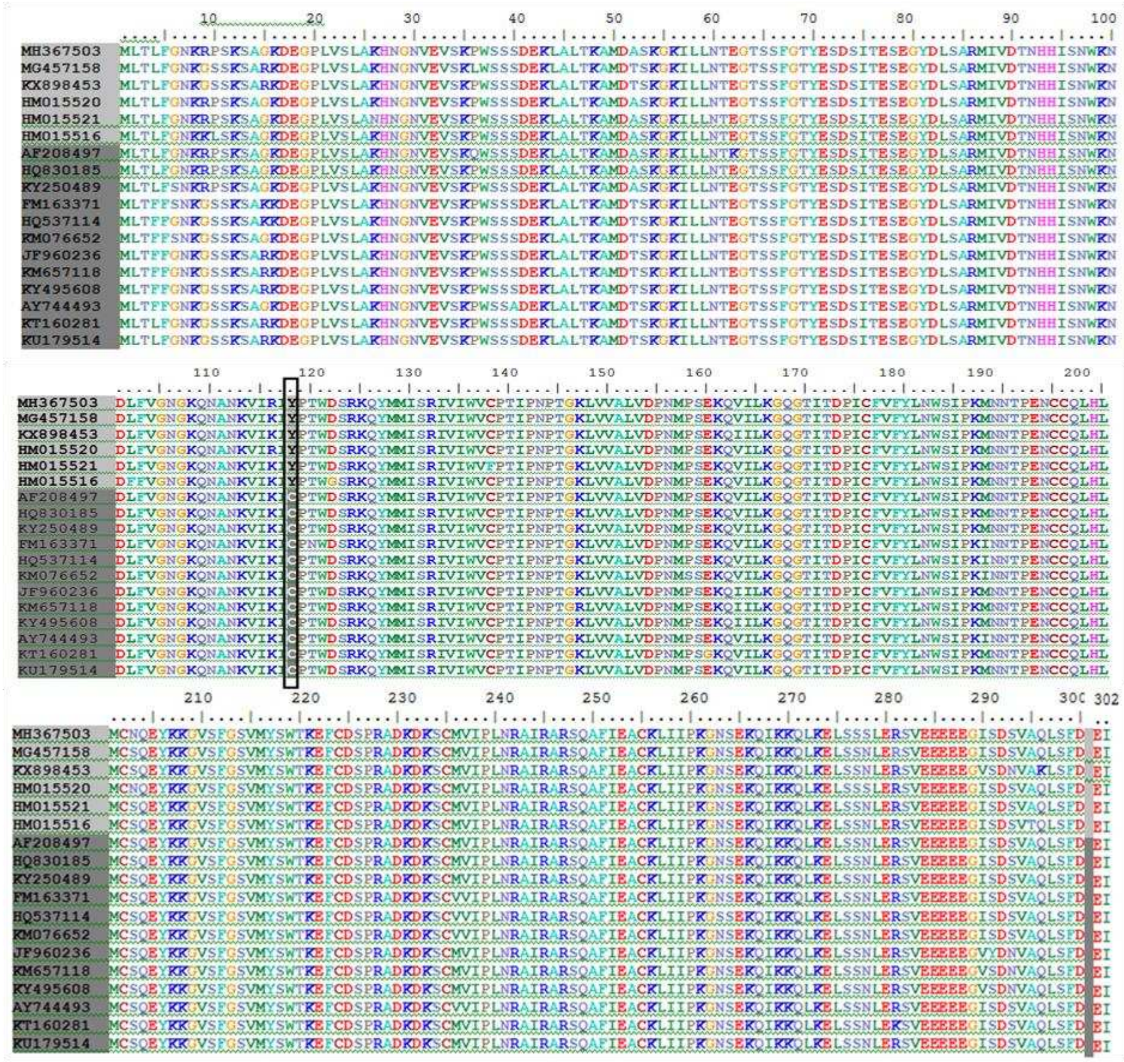
Appendix 2a. *Amino acid sequencing of the NSm open reading region of MH367503 (TSWVAntRB) isolate (302 AA)*



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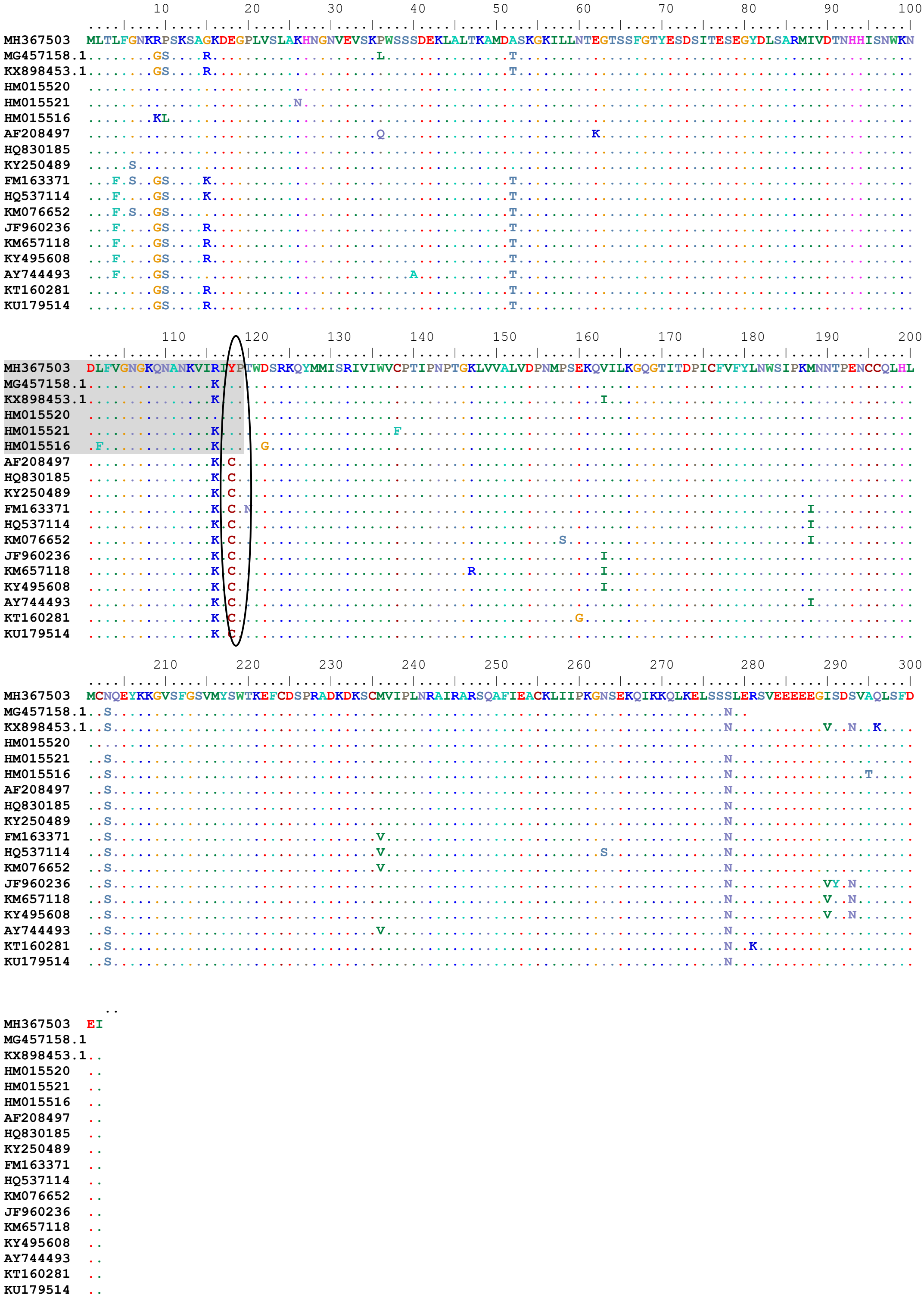
Appendix 2b. *Amino acid sequencing of the NSm open reading region of MH367503 (TSWVAntRB) isolate (302 AA) and consensus points wıth other isolates*



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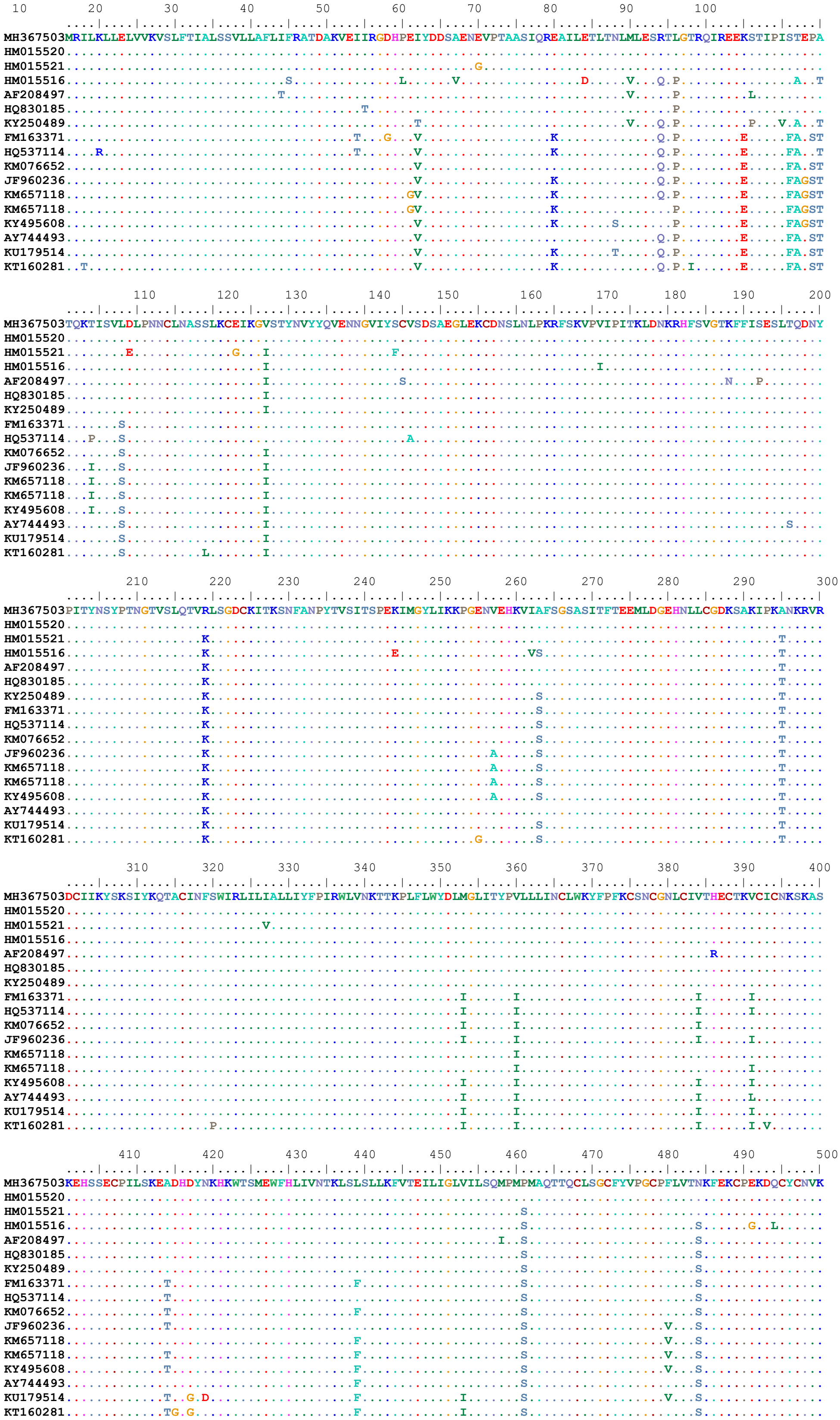
Appendix 3. *Amino acid sequencing of the Gn-Gc domain of MH367503 (TSWVAntRB) isolate*



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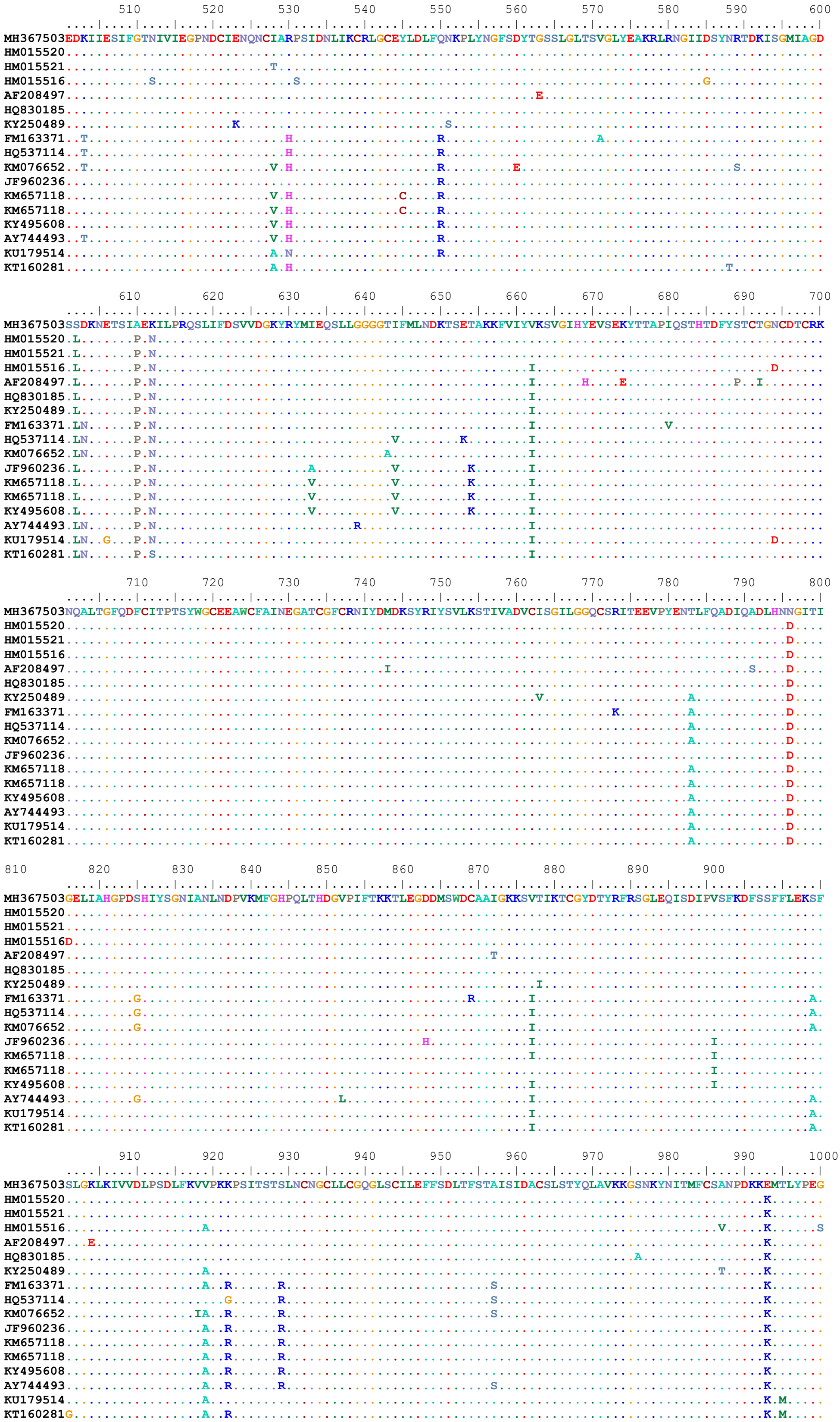

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                 1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MH367503NPDISVEILVNNVIIEEPENIIDQNDEYAHEEQQYNSDSSAWGFWDYIKSPFNFIASYFGSFFDTIRVALLIAFIFLVIYFCSILTSICKGYVKHKSYKS
HM015520....................................................................................................
HM015521....................................................................V...............................
HM015516.......V......VK....................................................V................A......I..E....
AF208497....P..V............................................................V...............................
HQ830185.......V............................................................V...............................
KY250489.......V......VK....................................................V.........................NE....
FM163371.......V.....VV.....................................................I.................T.......N.....
HQ537114.......V.....VV.....................................................I..T..............T.......NE....
KM076652.......V.....VV.....................................................I....S...A........T.......NE....
JF960236.......V......V.....................................................I.................T.......N.....
KM657118.......V......V......................................................I.................T.......N......
KM657118.......V......V.....................................................I.................T.......N.....
KY495608.......V......V.....................................................I.................T.......N.....
AY744493.......V.....VV.....................................................I........A........T.......NE....
KU179514.......V......V........................L............................V.................T.......NE....
KT160281.......V......V.....................................................I.................T.......NE....
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                  1110      1120      1130
        ....|....|....|....|....|....|....|
MH367503RSKIEDDDELEIKAPMLMKDTMTRRRPPMDFSHLV
HM015520...................................
HM015521...................................
HM015516.........P..R......................
AF208497.........P.........................
HQ830185...................................
KY250489.........P.........................
FM163371....V...DS.........................
HQ537114....V...DS.........................
KM076652........DS.........................
JF960236.........S.........................
KM657118........DS.........................
KM657118........DS.........................
KY495608........GS.........................
AY744493.........S.........................
KU179514........DS.........................
KT160281........DS......V..................
```

Appendix 4. *Results of haplotype analysis of 6 sequences using DnaSP Ver. 5.10.01*

#NEXUS
[File generated by DnaSP Ver. 5.10.01;   June 7, 2018]
[Haplotype Data from Data File: aaa.meg]
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[Number of Sites in the complete Data File: 4825]
[Selected Region: 1-4825]
[Sites with alignment gaps: not considered]
[Invariable sites: included]

BEGIN TAXA;
DIMENSIONS NTAX=1;
TAXLABELS
Hap_1;
END;

[Hap# Freq. Sequences]
[Hap_1: 6   1-6]

[Hap# Freq. Sequences]
[Hap_1: 6   MH367503.1_TSWVAntRB MH367506.1_TSWVAntRB MH367505.1_TSWVAntRB MH367501.1_TSWVAntRB MH367504.1_TSWVAntRB MH367502.1_TSWVAntRB]

BEGIN CHARACTERS;
DIMENSIONS NCHAR=4825;
FORMAT DATATYPE=DNA MISSING=? GAP=- MATCHCHAR=.;
MATRIX
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TTACACAAGCTCCTCTACCTTAGGCTGTTGAACTCAAAATGTTGACTCTTTTCGGTAACAAGA

GGCCTTCTAAGTCTGCCGGAAAGGATGAAGGTCCTTTAGTTTCACTTGCTAAACATAATGGC
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GGACGCATCCAAAGGAAAGATACTGTTGAACACTGAGGGAACATCTTCCTTTGGAACCTATG
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53215339

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1702_53215339